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Molecular characterization and antimicrobial resistance of STEC strains isolated from healthy cattle in 2011 and 2013 in Spain

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SUMMARY

Prevention of Shiga toxin-producing *Escherichia coli* (STEC) foodborne outbreaks is hampered by its complex epidemiology. We assessed the distribution of virulence genes (VGs), main serogroups/serotypes for public health [haemolytic uraemic syndrome (HUS)-related], antimicrobial resistance (AMR) profiles and pulsed-field gel electrophoresis (PFGE) patterns in a collection of STEC isolates obtained from cattle hide ($n = 149$) and faecal ($n = 406$) samples collected during a national survey conducted in Spain in 2011 and 2013. Isolates were cultured using McConkey and CT-SMAC agar after enrichment, and confirmed as STEC by PCR. STEC prevalence in hides (15·4%) was higher than in faeces (10·7%) and O157:H7 was more frequent in the former (2·7% *vs.* 0·99%). Non-O157 HUS-related serogroups were present albeit at low frequencies. The non-O157 isolates were more heterogeneous than O157:H7 in their VG patterns, with 25/64 presenting VGs from both STEC and enterotoxigenic pathotypes (hybrid isolates). Of the STEC isolates, 62·5% were resistant at least to one antimicrobial, and no differences in AMR between O157:H7 and non-O157 were detected. All isolates had different profiles by PFGE and did not form a cluster. Overall, our results demonstrated that STEC in the cattle reservoir is still a matter of concern for human health due to the presence of HUS-related serogroups, the occurrence of certain VGs, AMR and the additional risks that hybrid isolates may pose, and thus warrants further investigation.

Key words: Molecular epidemiology, Shiga-like toxin-producing *E. coli*, public health, pulsed-field gel electrophoresis (PFGE), zoonotic foodborne diseases.

INTRODUCTION

Escherichia coli are commensal bacteria that belong to the intestinal microbiota of warm-blooded mammals [1]. Of the six intestinal *E. coli* pathotypes for humans, the enterohaemorrhagic *E. coli* (EHEC), considered a subgroup within the Shiga toxin-producing *E. coli* (STEC), is the only one for which an animal reservoir (ruminants, mostly cattle) has been identified [2]. Infection with STEC strains can occur through the consumption of undercooked meat, contaminated water and even contact with another infected individual [1].

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In Europe, 3657 cases of disease due to STEC infection were reported in 2010 (1 detected case/100 000 inhabitants) [3]. However, in 2011 this number increased to 9487 STEC cases (2·58 cases/100 000 inhabitants), mostly due to the German outbreak caused by a hybrid STEC-enteroaggregative pathotype [4]. Since then, rates of STEC cases have remained at higher levels than before the outbreak (at 1·59 and 1·56 cases/100 000 inhabitants in 2013 and 2014, respectively) [5, 6]. To characterize STEC strains, certain specific virulence genes (VGs) of this pathotype may be targeted, including the Shiga toxin genes *stx1* and *stx2*, the intimin gene *eae*, and the EHEC-haemolysin or *ehx*A gene [1]. Altogether, VGs contribute to the pathogenic potential of STEC strains, although not all STEC are able to produce disease in humans [7]. Additionally, some STEC strains can carry more VGs typically associated with other pathotypes as demonstrated by the 2011 German outbreak [4]. A higher expression of VGs, particularly in *stx2*-positive strains, has been associated with a higher pathogenic potential for humans [8]. The O157:H7 serotype has been typically associated with haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS). However, other non-O157 serogroups have also been associated with human disease and an increase in its incidence has been observed in recent years [9]. The most important serogroups have been referred to as the 'big six' non-O157 STEC (O26, O103, O45, O111, O121, O145) [10, 11], but other non-O157 STEC serogroups have also been identified as pathogenic agents in some outbreaks, including O91, O113 [12, 13] and more recently O104 in the German outbreak [4]. Although surveillance for the presence of STEC in food animals is currently performed in several countries, according to EFSA, most European countries are focused on the detection of the STEC O157:H7 serotype, while information on the distribution of other serogroups of importance (e.g. the 'big six') is scarcer [3].

Even though use of antimicrobials in STEC infections is controversial, it may be required in certain cases, and the presence of genes associated with multidrug-resistant (MDR) phenotypes in STEC strains can compromise its success [14, 15]. Given the potential of domestic ruminants to act as reservoirs of pathogenic STEC, the increase in antimicrobial resistance (AMR) observed in livestock in the last two decades (attributed to the extensive use of antimicrobials in veterinary medicine [16, 17]) is being carefully monitored.

