Occurrence and characterization of *stx* and/or *eae*-positive *Escherichia coli* isolated from wildlife, including a typical EPEC strain from a wild boar.

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ABSTRACT

Shiga toxin-producing *E. coli* (STEC) and enteropathogenic *E. coli* (EPEC) strains are food-borne pathogens associated with acute diarrhea. Haemolytic-uremic syndrome (HUS) is often a complication of STEC infection. In order to examine the occurrence, serotypes, virulence and antimicrobial-resistance profiles of STEC and EPEC in wildlife, 326 faecal *E. coli* strains from 304 clinically healthy animals were analyzed. For this approach *stx*$_1$, *stx*$_2$ and *eae* genes, as well as accessory virulence determinants (*ehx*, *hlyA*, *saa*, *tia*, *bfp*, *subAB*) were PCR-screened and sequenced. Serotyping was performed employing all available O (O1-O185) and H (H1-H56) antisera. Genetic diversity was analyzed by XbaI-PFGE and phylotyping. Thirteen STEC (4.3%) and 10 EPEC (3.3%) were identified among 12 deer, 3 mouflon, 6 wild boars and 2 birds. Nine STEC showed seropathotypes B (O145:[H28]) and C (O22:H8, O128:[H2]) associated with HUS, and D (O110:H28, O146:H21, O146:[H28], ONT:H8) associated with human diarrhea. Although most isolates harbored *stx*$_{2b}$ and *stx*$_{1c}$ variants, *stx*$_{2a}$ and *stx*$_{1a}$ (related with severe disease) were also detected. Additionally, the *eae* gene was present in one *stx*$_{2a}$–positive O145:[H28] STEC from a deer and 11 STEC harbored *subAB* genes (mainly the *subAB*$_2$ variant). EPEC isolates showed 7 different intimin variants (β1, β2, γ1, ε1, ζ1, ι1-A, κ). Interestingly, the O49:[H10] *eae*-κ EPEC isolated from a wild boar was *bfpA*-positive showing a combination of serotype/virulence profile previously detected among human clinical tEPEC. Based on present results, wild ruminants, wild boars and to a lesser extent birds would be carriers of potentially pathogenic STEC and EPEC strains.

Keywords: STEC, EPEC, wildlife, *sub*AB, *Escherichia coli*.

INTRODUCTION

In humans, infections with Shiga toxin (Stx)-producing *E. coli* (STEC) causes illness ranging from mild diarrhea to haemorrhagic colitis and haemolytic uraemic syndrome (HUS). Domestic ruminants, such as cattle, goats and sheep, are recognized as their major natural reservoirs. However, serologically diverse STEC types have been also isolated from wild animals (Asakura *et al*., 1998; Sánchez *et al.*, 2009; Mora *et al.*, 2012). Furthermore, different sporadic cases and even outbreaks have been linked to food-borne transmission through the consumption of deer meat (Rabatsky-Ehr *et al.*, 2002; Rounds *et al.*, 2012) or fresh products from fields contaminated with faeces of free-living animal (Laidler *et al.*, 2013).

Although O157:H7 *E. coli* has been the serotype most frequently implicated in human disease, more than 400 O:H types of STEC have been associated with infections. However, most non-O157 STEC lack the biochemical characteristics differentiating them from *E. coli* strains present in the normal flora, likely resulting in the underestimation of its incidence in human illness (Blanco *et al*. 2004a).

In addition to Shiga toxins, STEC can synthesize the adhesin intimin (encoded by *eae*), a plasmid-encoded enterohemolysin (EhxA), or an autoagglutinating protein (Saa), among other virulence factors (Gyles, 2007). Furthermore, the subtilase (SubAB) is a cytotoxin elaborated by some STEC strains usually lacking the locus of enterocyte effacement (LEE), associated with lethality in mice and human cells and the enhancement of the *E. coli* survival in macrophages (Paton *et al.*, 2004). Three variants of SubAB have been described: a plasmid-borne *subAB*$_{1}$ operon, usually co-located with the *saa* gene, and two chromosomal variants (*subAB*$_{2\text{-}1}$ and *subAB*$_{2\text{-}2}$). The *subAB*$_{2\text{-}1}$ operon is located on the chromosomal pathogenicity island SEPAI, close to the *tia* gene encoding an invasin in enterotoxigenic *E. coli* (ETEC), while the *subAB*$_{2\text{-}}$

$_2$ is located next to a gene encoding an outer membrane efflux protein and associated genes of a type one secretion system (Paton *et al.*, 2004, Michelacci *et al.*, 2013; Funk *et al.*, 2015).

