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SHORT COMMUNICATION

Escherichia coli O157:H7 in wild boars (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) sharing pastures with free-ranging livestock in a natural environment in Spain

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Background: Wild ungulates have greatly increased in abundance and range throughout Europe. This new situation presents a concern for public health because many wild ungulates are known reservoirs of zoonotic pathogens.
Objectives: In this work, we tested for the presence of the zoonotic pathogen *Escherichia coli* O157:H7 in free-ranging livestock and sympatric wild boars (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*) in NE Spain from 2009 to 2011. In addition, antimicrobial resistance and virulence factors were assessed.
Animals and methods: In total, individual fecal samples were obtained from 117 hunter-harvested wild boars and 160 Iberian ibexes. Fifty-five samples were obtained from cattle (5 herds, 380 animals in total) and four from the only horse herd in the Natural Park 'Ports de Tortosa i Beseit' (32 animals). Fecal samples were processed according to the ISO 16.654:2001 protocol to obtain *E. coli* O157 based on immunomagnetic separation. In addition, a multiplex polymerase chain reaction (PCR) targeting nine virulence factors characteristic of human pathotypes was performed. The prevalence was compared between host species with Fisher's exact test.
Results: Four wild boars (3.41%, 95% CI = 0.94–8.52) and two Iberian ibexes (1.25%, 95% CI = 0.15–4.4) carried *E. coli* O157:H7, which was not found in livestock feces ($n = 59$, 95% CI = 0–8.94). All *E. coli* O157:H7 isolates were susceptible to all antimicrobial agents tested.
Conclusions: These results indicate that when prevalence in co-habiting livestock is low, wild ungulates do not seem to play an important role as reservoirs of *E. coli* O157:H7.

Keywords: wild boar; *Sus scrofa*; Iberian ibex; *Capra pyrenaica*; antimicrobial resistance; STEC; VTEC

1. Introduction

In recent decades, the likelihood of wild ungulates coming into contact with humans or livestock has increased throughout Europe, mainly due to intensive game management (Gortazar et al. 2006) and large population increases in most European ungulate species (Apollonio et al. 2010). In addition, wild ungulates have been recognized as reservoirs of food- and vector-borne pathogens (Gortazar et al. 2007).

Some *Escherichia coli* strains are known to be etiologic agents of food-borne diseases, for example Shiga-toxin-producing *E. coli* (STEC) (Garcia et al. 2010). Miko et al. (2009) underline that wild animals and their meat are underestimated as reservoirs for STEC and as possible sources for human infections. The number of cases of human STEC infection in the European Union has increased for three consecutive years, with O157:H7 being the most frequently reported serotype associated with human disease (European Food Safety Authority 2012). This serotype is part of the gut microbiota of many animal species, and ruminants have been identified as a major reservoir, particularly cattle (Hussein 2007). Wildlife has also been connected to food-borne outbreaks of *E. coli* O157:H7, such as the case of feral swine grazing near a spinach field (Jay et al. 2007) or the black-tailed deer jerky (Keene et al. 1997).

Wild boars have been previously described as carriers of *E. coli* O157:H7 (Wahlstrom et al. 2003; Sanchez et al. 2010; Mora et al. 2012) and other STEC strains that are potential human pathogens (Miko et al. 2009; Wacheck et al. 2010). Furthermore, STEC has also been isolated from wild boar meat and meat products from Spain (Diaz-Sanchez et al. 2012). The carriage of *E. coli* O157:H7 has never been assessed specifically in the Iberian ibex, but a study on its close relative the alpine Ibex (*Capra ibex*) by Obwegeser et al. (2012) found a considerable prevalence of STEC (6 out of 27).

