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Variation in the prevalence of non-O157 Shiga toxin-producing *Escherichia coli* in four sheep flocks during a 12-month longitudinal study

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) are an important group of emerging pathogens, with ruminants recognised as their main natural reservoir. The aim of this longitudinal study was to provide information on the prevalence and existence of seasonal variation in the occurrence of non-O157 STEC in four sheep flocks over a 12-month period. A total of 504 faecal samples from 48 adult sheep in four flocks were collected and examined for STEC using both phenotypic and genotypic methods. STEC were isolated from 407 (80.8%) faecal samples representing all the animals sampled. The overall monthly prevalence of STEC varied between 71.1 and 94.4% and no seasonal variation in the occurrence of STEC could be observed over the study period. A total of 521 STEC isolates were characterised. The PCR procedure indicated that 275 (52.8%) isolates carried the stx_1 gene, 44 (8.4%) carried the stx_2 gene and 202 (38.8%) contained both of these genes. The *eae* and *ehxA* genes were detected in 4 (0.8%) and 368 (70.6%) isolates, respectively. The isolates belonged to 28 O serogroups, although 72.4% were restricted to only 10 serogroups (O5, O6, O76, O87, O91, O123, O128, O146, O166 and O176). None of the isolates belonged to the O157 STEC serogroup. STEC isolates of serogroups O33, O53, O105 and O162 have not previously been reported in sheep. This is the first study to report the maintenance of high frequencies of non-O157 STEC infection in sheep flocks over long time periods.

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1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) have emerged as important food-borne pathogens. Human diseases ranging from mild diarrhoea to haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS) can be caused by STEC, typically affecting infants, young children and the elderly (Paton and Paton, 1998). The pathogenic capacity of STEC resides in two potent cytotoxins called Shiga toxins (Stx1, Stx2 and their variants), encoded by genes (stx_1 and stx_2) located on temperate phages (Paton and Paton, 1998). Another virulence-associated factor is an outer membrane protein called intimin, encoded by the *eae* gene, and responsible for intimate attachment of STEC to the intestinal epithelial cells, which causes attaching and effacing (A/E) lesions in the intestinal mucosa (Kaper et al., 1998). A factor that may also affect virulence of STEC is the enterohaemolysin (Ehly), encoded by a plasmid-borne

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gene known as *ehxA* (Schmidt et al., 1995). Serogroup O157 especially represents a major public health concern worldwide. However, in some countries non-O157 STEC strains are more prevalent in meat-producing animals and as contaminants in foods, and therefore humans are more likely to become exposed to such strains (Djordjevic et al., 2004).

Although healthy cattle are the best-recognised animal reservoir for STEC (Blanco et al., 2004), sheep are an important source of these organisms for humans in some countries (Blanco et al., 2003; Rey et al., 2003; Cookson et al., 2006). Several longitudinal studies of STEC have been performed on cattle, most of them focused only on STEC O157 (Mechie et al., 1997; Schouten et al., 2005), and another one has recently referred to non-O157 STEC in goats (Orden et al., 2008). Nevertheless, apart from the findings of studies of STEC O157 (Chapman et al., 1997; Kudva et al., 1997), little is known about the variation in the prevalence of STEC in sheep flocks over long time periods. Therefore, a 12-month longitudinal study was conducted to monitor the prevalence of non-O157 STEC and to provide information on the existence of seasonal variation in their occurrence in the context of a typical sheep flock in the South-West of Spain. A further objective was to characterise all STEC isolates recovered from sheep over the study period.

2. Materials and methods

2.1. Sheep flocks and sample collection

The study was conducted with four Merino sheep flocks (flocks 1–4) in the Extremadura region in the South-West of Spain. In this region, summers are typically long, hot and dry. The average temperature for July is usually over 26 °C, with the maximum temperature often exceeding 40 °C. Winters are normally mild and moist, with an average temperature of 7.5 °C. Annual precipitation is irregularly distributed and usually ranges from 300 to 800 mm, with large variations between years. Sheep flocks are managed under semi-extensive conditions, with grazing in a semiarid natural pastureland and dietary supplementation during poor forage production periods (i.e. summer and the coldest winter months) and during the lactation period. The flocks selected were not in adjacent pastures and there was no contact between sheep from different flocks. Flocks 1 and 2 had sizes of 344 and 300 sheep and grazing densities of 36 and 27 sheep per hectare, respectively. Flocks 3 and 4 had sizes of 691 and 958 sheep, respectively, but a grazing density of only 3 sheep per hectare.

