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Shiga toxin-producing *Escherichia coli* O157, O26 and O111 in cattle faeces and hides in Italy

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ABSTRACT

Introduction: Ruminants are regarded as the natural reservoir for Shiga toxin-producing *Escherichia coli* (STEC), especially of serogroup O157.

Materials and methods: During 2011 and 2012, 320 samples (160 faecal samples from the rectum and 160 hide samples from the brisket area) were collected from 160 cattle at slaughter in Northern Italy during warm months (May to October). Cattle were reared in different farms and their age at slaughter ranged between nine months and 15 years, most of them being culled cattle (median age: six years; average age: 4.6 years). Samples were tested by immunomagnetic-separation technique for *E coli* O157 and O26 and by a screening PCR for *stx* genes followed by cultural detection of STEC. The virulence genes *stx1*, *stx2*, *eae*, and *e-hlyA* were detected and among stx2-positive isolates the presence of the stx2a and stx2c variants was investigated.

Results: Twenty-one of 160 cattle (13.1 per cent; 95 per cent CI 8.3 to 19.4 per cent) were found to be faecal carriers of STEC. STEC O157 was found in 10 (6.3 per cent) samples, STEC O26 in six (3.8 per cent) and STEC O111 in one (0.6 per cent). Four isolates (2.5 per cent) were O not determined (OND). Six out of 160 (3.8 per cent; 95 per cent CI 1.4 to 8.0 per cent) hide samples were positive for STEC; four hides (2.5 per cent) were contaminated by STEC O157 and two (1.3 per cent) by STEC O26. In three cattle (1.9 per cent) STEC from both faeces and hides were detected. Among STEC O157, 87.5 per cent of them carried the *stx2c* gene and 12.5 per cent carried both *stx1* and *stx2c* genes. No O157 isolate harboured stx2a variant. STEC O26 and O111 carried the *stx1* gene only. One OND strain carried both the *stx2a* and *stx2c* genes.

Conclusions: This study shows that STEC O157 from cattle can harbour the stx2c variant, which is associated with haemolytic uraemic syndrome in humans, and that cattle hides may be a source of human pathogenic STEC O157 and O26 in the slaughterhouse environment.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) are foodborne pathogens that cause different clinical illnesses in humans, ranging from uncomplicated non-bloody diarrhoea to severe diseases such as haemorrhagic colitis (HC) and the life-threatening haemolytic uraemic syndrome (HUS) (Karmali and others 2010). The main virulence characteristic of STEC resides in the production of Shiga toxins 1 and 2 (Stx1, Stx2) encoded by lambdoid phages integrated in the bacterial chromosome (Melton-Celsa and O'Brien 1998). Toxins bind to the globotriaosylceramide (Gb3) receptor on the target endothelial cells, are internalised and their A subunit fragment causes inhibition in protein synthesis leading to cell death (O'Brien and others 1992, Monnens and others 1998). Different subtypes of Stx1 and Stx2 have been described, and several epidemiological studies have documented that Stx2 is more often associated with severe human disease than Stx1 (Boerlin and others 1999). Among the different Stx2 variants, Stx2a and Stx2c are more often associated with HUS than other subtypes (Eklund and others 2002, Friedrich and others 2002, Caprioli and others 2005, Persson and others 2007).

Additionally, most strains associated with severe human disease possess a pathogenicity island termed the locus of enterocyte effacement (LEE), encoding the adhesin intimin and its translocated intimin receptor (Tir), that enable the intimate attachment of virulent STEC to enterocytes (Nataro and Kaper 1998). Besides these two genes, the LEE encodes components of a type III secretion system and translocated effector molecules that are responsible for the 'attaching and effacing' (AE) histopathological lesion in the intestinal epithelium (Nataro and Kaper 1998, Caron and others 2006). Another virulence marker for most STEC is a plasmid-determined gene named *ehly-A*, encoding for an enterohaemolysin. This haemolysin can be detected phenotypically on specialised

media (as EHLY-agar) but PCR detection of the *ehly-A* gene is generally employed (EFSA 2007).

