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Seropathotypes, Phylogroups, Stx Subtypes, and Intimin Types of Wildlife-Carried, Shiga Toxin-Producing *Escherichia coli* Strains with the Same Characteristics as Human-Pathogenic Isolates

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The objectives of this study were to investigate the presence of Shiga toxin-producing *Escherichia coli* (STEC) strains in wildlife that have spread in Europe, living near human settlements; to analyze their epidemiological role in maintenance and transmission to domestic livestock; and to assess the potential health risk of wildlife-carried strains. STEC strains were recovered from 53% of roe deer, 8.4% of wild boars, and 1.9% of foxes sampled in the northwest of Spain (Galicia). Of the 40 serotypes identified, 21 were classified as seropathotypes associated with human disease, accounting for 81.5% of the wildlife-carried STEC strains, including the enterohemorrhagic serotypes O157:H7-D-*eae*-γ1, O26:[H11]-B1-*eae*-β1, O121:H19-B1-*eae*-ε1, and O145:[H28]-D-*eae*-γ1. None of the wildlife-carried strains belonged to the highly pathogenic serotype O104:H4-B1 from the recent Germany outbreak. Forty percent of wildlife-carried STEC strains shared serotypes, phylogroups, intimin types, and Stx profiles with isolates from human patients from the same geographic area. Furthermore, wildlife-carried strains belonging to serotypes O5:HNM-A, O26:[H11]-B1, O76:H19-B1, O145:[H28]-D, O146:H21-B1, and O157:H7-D showed pulsed-field gel electrophoresis (PFGE) profiles with >85% similarity to human-pathogenic STEC strains. We also found a high level of similarity among STEC strains of serotypes O5:HNM-A, O26:[H11]-B1, and O145:HNM-D of bovine (feces and beef) and wildlife origins. Interestingly, O146:H21-B1, the second most frequently detected serotype in this study, is commonly associated with human diarrhea and isolated from beef and vegetables sold in Galicia. Importantly, at least 3 STEC isolates from foxes (O5:HNM-A-*eae*-β1, O98:[H21]-B1-*eae*-ζ1, and O146:[H21]-B1) showed characteristics similar to those of human STEC strains. In conclusion, roe deer, wild boar, and fox in Galicia are confirmed to be carriers of STEC strains potentially pathogenic for humans and seem to play an important role in the maintenance of STEC.

Currently, the role of wildlife as reservoirs of zoonotic diseases is a major public health concern (11, 25). As in other parts of Europe, populations of foxes (*Vulpes vulpes*) in Spain have experienced such a growth in recent decades that they have spread almost throughout the Iberian Peninsula, invading human settlements in both rural and urban areas (12). A similar population growth is happening with roe deer (*Capreolus capreolus*) and wild boar (*Sus scrofa*) in certain geographic areas.

Shiga toxin-producing *Escherichia coli* (STEC) strains are recognized as important human pathogens. Although ruminants, especially cattle, are the main reservoirs of STEC (1, 4, 5, 10, 22, 30, 31, 35, 38), wildlife, including game animals, also play an important role as carriers of these pathogens (29).

At present, a large variety of STEC serotypes have been described. However, certain STEC serotypes isolated from animals or food have never been associated with disease. Accordingly, Karmali et al. (24) previously classified STEC strains into five seropathotypes (seropathotypes A to E) in relation to their association with hemolytic-uremic syndrome (HUS) and outbreaks. Furthermore, the ability of STEC to cause human diseases depends on the Shiga toxin gene subtype, with isolates producing Stx2a and, to a lesser extent, Stx2d being more commonly associated with HUS. In addition to Stx2a and Stx2d, Stx2c has also emerged as an important subtype associated with severe illness (15).

The objectives of the present study were (i) to investigate the presence of STEC strains in roe deer, wild boars, and foxes in the region of Galicia in the northwest of Spain; (ii) to analyze the epidemiological role of roe deer, wild boars, and foxes in the maintenance and transmission of STEC to domestic livestock; and (iii) to assess the potential health risk of wildlife-carried STEC isolates according to their seropathotypes, phylogenetic groups, and *stx* subtypes.

MATERIALS AND METHODS

Sample collection, culture, and STEC screening. Fecal samples were collected from wildlife killed by hunters during the 2009-2010 hunting season (April to October for roe deer, September to January for wild boars, and September to February for foxes) in A Coruña, Lugo, and Pontevedra (three provinces of Galicia). A total of 179 roe deer (*Capreolus capreolus*), 262 wild boars (*Sus scrofa*), and 260 foxes (*Vulpes vulpes*) were sampled by rectal swabs with Stuart transport medium, and samples were cultured within 24 h of collection.

