Presence and characteristics of sorbitol-negative *Escherichia coli* O157 in healthy sheep faeces

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ABSTRACT: The presence of sorbitol-negative *Escherichia coli* O157 was investigated in healthy Awassi sheep faeces from 175 randomly selected animals in Burdur province of Turkey. Out of 175 animals, 16 (9.1%) were faecal shedding of sorbitol-negative *E. coli* O157. Out of the 15 flocks included in the study, 7 (47%) had at least one sheep positive for sorbitol-negative *E. coli* O157. The isolation rate of sorbitol-negative *E. coli* O157 ranged from 8.3 to 60% among the animals tested in the flocks. A total of 16 ovine sorbitol-negative *E. coli* O157 strains were characterized by a multiplex PCR. Results showed that 6 (37.7%) strains carried *stx1* gene, 3 (18.8%) *stx2* gene and 1 (6.3%) both *stx1* and *stx2* genes. Intimin (*eaeA*) gene was detected in 4 (25%) of the strains. None of the strains encoding for *stx* genes was positive for *eaeA* gene. The results demonstrate that the majority of sorbitol-negative *E. coli* O157 strains (62.5%) isolated from Awassi sheep in Burdur province of Turkey are Shiga toxin-producing *E. coli* that have a potential as human pathogens.

Keywords: sorbitol-negative *Escherichia coli* O157; sheep; virulence factors

Food-borne transmission of sorbitol-negative *Escherichia coli* O157 is an important source of infection in humans (Karmali et al., 1985; Carter et al., 1987; Karmali, 1989; Olsvik et al., 1991; Heuvelink et al., 1998). Domestic ruminants, mainly cattle, sheep and goats, have been implicated as the principal reservoir (Beutin et al., 1993; Kudva et al., 1997; Heuvelink et al., 1998). The isolation of sorbitol-negative *E. coli* O157 from the faeces of healthy sheep supports the epidemiological evidence of a link between human disease and consumption of manure-contaminated products (Heuvelink et al., 1998; Djordjevic et al., 2001; Blanco et al., 2003; Rey et al., 2003).

The pathogenicity of sorbitol-negative *E. coli* O157 is associated with a number of virulence factors, including two phage encoded cytotoxins called Shiga toxins 1 and 2 (*stx1* and *stx2*), and an outer membrane protein called intimin which is encoded by the chromosomal gene *eaeA* (Jerse et al., 1990; Paton and Paton, 1998; Boerlin et al., 1999; Schmidt et al., 2001). Shiga toxin producing *E. coli* O157:H7 belonging to enterohaemorrhagic *E. coli* (EHEC) are responsible for a number of human diseases, including haemorrhagic colitis, bloody or non-bloody diarrhoea, and haemolytic-uraemic syndrome (Karmali et al., 1985; Ryan et al., 1986; Carter et al., 1987; Karmali, 1989; Salmon et al., 1989; Paton and Paton, 1998). Intimin is a surface protein essential for the formation of attaching and effacing (A/E) lesions on gastrointestinal epithelial cells. Severe diarrhoea (especially haemorrhagic colitis) and haemolytic-uraemic syndrome were closely associated with Shiga toxin-producing *Escherichia coli* (STEC) types carrying the *eaeA* gene for intimin (Paton and Paton, 1998; Boerlin et al., 1999; Schmidt et al., 2001).

In sheep, in contrast to cattle, the number of epidemiological studies carried out on EHEC strains is very limited (Kudva et al., 1996, 1997; Heuvelink et al., 1998; Blanco et al., 2003; Rey et al., 2003). To our knowledge, there is no study on the genotypic char-

acteristics of sorbitol-negative *E. coli* O157 strains isolated from sheep in Turkey. The objectives of this study were: (i) to investigate the presence of sorbitol-negative *E. coli* O157 in faeces of healthy sheep in Burdur province of Turkey; (ii) to detect the virulence-associated genes encoding intimin and Shiga toxins together with K99 and F41 fimbriae, and heat-stable enterotoxin (STa) by using PCR assay; and (iii) to determine whether the isolated strains are EHEC or enteropathogenic (EPEC).

