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5 **A longitudinal study of verotoxin-producing *Escherichia coli***

6 **in two dairy goat herds**

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## 23 Abstract

24

25 A longitudinal study was conducted on two dairy farms to investigate the pattern  
26 of shedding of VTEC in goats. Faecal samples were taken from 20 goat kids once  
27 weekly during the first four weeks of life and then once every month for the next five  
28 months of life, and from 18 replacement animals and 15 adults once every month for 12  
29 months. The proportion of samples containing VTEC was higher for replacement  
30 animals and adults (85.7% and 78.7%, respectively) than for goat kids (25.4%). About  
31 90% of the VTEC colonies isolated from healthy goats belonged to five serogroups (O33,  
32 O76, O126, O146 and O166) but the most frequent serogroups of these isolates, except  
33 one, were different in the two herds studied. *E. coli* O157:H7 was found in three goat  
34 kids on only one occasion. None of the VTEC isolates, except the three *E. coli* O157:H7  
35 isolates, was *eae*-positive. The patterns of shedding of VTEC in goat kids were variable,  
36 but, in contrast, most of the replacement animals and adults were persistent VTEC  
37 shedders. Our results show that isolates of VTEC O33, O76, O126, O146 and O166 are  
38 adapted for colonising the intestine of goats but that, in contrast, infection with VTEC  
39 O157:H7 in goats seems to be transient.

40

41 *Keywords:* Verotoxin-producing *Escherichia coli*; Dairy goats; Longitudinal study

42

## 43 1. Introduction

44

45 Verotoxin-producing *Escherichia coli* (VTEC) produces two families of  
46 verotoxins, VT1 and VT2 (Mainil, 1999). VTEC can cause severe diseases in humans such  
47 as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). Those VTEC

48 strains that are able to induce HC and HUS are called enterohaemorrhagic *E. coli* (EHEC)  
49 (Mainil, 1999). The predominant EHEC serotype associated with human infection and  
50 death is O157:H7 (Mainil, 1999). It is not known whether all variants of VTEC are equally  
51 pathogenic for humans and it is possible that VT production alone may not be sufficient  
52 for VTEC to cause disease (Beutin et al., 1995). One factor that affects the virulence of  
53 VTEC is the possession of the *eae* gene (Beutin et al., 1995).

54 Several longitudinal studies of VTEC have been performed on cattle and sheep,  
55 most of them focused only on *E. coli* O157:H7 (Kudva et al., 1997; Mechie et al., 1997;  
56 Schouten et al., 2005). These studies have shown that shedding of VTEC by cattle and  
57 sheep may be affected by factors such as age, diet and feeding, stress, housing and  
58 seasonal effects. However, to our knowledge, no longitudinal studies in goats with this  
59 type of *E. coli* have been published. This study was designed to investigate the pattern  
60 of shedding of VTEC, including a selective enrichment protocol for the detection of  
61 serotype O157:H7, in goat kids over a six-month period and in replacement and adult  
62 goats over a time period of 12 months. A further objective of the study was to  
63 characterise the VTEC colonies isolated.

64

## 65 **2. Materials and methods**

66

### 67 *2.1. Herds and specimen collection*

68

69 The study was conducted on two Murciano-Granadina goat herds in the Murcia  
70 region of southeastern Spain. The practice of artificial rearing, in which goat kids are  
71 withdrawn after parturition and fed pasteurised colostrum and milk replacer, was  
72 implemented in one of the herds (herd A). In contrast, in the other herd studied (herd B)

73 goat kids were fed with colostrum and milk from their dams. Faeces samples were taken  
74 from a total of 20 goat kids (nine from herd A and 11 from herd B) once weekly during  
75 the first four weeks of life and then once every month for the next five months of life.  
76 Faeces samples were also taken from a total of 18 replacement animals (eight from herd  
77 A and 10 from herd B) and 15 adults (seven from herd A and eight from herd B) once  
78 every month for 12 months. Replacement animals and adults in herd A were sampled  
79 from June 2004 until May 2005, while animals in herd B were studied from November  
80 2004 until October 2005. Goat kids of both herds were studied during the first six  
81 months of each study. At the beginning of the study period replacement animals were  
82 between six and 18 months old, while adults were older than 18 months.

83

#### 84 2.2. Isolation and characterisation of VTEC colonies

85

86 For isolation of non-O157 VTEC, the faecal samples were plated on to  
87 MacConkey agar. After overnight incubation, four colonies from each sample with the  
88 typical appearance of *E. coli* were randomly chosen. These isolates were identified as *E.*  
89 *coli* by biochemical tests. For isolation of VTEC O157:H7, a selective enrichment  
90 protocol involving immunomagnetic separation was performed as described previously  
91 (Cortés et al., 2005).