Enteropathogenic *E. coli* (EPEC) strains are defined as intimin (*eae*)-containing diarrheagenic *E. coli* that do not possess the *stx* genes. EPEC is further divided into two subtypes, typical (tEPEC) and atypical (aEPEC) depending on the presence or absence of the bundle-forming pilus (BFP), respectively. EPEC is an important cause of diarrhea in children, and while tEPEC is more dominant in developing countries, aEPEC seems to be more important in developed countries (Blanco *et al.*, 2006). Humans have been described as the only reservoir of tEPEC with few exceptions (Chandran & Mazumder, 2013), however aEPEC have been isolated from humans as well as from a wide variety of animals (Blanco *et al.*, 2005; Chandran & Mazumder, 2013).

The objectives of this study were to investigate the occurrence of STEC and EPEC in wildlife and to establish the serotypes, associated virulence markers, genetic relatedness and antimicrobial-resistance patterns of these isolates.

METHODS

E. coli collection

A total of 326 *E. coli* strains were included in the present work. All were recovered in previous studies, some of them published, focused on antimicrobial resistance genotyping (Alcalá *et al.*, 2016; Alonso *et al.*, 2016). These isolates were obtained from faeces or intestine portions with faecal contain collected between 2013-2015 in different geographic locations of Spain (Aragón, Castilla - La Mancha and Cádiz) from 304 clinically healthy wild animals belonging to the following species: 90 wild boars (*Sus scrofa*), 80 deer (79 red deer - *Cervus elaphus* - and 1 roe deer – *Capreolus capreolus* -

103), 79 wild birds of different species, 19 rodents (13 wood mice – *Apodemus sylvaticus* -
104 and 6 black rats – *Rattus rattus* -), 16 European rabbits (*Oryctolagus cuniculus*), 5
105 minks (*Mustela lutreola*), 4 European hedgehogs (*Erinaceus europaeus*), 3 mouflon
106 (*Ovis musimon*), 2 foxes (*Vulpes vulpes*), 2 martens (*Martes martes*), 2 badgers (*Meles*
107 *meles)*, 1 otter (*Lutra lutra*) and 1 genet (*Genetta genetta*).
108 The initial isolation was performed on Levine agar plates. Up to two *E. coli* colonies per
109 sample were randomly selected and identified by classical biochemical methods (gram-
110 staining, triple sugar iron, indol) and a species-specific PCR (*uid*A, Table S1).
111 Antibiotic susceptibility was determined by the disk-diffusion method according to the
112 Clinical Laboratory Standards Institute criteria in all isolates (CLSI, 2015). The
113 following antimicrobial agents were tested: ampicillin, amoxicillin/clavulanate,
114 ceftazidime, ceftriaxone, cefoxitin, imipenem, nalidixic acid, ciprofloxacin, gentamicin,
115 amikacin, tobramycin, streptomycin, chloramphenicol, sulfonamides,
116 trimethoprim/sulfamethoxazole and tetracycline. *E. coli* ATCC 25922 was used as
117 control strain. When isolates from a given sample exhibited an identical phenotypic
118 resistance pattern, only one colony was selected and stored at -80ºC.
119 Thus, a collection of 326 *E. coli* isolates from wild animals was available from previous
120 studies and was included in the present work.
121 *Detection and characterization of STEC and EPEC strains*
122 All the 326 *E. coli* strains were screened for the presence of *stx*$_1$, *stx*$_2$ and *eae* genes by
123 PCR. Subtypes of *stx*$_1$ were determined by multiplex-PCR, *stx*$_2$ by sequencing the most
124 variable part of the *stxAB*$_2$ operon and *eae* variants by PCR and sequencing. The
125 nomenclature proposed by Scheutz *et al.* (2012) was used in this study for the
126 designation of *stx*$_1$ and *stx*$_2$ subtypes. The primers used for virulence genotyping and

stx$_1$, *stx*$_2$, *eae* and *subAB* subtyping are described in Table S1 of the supplementary material.

All *stx* and/or *eae*-positive isolates were investigated for other virulence-associated factors, such as *ehx*, *hlyA*, *saa*, *tia* and *subAB*, using previously described primer combinations. Additionally, the bundle-forming pilus encoding gene cluster (*bfp*) was also screened in *eae*-positive strains. The plasmid-borne (*subAB*$_1$) and chromosomal (*subAB*$_2$) variants of Subtilase cytotoxin were identified using the primer pairs SubAF-RTsubABR and subA_startF/RTsubABR, respectively (Table S1).

O and H typing

E. coli isolates were characterized with regard to O:H serotype using the method previously described by Guinée *et al*. (1981) with all available O (O1–O181) and H (H1–H56) antisera. Nonmotile isolates were designated as HNM and as nontypeable (HNT) those which did not react with any antisera. HNM and HNT isolates were further tested by PCR to detect the presence of the flagellar genes as described elsewhere, and positive results were denoted in brackets [H] (Mora *et al*., 2012; Table S1).

Molecular typing

The genetic relatedness and diversity among STEC and EPEC isolates was analyzed by XbaI (BioLabs, New England) macrorestriction followed by pulsed-field gel electrophoresis (PFGE). PFGE conditions were as follows: 6 V/cm with pulse-times of 1-30 s for 23 h at 14ºC. A dendrogram was generated by the BioNumerics software 2.0 (Applied Maths, Belgium) (UPGMA algorithm; Dice coefficient; 1% tolerance).