MATERIAL AND METHODS

Specimen collection and *E. coli* strains

Fifteen Awassi sheep flocks in Burdur province, Turkey, were selected for this study. The flock size ranged from 80 to 540 animals and the animals in all flocks were clinically healthy adults. Faeces from 175 randomly selected animals, representing at least 5% of each flock, were collected from November 2004 to April 2005. Ovine faecal samples of unknown sorbitol-negative *E. coli* O157 infection statuses were collected via rectal retrieval in the morning. A new glove was used for collecting each sample to avoid cross-contamination during sampling. Samples were placed in sterile screw-top vials and transported to laboratory on ice and held at 4°C until processing (within 24 h).

As a control strain of *E. coli* O157:H7, RSKK232 (Refik Saydam Culture Collection, Ankara, Turkey) was used for all isolation procedures. In PCR assays, *stx1*- and *eaeA*-positive *E. coli* O157:H7 strain (RSKK232) and K99-, F41-, STa-positive *E. coli* vaccine strain (0101:H–:K99+, F41+, Sta+; Pendik Veterinary Control and Research Institute, Istanbul Turkey) were used as positive controls, and non-O157:H7 *E. coli* strain (ATCC 25922) which harboured no *eaeA*, *stx1*, *stx2,* K99, F41 and STa genes was used as negative control.

Isolation and identification of sorbitol-negative *E. coli* O157 colonies

The method was modified according to McDonough et al. (2000). Briefly, one gram of faeces was added to 9 ml of Tripticase soy broth (TSB) with cefixime (50 ng/ml) and potassium telluride (2.5 µg/ml; Sigma Chemical Co, St. Louis, USA), and the components were mixed on a vortex mixer and incubated at 37°C for 18 to 24 hours. After incubation, serial 10-fold dilutions were made in plain TSB in a microtitre plate, and then 0.1 ml of the 10^{-3} and 10^{-5} dilutions was plated onto 150-mm Sorbitol MacConkey agar (Oxoid Ltd, Hampshire, England) containing cefixime (50 ng/ml) and potassium telluride (2.5 µg/ml) supplements (SMACct) and was evenly spread. The SMACct plates were incubated at 37°C for 18 to 24 h, when sorbitol-negative colonies were picked for identification; the SMACct plates were reincubated for an additional 24 hours, and then additional colonies were picked, if present, for screening. Up to 10 sorbitol-negative colonies picked from the plates were subcultured onto MacConkey Agar plates (Oxoid) and incubated overnight at 37°C. Sorbitol-negative, lactose-positive isolates were identified by the following reactions: production of indole, hydrogen sulphide and urease, acid from glucose, gas from glucose, L-tryptophane deaminase, lysine decarboxylase and β-glucuronidase (Koneman et al., 1992; Heuvelink et al., 1998). All sorbitol-negative *E. coli* colonies were then characterized by using the *E. coli* O157 latex agglutination assay (Oxoid).

Detection of virulence genes by multiplex PCR

A multiplex PCR described by Franck et al. (1998) was used for the detection of *stx1*, *stx2*, *eaeA*, K99, F41 and STa genes in the sorbitol-negative *E. coli* O157 strains. Primers were chosen from published sequences (Franck et al., 1998) (Table 1). The assay was performed in a final volume of 50 µl reaction mixture consisting of 10X PCR buffer (Promega Co, Madison, WI, USA), 1.5mM $MgCl_2$, 0.5µM each of the primers, 250µM of each deoxynucleoside triphosphates, 1.25 U of Taq DNA polymerase (Promega) and 10 µl of DNA template. Bacterial DNA for amplification was obtained by suspending colonies of bacteria grown overnight on MacConkey agar in 100 µl of sterile double-distilled water and boiling at 100°C for 10 min without further treatment. The amplification was carried out in a MJ Research thermal cycler under the following conditions: 25 cycles beginning with 30s DNA denaturation at 94°C, primer annealing at 50°C for 45 s, followed by extension for 1 min 30 s at 70°C. The extension time was ramped for an additional 3 s per cycle, and a final extension for 10 min at 70°C was performed (Franck et al., 1998). The products

Table 1. Primer sequences used in a multiplex PCR assay and the expected sizes of the products (Franck et al., 1998)

Virulence factor	Primer sequence 5'–3'	Size of product (bp)
stx1	forward-TTCGCTCTGCAATAGGTA reverse-TTCCCCAGTTCAATGTAAGAT	555
eaeA	forward-ATATCCGTTTTAATGGCTATCT reverse-AATCTTCTGCGTACTGTGTTCA	425
F41	forward-GCATCAGCGGCAGTATCT reverse-GTCCCTAGCTCAGTATTATCACCT	380
K99	forward-TATTATCTTAGGTGGTATGG reverse-GGTATCCTTTAGCAGCAGTATTTC	314
STa	forward-GCTAATGTTGGCAATTTTTATTTCTGTA reverse-AGGATTACAACAAAGTTCACAGCAGTAA	190
stx2	forward-GTGCCTGTTACTGGGTTTTTCTTC reverse-AGGGGTCGATATCTCTGTCC	118

(7 µl) were electrophoresed in 1.5% agarose gel, stained with ethidium bromide and photographed under UV light.