For isolation, fecal swabs were plated directly onto lactose MacConkey agar (L-MAC) (catalog no. CM0115; Oxoid Ltd.) and onto sorbitol Mac-

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TABLE 1 Primers used in this study for PCR

Gene	Primer	Oligonucleotide sequence (5′–3′)	Fragment size (bp)	Annealing temp (°C)	Reference
stx_1	VT1-F1	TCGCTGAATGTCATTCGCTCTGC	539	55	34
	VT1-NR2	TCAGCAGTCATTACATAAGAAC			
stx_2	VT2-F1	TTTCTTCGGTATCCTATTCCC	358	55	34
	VT2-F2	TGTCTTCAGCATCTTATGCAG			
	VT2-R	CTGCTGTCCGTTGTCATGGAA			
eae	EAE-V3F	CATTGATCAGGATTTTTCTGGT	510	55	34
	EAE-MBR	TCCAGAATAATATTGTTATTACG			
wzx-wzy O26	*wzx-wzy O26F*	AAATTAGAAGCGCGTTCATC	532	60	14
	wzx-wzy O26R	CCCAGCAAGCCAATTATGACT			
O157 *rfbE*	O157-AF	AAGATTGCGCTGAAGCCTTTG	497	55	13
	O157-AR	CATTGGCATCGTGTGGACAG			
fliC-H7	H7-F	GCGCTGTCGAGTTCTATCGAGC	625	55	18
	H7-R	CAACGGTGACTTTATCGCCATTCC			
fliC-H8	H8-F	TAACAGCGCAAAAGACGATG	393	58	This study
	H8-R	CCGAGAAGTTTTCGCATCAAT			
fliC-H11	fliCRH11-1	ACTGTTAACGTAGATAGC	248	54	14
	fliCRH11-2	TCAATTTCTGCAGAATATAC			
fliC-H21	H21-F	GGCGATTGCTAACCGTTTTA	549–556	58	This study
	H21-R	CGTAAGTGAACCATCCGCAG			
fliC-H28	H28-F	ACGAAATCAAATCCCGTCTG	856	66	This study
	H28-R	GCCGATTGAAGAGACTCAGC			
terD	TerD1	AGTAAAGCAGCTCCGTCAAT	434	64	2
	TerD2	CCGAACAGCATGGCAGTCT			

Conkey agar (catalog no. CM0813; Oxoid Ltd.) supplemented with cefixime (0.05 mg/liter) and potassium tellurite (2.5 mg/liter) (CT-SMAC) and incubated at 37°C for 18 to 24 h. Confluent growth zones of all plates were screened, as previously described (30), for the stx_1 and stx_2 genes by duplex PCR using the primers shown in Table 1. For each PCR-positive culture, 10 *E. coli*-like colonies obtained from L-MAC and/or CT-SMAC plates were plated onto tryptone soy agar (TSA) (Cultimed; Panreac) (at 37°C for 18 to 24 h). In order to obtain the STEC isolates, individual colonies from TSA plates (at 37°C for 18 to 24 h) were analyzed for stx_1 and stx_2 genes by duplex PCR and *eae* genes by single PCR with the primers shown in Table 1. If no positive single colony was found among the first 10 colonies, at least 10 more were tested. When positive colonies from a given sample exhibited different genetic characteristics in terms of the presence or absence of virulence genes, one colony of each was selected and stored in nutrient broth (Cultimed; Panreac) with 0.75% nutrient agar (Cultimed; Panreac) at room temperature for further characterization.

O and H typing. The determination of O and H antigens was carried out according to a method previously described by Guinée et al. (21), with all available O (O1 to O181) and H (H1 to H56) antisera. Isolates that did not react with H antisera were classified as nontypeable (HNT), and those that were nonmotile were denoted HNM.

HNM strains were tested by PCR to detect the presence of the flagellar genes H8, H11, H21 and H28, and positive PCR results were denoted in square brackets ([H]). Also, O26:H11 and O157:H7 strains were confirmed by PCR. The primers used are shown in Table 1.

Seropathotype classification. Isolates were assigned by serotype to seropathotypes classified previously as seropathotypes A to E by Karmali et al. (24), based on data from previous reports (9, 20, 23) and based on Internet databases (http://www.lugo.usc.es/ecoli/SEROTIPOSHUM.htm). We also checked our private STEC database to obtain additional information.

Phylogenetic analysis. Strains were assigned to one of the four main phylogenetic groups of *E. coli* (phylogroups A, B1, B2, and D) by using a multiplex PCR-based method described previously by Clermont et al. (8).

Shiga toxin subtyping. Currently, there are three stx_1 subtypes (stx_{1a}, stx_{1c}, and stx_{1d}) and seven stx_2 subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, stx_{2f}, and stx_{2g}) according to the subtyping nomenclature proposal put forth at the 7th International Symposium on Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* Infection held in 2009 in Buenos Aires, Argentina. Stx subtypes were assigned by the multiplex PCR-based method recommended by the Joint European Reference Laboratory for VTEC (EU-RL VTEC) and European Center for Disease Prevention and Control External Quality Assurance (ECDC EQA), 2010 to 2011 (15, 36).

Intimin typing. The *eae* gene encoding intimin was typed by PCR and sequencing as previously described (6, 33).

Presence of *ter* (tellurite resistance) genes. All strains were tested by PCR to detect the presence of the *terD* gene using primers shown in Table 1.