Ruminants are regarded as the natural reservoir for STEC (Chapman and others 1997, Blanco and others 2003, Karmali and others 2010). Cattle are the most important source of human infections with STEC O157, as they are asymptomatic carriers of the microorganism which is a transient member of their common gut micro flora (Caprioli and others 2005). Unlike O157 STEC, some non-O157 STEC such as STEC O5, O26, O111 and O118 can be isolated from calves with diarrhoea (Dorn and others 1993, Wieler and others 1996). STEC human infections may be either foodborne or waterborne, following ingestion of food and water contaminated by faeces of ruminants, or may be traced back to direct contact with infected animals or person-to-person transmission (Duffy and others 2014). Foods of bovine origin, especially undercooked ground beef, have been responsible for nearly 75 per cent of human STEC O157 outbreaks in the USA (Vugia and others 2007). In EU member states, except for Portugal, a notification rate of 1.15 cases/100,000 population was recorded in 2012, for a total of 5671 confirmed cases, with increasing trends during 2008–2012 observed in different countries, including Italy. In Europe, serogroups O157 and O26 were the most prevalent, representing 56 and 12 per cent of the STEC isolates, respectively. However, in Italy, STEC O26 was the most commonly isolated serogroup in 2012, being responsible for 40 per cent of confirmed human cases, followed by STEC O157 and STEC O111, isolated from 33 and 12 per cent of cases, respectively (EFSA and ECDC 2014).

In EU member states, data on STEC in cattle and beef products are poor. As reported by the European Food and Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC), only a few countries have been monitoring STEC in cattle and beef meat products, with low isolation rates (1.3 per cent of positive samples for STEC and 0.1 per cent for STEC O157, respectively) and low levels of microorganisms (EFSA and ECDC 2014).

Different studies have demonstrated that cattle hides are an important source of carcase contamination at slaughter and therefore STEC persistence on hides is of great concern (Arthur and others 2004). Cattle can acquire hide contamination not only in the farm environment, but also during transportation and following arrival at the slaughter plant when they move through common holding areas (lairage). Reduction of cattle hide contamination by pathogenic bacteria, including STEC, is therefore essential, and one of the most effective strategies is probably a pre-harvest antimicrobial intervention at the farm to reduce contamination in both the lairage and slaughterhouse environments (Arthur and others 2011).

The aim of this study was to investigate the role of cattle at slaughter as faecal shedders of STEC O157 and O26 in Northern Italy, and to investigate the contamination of cattle hides which can be an important source of these pathogens in the slaughterhouse environment. STEC strains isolated from cattle faeces and hides were tested for the main virulence genes (*stx1, stx2, eae, ehly-A*) and, among the Stx2-positive isolates, the distribution of Stx2a and Stx2c subtypes was investigated.

MATERIALS AND METHODS

Samples collection

During the years 2011 and 2012, faecal and hide samples were collected from 160 cattle at lairage in one abattoir in Northern Italy. Cattle were reared in 160 different dairy herds located in the Lombardy region; their age at slaughter ranged between 9 and 15 years, most of them being culled cattle (median age: six years; average age: 4.6 years). In detail, six animals (3.75 per cent) were less than 12 months of age, 12 (7.5 per cent) were between 13 and 24 months, and 142 (88.75 per cent) were over 24 months. For each animal, the date of birth and type of transport (single or mixed with other cattle from the same herd) were recorded. The cattle were transported a relatively short distance (maximum 60 km) and held in lairage for no more than six hours.

A total of 80 animals were sampled each year. Samples were collected during the warmer months (from May to October), when STEC shedding rate by cattle increases (Caprioli and others 2005). Faecal samples were collected from the rectum by using a sterile glove and stored in sterile containers. Sample size could vary from 10 g to approximately 30 g, depending on the fullness of the rectum. Hide samples were collected by swabbing 100 cm^2 of the brisket area using a sterile sponge moistened with 10 ml of sterile BPW (Oxoid, Basingstoke, UK) and placed in sterile bags. We selected the brisket area instead of elsewhere on the body because of its distance from the rectum, thus reducing the possibility of local faecal contamination from the rectum, while increasing the possibility of contamination by direct contact of the brisket with the ground and floors during farming, transport and resting at lairage. Moreover, the brisket hide is cut during dehiding at slaughter and represents a likely source of contamination of the carcase (EFSA 2007). All samples were stored under refrigeration conditions and sent to the laboratory on the day of collection.

