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Prevalence and characterization of Vero cytotoxin-producing *Escherichia coli* isolated from diarrhoeic and healthy sheep and goats

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SUMMARY

Faecal samples from 146 diarrhoeic lambs and goat kids, and from 511 healthy sheep and goats were screened for the presence of Vero cytotoxin-producing *Escherichia coli* (VTEC). In healthy sheep and goats, VTEC were isolated in 24·4 and 16·2% of the animals, respectively. Moreover, VTEC were detected in 3·1 and 5·9% of the diarrhoeic lambs and goat kids, respectively. These data suggest that VTEC seems not to be associated with diarrhoea in lambs and goat kids. Only four VTEC strains were *eae*-positive. The absence of the *eae* gene in most of these VTEC strains could indicate that these strains are less virulent for humans that the classical *eae*-positive enterohaemorrhagic *E. coli* types. However, almost half (42·9%) and 12·2% of VTEC strains isolated from healthy sheep and goats, respectively, belonged to serotypes associated with severe diseases in humans.

INTRODUCTION

Vero cytotoxin-producing *Escherichia coli* (VTEC) strains may produce two families of Vero cytotoxins: VT1, a homogeneous group of toxins virtually identical to the VT of *Shigella dysenteriae* type 1 and VT2, a heterogeneous group of toxins [1]. VTEC may cause severe diseases in humans, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) [2, 3]. Those VTEC strains which are able to induce HC and HUS are called enterohaemorrhagic *E. coli* (EHEC) [4].

The predominant EHEC serotype associated with human infection and death is O157:H7 [2–4]. In addition, outbreaks of disease have been traced to non-O157 VTEC serotypes, especially in Europe, Australia and Asia [5–7].

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It is not known whether all variants of VTEC are equally pathogenic for humans and it is possible that Vero cytotoxin production alone may not be sufficient for VTEC to cause disease [8, 9]. A factor that may affect virulence of VTEC is the possession of the *eae* gene necessary to cause attaching and effacing lesions in the intestinal mucose [8–10]. Besides Vero cytotoxins and the *eae* gene, a specific plasmid-encoded haemolysin called EHEC haemolysin (previously referred to as enterohaemolysin, EntHly) which is encoded by *hlyA* gene [11] might contribute to the virulence of VTEC for humans [9].

VTEC have been reported to be more prevalent in healthy sheep and goats than in healthy cattle [12]. However, small ruminants have been the subject of fewer surveys than cattle, especially VTEC of other serotypes than O157. Little is known about the role of VTEC in the diarrhoea of small ruminants due to, among several factors, the limited number of

epidemiological studies carried out on VTEC strains from diarrhoeic lambs and goat kids [13–15]. Moreover, the presence of the *eae* gene in the VTEC strains was not determined in any of these studies.

This study was designed to determine the prevalence of VTEC in healthy and diarrhoeic small ruminants. Other objectives of the current study were to investigate the presence of the *eae* and *hlyA* genes and the type of VT synthesized by these strains, as well as their serotype.

MATERIALS AND METHODS

Sampling and isolation of *E. coli* strains

Faecal samples were collected from 129 diarrhoeic lambs in 32 outbreaks of neonatal diarrhoea. Two-hundred and fifty-eight (116 from lambs and 142 from adults) additional samples from non-diarrhoeic sheep were collected from 11 farms. Faecal samples were collected from 17 diarrhoeic goat kids from 7 outbreaks of neonatal diarrhoea. Two-hundred and fifty-three (94 from goat kids and 159 from adults) additional samples were collected from non-diarrhoeic goats on 10 farms. The lambs and goat kids included in this study were up to 4 weeks of age, while the adults were older than 6 months. The farms studied were located in the central region of Spain and there was no epidemiological links between them. Samples from diarrhoeic animals were selected by veterinarians. Only faecal samples obtained within 48 h of onset of clinical signs from non-treated animals were included in this study. Healthy animals were randomly selected, from farms that did not have a history of outbreaks of diarrhoea in neonates for at least 1 year preceding sample collection. Animals were observed for 1 week after sample collection for evidence of diarrhoea.