The longitudinal study started in November 2003. During the first sampling visit, 12 healthy ewes around 1 year of age in each flock were randomly selected for sampling and subsequent monthly sampling visits were carried out until October 2004. On each sampling occasion, rectal swabs (one per animal) were collected from the same selected individuals and transferred to tubes containing Cary-Blair transport medium (Deltalab, Barcelona, Spain), transported to the laboratory under refrigeration and placed in culture media within 24 h of collection. Overall, 504 faecal samples instead of 576 were collected, due to problems with sample collection in the flocks on some occasions.

2.2. Culture and STEC screening

Faecal samples were plated directly onto MacConkey (MAC) agar (Oxoid, Basingstoke, England). Following overnight incubation, bacterial growth from the first streaking area of the culture plate was tested for stx_1 and stx_2 genes by PCR. For each PCR-positive culture, 10 *E. coli*-like colonies obtained from MAC plates were tested for stx_1 and stx_2 genes in order to obtain the STEC isolates for further characterisation. If no single colony was found to be positive among the first 10 colonies, at least 40 more were tested. If still none of the assayed coliform colonies was positive in the PCR procedure, the sample was reported as PCR-positive without STEC isolation.

All PCR-positive isolates were tested for Shiga toxin production by cytotoxicity assays on Vero cells. One loopful of each isolated colony was inoculated in 50 ml Erlenmeyer flasks containing 5 ml of tryptone soy broth (Oxoid) (pH 7.5) with mitomycin C (0.5 μg/ml), incubated for 20 h at 37 °C (shaken at 200 rpm) and then centrifuged (6000 × *g*) for 30 min at 4 °C. The Vero cell culture assay was performed with nearly confluent cell monolayers grown in plates with 24 wells. At the time of assay, the growth medium (RPMI with 50 U/ml of polymyxin B sulfate) (Sigma–Aldrich, Madrid, Spain) was changed (0.5 ml per well) and 75 μl of undiluted culture supernatant was added. Cells were incubated at 37 °C in a 5% CO_2 atmosphere and the morphological changes in cells were observed after 24 and 48 h of incubation using a phase-contrast inverted microscope.

All STEC isolates were confirmed biochemically as *E. coli* by the API 20E system (bioMérieux, Marcy L'Etoile, France) and tested for *eae* and *ehxA* genes by PCR. When isolates from a given sample exhibited similar genetic characteristics in terms of the presence or absence of virulence genes, only one colony was selected and stored at −80 °C until further characterisation. Otherwise, when isolates with different genetic characteristics were obtained, one colony of each was selected and stored for further characterisation.

2.3. Detection of virulence genes by PCR

For PCR, a loopful of bacterial growth was suspended in 0.5 ml of sterile distilled water, boiled for 5 min to release the DNA, and centrifuged at 10,000 rpm for 5 min. The supernatant was added directly to the PCR mixture. Base sequences and predicted sizes of amplified products for the specific oligonucleotide primers used in this study are shown in Table 1. Multiplex PCR was used only for detection of stx_1 and stx_2 genes. Amplification of bacterial DNA was performed using 30 μl volumes containing 7 μl of the prepared sample supernatant; the oligonucleotide primers (60 ng for stx_2 primers, 90 ng for stx_1 and *ehxA* primers, and 150 ng for *eae* primers) (Invitrogen, Madrid, Spain); 0.2 mM (each) dATP, dGTP, dCTP, and dTTP (PCR Nucleotide Mix dNTP Set, GE Healthcare, Madrid, Spain); 10 mM Tris–HCl (pH 8.8); 1.5 mM $MgCl_2$; 50 mM KCl; and 1 U of *Taq* DNA Polymerase (GE Healthcare). The conditions for the PCR were 94 °C for 2 min (initial denaturation of DNA) followed by 35 cycles of 94 °C for 1 min (denaturation), 55–60 °C (Table 1) for 1 min (primer annealing), and 72 °C for 1 min (DNA synthesis) performed with an iCycler™ Thermal Cycler (Bio-Rad, Hemel Hempstead, United Kingdom). The amplified products were visualised by standard submarine gel electrophoresis using 10 μl of the final reaction mixture on a 2% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA). The samples were electrophoresed for 20–40 min at 130 V. Amplified DNA fragments of specific sizes were located by UV fluorescence after staining with ethidium bromide. Molecular size markers (ΦX174 DNA/HaeIII Markers, Promega, Madison, United States) were included in each gel. Reference *E. coli* strains used as controls were EDL 933 (human, O157:H7, stx_1, stx_2, *eae*, *ehxA*; ATCC No. 43895) and K12-185 (negative for stx_1, stx_2, *eae* and *ehxA* genes) (Blanco et al., 2004).