Here, we analysed a collection of *E. coli* isolates obtained through a nationwide sampling performed in domestic cattle in Spain in two years (2011 and 2013) in an attempt to (i) establish the prevalence of STEC using hide and faecal samples of cattle origin, (ii) identify the most important serogroups/serotypes in STEC isolates from cattle origin, (iii) assess the presence of VGs of interest in STEC isolates from O157 and non-O157 serogroups, and (iv) determine the AMR profiles and the genetic relationships in the STEC collection by pulsed-field gel electrophoresis (PFGE), evaluating potential associations between carriage of VGs and presentation of AMR.

MATERIALS AND METHODS

Sampling and isolation

The sampling strategy was performed according to the literature [18], i.e. collecting faecal and hide samples in healthy beef cattle at slaughter (age 1–2 years) in Spain in the context of the Spanish monitoring programme on zoonoses and AMR in compliance with Council Directive 99/2003/EC. Fifteen (2011) and 19 (2013) slaughterhouses were selected so that they would represent more than 50% of the slaughtering capacity in the country. Sampled animals were subjected to routine pre- and post-mortem inspections and no clinical/pathological signs were noted in any case. Animals from which samples originated came from 337 farms located in 11 different regions. In total, 406 faecal samples and 149 hide swabs were collected in 2011 and 2013, respectively. All samples were collected at the post-mortem veterinary inspection points by one of the authors (C.B.). Samples were processed upon arrival at the laboratory in the following 24 h, as described previously [19]. Briefly, samples were processed in pools composed of faeces of two animals ($n = 203$ pooled faecal samples) belonging to the same farm (one pooled sample/farm). After a non-selective enrichment in buffered peptone water (18 h/37 °C), the aggregated sample was streaked in McConkey and CT-SMAC agar using a sterile swab and plates were incubated for 24 h at 37 °C. The bacterial mass was then tested using a polymerase chain reaction (PCR) for Shiga toxin detection genes (*stx1*/*stx2*). Shiga toxin-positive plates were divided in five parts that were subcultured in the same agar medium. After incubation the PCR was repeated on bacterial growth recovered from all five plates and if one of them was positive 10 colonies/plate were subcultured and analysed again using the same PCR for *stx* confirmation. When a colony was positive for *stx1, stx2* or both, the isolate

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was classified as STEC. In the case of hides, the protocol was essentially the same but only one hide sample per animal and farm was tested.

Characterization of STEC isolates

Isolates confirmed as STEC were tested for the presence of nine VGs (*stx1, stx2, eae, ehx*A, *agg*R, *bfp*A, *inv*A, *est, elt*) using real-time PCRs as described previously [18, 20]. In addition, four O157:H7 serotype-related genes (wzx_{O104}, $fliC_{H4}$, rfb_{O157}, $fliC_{H7}$) and eight non-O157 serogroup-related genes (wzy_{O91}, wzx_{O26}, wzx_{O111}, wzx_{O103}, wzx_{O145}, wzx_{O113}, wzx_{O45}, wzx_{O121}), also found in association with HUS, were investigated using multiplex real-time PCR and conventional PCR, respectively, as described previously [18, 20, 21].

STEC isolates were also tested for AMR against 14 agents (ciprofloxacin, CIP; sulfamethoxazole, SMX; gentamicin, GEN; ampicillin, AMP; cefotaxime, FOT; ceftazidime, TAZ; tetracycline, TET; streptomycin, STR; trimethoprim, TMP; chloramphenicol, CHL; florfenicol, FFN; kanamycin, KAN; nalidixic acid, NAL; colistin, COL) by broth microdilution. For interpretation of the results, epidemiological cut-offs from the European Committee on Antimicrobial Susceptiblity Testing (EUCAST) were applied. The profile of resistances per isolate (resistotype) and the total number of antimicrobials to which an isolate was resistant were recorded for further analysis.

Isolates were further characterized using PFGE as described previously [22] using the *Xba*I restriction endonuclease. A *Salmonella* strain (H9812, serotype Braenderup) was included as standard in three lanes within each gel. Profiles were analysed using Bionumerics software v. 6.6 (Applied Maths, Belgium) and a phylogenetic tree was generated using the Dice coefficient. Isolates belonged to the same PFGE cluster if they presented not more than one band difference with the *Xba*I restriction.

