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Short communication

Pheno-genotypic characterisation of *Escherichia coli* O157:H7 isolates from domestic and wild ruminants

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ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 represents a major public health concern worldwide, with ruminants recognised as their main natural reservoir. The aim of this work was to determine the phenotypic features and genetic relationships of 46 *E. coli* O157:H7 isolates obtained from sheep, cattle and deer faeces and from unpasteurised goat milk in Spain over a period of 11 years. Characterisation was performed by polymerase chain reaction (PCR), phage typing and pulsed-field gel electrophoresis (PFGE). An atypical *E. coli* O157:H7 strain (sorbitol-fermenting and β -glucuronidase positive) originating from deer faeces was detected. Genes encoding Shiga toxins were detected in 69.6% of isolates, all of them carrying only the *stx*₂ gene. The isolates were from nine different phage types, although 67.4% were restricted to only three: PT14, PT34 and PT54. PT54 was the most prevalent phage type and contained isolates from cattle, sheep and deer. Majority of the isolates were from phage types previously found in strains associated with human infection. XbaI-PFGE identified 33 different types and 11 groups of closely related types (more than 85% similarity), one of which included 21 (45.7%) isolates originating from different animal species, including deer. These results indicate common origin or inter-species spread of genetically similar *E. coli* O157:H7 isolates and contribute to earlier investigations identifying deer as a natural source of *E. coli* O157:H7. The study also highlights the emergence of phenotypic variants of *E. coli* O157:H7, which may not be identified by routine culture methods or by biochemical tests used to characterise serotype O157:H7.

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1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 represents a major public health concern worldwide. Human diseases ranging from mild diarrhoea to haemor-

rhagic colitis and the life-threatening haemolytic uraemic syndrome can be caused by STEC O157:H7, typically affecting children, the elderly and immunocompromised patients (Centers for Disease Control and Prevention, 2001). The pathogenic capacity of STEC resides in a number of virulence factors, including Shiga toxins (Stx1 and Stx2), intimin and the enterohaemolysin (Gyles, 2007). Unlike other *E. coli* strains, O157:H7 strains neither ferment sorbitol nor exhibit β -glucuronidase (GUD) activity after

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overnight incubation, and these differences facilitate the detection of this organism (Kehl, 2002).

Healthy domestic ruminants can harbour STEC O157:H7 in their faeces and are thus natural reservoirs of this pathogen (Rey et al., 2003; Blanco et al., 2004; Orden et al., 2008), although STEC O157:H7 strains have also been isolated from wild ruminants, especially from deer (Renter et al., 2001; García-Sánchez et al., 2007). Sources of human infection include undercooked meat, unpasteurised milk and dairy products, vegetables or water and contact with animal carriers or the environment (Gyles, 2007).

The aim of the current study was to characterise a collection of *E. coli* O157:H7 isolates obtained from domestic and free-ranging wild ruminants in Spain over a period of 11 years, with the objective of determining their phenotypic features and genetic relationships and therefore contributing to the knowledge of the epidemiology of this pathogen.

2. Materials and methods

2.1. *E. coli* O157:H7 isolates

A total of 46 *E. coli* O157:H7 isolates obtained from faeces of different healthy ruminants (e.g., sheep, extensive and beef cattle and red deer (*Cervus elaphus*)) and from unpasteurised goat milk over a period of 11 years (1997–2008) were included in the present study. All of them originated from the Extremadura region in the southwest of Spain and comprised (1) 24 isolates epidemiologically related in different groups (15 isolates from sheep, seven isolates from extensive cattle and two isolates from deer) and (2) 22 isolates not known to be epidemiologically related (14 isolates from sheep, four isolates from beef cattle, three isolates from deer and one isolate from unpasteurised goat milk). Epidemiologically related isolates were *E. coli* O157:H7 isolates obtained from different animals at a single sheep flock, cattle herd or game estate during the same periods. Some of the isolates included in the present study were obtained from previously published studies (Rey et al., 2003, 2006; García-Sánchez et al., 2007; Sánchez et al., 2009), and the procedures for their isolation are described in detail in the reports of those studies.

2.2. Biotyping, serotyping and phage typing

All *E. coli* O157:H7 isolates were confirmed biochemically as *E. coli* by the API 20E system (bioMérieux, Marcy L'Etoile, France). Fermentation of sorbitol and GUD activity were investigated on sorbitol MacConkey agar (Oxoid, Basingstoke, England) and Chromocult Coliform agar (Merck, Darmstadt, Germany), respectively, after overnight incubation at 37 °C.

The identification of O and H antigens was carried out as described by Guinée et al. (1981) using O157 antiserum and the full range of H antisera from H1 to H56. All antisera were absorbed with corresponding cross-reacting antigens to remove non-specific agglutinins. O157 antiserum was produced in the Laboratorio de Referencia de *E. coli* (Lugo, Spain) and H antisera were obtained from the Statens Serum Institut (Copenhagen, Denmark).