2.4. Determination of O antigen

The identification of O antigen in STEC isolates was carried out as described by Guinée et al. (1981) using the full range of O antisera from O1 to O185. Antisera were produced in the Laboratorio de Referencia de *E. coli* (Lugo, Spain) and were absorbed with corresponding cross-reacting antigens to remove non-specific agglutinins.

2.5. Statistical analysis

In order to assess a possible seasonal influence in the occurrence of STEC, logistic regression methods were used to test the association between season (December–February = winter; March–May = spring; June–August = summer; September–November = autumn) and the dependent variable of positive or negative individual, controlling by the effect of "flock" in the model. The generalised estimating equations (GEE) approach was used to adjust for correlation of the multiple sampling occasions for each flock (SPSS Version 17.0; SPSS Inc., Chicago, USA) as described by Fossler et al. (2005) using "individual" as the subject effect in the repeated statement and "month" as the within-subject effect. Statistical significance was defined with p values < 0.05.

Table 1
Primer sequences and predicted lengths of PCR amplification products.

Gene	Primer	Oligonucleotide sequence (5′–3′)	Product size (bp)	Annealing temperature (°C)	Reference
stx_1	VT1-A VT1-B	CGCTGAATGTCATTCGCTCTGC CGTGGTATAGCTACTGTCACC	302	55	Blanco et al. (2003)
stx_2	VT2-A VT2-B	CTTCGGTATCCTATTCCCGG CTGCTGTGACAGTGACAAAACGC	516	55	Blanco et al. (2003)
ehxA	HlyA1 HlyA4	GGTGCAGCAGAAAAAGTTGTAG TCTCGCCTGATAGTGTTTGGTA	1551	60	Schmidt et al. (1995)
eae[a]	EAE-1 EAE-2	GAGAATGAAATAGAAGTCGT GCGGTATCTTTCGCGTAATCGCC	775	55	Blanco et al. (2003)

[a] *eae* gene detection. Universal oligonucleotide primer pair EAE-1 and EAE-2 with homology to the 5′ conserved region of *eae* gene (detects all *eae* variants currently reported).

3. Results

3.1. Summary and characterisation of STEC isolates

STEC were isolated from 407 (80.8%) out of 504 faeces originating from the 48 (100%) animals sampled. Up to two different strains, with different virulence profiles and/or serogroups, were isolated from 104 (20.6%) samples and even three different strains were isolated from 5 (1.0%) samples.

A total of 521 STEC isolates were characterised in the present study. All were cytotoxic to Vero cells. The PCR procedure indicated that 275 (52.8%) isolates carried the stx_1 gene, 44 (8.4%) carried the stx_2 gene and 202 (38.8%) contained both of these genes. The *eae* and *ehxA* genes were detected in 4 (0.8%) and 368 (70.6%) isolates, respectively.

The STEC isolates were from a total of 28 O serogroups, although 72.4% were restricted to only 10 serogroups (O5, O6, O76, O87, O91, O123, O128, O146, O166 and O176) (Table 2). In 109 of the isolates O antigen was found non-typeable (ONT). Nevertheless, the most frequent serogroups were different in the four flocks studied. In flock 1, these serogroups were O6, O91, O146 and O176 (93/143, 65.0%); in flock 2, they were O5, O91, O166 and O176 (98/140, 70.0%); in flock 3, they were O76, O91, O146 and O176 (58/128, 45.3%); and, in flock 4, they were O5, O76, O87 and O123 (85/110, 77.3%). None of the isolates belonged to the O157 STEC serogroup. Although there were 48 different associations between serogroup and virulence genes among the 521 isolates, 71.6% were restricted to only 10. The most commonly found association was O176 stx_1 *ehxA* (53 isolates), followed by O146 stx_1 stx_2 *ehxA* (48 isolates) and O166 stx_1 *ehxA* (42 isolates) (Table 2).