92 All *E. coli* colonies were tested for VT production by cytotoxicity assays on Vero  
93 cells, as described previously (Cortés et al., 2005). One VTEC isolate from each sample  
94 was selected and analysed by PCR for possession of the *vt1*, *vt2* and *eae* genes, and the  
95 type of *eae* gene was determined for all *eae*-positive VTEC isolates, as described  
96 previously (Cortés et al., 2005).

97 The determination of O antigens was carried out in one VTEC isolate from each  
98 sample in the Laboratorio de Referencia de *E. coli* (Lugo, Spain). *E. coli* O157 isolates  
99 were tested for the presence of H7 antigen. Phage typing of VTEC O157:H7 isolates was  
100 performed by the method of Khakhria et al. (1990) in the Centro Nacional de  
101 Microbiología (Majadahonda, Madrid, Spain).

102 Using pulsed field gel electrophoresis (PFGE), 57 VTEC isolates belonging to  
103 the five serogroups persistently detected in the herds (O33, O76, O126, O146 and  
104 O166) were analysed to determine if the isolates of the same serogroup shed by the  
105 same animal over the period of the study belonged to specific clones. *Xba*I-digested  
106 genomic DNA was analysed in 1% agarose gels in 0.5x Tris-boric acid-EDTA (TBE)  
107 buffer at 14°C in a CHEF MAPPER system (BioRad, Hemel Hempstead, United  
108 Kingdom) using a standard protocol for PFGE [[http://www.foodborne-](http://www.foodborne-net.de/content/e25/e70/e580/index_ger.html)  
109 [net.de/content/e25/e70/e580/index\\_ger.html](http://www.foodborne-net.de/content/e25/e70/e580/index_ger.html)].

110

### 111 **3. Results**

112

#### 113 *3.1. Summary and characteristics of VTEC colonies isolated*

114

115 Five hundred and sixty-one faecal samples were tested. Three hundred and sixty-  
116 one samples tested positive for VTEC (45 from goat kids, 180 from replacement  
117 animals and 136 from adults). The proportion of VTEC-positive samples isolated from  
118 replacement animals and adults (85.7% and 78.2%, respectively) was higher than that  
119 isolated from goat kids (25.4%). One VTEC isolate from each sample was selected and  
120 further characterised.

121 VTEC isolates belonged to 12 different serogroups, and 25 isolates could not be  
122 typed (Table 1). Three hundred and nineteen of the 361 VTEC isolates (88.4%) belonged  
123 to five serogroups (O33, O76, O126, O146 and O166) but the most frequent serogroups of  
124 these isolates, except one, were different in the two herds studied. In herd A these  
125 serogroups were O33 and O146 (111 of 130, 85.4%), and in herd B they were O76, O126,  
126 O146 and O166 (202 of 231, 87.4%).

127 PCR showed that 63.7% of the VTEC isolates carried the *vt1* gene, 0.8%  
128 possessed the *vt2* gene and 35.5% carried both *vt1* and *vt2* genes (Table 1). An  
129 association among serogroups and types of VT was found (see Table 1). The O157  
130 VTEC isolates were H7-positive, produced VT2, belonged to phage type 32-variant and  
131 were isolated from three goat kids in herd B during their first month of life on only one  
132 occasion. Only the three O157:H7 VTEC isolates were *eae*-positive, and they possessed  
133 intimin  $\gamma 1$  (Table 1).

134

### 135 3.2. Shedding patterns of VTEC

136

137 The time of onset of shedding of VTEC in goat kids was different in the two  
138 herds studied. Shedding of VTEC by goat kids in herd B began in the first week, while  
139 in herd A it began in the fourth month (Fig. 1). In 17 of the 20 goat kids studied VTEC  
140 colonies were detected in at least one of the samples (Fig. 1). In 10 goat kids the  
141 temporal pattern of VTEC shedding was transient, while in one animal in herd A and  
142 six goat kids in herd B VTEC isolates were detected in at least three of the six months  
143 sampled. Moreover, in the two herds studied the rates of VTEC excretion were higher  
144 during the fifth and sixth month of life than in the first four months of life.

145 The frequency of VTEC isolation in herd A varied between 62.5 and 100% in  
146 replacement animals and between 40 and 85.7% in adults (Table 2), while in herd B it  
147 varied between 70 and 100% in replacement animals and between 75 and 100% in  
148 adults (Table 2). Eighteen replacement animals and 12 adults (100 and 80%,  
149 respectively) shed VTEC on at least six occasions. Thus, most of the replacement  
150 animals and adults were persistent VTEC shedders. No seasonal variation in the  
151 shedding of VTEC was found.