All STEC and EPEC isolates were classified in the seven main phylogenetic groups (A, B1, B2, C, D, E, and F) using the Clermont multiplex PCR method, as previously described (Clermont *et al.*, 2013).

RESULTS

Thirteen STEC (4.3%) and 10 EPEC (3.3%) were identified among the 304 animals studied. Specifically, the 23 strains were recovered from 12 deer, 3 mouflon, 6 wild boars and 2 wild birds (Figure 1).

Among the 13 STEC strains, 9 different serotypes were identified. The 6 strains isolated from deer showed 4 different serotypes, with O27:H30 (n=3) as the most common one. The 3 isolates recovered from wild boar faeces belonged to different serotypes (O5:HNM, O146:H21 and O146:[H28]), and the 3 isolates of mouflon to O128:[H2] (n=2) and O146:H21 (n=1). Finally, the STEC strain isolated from a stork belonged to O22:H8 serotype.

The STEC strains were classified into seropathotypes according to the classification of Karmali *et al.* (2003), based on their clinical and epidemiological features and on different published data (Blanco *et al.*, 2004b; Girardeau *et al.*, 2005; Coombes *et al.*, 2008). Thus, 9 STEC isolates showed to belong to the seropathotypes B (O145:H28) and C (O22:H8, O128:H2) associated with HUS, and D (O110:H28, O146:H21, O146:H28, ONT:H8) associated with human diarrhea.

The PCR screening indicated that 4 strains harbored both *stx*$_1$ and *stx*$_2$ genes and 9 only *stx*$_2$. Two *stx*$_1$ subtypes (*stx*$_{1a}$ and *stx*$_{1c}$) and 3 *stx*$_2$ subtypes (*stx*$_{2a}$, *stx*$_{2b}$, and *stx*$_{2c}$) were detected among the 13 STEC strains, with a total of 5 different *stx*$_1$ and/or *stx*$_2$ subtype combinations, and *stx2b* alone being the most common profile (8 strains). No significant association was found between the carrier host and the *stx* subtype.

Regarding accessory virulence determinants associated with STEC isolates, *eae* gene (γ1 variant) was present in one *stx*$_{2a}$–positive O145:[H28] strain from a red deer. Nevertheless, the presence of subAB cytotoxin (mainly codified by the chromosomal s*ubAB*$_2$ gene) was identified in 11 out of 13 STECs (all of them *eae*-negative). Unlike

177 isolates harboring plasmidic s*ubAB1* gene (n=2), those carrying the chromosomal
178 s*ubAB2* variant were all positive for the *tia* gene (n=9) (Figure 2). The *ehxA* determinant
179 was detected in 8 STECs, *saa* only in one isolate and none of them carried the *hlyA*
180 gene.
181 The 10 EPEC identified in this study belonged to 9 different serotypes and showed 7
182 intimin types: β1 (1 strain), β2 (2 strains), γ1 (1 strains), ε1 (2 strains), ζ1 (2 strains), κ
183 (1 strain) and ι1-A (1 strain). Additionally, the strain O49:[H10] *eae*-κ isolated from a
184 wild boar was *bfpA* positive and therefore typical EPEC (tEPEC) (Figure 2).
185 PFGE analysis of the STEC and EPEC isolates demonstrated a high heterogeneity even
186 within isolates of the same serotype and virulence gene profile (Figures 2). Only a small
187 cluster of two O128:H2 strains showed a similarity ≥85%.
188 Phylotyping showed that the 13 STEC belonged to 3 phylogroups (8 strains B1, 4
189 strains E and 1 strain F) and the 10 EPEC to 4 phylogroups (5 strains B1, 3 strains B2, 1
190 strain A and 1 strain E) (Figure 2).
191 Only 2 O128:H2 STECs from mouflon exhibited resistance to antimicrobials
192 (ampicillin, tetracycline and trimethoprim-sulfamethoxazole) (Figure 2).
193
194 **DISCUSSION**
195 In the present study, we analyzed the occurrence of STEC and EPEC within a collection
196 of 326 *E. coli* isolates from different wild animal species. Also, isolates from these
197 pathotypes were molecularly characterized in order to examine the genotypic diversity
198 of these potentially pathogenic *E. coli* strains circulating in wildlife.
199 Among the studied bacterial collection, STEC were identified from 7.6% of deer, 3.3%
200 of wild boars, 1.3% of wild birds and 100% of mouflon isolates examined, all of them
201 serotyped as non-O157. Although this study involved isolates of many different animal