PFGE. XbaI pulsed-field gel electrophoresis (PFGE) analysis of 17 representative wildlife-carried strains was performed as previously described (32). Profiles were analyzed with BioNumerics fingerprinting software (Applied Maths, St-Martens-Latem, Belgium) and compared with PFGE profiles of strains belonging to the same STEC serotypes from our database. Dendrograms were generated by the unweighted-pair group method using arithmetic averages, based on the Dice similarity coefficient with a 1.0% band position tolerance.

Statistical analysis. Comparisons of proportions were tested by using Fisher's exact test. For each comparison, a P value of <0.05 was considered to denote a significant difference.

RESULTS

STEC strains from roe deer, wild boar, and fox. PCR screening of the confluent growth zone on L-MAC and CT-SMAC plates showed that 200 of the 701 wildlife-carried samples (28.5%) were positive for the stx_1 and/or stx_2 gene (120 from roe deer, 64 from wild boars, and 16 from foxes). STEC strains were recovered from 52.5% of the roe deer (94/179), 8.4% of the wild boars (22/262), and 1.9% of the foxes (5/260), with a total of 135 STEC strains being isolated from 121 animals (104 strains from roe deer, 25 from wild boars, and 6 from foxes). STEC strains isolated from the same animal are shown in Table 2. Interestingly, 41 of the 135 STEC strains (30%) could be recovered only due to the use of CT-SMAC.

O:H typing and seropathotypes. A total of 40 serotypes were established for the 135 STEC strains in the study (Table 2). The 104 strains isolated from roe deer showed 24 different serotypes, but 69% belonged to only 4 serotypes (27 strains belonged to O146:[H28], 22 strains belonged to O146:[H21], 13 strains belonged to O2:H6, and 10 strains belonged to O75:[H8]). The 25 strains isolated from wild boars belonged to 17 different serotypes, with only O75:[H8], O146:[H21], and O103:H28 being identified more than once (5, 4, and 2 isolates, respectively). Finally, the 6 strains isolated from foxes belonged to 6 different serotypes. Six serotypes were commonly detected among STEC strains isolated from roe deer and wild boars (O2:H6, O26:[H11], O75:[H8], O146:[H21], O146:[H28], and O157:H7). Serotype O146:[H21] was also detected in one strain of fox origin. Interestingly, 104 of the 135 STEC strains (77%) showed any of the four H types 21 (34 strains), 28 (33 strains), 8 (22 strains), and 6 (15 strains). To our knowledge, serotypes O11:HNT, O51:H49, O91:H6, O98:[H21], and O103:H28 are new serotypes not previously reported for STEC strains.

The assignment of isolates to seropathotypes according to the classification described previously by Karmali et al. (24) takes into account their reported frequencies in human illness and association with outbreaks and severe disease (hemorrhagic colitis [HC] and HUS). Accordingly, the 40 STEC serotypes remained distributed into the five seropathotypes, with 81.5% of the strains (110 out of 135) belonging to seropathotypes associated with human disease (seropathotypes A, B, C, and D) (Table 2). Specifically, 25 strains (18.5%) were grouped into seropathotypes linked to HUS (seropathotypes A, B, and C), and 85 strains (63%) were grouped into seropathotype D, associated with uncomplicated diarrhea. No significant differences in the prevalences of seropathotypes were found among the three wild species (P values of >0.05).

Phylogenetic typing. Phylogroup B1 was the most prevalent (51%) among the 135 wildlife-carried STEC strains, followed by phylogroups D (29%), B2 (17%), and A (3%) (Table 3). Significant differences were found for phylogroup A in relation with roe deer and wild boar origins versus fox origins (0% and 4% versus 50%; $P = 0.00$ and $P = 0.02$, respectively) and for phylogroups B1 and B2 in relation with roe deer versus wild boar origins (47% versus 68% [$P = 0.03$] and 21.1% versus 4% [$P = 0.03$]), respectively.

Shiga toxin, intimin typing, and the presence of *ter*. Of the 135 STEC strains presently characterized, 20 (14.8%) were stx_1 positive, 80 (59.2%) were stx_2 positive, and 35 (26%) were stx_1 stx_2 positive. Table 4 shows the *stx* prevalence in relation to the origin of isolation. Significant differences were found in the prevalence of the stx_1 stx_2 type in relation to roe deer origins versus wild boar origins (22.1% versus 44%; $P = 0.03$).

Two stx_1 subtypes (stx_{1a} and stx_{1c}) and six stx_2 subtypes (stx_{2a}, stx_{2b}, stx_{2c}, stx_{2d}, stx_{2e}, and stx_{2g}) were detected with a total of 14 different stx_1 and stx_2 subtype combinations. The most frequently detected subtypes were stx_{2b} (61 strains), stx_{1c} stx_{2b} (30 strains), stx_{1c} (13 strains), and stx_{1a} (7 strains), accounting for 111 strains (82.2%) (Tables 2 and 4). Significant differences in the prevalences of stx_{1a} (3.8% for roe deer versus 33% for fox [$P = 0.03$]) and stx_{2b} (54.8% for roe deer versus 16% for wild boar [$P = 0.00$] and 54.8% for roe deer versus 0% for fox [$P = 0.01$]) are shown in Table 4.