Statistical analysis

Prevalence of STEC in all sampled animals was calculated per year. In 2011 prevalence was estimated to account for the pooling factor, and assessment of differences in the prevalence between years of study was performed based on overlapping of 95% confidence intervals (CIs).

The proportion of isolates harbouring different VGs and serotype-related genes in STEC isolates and the total number of resistances against the most important antimicrobial agents (CIP, SMX, GENT, AMP, TET, STR, TMP, CLOR, FFC, KAN, NAL) per isolate were calculated in each of the years of the study, and the association between the presence of VGs, serotype-related genes and resistotypes was assessed using Pearson's χ^2 and Fisher's exact tests, while the association of those with total number of resistances was performed using Kruskal–Wallis and Mann–Whitney *U* tests. All the statistical calculations were performed using SPSS v. 20 (IBM Corp., USA), WINPEPI (PEPI-for-Windows) v. 11.35 and R v. 3.2.1 (https://www.r-project.org/) software.

RESULTS

Using the described methods for isolation and confirmation of STEC, in 2011 a total of 41 STEC isolates were recovered from 203 pooled faecal samples (10·7%, 95% CI 7·7–14·2) collected from 15 slaughterhouses. In 2013, 23 STEC isolates were detected from 149 hide samples (15·4%, 95% CI 10·0–22·3) collected from 19 slaughterhouses. No significant differences in the prevalence recorded each year were observed.

Four (9·8%) of the 41 STEC isolates cultured in 2011 belonged to the O157:H7 serotype (prevalence 0·99%, 95% CI 0·27–2·52) and in 2013 four (17·4%) of the 23 STEC isolates were O157:H7 (prevalence 2·70%, 95% CI 0·70–6·70%); no significant differences in the annual prevalence of O157:H7 were detected (overlapping of 95% CIs). Some isolates belonging to the non-O157 serogroups under study (two O26, two O111, one O91, one O145, one O104) were detected sporadically in both years of study. The only wzx_{O104}-positive isolate was further identified as O104:H7. The proportion of positive samples belonging to each of the alternative serogroups did not vary significantly depending on the year ($P > 0{\cdot}05$). $fliC_{H4}$ and the remaining serogroup-related genes (wzx_{O121}, wzx_{45}, wzx_{O103}, wzx_{O113}, wzx_{O121}) were not found. In total, 49 isolates did not belong to any of the serogroups tested.

Of the VGs under study, *ehx*A ($n = 56$, 87·5%), *stx2* ($n = 46$, 71·88%), *eae* ($n = 44$, 68·75%), *stx1* ($n = 40$, 62·5%), *est* ($n = 25$, 39·06%) and *bfp*A ($n = 1$, 1·56%) were the only ones detected in the STEC collection (Table 1). Significant differences in the prevalence of positive samples between years for each VG were only found for *ehx*A (2011, 95·2%; 2013, 66·7%; $P = 0{\cdot}003$).

The most frequent pattern of VGs were *stx2* and *stx1*/*stx2* (Table 2). Of the O157:H7 isolates, 7/8 isolates showed the *stx1*/*stx2*/*eae*/*ehx*A pattern. The non-O157 group was more heterogeneous, with only

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Table 1. *Proportion of virulence genes (VGs) in O157 and non-O157* Escherichia coli *isolates recovered from cattle samples collected in 2011 (faeces, n = 406) and 2013 (hides, n = 149)*

	O157:H7 ($N = 8$)			Non-O157 ($N = 56$)		
VG	2011 ($n = 4$)	2013 ($n = 4$)	Total	2011 ($n = 37$)	2013 ($n = 19$)	Total
stx1	3	4	7 (87·5%)	19	14	33 (58·9%)
stx2	4	4	8 (100%)	23	13	36 (64·3%)
eae	4	4	8 (100%)	27	9	36 (64·3%)
*ehx*A	4	4	8 (100%)	36	11	47 (83·9%)
est (ST)	0	0	0 (0%)	21	4	25 (44·6%)
*bfp*A	0	0	0 (0%)	0	1	1 (1·8%)

6/56 strains sharing the same genotype (*stx1*/*stx2*/*eae*/*ehx*A). The *est* gene was only found in non-O157 isolates ($n = 25$, 39·1%) (Table 2) while the prevalence of *eae* was significantly higher in the O157:H7 group ($P = 0·045$).