In this work, we carried out an intensive two-year sampling of both free-ranging game ungulates (wild boar – *Sus scrofa* - and Iberian ibex – *Capra pyrenaica*) and livestock herds sharing habitat in a game reserve in NE Spain in order to explore the presence of *E. coli* O157:H7 due to its zoonotic relevance. A previous study demonstrated the spill-over of *Salmonella enterica* (Mentaberre et al. 2013) between livestock and wild boars in the same area. Wild boar is a very abundant and popular game species in Europe and its meat is much appreciated, while the Iberian ibex is an endemic species of the Iberian Peninsula

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and is also greatly hunted and consumed. According to the official statistics, more than 222,000 wild boars and 5500 Iberian ibexes were hunted in Spain in 2012 (Ministerio de Agricultura, Alimentación y Medio Ambiente 2014).

2. Material and methods

2.1. Study area

The study area is located within the National Game Reserve and Natural Park 'Ports de Tortosa i Beseit' (NGR hereafter), in northeastern Spain. It is a calcareous mountain region with high topographical complexity that results in a rugged and abrupt terrain with numerous ravines and steep slopes. About 28% of the surface is above 1000 m above sea level, with the highest peak being Mont Caro (1442 m). The predominant habitat is pine grove (39%) followed by oak grove (15%). Wildlife and livestock share pastures in some canyons in the study area.

2.2. Animal sampling

2.2.1. Wild boar

In total, 117 individual fecal samples were obtained from hunter-harvested wild boars during the regular hunting season (October to January) from 2009 to 2011. The location of each hunting session was recorded, as well as the sex and age of each animal. Age was estimated based on tooth eruption pattern and replacement as well as dental attrition (Boitani & Mattei 1992). Feces were collected directly from the rectum and stored in sterile containers. They were refrigerated and sent to the laboratory within the subsequent 24 h.

2.2.2. Iberian ibex

A total of 160 Iberian ibexes were either hunter-harvested ($n = 130$), box-trapped ($n = 28$) or found sick ($n = 2$) from 2009 to 2011, and fecal samples were obtained directly from the rectum. Due to the characteristics of the hunting method, fecal samples had to be stored at −18 °C until being sent to the laboratory. However, samples collected from captured and sick animals were stored refrigerated (4 °C) and sent to the laboratory within 24 h after collection. *E. coli* are known for their cold-shock response (Phadtare 2004) and thus, we can assume that the isolation of this micro-organism from feces is not highly affected by storage at this temperature.

2.2.3. Free-ranging livestock

Fifty-nine samples from livestock were collected from 2010 to 2011. Previously, information had been obtained from the Reserve's managers on herd location. The farming conditions in the NGR are free-ranging (extensive) with supplemental feeding in the dry season (summer) and herds are small, which sometimes made it difficult to locate the animals (e.g., 60 animals in 1823 ha area). Fifty-five samples were obtained from cattle (5 herds, 380 animals in total) and four from the only horse herd in the NGR (32 animals). Livestock sampling was preferably performed on days that wild boars were also sampled. When livestock were located, animals were counted and observed until defecation. Then, feces were collected and stored in a sterile container and refrigerated until being sent to the laboratory within the subsequent 24 h.

2.3. Microbiological analyses

2.3.1. E. coli *O157:H7*

Fecal samples were processed according to the ISO 16.654:2001 protocol to obtain *E. coli* O157. This protocol applies immunomagnetic separation to select O157 positive *E. coli*. One suspected colony per sample was boiled in 600 μl of double-distilled water and confirmed by polymerase chain reaction (PCR) as *E. coli* O157:H7 as described previously (Desmarchelier et al. 1998; Heininger et al. 1999). In addition, a multiplex PCR targeting nine virulence factors characteristic of human pathotypes (virulence factors (VFs): stx_1, stx_2, *eae*, *ehx*A, heat-stable enterotoxin (ST), heat-labile enterotoxin (LT), *bfp*A, p*Inv* and *agg*R) was performed using all isolates as previously described (Cabal et al. 2013).

Pulsed field gel electrophoresis (PFGE) was performed with *Xba*I (Ribot et al. 2006) and DNA PFGE patterns were analyzed using the BioNumerics 6.5 software (Applied Math, St-Martens-Latem, Belgium).