Fourteen strains out of 135 (10.4%) carried the *eae* gene: 7 (6.7%) from roe deer, 4 (16%) from wild boars, and 3 (50%) from foxes. Strains positive for *eae* were significantly more frequently found among those grouped into HUS-associated seropathotypes A, B, and C ($n = 10$) than among those assigned to seropathotypes D and E ($P = 0.00$). Four intimin types were established: $\beta 1$ (8 strains), $\gamma 1$ (4 strains), $\zeta 1$ (1 strain), and $\varepsilon 1$ (1 strain).

The *terD* gene was present in 75 strains (55%), being significantly more frequently found among *eae*-positive STEC strains (13/14; 93%) than among *eae*-negative STEC strains (62/121; 51%) ($P = 0.00$).

PFGE comparison. The XbaI macrorestriction profiles obtained by PFGE of 17 representative wildlife-carried strains were compared with those of 17 STEC strains from our BioNumerics database (13 from human patients, 2 from bovines, and 2 from minced beef) to obtain the dendrogram shown in Fig. 1. The selection of the 17 representative wildlife-carried strains was based on their serotypes and gene profiles in comparison with human, animal, and food STEC strains also isolated in Galicia (34). The 34 STEC strains showed clusters of similarity of $>85\%$, which grouped wildlife- and human-carried strains of serotypes O5:HNM-A, O26:[H11]-B1, O76:H19-B1, O145:[H28]-D, O146:[H21]-B1, and O157:H7-D (Fig. 1).

DISCUSSION

To date, few studies on the prevalence of STEC strains other than O157 strains isolated from wild animals have been reported (7, 17, 27, 39, 40), and comparisons are difficult due to the differences in animal species studied and in the analytical methods used.

In the present study, non-O157 STEC strains were isolated from 52.5% of roe deer, 8.4% of wild boars, and 1.9% of foxes sampled in Galicia. These prevalences are higher than those reported previously by other studies of roe deer, from 0% (7, 27) in Italy and Norway to 5% (39) in the southwest of Spain, and of wild boars, 5.2% (40) in the southwest of Spain. The prevalence found for roe deer in this study is even higher than those for other small wild ruminants, such as red deer, with reported prevalences of 0% (7, 27), 10.5% (17), and 24.7% (39); fallow deer, with a reported prevalence of 33.3% (39); or mouflon, with a reported prevalence of 36.4% (39).

However, the prevalences of STEC O157:H7 isolates detected in Galicia (0.56% for roe deer, 0.38% for wild boar, and 0% for fox) were similar to or even lower than those reported previously by other studies for roe deer and wild boar, 0% and 3.3%, respectively (19), in the southwest of Spain, or for other small wild ru-

TABLE 2 Characterization of the STEC strains isolated in this study

Seropathotype and description	Serotype[a]	Phylogroup	*stx* and *eae* type	Host(s) (no. of strains)[b]
A, HUS and outbreak associated	O157:H7	D	stx_{2c} *eae*-γ1	RD (1), WB (1)
B, HUS and outbreak associated	O26:[H11]	B1	stx_{1a} *eae*-β1	RD (4), WB (1)[n]
	O121:H19	B1	stx_{2a} *eae*-ε1	RD (1)
C, HUS associated, not associated with outbreaks	O2:H6	B2	stx_{2b}	RD (12),[d,f,k] WB (1)[l]
	O2:H6	B2	stx_{1c} stx_{2b}	RD (1)[d]
	O5:HNM	A	stx_{1a} *eae*-β1	F (1)
	O128:H2	B1	stx_{1c} stx_{2b}	WB (1)
	O145:[H28]	D	stx_{1a} stx_{2d} *eae*-γ1	RD (1)
D, diarrhea associated, not associated with HUS, not associated with outbreaks	O9:[H21]	A	stx_{2e}	F (1)
	O75:[H8]	B1	stx_{1c} stx_{2b}	RD (6),[e,f,g] WB (5)[l,m,n]
	O75:[H8]	B1	stx_{1c}	RD (3)
	O75:[H8]	B2	stx_{1c}	RD (1)
	O76:H19	B1	stx_{1c}	WB (1)
	O98:[H21][c]	B1	stx_{1a} *eae*-ζ1	F (1)
	O117:H7	B1	stx_{2c}	WB (1)
	O146:[H21]	B1	stx_{1c} stx_{2b}	RD (9),[h,k] WB (3), F (1)
	O146:[H21]	B1	stx_{1c} stx_{2a} stx_{2b}	RD (1)
	O146:[H21]	B1	stx_{1c}	RD (5)[i]
	O146:[H21]	B1	stx_{2b}	RD (7),[h] WB (1)
	O146:[H28]	D	stx_{2b}	RD (26),[j] WB (1)
	O146:[H28]	D	stx_{2d}	RD (1)
	O174:H8	B1	stx_{2b}	RD (2)
	O174:[H8]	B1	stx_{1c}	RD (1)
	ONT:H1	D	stx_{2b}	WB (1)
	ONT:[H8]	B1	stx_{2b}	RD (2)
	ONT:[H8]	B1	stx_{1c} stx_{2b}	RD (1)
	ONT:H16	A	stx_{2g}	F (1)[o]
	ONT:H21	B1	stx_{1c} stx_{2b}	RD (1)
	ONT:[H28]	D	stx_{1a} stx_{2a}	WB (1)
	ONT:HNT	B1	stx_{2b}	RD (1)
E, not associated with diarrhea, not associated with HUS, not associated with outbreaks, animal and food association only	O2:H21	B2	stx_{2b}	RD (1)[e]
	O2:HNM	B2	stx_{2b}	RD (2)[g,i]
	O8:H10	B2	stx_{2b}	RD (1)
	O11:H45/H48	D	stx_{2a}	WB (1)
	O11:HNT	D	stx_{2a}	RD (2)
	O21:H21	B1	stx_{2b}	RD (2)
	O36:H14	D	stx_{2g}	RD (2)
	O51:H49	B1	stx_{2e} *eae*-β1	WB (1)[m]
	O52:H45	D	stx_{1a} stx_{2c} *eae*-γ1	WB (1)
	O54:H21	B1	stx_{2e}	WB (1)
	O74:[H28]	B1	stx_{2g}	RD (1)
	O75:H40	B1	stx_{1c} stx_{2b}	RD (1)
	O91:H6	B2	stx_{1c} stx_{2b}	RD (1)[j]
	O91:[H8]	B2	stx_{1c}	RD (1)[j]
	O100:HNM	A	stx_{2e}	WB (1)
	O103:H28	B1	stx_{2a} stx_{2g}	WB (1)
	O103:H28	B1	stx_{2g}	WB (1)
	O110:H45	B2	stx_{1c}	RD (1)[d]
	O110:H45	B2	stx_{2b}	RD (1)
	O141:H2	B1	stx_{1a} stx_{2d}	RD (1)
	O177:H11	B1	stx_{2a} *eae*-β1	F (1)[o]

