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26 **Abstract**

27       The aim of this work was to determine the prevalence and characteristics of  
28 *Escherichia coli* O157:H7 and non-O157 Shiga toxin-producing *E. coli* (STEC) in  
29 free-ranging wild boars killed during the hunting season in southwest Spain.  
30 Faecal samples from 212 wild boars (*Sus scrofa*) were collected and examined for  
31 STEC. Characterisation of isolates was performed by PCR, serotyping, phage  
32 typing, and pulsed-field gel electrophoresis (PFGE). *E. coli* O157:H7 and non-  
33 O157 STEC were isolated from 7 (3.3%) and 11 (5.2%) animals, respectively, and  
34 the resulting 19 isolates were characterised. The PCR procedure indicated that 4  
35 isolates carried the *stx*<sub>1</sub> gene, 12 carried the *stx*<sub>2</sub> gene, and 1 contained both of  
36 these genes. The *ehxA*, *eae*, and *saa* genes were detected in 13, 8, and 1 of the  
37 isolates, respectively. The *eae*-positive isolates comprised the types *eae*- $\gamma$ 1 and  
38 *eae*- $\zeta$ . The isolates belonged to 11 O:H serotypes, including 4 new serotypes not  
39 previously reported within STEC strains, and the majority of them were from  
40 serotypes previously associated with human infection. *E. coli* O157:H7 isolates  
41 belonged to phage types associated with severe human illness: PT14, PT34, and  
42 PT54. Indistinguishable PFGE types were found in *E. coli* O157:H7 isolates  
43 recovered from a wild boar and from a human patient with diarrhoea living in the  
44 same geographic area.

45

46 **Keywords:** Shiga toxin-producing *Escherichia coli* (STEC); *E. coli* O157:H7;  
47 Wild boars; Serotyping; Phage typing; Pulsed-field gel electrophoresis

48

49

## 50 1. Introduction

51 Shiga toxin-producing *Escherichia coli* (STEC) have recently emerged as  
52 important food-borne pathogens, especially serotype O157:H7. Human diseases  
53 ranging from mild diarrhoea to haemorrhagic colitis and haemolytic uraemic  
54 syndrome (HUS) can be caused by STEC, typically affecting children, the elderly,  
55 and immunocompromised patients (Centers for Disease Control and Prevention,  
56 2001). The pathogenic capacity of STEC resides in a number of virulence factors,  
57 including Shiga toxins (Stx1 and Stx2), intimin, enterohaemolysin, and the STEC  
58 autoagglutinating adhesin (Saa) (Gyles, 2007).

59 Healthy domestic ruminants such as cattle, sheep, and goats can harbour  
60 STEC and *E. coli* O157:H7 in their faeces and are thus natural reservoirs of these  
61 pathogens (Blanco et al., 2004b; Orden et al., 2008; Sánchez et al., 2009b).  
62 However, *E. coli* O157:H7 and non-O157 STEC strains have also been isolated  
63 from wild ruminants (Renter et al., 2001; García-Sánchez et al., 2007; Sánchez et  
64 al., 2009a), and deer have been implicated in the food-borne transmission of *E.*  
65 *coli* O157:H7 to humans in Japan (Nagano et al., 2004) and in the United States  
66 (Keene et al., 1997; Rabatsky-Ehr et al., 2002). Recent experimental and  
67 epidemiologic studies suggest that domestic pigs are biologically competent hosts  
68 and a potential reservoir of *E. coli* O157:H7 (Feder et al., 2003; Cornick and  
69 Helgerson, 2004). Actually, *E. coli* O157:H7 strains have been identified in wild  
70 boars in Sweden (Wahlström et al., 2003) and feral swine in the United States,  
71 where feral swine near spinach fields shed the outbreak strain in a nationwide  
72 spinach-associated outbreak of *E. coli* O157:H7 (Jay et al., 2007). Nevertheless,  
73 there are no reports of the isolation of STEC other than *E. coli* O157:H7 from  
74 wild boars and, to our knowledge, the occurrence of *E. coli* O157:H7 and non-

75 O157 STEC in wild boars in Spain is not known. The objectives of this study  
76 were to establish the prevalence of *E. coli* O157:H7 and non-O157 STEC in free-  
77 ranging wild boars killed during the hunting season in southwest Spain and to  
78 characterise them, in order to determine if wild boars represent a potential risk to  
79 public health.