202 species, those positive for STEC have been previously reported as carriers of *stx*$_1$ and/or
203 *stx*$_2$ genes in different surveys (Asakura *et al.*, 1998; Sánchez *et al.*, 2009; Borges *et al.*,
204 2017). Present data reinforces the role of these wild species, especially deer, mouflon
205 and wild boars, as reservoirs of potentially pathogenic STEC strains.
206 Comparing our results with those published by other authors, we found similarities in
207 the distribution of serotypes by source. As an example, the frequent detection of
208 O27:H30 serotype among STEC isolated from deer is in accordance with a previous
209 study that identified this as one of the most prevalent serotype in red deer (Díaz-
210 Sánchez *et al.*, 2013). It is worth mentioning that analyzed animals, as in the present
211 study, were resident in South-Central Spain. However, O27:H30 serotype has also been
212 detected in deer meat from Central Europe (Martin & Beutin, 2011). These observations
213 suggest a potential association between the serotype O27:H30 and deer source. In
214 addition, O146:H21 and O146:H28 serotypes, identified in 3 isolates from different
215 individuals in this study, have been recurrently identified within STEC isolates from
216 wild boar, mouflon and deer faeces, meat and products (Sánchez *et al.*, 2009; Martin &
217 Beutin, 2011; Mora *et al.*, 2012), indicating that STEC isolates belonging to these
218 serotypes are usual colonizers of large game animals. Similar findings were made for
219 O128:H2 serotype, frequently associated with STEC from sheep and goats (Martin &
220 Beutin, 2011; Sánchez *et al*., 2012). In the present study, 2 out of 3 STEC isolated from
221 mouflon belonged to this serotype, which might suggest that both domestic and wild
222 ovine represents a reservoir of STEC O128:H2 strains.
223 It is important to highlight that 9 out of the 13 STEC isolates detected in this study,
224 belonged to seropathotypes previously linked to human disease, including O145:H28,
225 O128:H2 or O22:H8 implicated in HUS (Karmali *et al.*, 2003; Girardeau *et al.* 2005).

When we characterized the Shiga toxin genes carried by STEC strains, *stx*$_2$ was the predominant detected determinant, alone or in combination with *stx*$_1$. This result is in agreement with previous studies performed in wildlife species (Asakura *et al.*, 1998; Mora *et al.*, 2012). Among the three known Stx1 subtypes and seven Stx2 subtypes (Scheutz *et al.* 2012), only Stx1a, Stx2a, Stx2c, and Stx2d have most often been implicated in human illness (Bielaszewska *et al.* 2006; Persson *et al.* 2007). When we analyzed the Shiga toxin gene subtypes present in the 13 STEC strains of this study and their clinical associations, we found that 5 STEC strains were positive for any of the *stx*$_{1a}$, *stx*$_{2a}$ or *stx*$_{2c}$ linked with severe illness. In particular, the highly virulent *stx*$_{2a}$ subtype was identified in an *E. coli* isolate from a deer belonging to seropathotype B (O145:H28 associated to HUS), which additionally carried the *eae*-γ1 gene (Marejková *et al.*, 2013).

Although most of the studied wild animals carried *eae*-negative STECs harboring the low virulent *stx*$_{2b}$ subtype, alone or in combination with other variants (*stx*$_{1a}$ or *stx*$_{1a}$ plus *stx*$_{1c}$), the *subAB* determinant was additionally detected in all but one isolates. This finding is in agreement with previous surveys carried out among wild ruminants, wild boars and game meat (Sánchez *et al.*, 2013), which demonstrated the existence of a significant association between *stx*$_{2b}$ subtype and *subAB*-positive STEC. The presence of *subAB* genes might increase the pathogenicity of these STEC in humans. In fact, different authors have reported bloody diarrheal cases affecting humans, in which the causative agent was considered *eae*-negative, *ehx*- and *subAB*-positive STEC belonging to O128:H2 and O76:H19 serotypes carrying *stx*$_{2b}$ alone or in combination with other *stx*$_1$ gene variants (Sánchez *et al.*, 2012; Sánchez *et al.*, 2014). It is also important to remark that most of the *subAB*-positive strains detected in the present study harbored the *subAB*$_2$ variant associated, in all cases, with the presence of *tia*. This latter gene,

previously described in enterotoxigenic *E. coli* (ETEC) (Fleckenstein *et al.*, 1996), encodes an outer membrane protein involved in the invasion of intestinal epithelial cells. The fact that the *subAB*$_2$ gene was always found in isolates that also had the *tia* determinant suggests that both are located on the same pathogenicity island as described by other authors (Tozzoli *et al.*, 2010). Conversely, the *subAB* genes carried by 2 STEC strains belonging to O146:H28 (isolated from wild boar) and ONT:H8 (isolated from deer) serotypes were subtyped as *subAB*$_1$. One of these isolates was also positive for the *saa* gene, which has been shown to be co-located on a plasmid close to the *subAB* operon (Paton *et al.*, 2004).