3.2. Variation in the prevalence of STEC over the study period

Over the 12-month study period, the monthly prevalence of STEC varied between 72.7 and 91.7% in flock 1, between 72.7 and 100% in flock 2, between 54.5 and 100% in flock 3 and between 50 and 91.7% in flock 4. The overall monthly prevalence of STEC, including samples originating from the four sheep flocks, varied between 71.1 and 94.4% (Fig. 1). However, regardless of the flock effect, no statistically significant seasonal differences in the occurrence of STEC were observed over the whole study period (Wald = 3.047, 3 df, $p = 0.384$).

4. Discussion

In the present study, the overall monthly prevalence of non-O157 STEC in sheep flocks varied between 71.1 and 94.4% over the 12-month study period. Prevalence rates of non-O157 STEC obtained in this study are comparable to those reported in Germany (66.7%) (Beutin et al., 1993), Norway (87.6%) (Urdahl et al., 2003), Australia (88.2%) (Fegan and Desmarchelier, 1999) and New Zealand (65.9%) (Cookson et al., 2006). Nevertheless, in previous studies from Spain (Blanco et al., 2003; Orden et al., 2003; Rey et al., 2003) there was a lower frequency of non-O157 STEC (between 24.4 and 35.5%) than in the current one. It is important to note that differences in STEC prevalence between studies were probably due to different criteria in the procedure for detection and isolation of STEC. Actually, the number of *E. coli*-like colonies tested from each PCR-positive sample in previous studies from Spain (1–10) was much more limited than in the present one (1–50). In addition, the occurrence of multiple non-O157 STEC strains in individual faecal samples in the current study has previously been reported by Beutin et al. (1993) in both sheep and cattle and by Kudva et al. (1997) in sheep.

Our sheep tested positive for non-O157 STEC over the whole study period, with high monthly prevalence rates (up to 94.4%) (Fig. 1) and no seasonal variation in the occur-

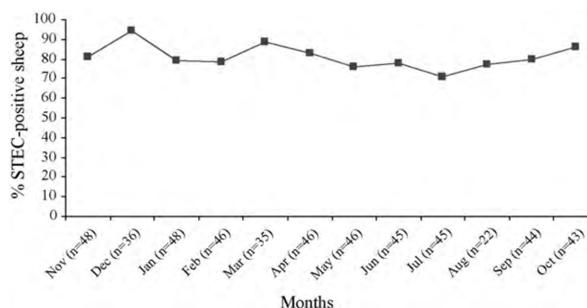

Fig. 1. Monthly prevalence of STEC in sheep faeces over the study period (from November 2003 to October 2004). For each month, the number of STEC-positive animals is indicated as a percentage of the total number of animals tested (n).

Table 2
Serogroups and virulence genes of STEC isolates recovered from sheep ($n = 521$).

Serogroup[a]	No. of STEC isolates	stx_1	stx_2	*eae*	*ehxA*
O2[b,c]	1	+	–	+	+
O5[b,c]	25	+	–	–	+
O5[b,c]	3	+	–	–	–
O5[b,c]	3	+	+	–	+
O6[b,c]	19	+	–	–	+
O6[b,c]	2	+	–	–	–
O15[b,c]	1	+	+	–	–
O20[b,c]	1	+	–	–	–
O21[b,c]	1	+	+	–	–
O33[d]	8	+	–	–	+
O35[b]	1	+	–	–	+
O52[b]	1	+	+	–	+
O53[d]	1	+	–	–	–
O76[b]	30	+	–	–	+
O76[b]	1	+	–	–	–
O76[b]	10	+	+	–	+
O76[b]	1	+	+	–	–
O79[b,c]	1	+	–	–	–
O87[b]	36	–	+	–	–
O91[b,c]	27	+	+	–	+
O91[b,c]	38	+	+	–	–
O105[b,c,d]	1	+	–	–	–
O112[b,c]	1	+	+	–	–
O113[b,c]	1	+	+	–	+
O117[b,c]	8	+	–	–	–
O118[b,c]	1	+	+	–	–
O119[b,c]	1	+	+	–	+
O123[b]	12	+	+	–	+
O125[b,c]	1	–	+	–	+
O128[b,c]	6	+	+	–	+
O128[b,c]	4	+	+	–	–
O136	2	+	–	–	–
O146[b,c]	48	+	+	–	+
O146[b,c]	2	–	+	–	+
O162[b,d]	1	+	–	–	–
O162[b,d]	1	+	+	–	+
O162[b,d]	1	+	+	–	–
O166[b]	42	+	–	–	+
O166[b]	13	+	+	–	+
O166[b]	2	–	+	–	+
O176	53	+	–	–	+
ONT[b,c]	1	+	–	+	+
ONT[b,c]	48	+	–	–	+
ONT[b,c]	26	+	–	–	–
ONT[b,c]	2	+	+	+	+
ONT[b,c]	10	+	+	–	+
ONT[b,c]	19	+	+	–	–
ONT[b,c]	3	–	+	–	–