80

## 81 **2. Materials and methods**

### 82 *2.1. Sample collection, culture, and STEC screening*

83 During the 2007-2008 hunting season (from mid-October to mid-February), a  
84 total of 212 European wild boars (*Sus scrofa*) killed by hunters in different game  
85 estates in the Extremadura region in southwest Spain were sampled. On each  
86 sampling occasion, one sample of rectal faeces per animal was collected from  
87 harvested individuals. Samples were transported to the laboratory under  
88 refrigeration in Cary-Blair transport medium (Deltalab, Barcelona, Spain) and  
89 placed in culture media within 24 h of collection.

90 For isolation of non-O157 STEC, faecal samples were plated directly onto  
91 MacConkey agar (Oxoid, Basingstoke, England). Following overnight incubation,  
92 10 suspect *E. coli* colonies were tested for the genes encoding Stx1 and Stx2  
93 toxins (*stx*<sub>1</sub> and *stx*<sub>2</sub> genes) by PCR as previously described (Rey et al., 2003). The  
94 resulting STEC isolates were confirmed biochemically as *E. coli* by the API 20E  
95 system (bioMérieux, Marcy L'Etoile, France) and tested for the genes encoding  
96 intimin (*eae* gene), enterohaemolysin (*ehxA* gene), and Saa (*saa* gene) as  
97 previously described (Sánchez et al., 2009a). When isolates from a given sample  
98 exhibited similar genetic characteristics in terms of the presence or absence of  
99 virulence genes, only one colony was selected and stored at -80 °C until further

100 characterisation. Otherwise, when isolates with different genetic characteristics  
101 were obtained, one colony of each was selected and stored for further  
102 characterisation. For isolation of *E. coli* O157:H7, faecal samples were examined  
103 by enrichment culture in modified buffered peptone water, concentration of *E.*  
104 *coli* O157 by immunomagnetic separation with Dynabeads anti-*E. coli* O157  
105 (Dynal, Oslo, Norway), and culture of magnetic beads onto cefixime tellurite  
106 sorbitol MacConkey agar (Oxoid). Following overnight incubation, 10 non-  
107 sorbitol-fermenting colonies were tested for the genes encoding O157 and H7  
108 antigens (O157 *rfbE* and *fliCh7* genes) by PCR as previously described (García-  
109 Sánchez et al., 2007). The resulting *E. coli* O157:H7 isolates were confirmed  
110 biochemically as *E. coli* by the API 20E system (bioMérieux) and tested for the  
111 genes encoding Stx1 and Stx2 toxins, intimin (*eae* and *eae-γ1* variant), and  
112 enterohaemolysin.  $\beta$ -glucuronidase (GUD) activity in *E. coli* O157:H7 isolates  
113 was investigated on Chromocult Coliform agar (Merck, Darmstadt, Germany)  
114 after overnight incubation. The *eae* type was determined for all *eae*-positive  
115 isolates as previously described (Rey et al., 2003).

116

## 117 2.2. Characterisation of isolates

118 The identification of O and H antigens in isolates was carried out as described  
119 by Guinée et al. (1981) using the full range of O (O1 to O185) and H (H1 to H56)  
120 antisera. All antisera were absorbed with corresponding cross-reacting antigens to  
121 remove non-specific agglutinins. The O antisera were produced in the Laboratorio  
122 de Referencia de *E. coli* (Lugo, Spain) and the H antisera were obtained from the  
123 Statens Serum Institut (Copenhagen, Denmark).

124 The phage typing of *E. coli* O157:H7 isolates was performed as described by  
125 Khakhria et al. (1990) using phages provided by the National Microbiology  
126 Laboratory (Public Health Agency of Canada, Winnipeg, Canada).