Typical EPEC strains are pathogenic for humans and have not been found in animals with only rare exceptions, namely the detection of *bfpA*-positive EPEC strains in dogs, coyotes and one deer (Ishii *et al.*, 2007; Chandran & Mazumder, 2013). The finding in the present study of a tEPEC isolate in a wild boar sample, could suggest the contact and acquisition from human sources. To our knowledge, this is the first report of the presence of typical EPEC in wild boars. Importantly, the serotype O49:H10 and intimin subtype *eae*-κ identified in this tEPEC has also been found among human clinical tEPEC isolated from faecal samples of 9 patients with diarrhea from the Hospital Lucus Augusti of Lugo (Galicia, northwest of Spain) between 2003 and 2014 (unpublished data).

Overall, the 10 EPEC strains detected in this work comprised a variety of 7 intimin variants, being β2, ζ1 and ε1 present in more than one isolate. The latter subtype (*eae*-ε1) has been widely identified in EPEC and STEC from different host species belonging to the O103:H2 serotype (Blanco *et al.*, 2005). This serotype has been frequently found in STEC causing HUS and hemorrhagic colitis (Karmali *et al.*, 2003) and, interestingly, was also detected in 2 EPEC recovered from deer in the present study. Shiga toxin-encoding

prophages can be stably maintained in the bacterial genome, but at least some strains of STEC can lose the prophage and thereby revert to the aEPEC state. Also, most human-pathogenic STEC originate from populations of EPEC lysogenized by one or more phages carrying genes encoding *stx*$_1$ and/or *stx*$_2$. Söderlund *et al.* (2016) analyzed this possibility within a collection of O103:H2 isolated from cattle. Phylogenetic comparison by SNP analysis indicated that while certain subgroups of aEPEC and STEC were closely related and had otherwise near identical virulence gene repertoires, they belonged to separate lineages, indicating the uptake or loss of Shiga toxin genes is a rare event in the natural cattle environment of these bacteria. Intimin γ1, frequently present among EPEC and STEC isolated from human patients, was identified in association with an aEPEC O55:H7 strain from a deer. This serotype is one of the most frequently reported among clinical aEPEC and evolutionary models postulated it as the one from which STEC O157:H7 are believed to have evolved (Feng *et al.* 2007).

Another aspect to be noted is the highly susceptible antimicrobial resistance patterns observed among STEC/EPEC strains from wild animals. We only found a multidrug-resistant profile (resistance to ampicillin, tetracycline and trimethoprim-sulfamethoxazole) in two genetically related STEC isolates, as defined by their PFGE-macrorrestriction profiles, recovered from mouflon. This is consistent with the fact that tetracyclines, β-lactams (mainly penicillins) and trimethoprim/sulphonamides are among the most widely employed antimicrobials in farm animals, from which resistant bacteria can emerge and spread to the environment. In fact, a recent study carried out in non-157 STEC farm- and abattoir-sourced isolates, reported 87% of multidrug resistance (MDR) to antimicrobials used in veterinary and agricultural practice (Kennedy *et al.*, 2017). Additionally, another work suggests that wild birds (57%) could act as carriers of

multidrug-resistant EPEC and STEC (Borges *et al*., 2017), leading to the assumption that MDR isolates may emerge in the environment in a near future as well.

As a final remark, it is important to mention that most of the wild animals involved in this study shared habitat resources with livestock species and, worryingly, some of them (hunted wild boars and some deer) were intended for human consumption. This encourages further study to elucidate the degree of human disease risk posed by these STEC and EPEC isolates carried by wild species.

CONCLUSION

Wild ruminants (deer and mouflon), wild boars and, to a lesser extent, birds are carriers of STEC and EPEC strains potentially pathogenic for humans, such as O145:H28 *stx*$_{2a}$ *eae*-γ1 implicated in HUS. Here, we first report a wild boar as carrier of a *bfpA*-positive O49:[H10] *eae*-κ strain of the same characteristics as tEPEC isolated from human diarrhea.