[a] STEC serotypes not previously reported are in boldface type; H types established by serotyping and/or PCR are denoted in square brackets ([H]).
[b] RD, roe deer; WB, wild boar; F, fox. Footnotes *d* to *o* represent STEC strains isolated from the same animal (12 animals total).
[c] Although serotype O98:[H21] is reported for first time for a STEC strain, we have detected it in both an animal strain and a human strain with the same virulence profile, so this strain has been assigned to seropathotype D.
[d] O2:H6-B2 stx_{2b}, O2:H6-B2 stx_{1c} stx_{2b}, and O110:H45-B2 stx_{1c}.
[e] O2:H21-B2 stx_{2b} and O75:[H8]-B1 stx_{1c} stx_{2b}.
[f] O2:H6-B2 stx_{2b} and O75:[H8]-B1 stx_{1c} stx_{2b}.
[g] O2:HNM-B2 stx_{2b} and O75:[H8]-B1 stx_{1c} stx_{2b}.
[h] O146:[H21]-B1 stx_{2b} and O146:[H21]-B1 stx_{1c} stx_{2b}.
[i] O2:HNM-B2 stx_{2b} and O146:[H21]-B1 stx_{1c}.
[j] O91:H6-B2 stx_{1c} stx_{2b}, O91:[H8]-B2 stx_{1c}, and O146:[H28]-D stx_{2b}.
[k] O2:H6-B2 stx_{2b} and O146:[H21]-B1 stx_{1c} stx_{2b}.
[l] O2:H6-B2 stx_{2b} and O75:[H8]-B1 stx_{1c} stx_{2b}.
[m] O51:H49-B1 stx_{2e} *eae*-β1 and O75:[H8]-B1 stx_{1c} stx_{2b}.
[n] O26:[H11]-B1 stx_{1a} *eae*-β1 and O75:[H8]-B1 stx_{1c} stx_{2b}.
[o] O177:H11-B1 stx_{2a} *eae*-β1 and ONT:H16-A stx_{2g}.

minants, such as white-tailed deer, with reported prevalences of 0.28% (37), 0.5% (16), and 2.4% (42); red deer, with a reported prevalence of 1.5% (19); or mouflon, with a reported prevalence of 0% (19).

As discussed above, the analytical methods used could influence data on prevalence. In the present study, 30% of the STEC strains could be recovered only with the use of CT-SMAC. Even though this medium was initially formulated for the selection of STEC O157 strains, we reported previously that it was also a very useful tool for the selective isolation of non-O157 STEC *eae*-pos-

TABLE 3 STEC distribution by phylogroup and origin of isolation

Phylogroup ($n = 135$) (no. of strains [%])	No. (%) of strains isolated from[a]:			*P* value[b]		
	RD ($n = 104$)	WB ($n = 25$)	F ($n = 6$)	RD vs WB	RD vs F	WB vs F
A (4 [3])	0	1 (4)	3 (50)	0.19	**0.00**	**0.02**
B1 (69 [51])	49 (47)	17 (68)	3 (50)	**0.03**	0.32	0.25
B2 (23 [17])	22 (21.1)	1 (4)	0	**0.03**	0.25	0.81
D (39 [29])	33 (32)	6 (24)	0	0.3	0.11	0.24

[a] RD, roe deer; WB, wild boar; F, fox.
[b] *P* values by Fisher's exact test. Significant differences are in boldface type.