Regarding the AMR profiles, 24 (37·5%) STEC isolates did not present resistance to any of the antimicrobials tested (2011, $n = 17$; 2013, $n = 7$) (Tables 3 and 4). Of the resistant isolates, the amount of overall AMR (number of antimicrobials to which the bacteria was resistant) ranged from 1 to 9, with those against SMX (62·5%), STR (57·8%), TET (57·8%) and TMP (50%) being the most frequent. Only three isolates presented resistance to nine antimicrobials. In total, 21 different resistotypes were identified. The resistotype including SMX, TET, STR, TMP, CLOR and FFC resistance was the most frequent ($n = 6$, 15%), followed by SMX-TET-STR-TMP ($n = 5$, 12·5%) and SMX-AMP-TET-STR-TMP ($n = 4$, 10%). All the other resistotypes were found in only 1–2 isolates.

Overall 66% of the non-O157 isolates were resistant to at least one antimicrobial, and the maximum number of AMRs in a single isolate was nine, compared to 50% for O157:H7 isolates and up to six AMRs. No significant differences in the total number of AMRs or the proportion of isolates resistant to each of the antimicrobials between O157:H7 and non-O157 isolates were observed. Three O157:H7 presented resistotypes that were not found in the rest of the STEC isolates (SMX/SMX, TET, STR, TMP, KAN/SMX, AMP, TET, STR, TMP, KAN). The number of AMRs was higher in isolates belonging to serogroups O111, O104, O91 and O26 than in the other non-O157 strains. The O111 and O104 isolates were resistant to seven antimicrobials (including CIP); followed by O91 ($n = 6$) with O26 presenting the lowest number of resistances ($n = 3$).

The presence of *stx2* was significantly associated with resistance to SMX, TET, STR and TMP ($P < 0·025$), and with the total number of resistances (median number of AMRs in *stx2*-negative isolates was 0 compared to 5 when this VG was present) ($P = 0·014$). The presence of *est* was also associated with an increased probability of harbouring a resistance against certain antimicrobials (SMX, TET, STR, TMP, CLOR, FFC; $P < 0·05$) and with a higher total number of AMRs ($P < 0·01$). Twenty of the 25 *est*-positive STEC were resistant to at least three antimicrobials. Significant differences in the proportion of isolates harbouring a particular VG and the observed AMR profiles were only observed for *stx1* ($P < 0·01$) and rfb_{O157} ($P = 0·04$) (Table 4). None of the other VGs was associated with a particular AMR pattern.

In the PFGE analysis the maximum similarity observed between isolates was 89·5% with a large variability in isolates belonging to the same serogroup and showing the same VG pattern (Fig. 1). None of the isolates formed a cluster. The highest similarity in isolates with a known serotype was observed in O157:H7 isolates ($n = 8$, 82·4%), followed by 73·7% represented by an O111 isolate. Isolates from the same serogroup were in general more closely related (Fig. 1).

DICUSSION

We analysed a collection of STEC isolates obtained through a sampling strategy with a national coverage in two separate years in an attempt to evaluate the prevalence of STEC in general and of serogroups of relevance for public health in particular, and compared these isolates in terms of VG profiles, AMR occurrence and PFGE profiles.

Sampling was conducted using different matrixes (hides and faeces) in the two years of study, which could be a source of variation of the results obtained in each of the years. Traditionally, faeces have been the sample of choice for STEC detection in cattle

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Table 2. *Year of isolation, and presence of virulence and serotype genes in each recovered isolate*