Faecal samples were plated on MacConkey agar. After overnight incubation, four colonies with the typical appearance of *E. coli* from each sample were randomly chosen. Isolates were identified as *E. coli* by biochemical tests, including hydrogen sulphide, citrate, urease and indole. These isolates were stored at room temperature in nutrient broth with 0·75% agar.

Production and detection of VT in Vero cells

All *E. coli* colonies were tested for VT production by cytotoxicity assays on Vero cells as described previously [13]. LREC-O157-156 (Laboratorio de Referencia de *E. coli*, Facultad de Veterinaria, Universidad de Santiago de Compostela, Spain) was used as positive control.

Table 1. *Prevalence of infection with VTEC in the different groups of sheep and goats studied*

Species and group of animals	No. of animals infected with VTEC/ No. examined (%)
Sheep	
Diarrhoeic lambs	4/129 (3·1)
Healthy sheep	63/258 (24·4)
Lambs	16/116 (13·8)
Adults	47/142 (33·1)
Goats	
Diarrhoeic goat kids	1/17 (5·9)
Healthy goats	41/253 (16·2)
Goat kids	4/94 (4·3)
Adults	37/159 (23·3)

Detection of *vt1*, *vt2* and *hlyA* sequences by PCR

All VTEC colonies were investigated for the presence of *vt1*, *vt2* and *hlyA* genes. Bacteria were harvested from an overnight culture on tryptic soy agar (TSA), suspended in 0·5 ml of sterile water, incubated at 100 °C for 10 min, and centrifuged. The supernatant was used in the PCR reactions. Detection of *vt1* and *vt2*, and *hlyA* sequences were performed with PCR techniques as described previously [11, 16, 17]. Control strain: LREC-O157-156 (O157:H7, $vt1^+$, $vt2^+$, $hlyA^+$) (Laboratorio de Referencia de *E. coli*, Facultad de Veterinaria, Universidad de Santiago de Compostela, Spain).

Detection of *eae* sequences by colony-blot hybridization

All VTEC colonies were investigated for the presence of the *eae* gene. Bacterial isolates were grown on trypticase soy broth (TSB) at 37 °C overnight and 2 μl of each culture were inoculated in Luria Bertani (LB) agar and further incubated at 37 °C for 18 h. Colonies were transferred onto nylon membranes (Hybond™-N, Amersham Life Sciences) and subjected to cell lysis and DNA denaturation [18]. The 1 kb *Sal*I–*Kpn*I fragment derived from the plasmid pCVD434 [19] was used as an *eae* probe. DNA probes were labelled with [α-^{32}P]dCTP by the random oligonucleotide primer system. Hybridizations were performed overnight at 65 °C in 7% SDS (sodium dodecyl sulphate), 0·5 M sodium phosphate, pH 7·2; and 1 mM EDTA buffer. Filters were washed with 2 × SSC (1 × SSC is 0·3 M

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Table 2. *Serotypes and virulence genes in VTEC strains recovered from healthy sheep*

Serotypes	No. of VTEC strains		*vt* genes			
	Lambs	Adults	*vt1*	*vt1* + *vt2*	*eae* gene	*hlyA* gene
O5:H$^-$	0	13	7	6	0	13
O6:H10	1	2	3	0	0	0
O21:H21	0	1	0	1	0	0
O21:H28	0	1	1	0	0	1
O26:H11	2	0	0	2	2	2
O71:H$^-$	1	0	0	1	0	0
O75:H8	0	1	0	1	0	1
O91:H$^-$	0	6	0	6	0	0
O128:H$^-$	3	3	3	3	0	0
O128:H16	0	1	1	0	0	0
O141:H$^-$	0	1	1	0	0	1
O146:H21	3	5	0	8	0	8
O163:H$^-$	1	0	0	1	0	1
O163:H11	0	1	0	1	0	0
O166:H$^-$	0	1	0	1	0	1
O166:H28	0	4	2	2	0	4
ONT*:H$^-$	4	5	3	6	0	3
ONT:H4†	1	2	2	0	0	2
Total	16	47	23	39	2	37

* NT, not typable.
† One VTEC strain of serotype ONT:H4 was positive in Vero cells but negative in PCR for *vt1* and *vt2* genes.