Microbiological analyses

Faecal samples (10 g) and brisket sponges were added to 90 ml of enrichment broth (BPW; Oxoid), incubated at 41.5±1°C for 18–20 hours, and tested for *Escherichia* coli O157 by ISO 16654:2001 method (ISO, 2001) and *E coli* O26 by the immunomagnetic separation (IMS) technique by using Dynabeads anti-*E coli* O157 or EPEC/STEC O26 (Invitrogen Dynal AS, Oslo, Norway), following the manufacturer's instructions. After O157 IMS, the beads–bacteria complexes were resuspended in 100 µl of phosphate buffered saline (PBS, Oxoid) and

duplicate 50 µl volumes were streaked on Cefixime-Tellurite Sorbitol MacConkey Agar (CT-SMAC; Oxoid) for *E coli* O157 detection. After O26 IMS, 50 µl of the resuspended beads were plated on Cefixime-Tellurite Rhamnose MacConkey Agar (CT-RMAC; Oxoid) and 50 µl on MacConkey agar (Oxoid). Plates were incubated at 37°C±1°C for 18–24 hours. When present, up to 10 sorbitol non-fermenting colourless colonies grown on CT-SMAC, rhamnose non-fermenting colourless colonies grown on CT-RMAC or pink colonies grown on MacConkey agar were selected and tested for indole production. Up to 10 colonies per medium were selected, if available. Indole-positive cultures were subjected to slide agglutination with *E coli* O157 Latex test kit (Oxoid) and *E coli* O26 antiserum (Denka Seiken, Tokyo, Japan). Agglutinating cultures were confirmed biochemically as *E coli* by using the API 20 E system (bioMérieux, Marcy l'Etoile, France). The *stx*, *eae* and *ehlyA* status of confirmed *E coli* isolates was determined by PCR as described below.

PCR screening of enrichment cultures and confirmation of single colonies

The BPW enrichment cultures of faeces and sponges were also screened by PCR to detect the *stx1* and *stx2* genes with primer pairs described by Karch and Meyer (1989). DNA extraction for the PCR screening was performed by boiling 1 ml of the enrichment broth cultures for 10 minutes. After boiling, centrifugation at 14,000 rpm for five minutes was performed and the supernatant was transferred to a sterile tube. The *stx*-positive cultures were plated onto MacConkey agar and up to 50 *E coli*-resembling colonies were screened for indole production; indole-positive colonies were pooled and tested by PCR for the *eae*, *stx1*, and *stx2* genes and the STEC plasmid-harboured e-*hlyA* genes (Paton and Paton 1998). *E coli* selected colonies were then resuspended in 500 µl of sterile water before boiling for 10 minutes. After boiling, centrifugation at 14,000 rpm for five minutes was performed and the supernatant was used as the template for PCR. All single colonies included in the *stx1*- and/or *stx2*-positive pools were tested individually to obtain pure STEC cultures.

E coli O157 and O26 strains isolated by IMS were also subjected to PCR for the identification of virulence genes (Paton and Paton 1998).

In each PCR assay a positive control (EDL933 *E coli* strain) and a negative control (sterile distilled water) were used.

Detection of stx2 subtypes

The genes encoding for the two toxin variants Stx2a and Stx2c were detected in the *stx2*-positive isolates according to the subtyping protocol described by Scheutz and others (2012). Strains ED 747 (*stx1a*- and *stx2a*-positive) and ED 748 (*stx2b*- and *stx2c*-positive) were used as positive controls.

Confirmation of STEC isolates

Finally STEC isolates were sent to the European Union Reference Laboratory-verotoxigenic *Escherichia coli* (VTEC) (Istituto Superiore di Sanità, Rome, Italy) for serogroup and virulence gene confirmation. Serogroups of the isolated strains were determined by slide agglutination. The isolates were initially tested with pool antisera (OK pool 1: anti-O157, O26, O103, O145, O111; OK pool 2: anti-O55, O119, O125, O127, O128; OK pool 3 anti-O86, O114, O121, O126, O142; Statens Serum Institut, Copenhagen, Denmark) and in case of a positive reaction the single antisera composing the pool were tested separately. For *eae*-negative strains, slide agglutination was conducted with single antisera (O91, O113, O104, O127, O128, O146, Statens Serum Institut). In the absence of a positive reaction with one of the antisera assayed the O-group was defined as 'O not determined' (OND).

The presence of *eae*, *stx1* and *stx2* genes was assessed by real-time PCR, by using primers and probes described in the literature (Nielsen and Andersen 2003, Perelle and others 2004).

RESULTS

Based on isolation of STEC, 24 of 160 cattle (15.0 per cent; 95 per cent CI 10.0 to 21.0 per cent) were found to be positive (Table 1). A total of 21 cattle were faecal shedders of the microorganisms and six were positive on their hide, three of them being concurrent STEC faecal carriers. Comprehensively, 27 STEC isolates were detected in the faecal and hide samples.