Iso	Year	*stx1*	*stx2*	*eae*	*ehx*A	*est*	*bfp*A	rfb_{O157}	$fliC_{H7}$	wzx_{O26}	wzx_{O111}	wzy_{O91}	wzx_{O145}	wzx_{O104}
1	2011	1	1	1	1	0	0	1	1	0	0	0	0	0
2	2011	1	0	1	1	0	0	0	0	0	0	0	0	0
3	2011	1	0	1	1	0	0	0	0	0	0	0	0	0
4	2011	1	0	1	1	0	0	0	0	0	0	0	0	0
5	2011	0	1	1	1	0	0	0	0	0	0	0	0	0
6	2011	1	0	0	1	1	0	0	0	0	0	0	0	0
7	2011	0	1	1	1	0	0	0	0	0	0	0	0	0
8	2011	1	1	1	1	0	0	1	1	0	0	0	0	0
9	2011	1	1	1	1	0	0	1	1	0	0	0	0	0
10	2011	1	0	1	1	0	0	0	0	0	0	0	0	0
11	2011	0	1	0	1	1	0	0	0	0	0	0	0	0
12	2011	1	0	1	1	0	0	0	0	0	0	0	0	0
13	2011	1	0	1	1	0	0	0	0	0	0	0	0	0
14	2011	1	0	1	1	1	0	0	0	0	0	0	0	0
15	2011	1	0	1	1	1	0	0	0	0	0	0	1	0
16	2011	1	0	0	1	1	0	0	0	0	0	0	0	0
17	2011	1	0	1	1	0	0	0	0	0	0	0	0	0
18	2013	1	1	1	1	0	0	1	1	0	0	0	0	0
19	2013	1	1	0	0	0	0	0	0	0	0	0	0	0
20	2013	1	0	1	1	0	0	0	0	1	0	0	0	0
21	2013	1	1	0	1	0	0	0	0	0	0	0	0	0
22	2013	1	0	1	1	0	0	0	0	0	0	0	0	0
23	2013	1	1	1	1	0	0	0	1	0	0	0	0	0
24	2013	1	1	0	1	0	0	0	0	0	0	0	0	0
25	2011	0	1	1	1	0	0	1	1	0	0	0	0	0
26	2011	1	1	1	1	0	0	0	0	0	0	0	0	0
27	2013	0	1	1	0	1	0	0	0	0	0	0	0	0
28	2011	1	0	1	1	0	0	0	0	0	1	0	0	0
29	2013	1	0	1	1	0	0	0	0	1	0	0	0	0
30	2011	1	0	0	1	1	0	0	0	0	0	0	0	0
31	2011	0	1	1	1	1	0	0	0	0	0	0	0	0
32	2011	0	1	1	1	1	0	0	0	0	0	0	0	0
33	2013	1	1	0	0	0	0	0	1	0	0	0	0	0
34	2013	0	1	1	1	0	0	0	0	0	0	0	0	0
35	2013	1	1	1	1	0	0	1	1	0	0	0	0	0
36	2011	0	1	1	1	1	0	0	0	0	0	0	0	0
37	2011	0	1	1	1	0	0	0	0	0	0	0	0	0
38	2011	0	1	0	0	0	0	0	0	0	0	0	0	0
39	2011	0	1	0	1	1	0	0	0	0	0	0	0	0

Table 2 (*cont.*)

Iso	Year	*stx1*	*stx2*	*eae*	*ehx*A	*est*	*bfp*A	rfb_{O157}	$fliC_{H7}$	wzx_{O26}	wzx_{O111}	wzy_{O91}	wzx_{O145}	wzx_{O104}
40	2011	0	1	0	1	1	0	0	0	0	0	0	0	0
41	2011	0	1	1	1	1	0	0	0	0	0	0	0	0
42	2011	0	1	1	1	1	0	0	0	0	0	1	0	0
43	2013	0	1	0	0	1	0	0	0	0	0	0	0	0
44	2011	0	1	1	1	0	0	0	0	0	0	0	0	0
45	2013	1	0	0	1	1	0	0	0	0	0	0	0	0
46	2011	0	1	1	1	1	0	0	0	0	0	0	0	0
47	2011	1	1	1	1	1	0	0	0	0	0	0	0	0
48	2011	0	1	1	1	1	0	0	0	0	0	0	0	0
49	2013	0	1	1	0	0	1	0	0	0	0	0	0	0
50	2013	1	1	1	1	0	0	1	1	0	0	0	0	0
51	2011	0	1	1	1	1	0	0	0	0	0	0	0	0
52	2013	1	1	1	1	0	0	1	1	0	0	0	0	0
53	2013	0	1	0	1	0	0	0	0	0	0	0	0	0
54	2011	0	1	0	1	1	0	0	0	0	0	0	0	0
55	2011	1	1	1	1	0	0	0	0	0	0	0	0	0
56	2013	1	1	1	0	0	0	0	1	0	0	0	0	0
57	2013	1	0	0	0	1	0	0	0	0	0	0	0	0
58	2013	1	1	1	1	0	0	0	1	0	0	0	0	0
59	2011	0	1	0	1	1	0	0	0	0	0	0	0	0
60	2013	1	0	0	1	0	0	0	1	0	0	0	0	1
61	2013	1	1	0	0	0	0	0	1	0	0	0	0	0
62	2011	1	1	1	1	0	0	0	0	0	1	0	0	0
63	2011	1	0	1	1	1	0	0	0	0	0	0	0	0
64	2011	1	1	0	1	1	0	0	0	0	0	0	0	0
Total		40 (62·5%)	44 (68·8%)	44 (68·8%)	55 (85·9%)	25 (39·1%)	1 (1·6%)	8 (12·5%)	15 (23·4%)	2 (3·1%)	2 (3·1%)	1 (1·6%)	1 (1·6%)	1 (1·6%)

Iso, Isolate ID.
Key for VGs and serogroups: 0, absence; 1, presence.