NaCl plus 0·03 M Na citrate, pH 7·0) 0·1% (w/v) SDS AT 65 °C for 20 min and autoradiographed. The *E. coli* strains E2348 (*eae*$^+$) and HS were used as positive and negative controls [19].

Serotyping

The determination of O and H antigens was carried out by the method described by Guinée and colleagues [20] and modified by Blanco and colleagues [21], employing all available O (O1–O175) and H (H1–H56) antisera. All antisera were obtained and absorbed with the corresponding cross-reacting antigens to remove the non-specific agglutinins. The O antisera were produced in the LREC (Lugo, Spain, http://www.lugo.usc.es/ecoli/kitsi.htm) and the H antisera were obtained from the Statens Serum Institut (Copenhagen, Denmark).

RESULTS

Prevalence of VTEC infection in sheep and goats

A total of 2628 *E. coli* colonies from 657 animals were investigated for Vero cytotoxin production in Vero cells. Table 1 shows the prevalence of VTEC infection in the different groups of sheep and goats studied.

vt1 and *vt2* genes

The mean number of VTEC isolates detected in each animal that had positive results for VTEC isolates were 2·3, 2·6, 1·5 and 2 in healthy sheep, healthy goats, diarrhoeic lambs and diarrhoeic goat kids, respectively. All the VTEC colonies were investigated for the presence of *vt1*, *vt2*, *hlyA* and *eae* genes. When the VTEC isolates from an animal showed the same characteristics (presence or absence of *vt*, *hlyA* and *eae* genes) it was assumed that they were the same strain. In all sheep and goats infected with VTEC only one different strain per animal was identified. In total, 109 VTEC strains were identified in this study: 63 isolated from healthy sheep (Table 2), 41 isolated from healthy goats (Table 3) and 5 from diarrhoeic lambs and goat kids (Table 4). PCR showed that 55 (50·4%) of VTEC strains carried *vt1* genes and 52 (47·8%) carried both *vt1* and *vt2* genes. Two strains positive by assay in Vero cells were negative for *vt1* and *vt2* genes in PCR assay (1 from a healthy sheep and 1 from a healthy goat).

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Table 3. *Serotypes, and virulence genes in VTEC strains recovered from healthy goats*

Serotypes	No. of VTEC strains		*vt* genes		*eae* gene	*hlyA* gene
	Goat kids	Adults	*vt1*	*vt1* + *vt2*		
O5:H$^-$	0	3	1	2	0	3
O18:H28	0	1	1	0	0	0
O21:HNT*	0	1	0	0	0	0
O58:H21	0	1	0	1	0	1
O76:HNT	0	1	1	0	0	0
O81:H$^-$	0	2	0	2	0	0
O81:H21	0	9	9	0	0	8
O87:H38	0	1	0	1	0	1
O128:H$^-$	0	1	1	0	0	0
O128:H2	0	1	0	1	0	1
O128:H19	0	1	1	0	0	1
O146:H21	0	2	1	1	0	2
O156:H25	1	0	1	0	1	1
O166:H28	3	5	8	0	0	3
O173:H8	0	1	1	0	0	0
O174:H8	0	1	1	0	0	0
ONT:H$^-$	0	2	0	2	0	0
ONT:H4	0	2	2	0	0	2
ONT:H21	0	2	1	1	0	2
Total	4	37	29	11	1	25

* NT, not typable. One VTEC strain of serotype O21:HNT was positive in Vero cells but negative in PCR for *vt1* and *vt2* genes.