2.3.2. Antimicrobial susceptiblity testing

Antimicrobial susceptibility was tested in all isolates by agar diffusion method to obtain the Inhibition Zone Diameter against amoxicillin-clavulanate, cefoxitin, amikacin, apramycin, imipenem and aztreonam while broth microdilution method was performed to determine the minimum inhibitory concentrations of sulfamethoxazole, gentamicin, ampicillin, ciprofloxacin, cefotaxime, ceftazidime, tetracycline, streptomycin, trimethoprim, chloramphenicol, florfenicol, kanamycin and nalidixic acid. The epidemiological cut-off values used were reported in Navarro-Gonzalez et al. 2013.

2.4. Statistical analysis

Prevalence and 95% CI of *E. coli* O157:H7 were calculated. The prevalence was compared between host species with Fisher's exact test and the significance level set at $\alpha = 0.05$. All statistical analyses were performed with R Software (R Development Core Team 2.14.0, 2011), specifically with package epiR for obtaining the 95% CI (Stevenson et al. 2012).

3. Results

Four wild boars out of 117 were positive for *E. coli* O157:H7 (3.41%, 95% CI = 0.94−8.52) and two Iberian ibexes out of 160 were positive (1.25%, 95% CI = 0.15−4.44), while this micro-organism was not isolated from any livestock sample (0%, 95% CI = 0−8.94). We detected no statistically significant difference among these host

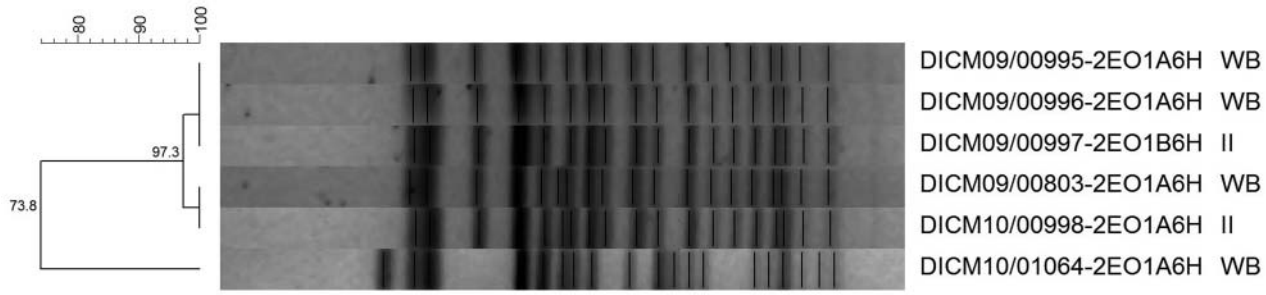

Figure 1. Pulse Field Gel Electrophoresis pattern of *E. coli* O157:H7 isolates from co-habiting wild boar (*Sus scrofa*) and Iberian ibex (*Capra pyrenaica*).
Note: WB: Wild boar. II: Iberian ibex.

groups (*P* values ranging from 0.24 to 1). All isolates were susceptible to the antimicrobial agents tested. With regards to VFs, all isolates were positive to *eae* and *ehxA*; and negative to ST, LT, *bfpA*, *agg*R and p*In*V (as expected for *E. coli* O157). Both isolates from Iberian ibex, as well as three isolates from wild boar were stx_1-negative and stx_2-positive. However, one isolate from wild boar was both stx_1 and stx_2-positive. This isolate showed a PFGE pattern (Figure 1) that distinguished it from the rest, which showed high similarity among them (97.3%).