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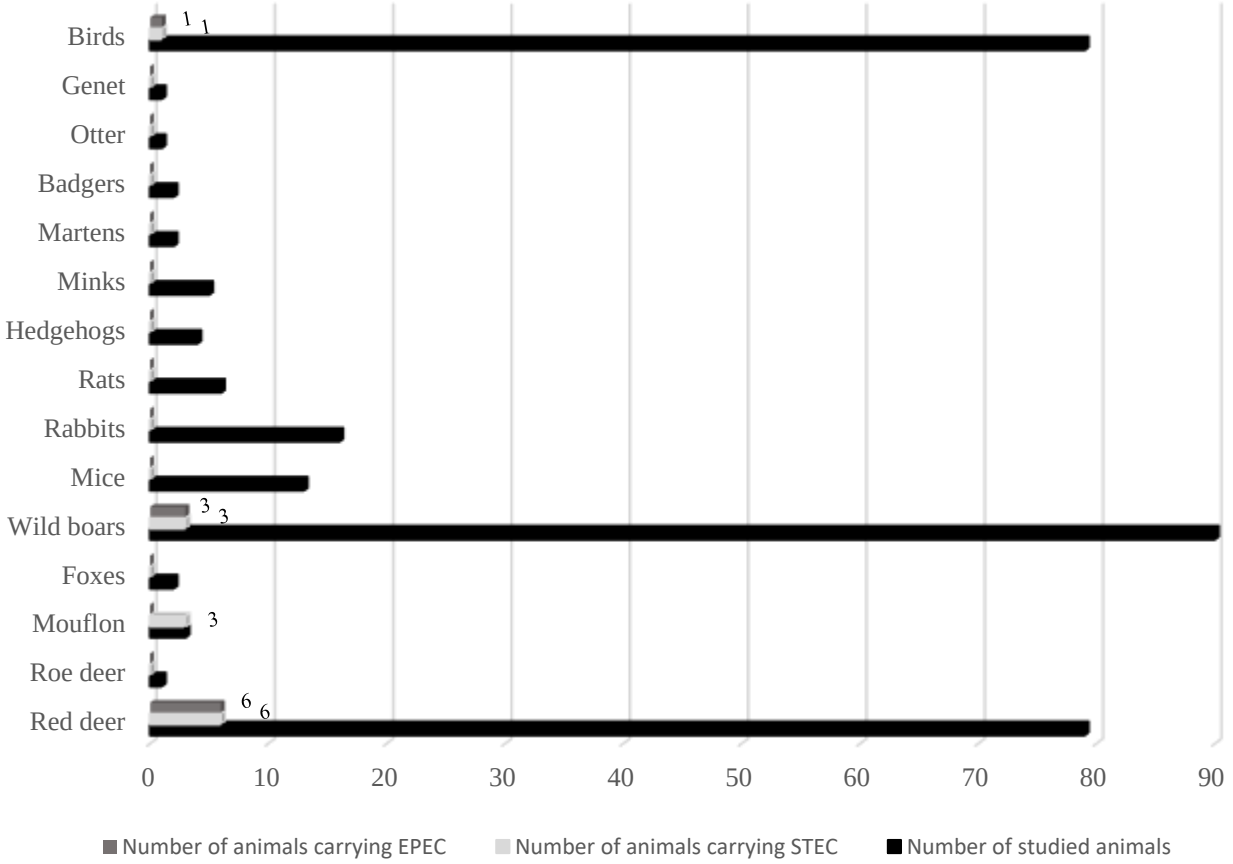
FIGURE LEGENDS

Fig. 1. Distribution of STEC and EPEC isolates identified among studied animal collection.

Fig. 2. PFGE patterns of XbaI digested genomic DNA from STEC and EPEC isolates detected among studied bacterial collection. Association between serotype, virulence genes, antibiotic resistance phenotype and phylogroup of each isolate is indicated on the right. [a] HNM and HNT isolates were further tested by PCR to detect the presence of the flagellar genes and positive results were denoted in brackets [H], [b] AMP: Ampicillin, TE: Tetracycline, SXT: Trimethoprim/Sulfamethoxazole.

Fig. 1.


Birds
Genet
Otter
Badgers
Martens
Minks
Hedgehogs
Rats
Rabbits
Mice
Wild boars
Foxes
Mouflon
Roe deer
Red deer
1
1
3
3
3
6
6
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10
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30
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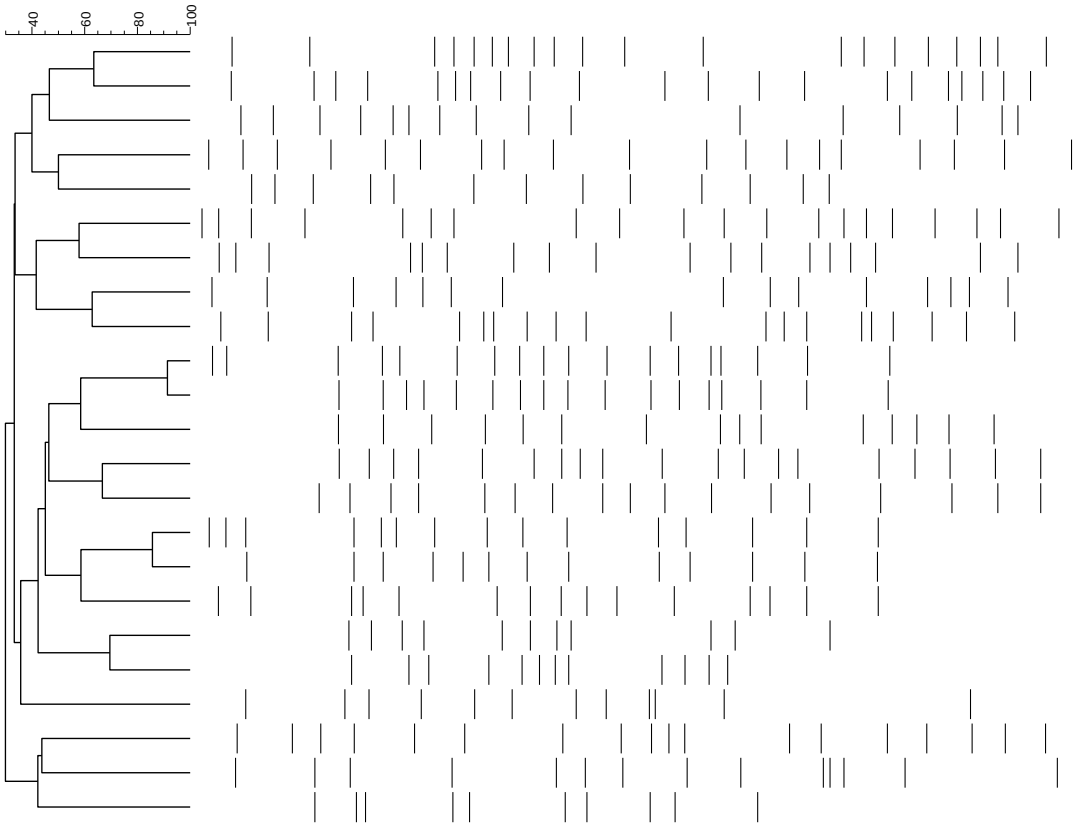
60
70
80
90
Number of animals carrying EPEC
Number of animals carrying STEC
Number of studied animals