Table 4. *Serotypes and virulence genes in VTEC strains recovered from diarrhoeic lambs and goat kids*

Serotypes	No. of VTEC strains (animal)	*vt* genes		*eae* gene	*hlyA* gene
		vt1	*vt1* + *vt2*		
O71:H$^-$	1 (lamb)	0	1	0	0
O75:H$^-$	1 (lamb)	0	1	1	1
O110:H$^-$	1 (lamb)	1	0	0	0
O166:H28	1 (goat kid)	1	0	0	1
ONT*:H$^-$	1 (lamb)	1	0	0	0
Total	5	3	2	1	2

* NT, not typable.

eae and *hlyA* genes

Only 4 (3·7%) of the 109 VTEC strains were positive for the *eae* gene. These strains were isolated from 4 animals (2 healthy lambs, 1 healthy goat kid and 1 diarrhoeic lamb). Thus, no VTEC strain isolated from adult animals was positive for the *eae* gene. One of the VTEC strains which possessed the *eae* gene was *vt1*$^+$ and three were *vt1*$^+$ *vt2*$^+$. In contrast, a high percentage (58·7%, 64 of 109) of VTEC strains were *hlyA*-positive, including all those that were *eae*-positive.

Serotypes of VTEC strains

VTEC strains isolated from healthy sheep belonged to 18 different serotypes (Table 2) and VTEC strains isolated from healthy goats belonged to 19 serotypes (Table 3). The most frequent serotypes among VTEC isolated from healthy sheep were O5:H$^-$, O91:H$^-$, O128:H$^-$, O146:H21 and ONT:H$^-$ (NT indicates not typable) and among VTEC isolated from healthy goats were O5:H$^-$, O81:H21 and O166:H28. VTEC strains isolated from diarrhoeic lambs and goat kids belonged to 5 serotypes (Table 4). The *eae*-positive

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VTEC strains belonged to serotypes O26:H11, O75:H$^-$ and O156:H25.

An association among certain serotypes and type of VT in VTEC from sheep and goats was found. Thus, all strains of VTEC O26:H11, O91:H$^-$ and O146:H21 from healthy sheep were *vt1*$^+$ *vt2*$^+$ and all strains of VTEC O81:H21 and O166:H28 from healthy goats carried the *vt1* gene. Moreover, VT production was closely associated with *hlyA* in certain VTEC serotypes (O5:H$^-$, O81:H21, and O146:H21).

DISCUSSION

The sampling procedure was different in healthy and diarrhoeic animals. Healthy animals were randomly selected, whereas for diarrhoeic animals the submission of samples was probably biased, since veterinarians may have tended to select severe cases of diarrhoea for sampling, and, thus, mild cases may be underrepresented in this study. In fact, liquid faeces were much more frequently submitted than semi-liquid or pasty faeces.

In this study we have found a VTEC prevalence rate of 24·4 and 16·2% among healthy sheep and goats, respectively. Higher prevalence rates of VTEC infection in healthy small ruminants than these found in this study were reported by Beutin and colleagues [22] (66·6% in sheep and 56·1% in goats), Adesiyun and Kaminjolo [23] (32·3% in sheep) and Fegan and Desmarchelier [24] (45% in sheep). However, Wray and colleagues [25] described a lower proportion of healthy sheep infected with VTEC (6·1%) than that found in this study. The differences in the occurrence of VTEC among these studies may be due to the patterns of shedding of VTEC are affected by diet, age, environmental conditions, and seasonal variation [26, 27].