RESULTS

Presence of sorbitol-negative *E. coli* O157 in healthy sheep

Out of 175 animals, 16 (9.1%) were faecal shedding of sorbitol-negative *E. coli* O157. Out of the 15 flocks included in the study, 7 (47%) had at least one sheep positive for sorbitol-negative *E. coli* O157. The isolation rate of sorbitol-negative *E. coli* O157 ranged from 8.3 to 60% among the animals tested in the flocks (Table 2).

Virulence genes

The multiplex PCR correctly determined the presence or absence of the genes of interest in all of the reference strains. The PCR analysis of 16 sorbitol-negative *E. coli* O157 strains from healthy sheep faeces showed that 6 (37.7%) strains carried the *stx1* gene, 3 strains (18.8%) had the *stx2* gene, and 1 strain (6.3%) had both *stx1* and *stx2* genes (Table 2). Shiga toxin genes were found in 10 (62.5%) out of 16 *E. coli* O157 strains. Intimin (*eaeA*) gene was detected in 4 (25%) out of the strains. None of the strains encoding for *stx* genes was positive for *eaeA* gene. The multiplex PCR did not detect any of F41, K99 and STa genes in 16 sorbitol-negative *E. coli* O157 strains isolated (Figure 1).

DISCUSSION

Although it is generally accepted that the cattle are a primary reservoir of sorbitol-negative *E. coli* O157, this pathogen has also been isolated from domestic ruminants, especially sheep and goats (Beutin et al., 1993; Kudva et al., 1996, 1997; Heuvelink et al., 1998; Blanco et al., 2003; Rey et al., 2003). However, very little is known about the epidemiology of this organism on sheep farms. Kudva et al. (1996) studied 35 free-ranging healthy sheep of a single flock in Idaho and reported that the incidence of faecal shedding varied from 31% of sheep in June to none in November. In Holland, O157 STEC strains were found in the faeces from 2 (3.8%) out of 52 ewes and 2 (4.1%) out of 49 lambs sampled at the main slaughterhouses, located at different geographic sites (Heuvelink et al., 1998). Rey et al. (2003) found STEC O157 strains in 1% of 697 healthy lambs in Spain. Gulhan (2003) reported that *E. coli* O157 was isolated from 26% of ewes and 20% of lambs sampled at the Van slaughterhouse in the east of Turkey. In the present study we found sorbitol-negative *E. coli* O157 strains in 9.1% of sampled healthy adults. While these differences may be attributed to the geographic variations and the use of more sensitive detection methods, they may also reflect an increasing distribution of sorbitol-negative *E. coli* O157 across sheep flocks over time. In the sheep flocks studied, the percentage of positive animals varied greatly, from 8.3 to 60%. Kudva et al. (1996, 1997) reported that variation in the occurrence of sorbitol-negative *E. coli* O157 positive sheep was observed, with animals being culture positive only in summer months but