Regarding the isolation methods, the screening PCR for *stx1* and *stx2* genes found 48 positive samples, but only 17 (35 per cent) tested positive by the subsequent cultural detection (Table 2). The IMS technique for STEC O157 and O26 found 22 positive samples, all of them confirmed by biochemical identification of the isolates.

As shown in Table 2, 48/320 samples (15.0 per cent) were positive for Stxs by screening PCR; 34/160 (21.3 per cent) were faecal samples and 14/160 (8.8 per cent) were hide samples. Among the 34 PCR-positive faecal samples, STEC were isolated from 21 samples (62 per cent); 10 by O157 IMS, six O26 IMS, one O111 STEC, and four OND STEC by plating PCR-positive broths and testing of individual colonies. STEC were not isolated from 13/34 (38 per cent) PCR-positive faecal samples. Of the 14 positive hide samples, six (43 per cent) yielded either O157 STEC (n=4) or O26 STEC (n=2). STEC were not isolated from eight (57 per cent) of the PCR-positive hide samples, neither by IMS nor by culturing PCR-positive broths. Based on isolation rates, 24/160 cattle (15.0 per cent) were STEC-positive, 21/160 (13.1 per cent; 95 per cent CI 8.3 to 19.4 per cent) being faecal shedders and 6/160 (3.8 per cent; 95 per cent CI 1.4 to 8.0 per cent) having hide contamination. STEC were isolated from both faecal and hides samples of 3/160 (1.9 per cent) animals, and only from hides of 3/160 (1.9 per cent) animals.

TABLE 1: Serogroups, isolation method and virulence factors of STEC isolated from faeces and hides of cattle at slaughter, with age of animals and mode of transportation to the abattoir

	Faecal samples						Hide samples					
Age	**Serogroup**	**Virulence factors**					**Serogroup**	**Virulence factors**				**Transport**
18 months	O157*†			*stx2c*	*eae*	*e-hlyA*						Mixed
23 months	OND†		*stx2a*									Mixed
2 years							O157*		*stx2c*	*eae*	*e-hlyA*	Mixed
3 years	O157*			*stx2c*	*eae*	*e-hlyA*						Mixed
3 years	O157*†	*stx1*		*stx2c*	*eae*	*e-hlyA*						Mixed
3 years	O157*	*stx1*		*stx2c*	*eae*	*e-hlyA*						Mixed
3 years	O157*			*stx2c*	*eae*	*e-hlyA*	O157*†		*stx2c*	*eae*	*e-hlyA*	Mixed
4 years	O26*†	*stx1*			*eae*	*e-hlyA*						Mixed
4 years							O26*†	*stx1*		*eae*	*e-hlyA*	Mixed
4 years							O157*†		*stx2c*	*eae*	*e-hlyA*	Mixed
4 years	O157*†			*stx2c*	*eae*	*e-hlyA*						Mixed
4 years	O26*	*stx1*			*eae*	*e-hlyA*						Single
4 years	OND†		*stx2a*									Mixed
4 years	OND†		*stx2a*	*stx2c*	*eae*	*e-hlyA*						Mixed
5 years	O26*	*stx1*			*eae*	*e-hlyA*						Mixed
5 years	O157*			*stx2c*	*eae*	*e-hlyA*						Mixed
5 years	O111†	*stx1*			*eae*	*e-hlyA*						Mixed
6 years	O26*†	*stx1*			*eae*	*e-hlyA*						Single
7 years	O26*	*stx1*			*eae*	*e-hlyA*	O26*†	*stx1*		*eae*	*e-hlyA*	Mixed
7 years	O26*†	*stx1*			*eae*	*e-hlyA*						Mixed
7 years	O157*†			*stx2c*	*eae*	*e-hlyA*						Single
7 years	OND†	*stx1*	*stx2a*									Mixed
7 years	O157*			*stx2c*	*eae*	*e-hlyA*	O157*		*stx2c*	*eae*	*e-hlyA*	Mixed
15 years	O157*†			*stx2c*	*eae*	*e-hlyA*						Mixed

*IMS=immunomagnetic separation with O157 or O26 magnetic beads
†Screening PCR=screening of enrichment broths for Stx1 and Stx2 and isolation of STEC from PCR-positive broths by plating and testing of 50 colonies per sample
OND=O serogroup not determined with OKO antisera for common pathogenic *Escherichia coli*
IMS, immunomagnetic separation; STEC, Shiga toxin-producing *Escherichia coli*