127 Pulsed-field gel electrophoresis (PFGE) was used to establish relatedness and  
128 diversity among *E. coli* O157:H7 isolates. PFGE was performed in accordance  
129 with the PulseNet-Europe protocol (<http://www.pulsenet-europe.org/docs.htm>).  
130 Genomic DNA was digested with XbaI (Roche Diagnostics, Mannheim,  
131 Germany) and analyzed in 1% agarose gels (Bio-Rad, Hemel Hempstead, United  
132 Kingdom) in 0.5× TBE buffer at 14 °C using the CHEF MAPPER system (Bio-  
133 Rad). The runtime was 21.3 h at 6 V/cm, with initial and final switch times of  
134 2.16 and 54.17 s, respectively. The XbaI-digested DNA from *Salmonella enterica*  
135 Braenderup H9812 was used as a molecular size marker. Resultant images were  
136 analyzed with the InfoQuestFP software (Bio-Rad). Isolates were allocated a  
137 different PFGE type when a genetic difference could be detected. Cluster analysis  
138 was performed using the Dice coefficient and the unweighted pair group method  
139 with arithmetic averages (UPGMA).

140

### 141 **3. Results and discussion**

142 Overall, STEC were detected in 17 (8.0%) of the animals sampled. *E. coli*  
143 O157:H7 was isolated from 7 (3.3%) animals and non-O157 STEC were isolated  
144 from 11 (5.2%). Two different strains were identified in samples from 2 animals,  
145 in one of which both *E. coli* O157:H7 and non-O157 STEC (O6:H10) were  
146 isolated. These results indicate a high prevalence of STEC infection in free-  
147 ranging wild boars in this area of Spain, with a higher prevalence rate of *E. coli*

148 O157:H7 (3.3%) than in a previous study in Sweden (1.5%) (Wahlström et al.,  
149 2003).

150 All isolates obtained in the present study (19 in total) were further  
151 characterised (Table 1). They belonged to 9 O serogroups and 11 O:H serotypes,  
152 including 4 new serotypes not previously reported within STEC strains. The  
153 majority of isolates were from serotypes previously found in STEC strains  
154 associated with human infection and 8 of them belonged to serotypes (O104:H–  
155 and O157:H7) associated in particular with causing HUS  
156 (<http://www.lugo.usc.es/ecoli>; <http://www.microbionet.com.au/vtactable.htm>).

157 The PCR procedure indicated that 4 isolates carried the *stx*<sub>1</sub> gene, 12 carried the  
158 *stx*<sub>2</sub> gene, and 1 contained both of these genes. The *ehxA*, *eae*, and *saa* genes were  
159 detected in 13, 8, and 1 of the isolates, respectively. The 8 *eae*-positive isolates  
160 comprised the types *eae*- $\gamma$ 1 (7 isolates) and *eae*- $\zeta$  (1 isolate), which are intimin  
161 subtypes previously reported within ovine, bovine, and human STEC strains (Rey  
162 et al., 2003; Blanco et al., 2004a; Blanco et al., 2004b). There were 12 different  
163 associations between serotype and virulence genes among the 19 isolates, being  
164 O157:H7 *stx*<sub>2</sub> *eae*- $\gamma$ 1 *ehxA* the most commonly found association (5 isolates),  
165 followed by O157:H7 *eae*- $\gamma$ 1 *ehxA*, O157:H21 *stx*<sub>2</sub> *ehxA*, and O23:H21 *stx*<sub>2</sub> (2  
166 isolates each). Two isolates among the 7 *E. coli* O157:H7 isolates were *stx*-  
167 negative, although they carried genes encoding other virulence-associated factors  
168 (intimin and enterohaemolysin). Whether this finding resulted from the loss of *stx*  
169 gene(s) from initially *stx*-positive strains remains unclear. However, whilst the  
170 human health risks associated with *stx*-negative *E. coli* O157:H7 strains may be  
171 less significant, the acquisition of *stx*-carrying phages from the environment  
172 cannot be excluded (Muniesa et al., 1999).