152

### 153 *3.3. Restriction endonuclease digestion patterns*

154

155 PFGE of *Xba*I-digested bacterial DNA from 57 isolates belonging to the five  
156 main serogroups revealed 34 unique restriction endonuclease digestion patterns,  
157 although each serogroup generated a cluster (similarity >85%) that grouped all the  
158 corresponding isolates (clusters I to V) (Fig. 2). One isolate of serogroup O146 was  
159 autodigested, so its pulsotype could not be obtained. A similar number of pulsotypes  
160 (five to eight) was detected in each of the five clusters; the 12 isolates of serogroup O76  
161 generated the fewest differences, with only five pulsotypes.

162

## 163 **4. Discussion**

164

165 To our knowledge, this is the first longitudinal study of VTEC performed on  
166 goats. In our study, only one VTEC isolate from each sample was selected for further  
167 characterisation because concurrent shedding of more than one VTEC strain is very  
168 uncommon in goats (Beutin et al., 1993; Orden et al., 2003; Cortés et al., 2005). In  
169 agreement with previous studies performed by our group (Orden et al., 2003; Cortés et

170 al., 2005), the proportion of samples containing VTEC was higher in replacement  
171 animals and adults than in goat kids.

172 About 90% of the VTEC colonies isolated from healthy goats belonged to five  
173 serogroups (O33, O76, O126, O146 and O166) but the most frequent serogroups among  
174 these isolates, except one, were different in the two herds studied. All these serogroups,  
175 except O33, have been previously described among the most frequent serogroups of  
176 caprine VTEC strains (Orden et al., 2003; Cortés et al., 2005).

177 *E. coli* O157:H7 was found in three goat kids on only one occasion. However, in  
178 contrast with the results of this work, previous cross-sectional studies (Beutin et al., 1993;  
179 Orden et al., 2003; Cortés et al., 2005) have been unable to detect serotype O157:H7 in  
180 healthy goats. The differences between studies in the detection of *E. coli* O157:H7 may  
181 be due to the transient nature of shedding of *E. coli* O157:H7 (Kudva et al., 1997;  
182 Mechie et al., 1997; Schouten et al., 2005). As far as we know, *E. coli* O157:H7 has  
183 been previously isolated from goats in Spain from milk samples (Rey et al., 2006) but  
184 not from faecal samples.

185 None of the VTEC colonies isolated from healthy goats in this study, except the  
186 three *E. coli* O157:H7 isolates, was *eae*-positive. This finding is in agreement with data  
187 obtained in earlier studies (Beutin et al., 1995; Orden et al. 2003; Cortés et al., 2005). It has  
188 been previously reported that the *eae* gene may be required for the expression of full  
189 virulence of VTEC in humans (Beutin et al., 1995). If this is true, the *eae*-negative VTEC  
190 types would present a minor health hazard for humans compared with classical EHEC  
191 types that possess the *eae* gene.

192 VTEC colonies were isolated more frequently from the goat kids of herd B than  
193 from those of herd A. Moreover, the time of onset of shedding of VTEC in goat kids  
194 was different in the two herds studied. Some goat kids as young as one week of age had

195 evidence of faecal VTEC excretion, indicating that dairy goats may be exposed to  
196 VTEC from birth. Changes in diet and animal management practices have been shown  
197 to affect the patterns of shedding of VTEC (Callaway et al., 2003). It is possible that the  
198 difference in the frequency of VTEC infection in goat kids between the two herds  
199 studied was due to different feeding and management practices. Moreover, during the  
200 course of the study there was an age-related change in the percentage of goat kids  
201 excreting VTEC. Thus, a higher frequency of VTEC isolation from goat kids was  
202 observed in the fifth and sixth months of life.

203 VTEC colonies were detected in at least one of the samples from all the  
204 replacement animals and adults studied. Most of the replacement animals and adults in  
205 this study were found to be persistent VTEC shedders. This may reflect continuous  
206 exposure and shows, in agreement with data obtained previously by our group (Orden et  
207 al., 2003; Cortés et al., 2005), that VTEC form part of the normal intestinal microbiota  
208 in replacement animals and adult goats. In contrast with studies performed in cattle and  
209 sheep (Kudva et al., 1997; Mechie et al., 1997), no seasonal variation in the shedding of  
210 VTEC was found in the goats studied.

211 When the isolates from the same animal belonging to the most prevalent  
212 serogroups were analysed by PFGE, the persistence of specific genetic subtypes that  
213 grouped in the same cluster was shown. The minor differences detected in the  
214 pulsotypes of colonies isolated from the same animal could be attributed to clonal  
215 turnover (Akiba et al., 2000).