The VTEC prevalences in healthy lambs and goat kids found in this study (13·8 and 4·3%, respectively) were much lower than those found in adults (33·1% in sheep and 23·3% in goats). These data suggest that VTEC may form part of the normal intestinal flora in adult sheep and goats. Muñoz and colleagues [15] found VTEC prevalences in healthy lambs and goat kids (6·1% in lambs and 0% in goats kids) lower than those found by us. However, Kudva and colleagues [27] and Fegan and Desmarchelier [24] detected VTEC in the 42·8 and 36% of the faecal samples from healthy lambs, respectively. Probably the higher prevalence of VTEC reported by Kudva and colleagues [27] may be, at least partially, due to the age of the animals considered as lambs. Thus, although Fegan and Desmarchelier [24] did not indicate the age of the lambs investigated, the lambs included in the study of Kudva and colleagues [27] were 8·5 months whereas in this study the lambs and goat kids were up to 4 weeks.

The VTEC prevalence in diarrhoeic lambs found in this study (3·1%) is similar to those found previously by us [13] and by Muñoz and colleagues [15] in diarrhoeic lambs (4·8 and 4·1%, respectively) but is lower than that found in healthy lambs in this study (13·8%). Moreover, in this study VTEC were detected in 5·9% of the diarrhoeic goat kids studied. This prevalence rate of VTEC infection is higher than that found previously by our group [14] and by Muñoz and colleagues [15] in diarrhoeic goat kids (1·8 and 0%, respectively) but it is similar to that found in this study among healthy goat kids (4·3%). Thus, according to this and other studies VTEC seem not to be associated with diarrhoea in lambs and goat kids. This hypothesis is supported by the fact that cases of natural and experimental enteric infections with VTEC have been reported very infrequently in small ruminants. To our knowledge, only Duhamed and colleagues [28] have described a naturally occurring case of enteric colibacillosis in a goat due to a VTEC strain (serotype O103:H2). The reason for the lower proportion of VTEC isolates in the diarrhoeic animals compared with the healthy ones is not clear. It is possible that a non-VTEC *E. coli* or other enteropathogen responsible for diarrhoea in sheep and goats outcompete the VTEC.

The *vt1*$^+$ *vt2*$^+$ and *vt1*$^+$ only were the predominant *vt* genotypes identified among healthy ovine and caprine VTEC strains, respectively. These results are in agreement with most of the previous findings in sheep [12, 22, 24, 27, 29]. To our knowledge, only Fagan and colleagues [30] and Paiba and colleagues [31] found that the dominant type of toxin produced by VTEC isolated from healthy sheep were VT1 only and VT2 only, respectively. These differences may be due to the reduced number of VTEC strains tested by Fagan and colleagues [30] or to a differences in the serotypes of the VTEC strains studied (Fagan and colleagues [30] did not indicate the serotypes of the VTEC strains investigated, and Paiba and colleagues [31], they only studied VTEC O157, which was not detected in this study). Moreover, in contrast with our results, Beutin and colleagues [22] found that most of the VTEC strains isolated from healthy goats were *vt1*$^+$ *vt2*$^+$.

In this study VTEC strains from diarrhoeic lambs were *vt1*$^+$ (2 strains) and *vt1*$^+$ *vt2*$^+$ (2 strains), and the only VTEC strain from the diarrhoeic goat kid was

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vt1$^+$. In previous studies performed by us [13, 14] all VTEC strains positive by PCR isolated from diarrhoeic lambs (6 strains) and from diarrhoeic goat kids (1 strain) were *vt1*$^+$.

Moreover, in this study two strains isolated from healthy sheep and goats were positive by tissue culture assay but negative for *vt1* and *vt2* genes in the PCR assay used. In a previous study we also detected one VTEC strain (serogroup O117) isolated from diarrhoeic lambs positive by tissue culture assay but negative by PCR [13], and Beutin and colleagues [9] found that most strains from sheep (20 of 21) and goats (10 of 12) positive with a *vt2* DNA probe were *vt2*-negative by PCR. As cited previously, VT2, in contrast to VT1, is a heterogeneous group of toxins. Several VT2 variants produced by human and animal *E. coli* have been and are still being described [32]. These variants have been recognized on the basis of partial neutralization by anti-VT2 immune serum and/or the absence of a positive PCR reaction [1]. Thus, it is probable that the VTEC strains found negative by PCR in this study produce VT2 variants.