Table 3. *Antimicrobial resistance values, concentration and breakpoints used for each antimicrobial and percentage of resistant isolates*

Antimicrobial	Abbreviation	AM range (mg/l)	Cut-off*	NR	%R
Ampicillin	AMP	0·5–32	8	45	29·6
Cefotaxime	FOT, CTX, CEFOT	0·06–4	0·25	64	0
Ceftazidime	TAZ, CAZ	0·25–16	0·5	64	0
Ciprofloxacin	CIP	0·008–8	0·064	59	7·8
Nalidixic acid	NAL	4–64	16	59	7·8
Gentamicin	GEN, GENT	0·25–32	2	57	10·9
Kanamycin	KAN	4–128	8	58	9·4
Streptomycin	STR	2–128	16	27	57·8
Chloramphenicol	CHL, CLOR	2–64	16	42	34·4
Florfenicol	FFN, FFC	2–64	16	52	18·8
Tetracycline	TET	1–64	8	27	57·8
Sulfamethoxazole	SMX	8–1024	64	24	62·5
Trimethoprim	TMP	0·5–32	2	32	50
Colistin	COL	2–4	2	64	0

NR, Number of non-resistant isolates; %R, percentage of resistance isolates.
* EUCAST (accessed 23 December 2015).

and higher prevalence values for samples obtained from faeces than from hides have been reported [23]. However, hides have recently been described as an important source of STEC [24]. In our study, the proportion of positive samples was higher when hides were used, although no significant differences in the prevalence of STEC or O157:H7 between years were observed in this study, depending on sample type. Still, comparison of the results is difficult due to the lack of parallel use of both matrixes.

Prevalence of STEC in our faecal samples was lower compared to the literature [25, 26]. These differences may be attributed to the use of different sampling units (slaughter batch, herd or single animals), the matrix used (faeces, hides, carcasses, ear) and the laboratory protocol [3]. The same applies to O157:H7, with our study reporting lower prevalence compared to previous reports [23, 24]. In this case the difference could be due to the different methodology used, since the majority of previous studies were based on inmunomagnetic separation for detection of O157:H7 strains, increasing diagnostic sensitivity [24]. Interestingly, our prevalence for some of the non-O157 HUS-related serogroups in this national study was lower compared to that reported by other authors [27–29]. Ekiri *et al.* and Dewsbury *et al.* reported serogroup O103 as the most frequently detected in cattle faeces although we did not find any O103 in our isolates [27, 28]. In contrast, Stromberg *et al.* reported serogroup O145 as the most frequent in hides [29], while we only detected 1·6% for this serogroup.

The *stx1*/*stx2* and *stx2* were the most abundant VGs in the STEC isolates recovered in this study for both O157:H7 and non-O157 strains (Tables 1 and 2) in agreement with previous reports [17, 30]. Moreover, the *eae* gene was present not only in O157:H7 but also in non- O157 isolates, including O26, O111, O191, O104 and O145 isolates, as reported by other authors [17, 31]. The high prevalence of *ehx*A in the faecally derived isolates could represent an additional risk since *ehx*A-positive strains have been associated with an increased probability of HUS [32], especially when combined with *stx2*/*eae* [33].

Lack of detection of *agg*R, *elt* and *inv*A was not surprising since these VGs are characteristic of pathotypes typically not associated with cattle [1, 34]. In contrast, the high frequency (31·3%) of resistant *est*-positive isolates, especially in isolates recovered from faeces, was unexpected since this VG is not typically found in STEC strains [34]. In agreement with our findings other authors have recently reported STEC/ETEC hybrids in humans (1%) and cattle (14%), although none of them belonged to the 'big six' serogroups and only three showed AMR to one antimicrobial (ampicillin) [35]. In a previous study performed in indicator *E. coli* isolated from faeces of Spanish cattle in 2009, a lower percentage of *est*-positive isolates was reported (<2% *vs.* 31·3% in this study) [20]. The increased prevalence of *E. coli* isolates carrying *est* in STEC isolates compared to indicator strains, particularly in serogroups of importance for public health (O145 and O91), highlights a potential