The phage typing was performed as described by Khakhria et al. (1990) in the Centro Nacional de Microbiología (Instituto de Salud Carlos III, Madrid, Spain) using phages provided by the National Microbiology Laboratory (Public Health Agency of Canada, Winnipeg, Canada). The 16 different phages used were capable of identifying 90 phage types.

2.3. PCR of *stx*₁, *stx*₂, *ehxA*, *eae*, O157 *rfbE* and *fliCh7* genes

All isolates were tested as previously described (Mora et al., 2004) with primers specific for the genes encoding Stx1 and Stx2 (*stx*₁ and *stx*₂), enterohaemolysin (*ehxA*), intimin (*eae* and *eae*- γ 1 variant), O157 antigen (O157 *rfbE*) and H7 antigen (*fliCh7*). Reference *E. coli* strains used as controls were EDL 933 (human, O157:H7, *stx*₁, *stx*₂, *eae*, *ehxA*) (ATCC No. 43895) and K12-185 (negative for *stx*₁, *stx*₂, *eae* and *ehxA* genes) (Blanco et al., 2004).

2.4. Pulsed-field gel electrophoresis (PFGE)

PFGE was performed in accordance with the PulseNet-Europe protocol (<http://www.pulsenet-europe.org/docs.htm>). Genomic DNA was digested with XbaI (Roche Diagnostics, Mannheim, Germany) and analysed in 1% agarose gels (Bio-Rad, Hemel Hempstead, United Kingdom) in 0.5 \times TBE buffer at 14 °C using the CHEF MAPPER system (Bio-Rad). The runtime was 21.3 h at 6 V cm⁻¹, with initial and final switch times of 2.16 and 54.17 s, respectively. The XbaI-digested DNA from *Salmonella enterica* Braenderup H9812 was used as a molecular size marker. The resultant images were analysed with the InfoQuestFP software (Bio-Rad). Isolates were allocated a different PFGE type when a genetic difference could be detected. Cluster analysis was performed using the Dice coefficient and the unweighted pair group method with arithmetic averages (UPGMA).

3. Results

3.1. Phenotypic properties and phage types

All but one of the isolates evaluated in the current study were biochemically typical of *E. coli* O157:H7 (non-sorbitol-fermenting and GUD negative) (Fig. 1). A single isolate, originating from deer faeces, fermented sorbitol and exhibited GUD activity after overnight incubation.

The 46 *E. coli* O157:H7 isolates were from a total of nine different phage types (Table 1). However, among those nine, three phage types accounted for 67.4% of isolates analysed: PT14 (four isolates), PT34 (three isolates) and PT54 (24 isolates). PT54 was the most prevalent phage type among both sheep (62.1%) and deer (80.0%) isolates and one of the most frequently found among cattle isolates (18.2%). Seven isolates reacted with typing phages but did not conform to a recognised pattern (RDNC/NT = reacts but does not conform/non-typeable).

Forty-three (93.5%) of 46 *E. coli* O157:H7 isolates expressed the H7 antigen and three (6.5%) were non-motile (NM). These NM isolates belonged to PT14 (one isolate) and PT34 (two isolates).