itive strains (3). In this study, however, only 14 strains were *eae* positive; all of them were able to grow in CT-SMAC. Tzschoppe et al. (43) previously detected the *terB* (tellurite resistance protein) genes in different groups of *E. coli*, although they were significantly more frequent among enterohemorrhagic *E. coli* (EHEC) strains (*eae*- and *stx*-positive *E. coli* strains) (87.2%) and EHEC-like strains (*eae*-positive, *stx*-negative *E. coli* strains belonging to serotypes associated with EHEC) (72.2%) than among EPEC (enteropathogenic *E. coli*) strains (12.5%), STEC strains (13.5%), and apathogenic *E. coli* strains (12.0%). We also found significant differences between *eae*-positive and *eae*-negative STEC strains (93% versus 51%), but the number of *eae*-negative STEC strains carrying *terD* was higher than that described previously by Tzschoppe et al. (43) (51% versus 13.5%). All wildlife-carried STEC strains recovered only in CT-SMAC were *terD* positive. Therefore, we highly recommend the use of this medium for STEC detection protocols.

A total of 40 serotypes were identified among the 135 STEC strains in the study. When we compared our results with data available in the literature on STEC serotypes isolated from wild animals, only O8:H10, O128:H2, O146:[H21], and O157:H7 have been commonly described (17, 40, 41). However, more information is available regarding STEC isolates from wild meat. Miko et al. (29) previously detected 38 serotypes among 140 STEC strains isolated from game meat (including 70 from roe deer and 18 from wild boars) in Germany. Eleven of those 38 serotypes were also detected in present study (O2:H6, O5:HNM, O21:H21, O26:[H11], O36:H14, O110:H45, O128:H2, O146:[H21], O146:[H28], O174:[H8], and ONT:HNT). Other serotypes also found in this study, namely, O11:H48, O74:[H28], O145:[H28], O157:H7, ONT:H21, and ONT:[H28], were detected previously in STEC strains isolated from game meat by Martin and Beutin (28). This highlights the fact that despite the heterogeneity of serotypes among wildlife-carried strains, we found that 68% of the strains showed any of the H types H8, H21, and H28 by serotyping or PCR, similarly to the 65% of strains from game meat reported previously by Miko et al. (29).

Of the 40 serotypes established among the 135 STEC strains in the present study, 21 were assigned to seropathotypes associated with human disease (seropathotypes A, B, C, and D), accounting for 81.5% of the STEC strains carried by wild animals, including enterohemorrhagic serotypes O26:[H11] (5 strains), O157:H7 (2 strains), O121:H19 (1 strain), and O145:[H28] (1 strain). When we analyzed the serotypes most frequently identified among human STEC strains isolated from patients of the Hospital Lucus Augusti of Lugo (Galicia, northwest of Spain) between 2003 and 2011 (34), we found that five were also detected among wildlife-carried STEC strains (O5:HNM, O26:[H11], O145:[H28], O146:[H21], and O157:H7), accounting for 26.7% of the wildlife-carried strains. A recent study of human-carried STEC in the Hospital Lucus Augusti of Lugo between May and December 2011 revealed an STEC prevalence of 2.9% (25/936) in human stool samples from patients of this hospital, who were affected mostly with gastrointestinal pathologies. The above-described five serotypes were identified in 13 of 25 (52%) STEC strains isolated from humans (our unpublished data). Interestingly, O146:[H21], the second most frequently detected serotype among wildlife-carried STEC strains in this study (20% of the strains), is not only frequently associated with human diarrhea in our health area but is also isolated from beef meat and from vegetables sold in our city (34). O146:[H21] was the only serotype detected in STEC strains of the three origins (22 from roe dear, 4 from wild boars, and 1 from fox). Other STEC serotypes detected in the Hospital Lucus Augusti and also found in 18 strains in this study were O75:[H8], O76:[H19], O98:[H21], and O121:H19. These four serotypes were identified in 3% of 263 human STEC strains isolated in the Hospital Lucus Augusti of Lugo between the years 2003 and 2010 (our unpublished data). Overall, the nine human-pathogenic

TABLE 4 *stx* prevalence in relation to origin of isolation

stx type(s) or most prevalent subtype(s) ($n = 135$) (no. of strains [%])	No. (%) of strains isolated from[a]:			*P* value[b]		
	RD ($n = 104$)	WB ($n = 25$)	F ($n = 6$)	RD vs WB	RD vs F	WB vs F
stx_1 (20 [14.8])	16 (15.4)	2 (8)	2 (33)	0.27	0.25	0.14
stx_2 (80 [59.2])	65 (62.5)	12 (48)	3 (50)	0.14	0.27	0.35
stx_1 stx_2 (35 [26])	23 (22.1)	11 (44)	1 (17)	**0.03**	0.61	0.23
stx_{1a} (7 [5.2])	4 (3.8)	1 (4)	2 (33)	0.67	**0.03**	0.08
stx_{1c} (13 [9.6])	12 (11.5)	1 (4)	0	0.24	0.49	0.81
stx_{2b} (61 [45.2])	57 (54.8)	4 (16)	0	**0.00**	**0.01**	0.40
stx_{1c} stx_{2b} (30 [22])	20 (19)	9 (36)	1 (17)	0.07	0.68	0.35

[a] RD, roe deer; WB, wild boar; F, fox.
[b] *P* values by Fisher's exact test. Significant differences are in boldface type.