216 In conclusion, most of the replacement animals and adult goats studied were  
217 persistent VTEC shedders, while the patterns of shedding of VTEC in the goat kids  
218 were variable. Our results also show that isolates of VTEC O33, O76, O126, O146 and

219 O166 are adapted for colonising the intestines of goats but that, in contrast, infection  
220 with VTEC O157:H7 in goats seems to be transient.

221

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223

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233

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276 Table 1

277 Serogroups and virulence genes in VTEC isolates recovered from goats

Serogroup	No. of VTEC isolates			<i>vt</i>			<i>eae</i> (type)
	Goat kids	Replacements	Adults	<i>vt1</i>	<i>vt2</i>	<i>vt1</i> + <i>vt2</i>	
O5	1	2	2	5	0	0	0
O17	0	0	1	0	0	1	0
O23	0	1	0	1	0	0	0
O33	6	27	42	75	0	0	0
O76	6	31	42	79	0	0	0
O81	0	3	2	5	0	0	0
O110	0	1	0	1	0	0	0
O116	0	0	1	0	0	1	0
O126	4	4	31	39	0	0	0
O146	11	61	3	0	0	75	0
O157	3	0	0	0	3	0	3 ( $\gamma$ 1)
O166	7	32	12	0	0	51	0
ONT <sup>a</sup>	7	18	0	25	0	0	0
Total	45	180	136	230	3	128	3

278 <sup>a</sup> Not typable.

279 Table 2

280 Frequencies of VTEC isolation in replacement animals and adults

Replacement animals				Adults			
Herd A		Herd B		Herd A		Herd B	
Animal no.	%	Animal no.	%	Animal no.	%	Animal no.	%
1	81.8 <sup>a</sup>	1	58.3	1	41.7	1	80 <sup>b</sup>
2	81.8 <sup>a</sup>	2	100	2	50	2	91.7
3	100 <sup>a</sup>	3	91.7	3	83.3	3	100
4	58.3	4	100	4	72.7 <sup>a</sup>	4	91.7
5	72.7 <sup>a</sup>	5	91.7	5	90.9 <sup>a</sup>	5	100
6	100	6	83.3	6	33.3	6	100
7	80 <sup>b</sup>	7	100	7	50 <sup>b</sup>	7	100
8	66.7	8	100			8	83.3
		9	100				
		10	75				

281 <sup>a</sup> Animals from which 11 samples instead of 12 were taken.282 <sup>b</sup> Animals in which 10 samples instead of 12 were taken.

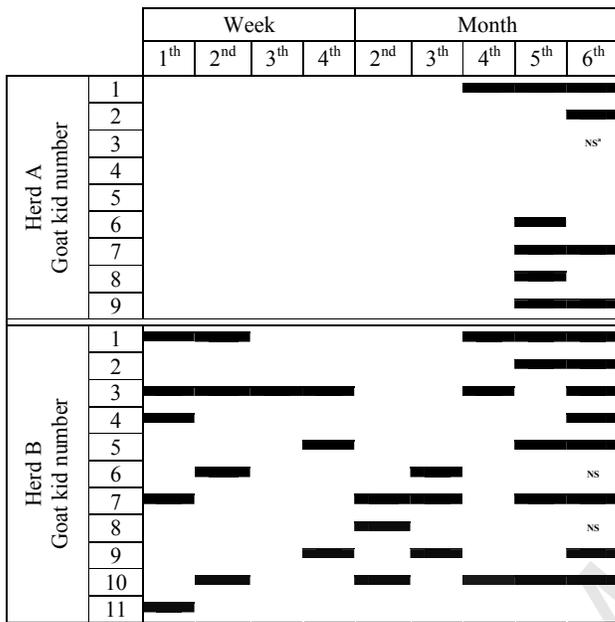
283 **Figure Legend:**

284 Fig. 1. Shedding of VTEC by goat kids.

285

286 Fig. 2. PFGE of *Xba*I-digested DNA from 56 VTEC isolates. Serogroup, designation of  
287 the isolate (animal number; a=adult, r=replacement-herd-month of isolation) and  
288 pulsotype are shown at right. This unweighted pair-group method with arithmetic mean  
289 dendrogram was generated using BioNumerics software (Applied Maths, St-Martens-  
290 Latem, Belgium) by using the Dice coefficient with a 0.5% lane optimization and 1.5%  
291 band position tolerance. The scale above the dendrogram indicates percentage  
292 similarity.

Figure 1



<sup>a</sup> Not sampled.

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Figure 2