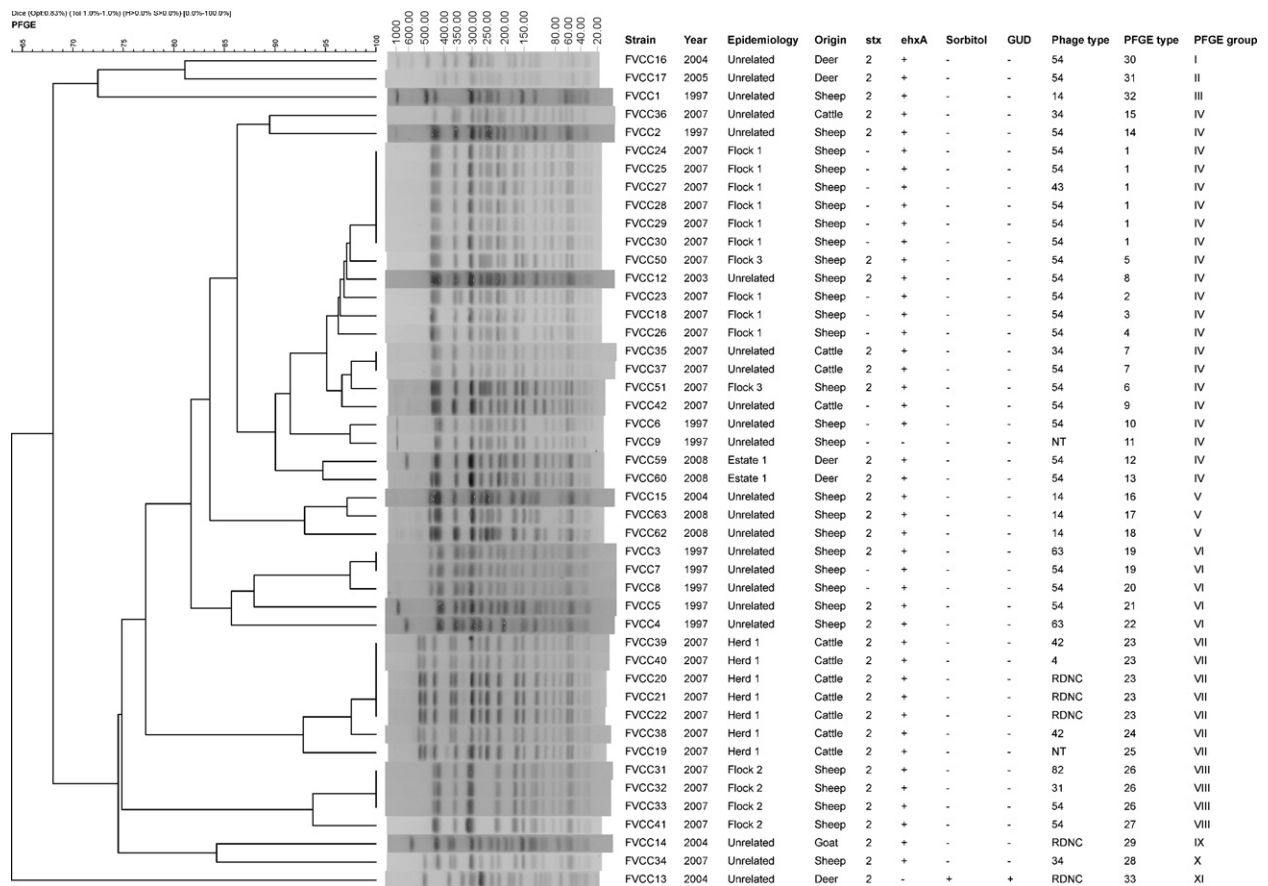


Fig. 1. XbaI-PFGE dendrogram based on the Dice coefficient indicating the genetic relatedness of the 46 *E. coli* O157:H7 isolates from domestic and wild ruminants. The scales at the top indicate the similarity indices (in percentages) and molecular sizes (in kilobases).

3.2. Gene detection: *stx*₁, *stx*₂, *ehxA*, *eae*, *O157 rfbE* and *fliCh7*

The PCR procedure indicated that all *E. coli* O157:H7 isolates carried the *eae*-γ1, *O157 rfbE* and *fliCh7* genes. In addition, genes encoding Shiga toxins were detected in 32 (69.6%) isolates, all of them carrying only the *stx*₂ gene, and 44 (95.7%) isolates contained the *ehxA* gene (Fig. 1).

3.3. PFGE types and cluster analysis

PFGE of XbaI-digested genomic DNA of the 46 *E. coli* O157:H7 isolates produced a dendrogram indicating 33 different PFGE types with 15–21 discernible fragments, ranging from approximately 33 to 1100 kb in molecular size (Fig. 1). Twenty-one types were identified among 29

Table 1
Phage types of *E. coli* O157:H7 isolates from different origins.

Phage type	No. of isolates				
	Total (n = 46)	Sheep faeces (n = 29)	Cattle faeces (n = 11)	Deer faeces (n = 5)	Goat milk (n = 1)
PT4	1	0	1	0	0
PT14	4	4	0	0	0
PT31	1	1	0	0	0
PT34	3	1	2	0	0
PT42	2	0	2	0	0
PT43	1	1	0	0	0
PT54	24	18	2	4	0
PT63	2	2	0	0	0
PT82	1	1	0	0	0
RDNC/NT ^a	7	1	4	1	1

^a Reacts but does not conform/non-typeable.

sheep isolates, six types in 11 cattle isolates and five types in five deer isolates, and no PFGE type contained isolates from more than one origin. However, these 46 isolates could be divided into 11 groups (I–XI, containing between 1 and 21 isolates per group) of closely related PFGE types (more than 85% similarity), according to the Dice coefficient of similarity (Fig. 1). Forty isolates (87.0%) were grouped in only five clusters (IV, V, VI, VII and VIII), and one of them (group IV) included 21 (45.7%) isolates originating from sheep, cattle and deer faeces, with PT54 being the predominant phage type in this cluster.