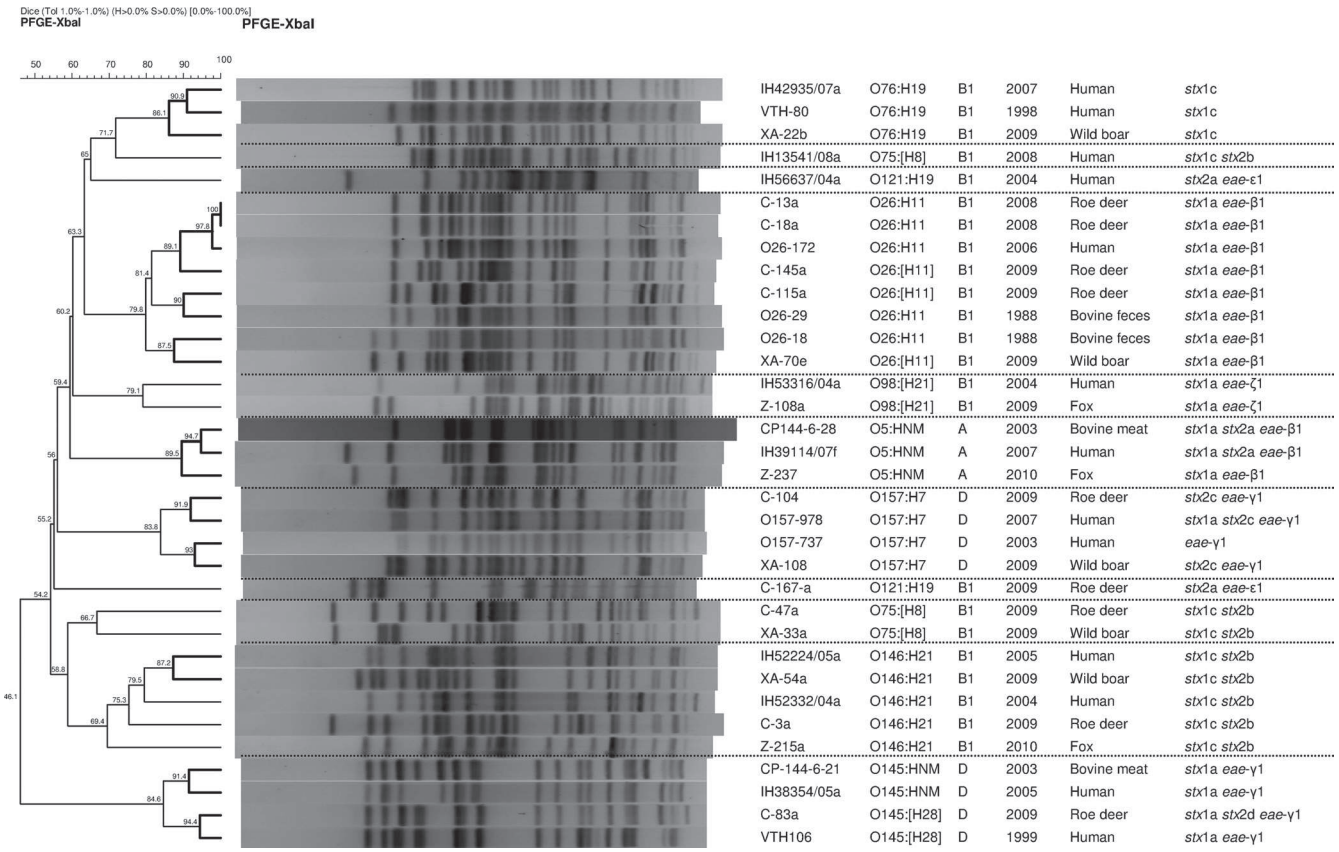

FIG 1 Comparison of 17 wildlife-carried strains from this study with 17 strains obtained from our BioNumerics database. Shown is a dendrogram of the XbaI macrorestriction profiles. Strain codes, serotypes, phylogroups, years of isolation, origins, and virulence genes (Stx subtypes and intimin types) are shown at the right side of the dendrogram.

STEC serotypes described above accounted for 54 (40%) of the wildlife-carried STEC strains. When we compared human- and wildlife-carried strains of the nine serotypes, we found that they shared the same phylogroups and intimin types and the same or similar profiles of *stx* subtypes (Fig. 1). Furthermore, human-pathogenic and wildlife-carried STEC strains belonging to serotypes O5:HNM-A, O26:[H21]-B1, O76:H19-B1, O145:[H28]-D, O146:[H21]-B1, and O157:H7-D showed PFGE profiles with >85% similarity (Fig. 1). Remarkably, although serotype O98:[H21] is reported here for first time for an STEC strain, we have detected it in both a strain isolated from an animal (fox) and a strain isolated from a human with the same virulence profile, so this serotype has been assigned to seropathotype D. This is also the first study to report the isolation of STEC in foxes, and although the prevalence detected was low (1.9%), 3 of the 6 strains recovered carried the *eae* gene, and at least 3 strains (O5:HNM, O98:[H21], and O146:[H21]) showed the same characteristics as human STEC strains. Moreover, the fox strain of serotype O5:HNM-A clustered with a human strain (similarity of >85%).