Strain	Origin	*stx* $_1$ subtype	*stx* $_2$ subtype	*eae* variant	Other virulence genes						Serotype	Phylo group	Resistance phenotype[b]
					subAB subtype	*bfp*	*ehx*	*hlyA*	*saa*	*tia*			
C7331	deer	–	–	ε1	–	–	+	–	–	–	O103:H2	B1	susceptible
C7332	deer	–	–	β1	–	–	+	–	–	–	O5:H21	B1	susceptible
C8417	mouflon	*stx* $_{1a}$, stx$_{1c}$	*stx* $_{2b}$	–	*subAB* $_2$	NT[a]	+	–	–	+	O146:H21	B1	susceptible
C7340	deer	*stx* $_{1a}$, stx$_{1c}$	*stx* $_{2c}$	–	*subAB* $_1$	NT	+	–	+	–	ONT:H8	B1	susceptible
C8020	wild boar	–	–	ι1-A	–	–	–	–	–	–	O2:H49	B2	susceptible
C7344	deer	–	*stx* $_{2a}$	γ1	–	NT	+	–	–	–	O145:[H28]	E	susceptible
C6978	wild boar	–	*stx* $_{2b}$	–	*subAB* $_1$	NT	+	–	–	–	O146:[H28]	F	susceptible
C7352	deer	–	–	γ1	–	–	–	–	–	–	O55:[H7]	E	susceptible
C7976	wild boar	–	–	κ	–	+	–	–	–	–	O49:[H10]	A	susceptible
C8415	mouflon	–	*stx* $_{2b}$	–	*subAB* $_2$	NT	+	–	–	+	O128:H2	B1	AMP, TE, SXT
C8419	mouflon	–	*stx* $_{2b}$	–	*subAB* $_2$	NT	+	–	–	+	O128:[H2]	B1	AMP, TE, SXT
C8103	owl	–	–	β2	–	–	–	–	–	–	ONT:H14	B2	susceptible
C7270	deer	–	–	ζ	–	–	+	–	–	–	O109:HNM	B1	susceptible
C7281	deer	–	–	ζ	–	–	+	–	–	–	O156:[H25]	B1	susceptible
C8416	deer	–	*stx* $_{2b}$	–	*subAB* $_2$	NT	–	–	–	+	O27:H30	E	susceptible
C8414	deer	–	*stx* $_{2b}$	–	*subAB* $_2$	NT	–	–	–	+	O27:H30	E	susceptible
C7820	deer	–	*stx* $_{2b}$	–	*subAB* $_2$	NT	–	–	–	+	O27:H30	E	susceptible
C6980	wild boar	*stx* $_{1a}$	*stx* $_{2b}$	–	*subAB* $_2$	NT	+	–	–	+	O146:H21	B1	susceptible
C8420	deer	–	*stx* $_{2b}$	–	*subAB* $_2$	NT	–	–	–	+	O110:H28	B1	susceptible
C6979	wild boar	*stx* $_{1a}$, stx$_{1c}$	*stx* $_{2b}$	–	*subAB* $_2$	NT	+	–	–	+	O5:HNM	B1	susceptible
C7356	deer	–	–	ε1	–	–	+	–	–	–	O103:H2	B1	susceptible
C7375	stork	–	*stx* $_{2b}$	–	–	NT	–	–	–	–	O22:H8	B1	susceptible
C8129	wild boar	–	–	β2	–	–	–	–	–	–	O33:H6	B2	susceptible

Table S1. Primer pairs used in this study for *E. coli* identification and virulence characterization.