The major genetic relatedness was observed in four groups containing isolates with indistinguishable PFGE types (100% similarity): IV (six isolates epidemiologically related from sheep and two isolates not known to be epidemiologically related from cattle), VI (two isolates not known to be epidemiologically related from sheep), VII (five isolates epidemiologically related from cattle) and VIII (three isolates epidemiologically related from sheep). The most divergent PFGE type was that of the atypical *E. coli* O157:H7 isolate obtained from deer faeces, which was distantly related to the other typical isolates (less than 64% similarity) (Fig. 1).

4. Discussion

The recognition of STEC O157:H7 has been largely facilitated by the availability of classical microbiological diagnostic procedures that are based on the characteristic phenotypic features of this pathogen, in particular, its inability to ferment sorbitol and lack of GUD activity after overnight incubation. However, other phenotypic variants of STEC O157 have been isolated during the past decade in Germany (Ammon et al., 1999), the Czech Republic (Bielaszewska et al., 1998), Finland (Saari et al., 2001), Italy (Bonardi et al., 1999), the United States (Hayes et al., 1995), Australia (Bettelheim et al., 2002) and Japan (Nagano et al., 2002). To our knowledge, the atypical STEC O157:H7 strain (sorbitol-fermenting and GUD positive) originating from deer faeces detected in the present study is the first phenotypic variant isolated in Spain and the first one originating from ruminants other than cattle in Europe, since such variants have been isolated before from deer in Japan and the United States (Dunn et al., 2004; Nagano et al., 2004). This atypical STEC O157:H7 strain was distantly related to the other typical strains (less than 64% similarity) (Fig. 1). Similarly, GUD-positive STEC O157:H7 strains isolated in Japan between 1996 and 2001, including human and deer strains, belonged to a single cluster only distantly related to the other typical STEC O157:H7 strains (less than 60% similarity by PFGE) (Nagano et al., 2002, 2004). Based on these findings, the authors suggested that such phenotypic variants may represent a distinct clone within STEC serogroup O157.

At least 90 phage types have been currently reported for STEC O157:H7 (Ahmed et al., 2001), but only seven of these (PT2, PT4, PT8, PT14, PT21/28, PT32 and PT54) account for the majority (more than 75%) of human strains isolated in Europe and Canada. Three of these phage types (PT4, PT14 and PT54) were identified in the present study, and they accounted for 63.0% of the isolates. PT54 was the most prevalent phage type and contained isolates from cattle,

sheep and deer. In fact, PT54 was the most prevalent phage type among isolates from deer. To our knowledge, this is the first report of the phage typing of STEC O157:H7 strains isolated from deer in the literature.

Fourteen (30.4%) *E. coli* O157:H7 isolates were *stx*-negative, although all of them carried genes encoding other virulence-associated factors (enterohaemolysin and/or intimin). Whether this finding resulted from the loss of *stx* gene(s) from initially *stx*-positive strains remains unclear. However, whilst the human health risks associated with *stx*-negative *E. coli* O157:H7 strains may be less significant, the acquisition of *stx*-carrying phages from the environment cannot be excluded (Muniesa et al., 1999).

Among the 46 *E. coli* O157:H7 isolates characterised in the current study, no PFGE type contained isolates from more than one origin, findings in contrast to those of Avery et al. (2004) and Mora et al. (2004), with some of the PFGE types containing *E. coli* O157:H7 isolates from more than one origin, even though none of the samples were known to be epidemiologically related. Nevertheless, the PFGE group IV in the present study included closely related isolates (more than 85% similarity) obtained from different animal species, including deer, over a period of 11 years (isolated in 1997, 2003, 2004, 2007 and 2008) with and without known epidemiological links (Fig. 1). On the one hand, these results indicate common origin or inter-species spread of genetically similar *E. coli* O157:H7 isolates. On the other hand, indistinguishable or closely related PFGE types were found in isolates recovered from samples from different animals at a single sheep flock, cattle herd or game estate during the same periods (Fig. 1). This high similarity suggests the existence of horizontal transmission among animals, which has been demonstrated to be important in maintaining *E. coli* O157:H7 infections on farms (Faith et al., 1996).

In conclusion, the current study contributes to the earlier investigations identifying deer as a natural source of *E. coli* O157:H7 and reports the isolation of a sorbitol-fermenting and GUD-positive strain from deer. These data highlight the emergence of phenotypic variants of *E. coli* O157:H7, which may not be identified by routine culture methods or by biochemical tests used to characterise serotype O157:H7. The study reports that the most common phage type among strains isolated from deer is also common among human strains, supporting the idea that ruminants are a principal reservoir. The current study also shows the natural occurrence of many genetic variants among *E. coli* O157:H7 isolated from domestic and wild ruminants in Spain but indicate common origin or inter-species spread of genetically similar isolates.

Conflict of interest statement

None of the authors of this paper have a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the article.

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