4. Discussion

The prevalence of *E. coli* O157:H7 in wild boars found by us is within the range of what has been previously reported in this species in other parts of Spain: 3.3% (Sanchez et al. 2010) and 0.38% (Mora et al. 2012). Sanchez et al. (2010) found indistinguishable PFGE types in *E. coli* O157:H7 isolates from a wild boar and a human patient and Mora et al. (2012) reported similarities between STEC from wildlife and humans, data that strongly suggest that wild boar and other wild animals play a role in human infection. In our study, three out of the four *E. coli* O157:H7 positive wild boars were piglets. Since in our sample only 10 animals were piglets, this represents a high percentage of positivity among piglets (30%). Further research is needed to elucidate if the carriage of this micro-organism in wild boar is age-dependent. However, it has to be taken into account that these samples were likely not independent, since these piglets could have been siblings. The fact that these positive piglets came from the same canyon and were sampled within one month of each other suggests that there may have been a common source of contamination, though momentary in time and space. This can also explain the fact that no livestock sample was positive for *E. coli* O157:H7. Although we cannot rule out the carriage of this pathogen by free-ranging livestock of our study area, we can assure that prevalence is low (95% CI = 0−8.94). Opposite to our results, Branham et al. (2005) found *E. coli* O157 in livestock but not in co-habiting white-tailed deer. Previous studies conducted in the same area on the same species found a higher prevalence and spill-over of *Salmonella* and *Campylobacter*, particularly between cattle and wild boar (Navarro-Gonzalez et al. 2012, 2014a, 2014b). Interestingly, the dynamics of *E. coli* O157:H7 seems to be different with regards to both prevalence and interspecific transmission. Our results suggest that under these circumstances where *E. coli* O157:H7 is absent or at low prevalence in livestock, wild ungulates may not be important reservoirs for humans and other species.

Regarding the VFs, all strains carried stx_2, *ehx*A and *eae* in agreement with previous studies performed in wild boar (Jay et al. 2007; Sanchez et al. 2010). It seems that the detection of stx_1 alone or in combination with stx_2 is less frequent than the carriage of stx_2 alone. This fact may indicate that in Spain, O157:H7 from wild animals are similar to those of cattle regarding their VFs combination (Cabal et al. 2013). However, in other countries, stx_2 was more frequently detected than the other two patterns in cattle (Ateba & Mbewe 2011).

With regards to *E. coli* O157:H7 in Iberian ibex, the prevalence found by us is lower than what has been described in Alpine ibex as Obwegeser et al. (2012) reported 6 positive ibexes out of 27 and this means a prevalence of 22.2% (95% CI = 8.62−42.26). The causes underlying this fact are not known to us, but can be related to the carriage of this pathogen by sympatric animal populations, both domestic and wild. In our study area, Iberian ibex may be just reflecting the low prevalence of this pathogen in sympatric wild boar and livestock. Nevertheless, it has to be considered that meat of both wild boar and Iberian ibex is consumed by hunters and their families and carcasses are usually processed under insufficient hygienic conditions. Some practices like skinning and evisceration in the field or in private barns or garages may increase the risk of fecal contamination from the animal's intestines to meat cuts. Also, the habit of abandoning the offal in the field may enhance environmental contamination and transmission to other animal species, e.g. scavengers.

Out of the two *E. coli* O157:H7 positive Iberian ibexes, one animal was hunted and apparently healthy, but one was found sick and died within a few hours. This animal showed hemorrhagic enteritis (data not shown). We were not able to determine the cause of death due to the advanced autolysis of the organs, and so further research is needed to determine if this micro-organism can cause disease in Iberian ibex. Even if the prevalence detected was low, there could be an impact of this potential disease on the local population, as has been suggested with regards to *Salmonella* in this population (Navarro-Gonzalez et al. 2014b).

The susceptibility of all the *E. coli* O157:H7 isolates to all antimicrobial agents tested is good news for this natural environment. Oppositely, antimicrobial resistance has been found in *Salmonella* and indicator *E. coli* from wildlife and livestock, including resistance to fluoroquinolones and cephalosporines (Navarro-Gonzalez et al. 2012, 2013), which is of concern for public health. Cabal et al. (2013) reported high frequency of antimicrobial resistance in *E. coli* O157:H7 from healthy cattle in Spain. This suggests that the *E. coli* O157:H7 strains from wildlife might be genetically different to those in livestock, and that the sources of infection might be different for livestock and wildlife.

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Disclosure statement

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