Fifteen of the 40 serotypes established in the present study were previously detected in minced beef collected from local stores in Lugo City between 1995 and 2009: O2:HNM, O5:HNM, O21:H21, O26:[H11], O75:[H8], O91:HNM, O145:HNM, O146:[H21], O157:H7, O174:HNM, ONT:H21, ONT:HNT, O52:H45, O54:H21, and ONT:[H8] (30, 34; our unpublished data). Eleven of those 15 serotypes accounted for 30.2% (29/96) of the STEC strains isolated from minced beef samples in Lugo City in a study carried out between 1994 and 2003. In that study, STEC strains were isolated in 12% of 785 minced beef samples (30). A comparison of the XbaI macrorestriction profiles obtained by PFGE of 17 representative wildlife-carried strains with STEC strains from our BioNumerics database showed that two minced beef strains of serotypes O5:HNM-A and O145:HNM-D were closely related to wildlife-carried strains (clusters of 89.5% and 84.6% similarity, respectively) (Fig. 1).

When we compared wildlife-carried serotypes with those found among 514 bovine STEC strains isolated between 1993 and 1999 in Galicia, 9 of the 40 serotypes established in the present study were identified in bovines: O26:[H11], O74:H28, O141:H2, O146:[H21], O157:H7, O174:HNM, O177:H11, ONT:H8, and ONT:H21. Serotypes O26:[H11] and O157:H7 were the most prevalent among bovine STEC strains (102/514; 20%) (5). Interestingly, two O26:[H11] strains isolated from bovine feces showed PFGE profiles with 90% and 87.5% similarities with roe deer and wild boar strains, respectively (Fig. 1). In view of the present results, if we accept that cattle are the primary animal reservoirs of STEC strains, there must be a successful transmission of strains between livestock and the wildlife species studied in our geographical area, as can be inferred from the high level of similarity among STEC strains of bovine (feces and beef) and wildlife origins of serotypes O5:HNM-A (CP144-6-28 and Z-237), O26:[H11]-B1 (C-115a, O26-29, XA-70e, and O26-18), and O145:HNM-D (CP-

144-6-21 and C-83a). Importantly, this transmission appears to be maintained over time, as clustered strains were isolated in different years (Fig. 1). Therefore, the epidemiological role of roe deer, wild boars, and foxes in the maintenance of STEC should be taken into account.

None of the wildlife-carried STEC strains analyzed belonged to the highly pathogenic serotype O104:H4 recently detected in Germany, in concordance with our previously reported data on STEC serotypes identified in Spain (34).

Although phylogroup B1 was the most prevalent phylogenetic group (51%), significant differences were detected in the origins of isolation for phylogroup B2 (21.1% for roe deer versus 4% for wild boars; $P = 0.03$) or phylogroup A (50% for fox versus 0% for roe deer; $P = 0.00$). Besides, phylogroup D revealed a substantial prevalence in roe deer (32%) and wild boars (24%). Curiously, phylogroups B2 and D, typically associated with extraintestinal pathogenic *E. coli* strains (32), accounted for 62 STEC strains (45.9%) in the present study. The distribution of phylogroups established here is clearly different from that described previously by Girardeau et al. (20) for 287 STEC strains from different sources (172 bovine, 50 food, and 11 environmental isolates), who found prevalences of 70% for phylogroup B1 and 19% for phylogroup A, with only two strains belonging to phylogroup B2.

When we analyzed the Shiga toxin gene subtypes present in the 135 STEC strains of this study and their clinical associations, we found that 15 STEC strains were positive for any of the stx_{2a}, stx_{2c}, and stx_{2d} subtypes linked with severe human illness (15). The stx_{2b} subtype, found in 61 STEC strains in this study, was proposed previously to designate a subtype of stx_{2c} that is not found in cases of severe illness (15). The combination of stx_{1c} and stx_{2b}, established for 30 STEC strains in the present study (20 from roe deer, 9 from wild boars, and 1 from fox), is found frequently for STEC strains from sheep, goats, and deer (animals, meat, and products) (28). The stx_{2e} subtype, identified in four STEC strains (3 from wild boars and 1 from fox), has rarely been implicated in diarrhea or severe illnesses in humans but is commonly associated with pig edema disease, pig meat and products, and wild boar (animal and meat) (15, 28). Finally, the stx_{2g} subtype detected alone (3 strains from roe deer, 1 from wild boar, and 1 from fox) or with other *stx* subtypes (1 strain from wild boar, stx_{2a} stx_{2g}) was originally isolated from STEC strains from cattle (26) and does not seem to be implicated in human illness (15).

Conclusions. In view of our results, the populations of roe deer, wild boar, and fox in the northwest of Spain are carriers of STEC strains that are potentially pathogenic for humans and contribute to the maintenance and transmission of STEC.

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