173 The *E. coli* O157:H7 isolates neither fermented sorbitol nor exhibited GUD  
174 activity after overnight incubation. Two isolates expressed the H7 antigen and 5  
175 were non-motile. The 7 *E. coli* O157:H7 isolates belonged to 3 phage types: PT14  
176 (3 isolates), PT34 (1 isolate), and PT54 (3 isolates) (Fig. 1). Interestingly, 6 of  
177 those 7 *E. coli* O157:H7 isolates belonged to two of the most common phage  
178 types associated with severe human illness in Europe and Canada (Mora et al.,  
179 2007). Six distinct PFGE types (1 to 6) with a high degree of similarity (>86%)  
180 were found among the 7 *E. coli* O157:H7 isolates recovered from wild boars (Fig.  
181 1). The PT14 and PT34 isolates shared 96% of similarity, with two  
182 indistinguishable PFGE types and other two which differed only in  $\leq 2$  restriction  
183 fragments (Fig. 1). These PFGE types were compared with those found by us in  
184 *E. coli* O157:H7 strains isolated from human patients with diarrhoea living in the  
185 same geographic area (Martínez et al., 2009b). It is interesting that the PFGE type  
186 of the O157:H7 *stx<sub>2</sub> eae- $\gamma$ 1 ehxA* isolate belonging to PT54 recovered from the  
187 faeces of a hunter-harvested wild boar in November (PFGE type 5) was  
188 indistinguishable from that found in an O157:H7 *stx<sub>2</sub> eae- $\gamma$ 1 ehxA* isolate  
189 belonging to the same phage type recovered from a patient in the same area in  
190 December (Fig. 1). In the absence of epidemiologic data, these 2 isolates were  
191 also tested with multilocus variable-number tandem repeat analysis (MLVA)  
192 which fully confirmed the PFGE results (Martínez et al., 2009a).

193 The common carriage of *E. coli* O157:H7 and non-O157 STEC indicates that  
194 hunted wild boars are a potential source of human infection, since the initial  
195 microbiological condition of the carcasses from hunted animals is generally  
196 affected by several highly variable factors and so contamination of the meat  
197 obtained from them with such pathogens would be frequent. Nevertheless, only

198 few reports have been published on the contamination of pork from wild boar or  
199 venison with STEC (Piérard et al., 1997; Miko et al., 2009). In addition, the  
200 finding of indistinguishable PFGE types (and MLVA types) in *E. coli* O157:H7  
201 isolates recovered from samples from a wild boar and from a human patient in the  
202 same area and period suggests that wild boars in this geographic area may play a  
203 role in human infections with *E. coli* O157:H7. However, further studies will be  
204 required to further elucidate the degree of zoonotic risk posed. These findings  
205 may also have implications in the development of programs for controlling STEC  
206 and *E. coli* O157:H7 at the farm level, since wild boar populations in the studied  
207 areas have access to the same pastures as cattle and sheep, and therefore  
208 interactions between them will likely lead to interspecies transfer.

209

#### 210 **Conflict of interest statement**

211 None of the authors of this paper has a financial or personal relationship with  
212 other people or organisations that could inappropriately influence or bias the  
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214

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230

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327  
328

329 **Table 1**

330 Association between serotype and virulence genes for isolates recovered from  
 331 wild boars

Serotype <sup>a</sup>	No. of isolates	Virulence genes profile <sup>f</sup>				
		<i>stx</i> <sub>1</sub>	<i>stx</i> <sub>2</sub>	<i>eae</i>	<i>ehxA</i>	<i>saa</i>
O6:H10	1	+				
O23:H21 <sup>d</sup>	2		+			
O104/O127:H <sup>-c, e</sup>	1		+		+	
O104/O127:H1/H12 <sup>d, e</sup>	1	+				+
O109:H <sup>-</sup>	1	+		+	+	
O127:H2 <sup>d</sup>	1		+		+	
O142:H8/H21 <sup>d, e</sup>	1	+				
O146:H21 <sup>b</sup>	1	+	+		+	
O157:H7 <sup>c</sup>	2			+	+	
O157:H7 <sup>c</sup>	5		+	+	+	
O157:H21	2		+		+	
ONT:H7 <sup>b</sup>	1		+			

332

333 <sup>a</sup> ONT, O antigen non-typeable.334 <sup>b</sup> Serotype previously found in human STEC strains.335 <sup>c</sup> Serotype previously associated with human STEC strains that have caused HUS.336 <sup>d</sup> Serotype not previously reported within STEC strains.337 <sup>e</sup> Each of the isolates cross-reacted with the respective O and H antisera.338 <sup>f</sup> +, gene detected by PCR.

339

340 **Fig. 1.** Dendrogram generated with InfoQuestFP software showing the PFGE-  
341 XbaI digestion types for *E. coli* O157:H7 isolates recovered from 7 wild boars and  
342 a human patient. The scales at the top indicate the similarity indices (in  
343 percentages) and molecular sizes (in kilobases).

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Dice (Opt 0.50%) (Tot 1.5%-1.5%) (H>0.0% S>0.0%) (0.0%-100.0%)  
**PFGE**