Only 3 of the 104 (2·9%) VTEC strains isolated from healthy small ruminants in this study were *eae*-positive and all of these strains were isolated from lambs and goat kids. These results are in agreement with data obtained by Beutin and colleagues [9, 29] and Fegan and Desmarchelier [24]. These authors found that most VTEC isolated from healthy sheep (97·4–100%) and all VTEC isolated from healthy goats were negative for *eae* sequences. Moreover, all *eae*-positive VTEC strains found by Fegan and Desmarchelier [24] were isolated from lambs. However, other authors [27, 30, 33] have found higher percentages (12·2–100%) of VTEC from healthy sheep positive for *eae* gene. Moreover, the VTEC strain isolated from the diarrhoeic goat kid was *eae*-negative and only one VTEC strain isolated from diarrhoeic lambs was *eae*-positive. To our knowledge, this is the first description of *eae*-positive VTEC strains in diarrhoeic lambs.

It has been previously reported that the *eae* gene may be required for the expression of full virulence of VTEC for humans [8, 10]. Considering this, the *eae*-negative VTEC types would present a minor health hazard for humans compared with the classical EHEC types which possess *eae* gene. This idea is supported by the fact that despite the high incidence of VTEC-positive small ruminants, human infections due to contact and transmission are rare [9]. However, VTEC strains that do not possess the *eae* gene should not be overlooked since De Azavedo and colleagues [34] reported that a significant proportion of VTEC strains isolated from patients with HC and HUS were *eae*-negative. Also Paton and colleagues [35] indicated that the *eae* gene is essential to cause HC and HUS for some VTEC strains but not for others.

About 60% of VTEC strains isolated in this study from healthy sheep and goats were *hlyA*-positive. As this gene is considered a potential virulence factor for humans [9], their presence in a high percentage of VTEC strains from sheep and goats might increase the pathogenicity of these strains for human beings. Of particular interest is the fact that the four *eae*-positive VTEC strains were also *hlyA*-positive. Beutin and colleagues found percentages of EHEC haemolysin-positive VTEC to be similar (62·8%) [22] or higher (88%) [29] in healthy sheep and higher in healthy goats (90%) [22] compared with those found in this study. Whereas Fegan and Desmarchelier [24] found a percentage of *hlyA*-positive VTEC from healthy sheep lower (26%) than that found in this study.

The most prevalent serotypes of VTEC strains from healthy sheep found in this study were O5:H$^-$, O91:H$^-$, O128:H$^-$, O146:H21 and ONT:H$^-$. All these serotypes, except O128:H$^-$, have been previously described among the most frequent serotypes of the ovine VTEC strains [12, 22, 27, 33]. Three (O128:H$^-$, O146:H21 and ONT:H$^-$) of the five most prevalent serotypes in this study were found in both lambs and adults but two (O5:H$^-$ and O91:H$^-$) were exclusively found in adults. However, Kudva and colleagues [27] and McCluskey and colleagues [12] found VTEC in lambs which belonged to serotypes O5:H$^-$ and O9:H$^-$. McCluskey and colleagues [12] did not indicate the age of the lambs investigated and, as mentioned previously, in our study the lambs were up to 4 weeks of age. In the study of Kudva and colleagues [27] the lambs were 8·5 months. Thus, more studies will be necessary before a serotype association with age can be made.

The most frequent serotypes among VTEC isolated from healthy goats were O5:H$^-$, O81:H21 and O166:H28. Beutin and colleagues [22] found serotype O5:H$^-$ the most prevalent among VTEC from healthy goats but these authors did not detect serotypes O81:H21 and O166:H28 in this animal species whereas in this study 41·5% (17 of 41) of the VTEC strains from healthy goats were of one of these two serotypes. This difference may be due to the number of samples tested, and strains isolated in the studies to the effect of selection criteria or to geographical differences.