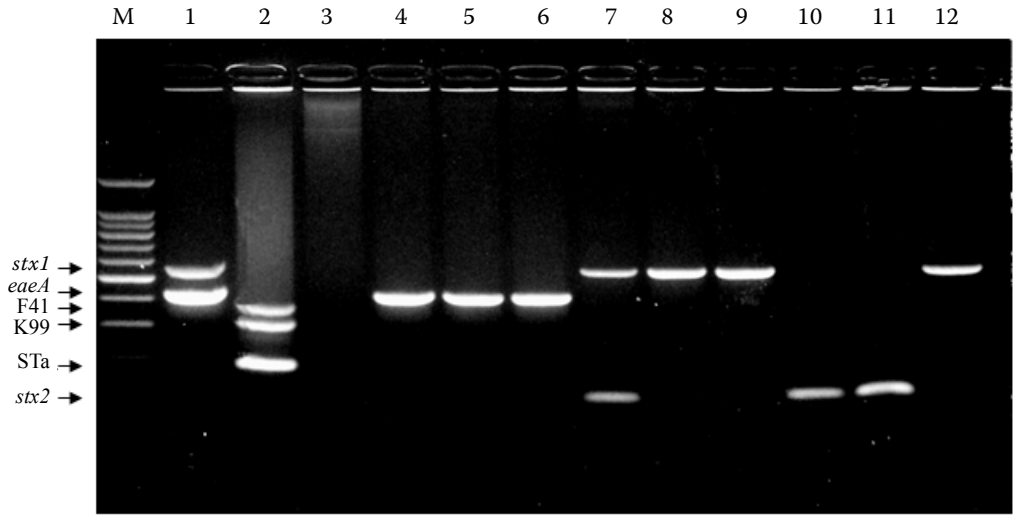

Figure 1. Multiplex PCR of control strains and sorbitol-negative *E. coli* O157 strains isolated from sheep showing the presence of virulence genes. Lane M = DNA molecular weight marker; lane 1 = RSKK232 *E. coli* O157:H7 (*stx1*- and *eaeA*-positive); lane 2 = *E. coli* vaccine strain (K99-, F41-, STa-positive); lane 3 = ATCC 25922 *E. coli* strain (*stx1*-, *stx2*- and *eaeA*-negative); lane 4–12 = sorbitol-negative *E. coli* O157 strains isolated from sheep. The relative positions in the gel of predicted size of PCR products are indicated by arrows on the left side

not in spring, autumn, or winter. In contrast, we detected sorbitol-negative *E. coli* O157 positive sheep in winter and spring months. This result may be explained on the basis of reports by Bolton et al. (1999) and Wang et al. (1996), who stated that sorbitol-negative *E. coli* O157 survived in faeces and/or contaminated grassland for several months during temperature fluctuations. Additionally,

Table 2. Distribution and virulence factors of sorbitol-negative *E. coli* O157 strains isolated from healthy Awassi sheep from 15 flocks in Burdur, Turkey

Flock No.	Flock size	Number of tested animals	Number of sorbitol-negative *E. coli* O157 positive animals (%)	Mark of strains	Virulence factors (No. of isolates)
1	200	10	0		
2	540	27	3 (11.1%)	1, 2, 3	*eaeA* (3)
3	360	18	2 (11.1%)	4, 7	*stx1 stx2* (1) *stx2* (1)
4	100	5	3 (60%)	5, 8, 11	*stx1* (1) *stx2* (2)
5	200	10	0		
6	80	4	0		
7	180	9	2 (22.2%)	6, 9	*stx1*(2)
8	220	11	0		
9	240	12	0		
10	240	12	1 (8.3%)	10	
11	100	5	0		
12	280	14	0		
13	100	5	1 (20%)	12	*eaeA* (1)
14	460	23	0		
15	200	10	4 (40%)	13, 14, 15, 16	*stx1* (3)
Total	3 500	175	16 (9.1%)		

we also considered that diet might influence the shedding of sorbitol-negative *E. coli* O157 from the guts as previously reported (Kudva et al., 1997; Heuvelink et al., 1998; Garber et al., 1999). Such persistence of sorbitol-negative *E. coli* O157 in faeces and contaminated grassland may allow the initial infection and re-infection of sheep, leading to increased overall carriage rates in flocks.