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Table 4. *Total number of resistances and antimicrobial resistance (AMR) patterns (resistotypes) found for each of the isolates tested*

Isolate	Resistotype*	AMR
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
7	0	0
8	0	0
9	0	0
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0
15	0	0
16	0	0
17	0	0
18	0	0
19	0	0
20	0	0
21	0	0
22	0	0
23	0	0
24	0	0
25	1 000 000 000	1
26	1 000 100 000	2
27	1 001 010 000	3
28	1 001 100 000	3
29	1 001 100 000	3
30	1 001 110 000	4
31	1 001 110 000	4
32	1 001 110 000	4
33	1 001 110 000	4
34	1 001 110 000	4
35	1 001 110 010	5
36	1 001 111 000	5
37	1 001 111 000	5
38	1 001 111 100	6
39	1 001 111 100	6
40	1 001 111 100	6
41	1 001 111 100	6
42	1 001 111 100	6
43	1 001 111 100	6
44	1 010 100 000	3
45	1 011 101 100	6
46	1 011 110 000	5
47	1 011 110 000	5
48	1 011 110 000	5
49	1 011 110 000	5
50	1 011 110 010	6
51	1 011 111 000	6
52	1 011 111 000	6
53	1 111 001 000	5
54	1 111 101 010	7
55	1 111 111 010	8
56	1 111 111 010	8
57	1 111 111 100	8
58	1 111 111 100	8
59	1 111 111 110	9
60	11 001 111 001	7
61	11 001 111 001	7
62	11 011 110 001	7
63	11 011 111 101	9
64	11 011 111 101	9

* AMR for each resistotype is shown in the following order: CIP-SMX-GENT-AMP-TET-STR-TMP-CLOR-FF-C-KAN-NAL. FOT, TAZ and COL were not used in the concatenation for obtaining each resistotype as all isolates were susceptible to these antimicrobials.
† Amount of overall AMR for each isolate.

risk given the severity of other *E. coli* hybrids reported since 2011 [4, 36–38]. Those hybrid strains could be the product of horizontal gene transfer events occurring in an animal carrying both pathotypes, as already described in calves suffering from colibacillosis [39]. In addition, a *stx2*/*bfp*A/*ehx*A STEC was also found, suggesting a possible hybrid EPEC/STEC strain. This is particularly interesting since animal EPEC strains usually lack the *bfp*A gene [1].

In general, the most prevalent AMRs (SMX, STR, TET, TMP) in our STEC strains were in agreement with those reported previously [17], but the percentage (50%) of O157:H7 isolates presenting at least one AMR was higher [40, 41]. In contrast with previous studies that found a lower proportion of AMR in O157:H7 isolates [17, 41], we did not find differences in the proportion of AMR present in O157 and non-O157 strains. However, these results should be interpreted with caution due to the limited number of O157:H7 isolates recovered in this study. Interestingly, the proportion of O157:H7 isolates resistant to AMP, STR, KAN, CHL, TET, SMX and TMP out of the 14 antimicrobials tested in the study was lower than that found in a collection of O157:H7 strains recovered from cattle in Spain in 2009, although this difference was not significant [18].

Of the non-O157 isolates, O104:H7 presented a high number of resistances ($n = 6$) and harboured *stx1* and *ehx*A. This rare serotype did not share the same flagellar antigen with the German outbreak strain. It had been detected previously in humans and cattle, but it did not harbour Shiga toxin genes [42, 43].