Only 6 of the 31 different serotypes described in healthy sheep and goats in this study and only 1 (O5:H$^-$) of the 3 most prevalent serotypes were found to occur in both animal species. These results are in agreement with the findings obtained by Beutin and colleagues [22] and suggest that many VTEC serotypes are strongly restricted to their host and that some VTEC serotypes may be among the most prevalent in one animal species but are rarely isolated from others.

On the other hand, the reduced number of VTEC strains isolated from diarhoeic lambs and goat kids found in this study and in previous studies [13, 14] showed a considerable heterogeneity in their serotypes.

In this study an association among certain serotypes and type of VT in VTEC from healthy sheep and goats was found. Thus, all strains of VTEC O91:H$^-$ and O146:H21 from healthy sheep were *vt1*$^+$ *vt2*$^+$ and all strains of VTEC O81:H21 and O166:H28 from healthy goats were *vt1*$^+$. Previous studies [12, 22, 27, 29] also found an association in healthy sheep among the serotypes O91:H$^-$ and O146:H21 and the *vt1* and *vt2* sequences. In contrast, VT production was not closely associated with serotype O5:H$^-$, one of the most prevalent serotypes in small ruminants, as 8 of 16 strains produced VT1 and the remaining 8 strains produced VT1 and VT2. Moreover, VT production was closely associated with *hlyA* in certain serotypes (O5:H$^-$, O81:H21, O146:H21). These results are in agreement with data obtained by Beutin and colleagues [22, 29], who found that VT production is closely associated with EHEC haemolysin (which is encoded by *hlyA* gene) in certain serotypes (i.e. O5:H$^-$, O146:H21). Although the influence of EHEC haemolysin on intestinal disease has not been defined, the results of this and other studies [22, 29, 36] indicate that EntHly is a suitable epidemiological marker for detection of some frequently occurring VTEC serotypes in humans and animals.

The serotype O157:H7 has been found in small ruminants by several groups [12, 26, 27, 37–41] although outbreaks due to this serotype from sheep and goats are very infrequent. Thus, to our knowledge, only one outbreak of infection in humans with *E. coli* O157 from sheep [41] and only a few from goats [38, 40] have been reported. In contrast to the papers previously cited but in agreement with data obtained by Beutin and colleagues [22, 29] and Djordjevic and colleagues [33], the serotype O157:H7 was not detected in this study. It may be because we did not use a selective enrichment protocol including the immunomagnetic separation method.

Although the serotype O157:H7 was not found, several serotypes of VTEC isolated in this study have been implicated as human pathogens causing HUS (i.e. O5:H$^-$, O26:H11, O91:H$^-$, O128:H2, O128:H$^-$) [42]. These five serotypes represented 42·9% (27 of 63) of VTEC isolated from healthy sheep and 12·2% (5 of 41) of VTEC isolated from healthy goats. Other authors have also found high percentages of VTEC strains isolated from sheep belonging to serotypes associated with diseases in humans [12, 22, 27, 29]. However, in contrast with the results of this study, Beutin and colleagues [22] found that most of the VTEC strains isolated from goats belonged to serotypes implicated as human pathogens.

We conclude that in Spain VTEC are rarely isolated from diarrhoeic small ruminants whereas VTEC prevalences in both healthy sheep and goat are high. Thus, VTEC seems not to be associated with diarrhoea in small ruminants. The high proportion of VTEC found among healthy sheep and goats indicates that these animal species represent an important reservoir of VTEC and a source of infection for humans. However, the absence of the *eae* gene in most of these VTEC strains could indicate that these strains are less virulent for humans than the classical *eae*-positive enterohaemorrhagic *E. coli* types.

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