Recently, a number of PCR-based assays have been developed for detection of sorbitol-negative *E. coli* O157 (Franck et al., 1998; Hu et al., 1999; Blanco et al., 2003; Kang et al., 2004). Most of these assays are designed for detection of *stx* and *eaeA* genes. In this study, a multiplex PCR assay developed by Franck et al. (1998) was used. The characterization of 16 sorbitol-negative *E. coli* O157 strains for virulence genes by the multiplex PCR showed that 37.6% of ovine field strains carried *stx1* gene, 18.8% *stx2* gene, and 6.3% *stx1* and *stx2* gene. The rate of STEC O157 strains was 62.5% and they were regarded as potential human pathogens, indicating that healthy sheep may serve as a reservoir of human pathogens (Beutin et al., 1993; Kudva et al., 1996; Kudva et al., 1997; Heuvelink et al., 1998). Intimin together with these phage-encoded toxins is considered essential for EHEC virulence in humans (Karmali et al., 1985; Ryan et al., 1986; Carter et al., 1987; Salmon et al., 1989; Paton and Paton, 1998; Boerlin et al., 1999; Blanco et al., 2004). The outer membrane protein, intimin, encoded by *eaeA* gene, has been shown to be necessary for the A/E activity of EHEC O157 (Jerse et al., 1990; Paton and Paton, 1998; Schmidt et al., 2001). In the present study, the gene encoding intimin together with either one or both of the Shiga toxin genes was amplified via the multiplex PCR. The *eaeA* gene was detected only in 4 (25%) out of 16 ovine sorbitol-negative *E. coli* O157 strains. This finding supports that *eaeA* was found less frequently in ovine STEC strains in comparison with characterized bovine strains (Djordjevic et al., 2001; Blanco et al., 2003; Rey et al., 2003). It might be possible that *eaeA*-positive *stx*-negative *E. coli* O157 strains lost their *stx*-encoding phages (Heuvelink et al., 1998; Rey et al., 2003). We also thought that these strains were most probably EPEC strains. Some of the sorbitol-negative *E. coli* O157 strains detected in this study did not carry *stx* or *eaeA* genes, which is in agreement with the other study that also reported the profiles in sorbitol-negative *E. coli* O157 strains from sheep (Kudva et al., 1997). None of the strains encoding for *stx* genes was positive for *eaeA* gene. Many of the STEC strains considered to be highly virulent have the capacity to produce A/E lesions on intestinal mucosa, a property encoded on a pathogenicity island termed the locus for enterocyte effacement (LEE) (Jerse et al., 1990; Paton and Paton, 1998). It has been reported that *eaeA* was a part of the LEE and *eaeA* was used as a diagnostic marker for LEE positive STEC strains (Paton and Paton, 1998). However, the presence of *eaeA* is not absolutely linked to virulence, because some STEC diseases in humans have been caused by LEE-negative strains (Paton and Paton, 1998, 2002; Schmidt et al., 2001). Unfortunately we were not able to perform any other test to confirm the absence or presence of the LEE. In addition, it has been stated that different intimin types were found in STEC O157 strains (Blanco et al., 2003, 2004). In this study, the generic *eaeA* primers were used in PCR; divergent *eaeA* genes were not amplified. Therefore it remains unclear whether these STEC O157 strains have the capacity to produce A/E lesions and to cause disease or not.

Boerlin et al. (1999) stated that adherence might be a more important factor in STEC-associated disease than Shiga toxins. However, it has been suggested that the *eaeA*-negative STEC strains may express additional adherence factors that allow them to colonize the intestinal tract (Paton and Paton, 2002; Blanco et al., 2004; Zweifel et al., 2005). Kudva et al. (1997) reported that some ovine sorbitol-negative *E. coli* O157 isolates that were tested negative for the toxin and *eae* genes were positive for the K99 and F107 gene loci and all of non-O157 STEC isolates were positive for *eaeA* and both the K99 and F107 gene loci. In this study, the presence of K99, F41 and STa genes which were considered to be virulence genes of EPEC strains was also investigated as well as the detection of *stx* and *eaeA* genes in the *E. coli* O157 positive strains isolated. But these genes were not detected in all *E. coli* O157 strains isolated from sheep. Osek et al. (2003) and Osek (2004) stated that porcine *E. coli* O157 were different from human and bovine STEC O157 in terms of virulence factor (presence of *stx1*, *stx2* and *eaeA*) genes and only few porcine O157 STEC isolates were *eaeA*-positive. It was also shown that a fimbrial gene cluster (*lpfAO113*) is widely distributed among porcine *eaeA*-negative *E. coli* isolates and may represent an important adherence factor in this group of pathogens (Osek et al., 2003). Therefore, further studies are needed to investigate this gene in *eaeA*-negative STEC strains.

The phage typing have become the most commonly used and the only internationally standardized subtyping method for STEC O157 (Allison et al., 2000; Mora et al., 2004). Unfortunately, we were unable to type our strains during analyses due to the lack of phages. Additionally the aim of our study described here was only to determine the virulence factors of ovine sorbitol-negative *E. coli* O157 strains. Finally, the lack of data on phage types of *E. coli* O157 strains isolated in this study makes definite discussion difficult. To clarify this, future studies focused on the phage types of *E. coli* O157 strains isolated from sheep are required.

This is the first report providing the genotypic characterization of ovine sorbitol-negative *E. coli* O157 strains in Turkey, and underlining the importance of the determination of virulence factors to assess the potential pathogenicity of these strains for humans. From the data presented in this study it can be concluded that Awassi sheep are natural hosts of sorbitol-negative *E. coli* O157 strains in Burdur province and the majority of these strains are STEC.

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