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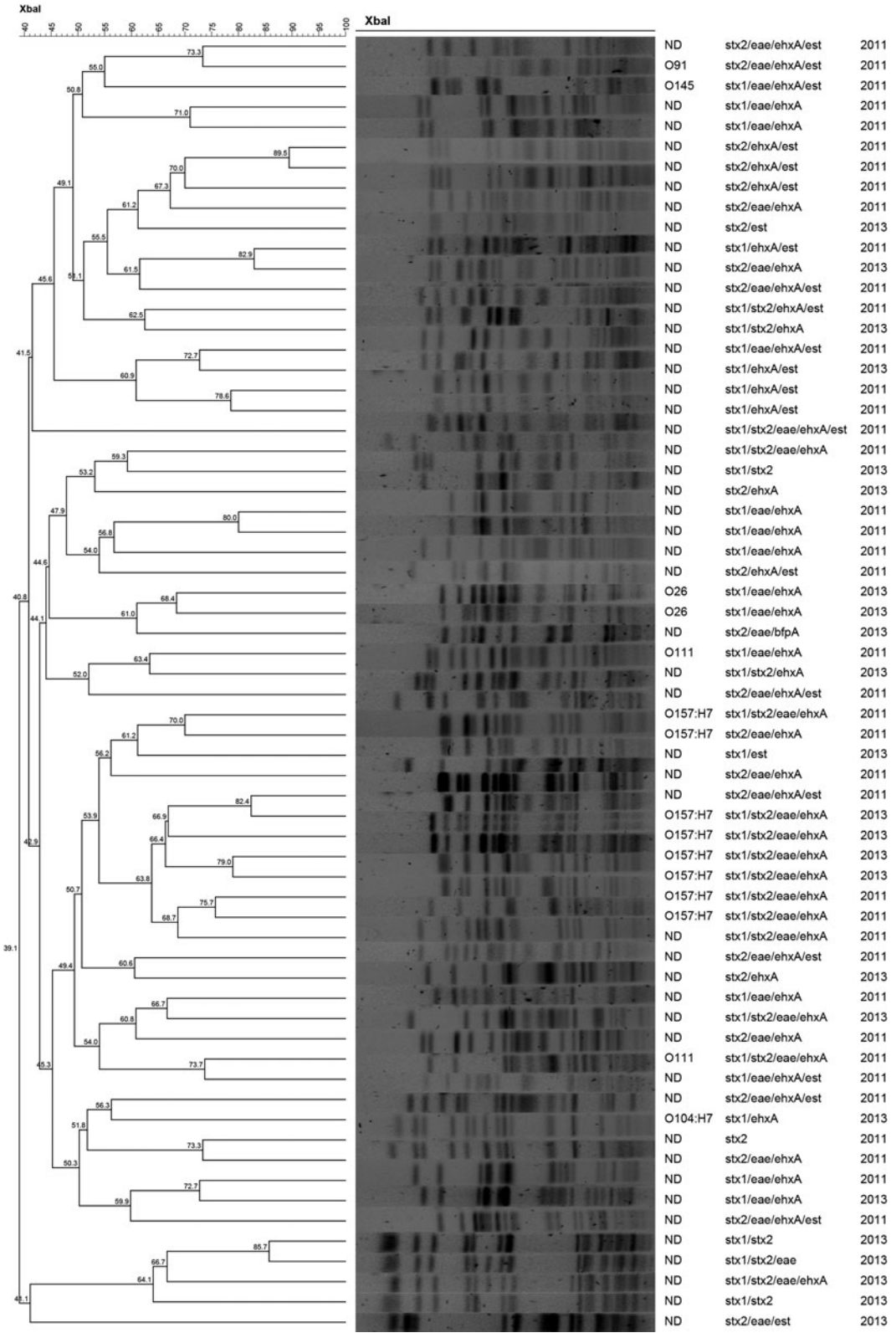

Fig. 1. PFGE patterns for the analysed isolates. Figure shows a dendrogram representing the degree of similarity (%) in all STEC isolates. For each isolate, the year of the sampling, its virulence gene pattern and its serogroup/serotype are also indicated. Isolates belonging to an unknown serogroup/serotype are identified as 'ND' (not determined).

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In contrast with other studies in which an association between STEC VGs and AMR was not found, we showed a relationship between the presence of *stx2* and AMR and between *est* and AMR [44]. In addition, and although AMR prevalence was in general low, the association between certain VGs and AMR highlights the importance of monitoring the situation in the cattle reservoir to prevent the emergence of MDR strains carrying VGs.

Of all molecular typing-based methodologies, PFGE remains the gold standard for the study of disease outbreaks and other epidemiological research [10]. The use of PFGE in this study revealed a high genetic diversity in STEC isolates as expected, given the lack of any epidemiological relationship between them, although it was even higher than that reported in cattle *E. coli* strains obtained in similar conditions in other studies [45].

In conclusion, our study demonstrates that the presence of STEC in healthy cattle remains a potential risk for public health, since both O157:H7 and other serogroups of relevance were found in a representative sample of cattle in Spain in two separate years. The high frequency of STEC/ETEC hybrid isolates confirms once again the plasticity of the *E. coli* genome, particularly for non-O157 isolates. This should be taken into consideration in future surveillance and genomic studies since it adds complexity to the current scenario, and could be missed by routine surveillance studies based on detection of typical STEC VGs. New technologies such as next-generation sequencing would help to elucidate the genetic relatedness of these isolates with the established pathotypes and its proximity to human pathogenic strains.

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DECLARATION OF INTEREST

None.

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