Target	Primers	Sequence (5'-3')	Size of product (bp)	Reference
Identification of *E. coli*				
uidA	uidA-F	ATCACCGTGGTGACGCATGTCGC	486	Heininger *et al.* 1999
	uidA-R	CACCACGATGCCATGTTCATCTGC		
Virulence Genotyping				
stx$_1$	stx1-F	CAGTTAATGTGGTGGCGAAGG	348	Vidal *et al.* 2005
	stx1-R	CACCAGACAATGTAACCGCTG		
stx$_2$	stx2-F	ATCCTATTCCCGGGAGTTTACG	584	Vidal *et al.* 2005
	stx2-R	GCGTCATCGTATACACAGGAGC		
eae	eae-F	TCAATGCAGTTCCGTTATCAGTT	482	Vidal *et al.* 2005
	eae-R	GTAAAGTCCGTTACCCCAACCTG		
bfp	bfp-F	AATGGTGCTTGCGCTTGCTGC	326	Gunzburg *et al.* 2001
	bfp-R	GCCGCTTTACCAACCTGGTA		
saa	SAA-DF	CGTGATGAACAGGCTATTGC	119	Paton and Paton 2002
	SAA-DR	ATGGACATGCCTGTGGCAAC		
tia	tia-Io	TCCATGCGAAGTTGTTATCA	1800	Tozzoli *et al.* 2010
	tia-up	GAAATGAAAAAGATTATTGCGG		
ehx	HlyA1	GGTGCAGCAGAAAAAGTTGTAG	1551	Schmidt *et al.* 1995
	HlyA4	TCTCGCCTGATAGTGTTTGGTA		
hlyA	hlyA-F	AACAAGGATAAGCACTGTTCTGGCT	1177	Yamamoto *et al.* 1995
	hlyA-R	ACCATATAAGCGGTCATTCCCGTCA		
subAB	RTsubABF	GCAGATAAATACCCTTCACTTG	231	Paton *et al.* 2004
	RTsubABR	ATCACCAGTCCACTCAGCC		
***stx1, stx2, eae* and *subAB* subtyping**				
stx$_{1a}$	stx1a-F1	CCTTTCCAGGTACAACAGCGGTT	478	Scheutz *et al.* 2012
	stx1a-R2	GGAAACTCATCAGATGCCATTCTGG		

[a] LREC: Laboratorio de Referencia de *Escherichia coli*

Target	Primers	Sequence (5'-3')	Size of product (bp)	Reference
***stx1, stx2, eae* and *subAB* subtyping (cont.)**				
stx$_{1c}$	stx1c-F1	CCTTTCCTGGTACAACTGCGGTT	252	Scheutz *et al.* 2012
	stx1c-R1	CAAGTGTTGTACGAAATCCCCTCTGA		
stx$_{1d}$	stx1d-F1	CAGTTAATGCGATTGCTAAGGAGTTTACC	203	Scheutz *et al.* 2012
	stx1d-R2	CTCTTCCTCTGGTTCTAACCCCATGATA		
stx$_{2a}$, *stx*$_{2b}$, *stx*$_{2c}$, *stx*$_{2d}$, *stx*$_{2g}$	F4	GGCACTGTC TGAAACTGCTCCTGT	627	Persson *et al.* 2007
	R1	ATTAAACTGCACTTCAGCAAATCC		
stx$_{2e}$, *stx*$_{2f}$	F4-f	CGCTGTCTGAGGCATC TCCGCT	625	Persson *et al.* 2007
	R1-e/f	TAAACTTCACCTG GGCAAAGCC		
eae	EAE-R11	TCTTCGGAGGGTTTTTTATT	1125	LREC[a], this study
	EAE-FBN	CAGGTCGTCGTGTCTGCTAAAAC		
eae	EAE-R12	CCAGACGAATATATACATATTC	1181	LREC, this study
	EAE-FBN	CAGGTCGTCGTGTCTGCTAAAAC		
subAB	subA_startF	CCCTGTAACATATTGACCAGCA		Michelacci *et al.* 2013
	SubAF	GTACGGACTAACAGGGAACTG		Paton *et al.* 2004
Dctection of flagellar genes				
fliC$_{H2}$	H2-F	AACGACGGCGAAACAATTAC	828	LREC, Mamani 2014
	H2-R	AGAACGCAACGAGTCAACCT		
fliC$_{H10}$	H10-F	AGCAAGTGGCAGTAGGTGCT	624	LREC, Mamani 2014
	H10-R	GCTGGATAATCTGCGCTTTC		
fliC$_{H25}$	H25-F	ATGAAATTGACCGCGTATCC	212	LREC, Mamani 2014
	H25-R	TTGCGGGATAGATGTGATAGC		
fliC$_{H28}$	H28-F	ACGAAATCAAATCCCGTCTG	856	LREC, Mora *et al.* 2012
	H28-R	GCCGATTGAAGAGACTCAGC		
fliC$_{H7}$	H7-F	GCGCTGTCGAGTTCTATCGAGC	625	Gannon *et al.* 1997
	H7-R	CAACGGTGACTTTATCGCCATTCC		

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