[a] ONT, O antigen non-typeable.
[b] Serogroups previously associated with human infection.
[c] Serogroups previously associated with HUS.
[d] Serogroups not previously reported in sheep.

rence of STEC. This finding is in agreement with a recent study of non-O157 STEC in dairy goats (Orden et al., 2008). However, this result contrasts with the fact that sheep have been shown to be colonised naturally by STEC O157:H7 in a transient and seasonal manner (Kudva et al., 1997), similar to cattle (Mechie et al., 1997). In a 16-month study performed in the United States, Kudva et al. (1997) reported that their sheep were culture positive for STEC O157:H7 only in the summer months but not in the spring, autumn, or winter. A similar trend was found in the United Kingdom when sheep were tested for STEC O157 over a 12-month period (Chapman et al., 1997). In the only study of non-O157 STEC performed with sheep (Hussein et al., 2003), the prevalence did not follow the seasonal trend previously reported for STEC O157:H7, with the highest prevalence rates (up to 26%) during winter and spring. Kudva et al. (1997) hypothesised that changes in diet and/or environment influenced the seasonal variation observed in the occurrence of STEC O157:H7. Nevertheless, despite important changes in feeding and management practices during the year, and also the extreme variations in temperature and precipitation which are typical of the South-West of Spain, no seasonal variation in the occurrence of non-O157 STEC could be observed in the current study.

STEC strains from more than 50 O serogroups have previously been isolated from sheep (Blanco et al., 2003) (http://www.microbionet.com.au/vtectable.htm; http://www.lugo.usc.es/ecoli). About 72% of the STEC isolates in the present study belonged to only 10 serogroups (O5, O6, O76, O87, O91, O123, O128, O146, O166 and O176), which are serogroups frequently represented among ovine STEC strains (Blanco et al., 2003; Rey et al., 2003; Zweifel et al., 2004). Notably, among ovine STEC isolates belonging to serogroups O5, O91 and O146, the same clones were obtained on sampling occasions separated by as much as 11 months at the flock level, as well as the individual-animal level, indicating persistent colonisation of sheep by these serogroups (Sánchez et al., 2009). STEC isolates of serogroups O33, O53, O105 and O162 had not previously been reported in sheep. With the exception of serogroup O176, the most frequent STEC serogroups identified in this study have previously been associated with human infection and five of them (O5, O6, O91, O128 and O146) have been associated with HUS (http://www.microbionet.com.au/vtectable.htm; http://www.lugo.usc.es/ecoli).

Of the 521 STEC isolates characterised in the present study, stx_1 was the predominant *stx* gene identified, a finding in agreement with several previous studies performed on sheep (Blanco et al., 2003; Urdahl et al., 2003; Djordjevic et al., 2004; Zweifel et al., 2004). Boerlin et al. (1999) reported that stx_2-carrying human STEC isolates were associated with severe disease, while stx_1-carrying isolates were associated with more uncomplicated diarrhoea and healthy individuals. Given that about 47% of STEC isolates in this study carried the stx_2 gene (most of them in combination with stx_1), although only 0.8% of them carried the *eae* gene, they cannot be excluded as potential human pathogens, because *eae*-negative STEC strains have also been associated with cases of HC and HUS (Paton et al., 1999).

5. Conclusions

The results of the present study demonstrate a very high prevalence of non-O157 STEC infection in sheep flocks, without evidence of seasonal variation over the year. To our knowledge, this is the first study to report the maintenance of high frequencies of non-O157 STEC infection in sheep flocks over long time periods. Pre-slaughter protocols and recommendations should be followed to reduce the likeli-

hood of these potential pathogens entering the food chain or being directly transmitted to humans.

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