All STEC isolates belonging to serogroups O157, O26 and O111 possessed the *eae* gene encoding the intimin adhesin responsible for the 'AE' colonisation mechanism, whereas three out of four OND isolates tested negative for the presence of the *eae* gene. The majority of STEC O157 possessed the *stx2* gene (85.7 per cent) alone and two STEC O157 (14.3 per cent) harboured both *stx1* and *stx2* genes. All STEC O26 isolates were *stx1*-positive. A total of 18 STEC isolates were *stx2*- positive; they were represented by 14 STEC O157 and four OND isolates. As shown in Table 1, all O157 isolates carried the *stx2c* gene and no one harboured the *stx2a* variant. One STEC OND isolate carried both the *stx2a* and *stx2c* sequences, while the three *E coli* OND *eae*-negative isolates harboured the *stx2a* variant alone (two isolates) or the *stx1* and *stx2a* genes (one isolate).

In our study, the faecal carriers ranged from 18 months to 15 years of age, but most of them (18/21; 86 per cent) were three to seven years old (median age: seven years). No faecal shedders were found among

TABLE 2: Results of screening PCR and confirmatory tests to detect presumptive STEC positive samples in cattle faeces and hides

	PCR screening on enrichment cultures					Number of *E coli* O157 and O26 detected by IMS (%)	
		Number of samples with STEC isolation (%)					
Samples	**N° of samples stx1/stx2-positive (%)**	**O157**	**O26**	**O111**	**OND**	**O157**	**O26**
Cattle faeces (N=160)	34 (21.3)	5 (3.1)	3 (1.9)	1 (0.6)	4 (2.5)	10 (6.3)	6 (3.8)
Cattle hides (N=160)	14 (8.8)	2 (1.3)	2 (1.3)	–	–	4 (2.5)	2 (1.3)

IMS, immunomagnetic separation; STEC, Shiga toxin-producing *Escherichia coli*

cattle younger than 12 months, two (2/12; 16.7 per cent) were identified in cattle aged 13–24 months, and 19 (19/142; 13.4 per cent) were found in cattle over two years.

Regarding the type of transport to the slaughterhouse, 151 cattle (94 per cent) were transported together with other cattle from the same herd (mixed transport) and nine (6 per cent) were transported alone to the slaughterhouse (single transport). A total of 21 out of 24 STEC-positive cattle were transported mixed and three animals (12.5 per cent) were transported singly. All hide-positive cattle were transported together with other animals, while faecal-positive cattle were transported both mixed (18/21; 85.7 per cent) and singly (3/21; 14.3 per cent).

DISCUSSION

Monitoring of ruminants is essential to evaluate risk factors associated with STEC infection in humans. According to the EFSA, faeces, hides and pre-chilled carcases are the best samples to monitor STEC at slaughter and to compare data among countries (EFSA 2007). Our study highlights the occurrence of the most important human STEC serogroups in cattle at slaughter in Northern Italy and, to the best of our knowledge, this is the first study concerning the contamination of cattle hide by STEC O157 and O26 in our country.

The carriage of STEC O157, O26 and O111 by cattle at slaughter was studied by different authors (Jenkins and others 2003, Pearce and others 2006, Joris and others 2011, Ekiri and others 2014). In the current study, 13.1 per cent of the rectal samples collected during warmer months (May–October) were positive for STEC by both IMS and screening PCR followed by confirmatory tests. Prevalence of STEC O157, O26, O111 and OND were 6.3, 3.8, 0.6 and 2.5 per cent, respectively. Comparing these results with previous findings, the faecal carriage of STEC O157 by cattle at slaughter in Northern Italy is higher than in 2003–2005 (3.3 per cent) but lower than in 1997–1998 when 16.1 per cent of dairy cull cows and 16.6 per cent of feedlot cattle harboured STEC O157 in their faeces (Bonardi and others 1999, 2007). In contrast, the faecal prevalence of STEC O26 observed in this study is higher than that reported in a previous study (0.5 per cent) (Bonardi and others 2007), thus suggesting an increasing circulation of this serogroup among Italian dairy cattle. The detection of STEC O26 in the faecal matter of slaughtered cattle is of the greatest importance, because in Italy for more than 10 years this serogroup has surpassed STEC O157 as the major cause of HC and HUS in paediatric patients (Tozzi and others 2003). Furthermore, STEC O26 is the second most commonly reported serogroup in EU patients after O157 (EFSA and ECDC 2014).

The detection of STEC O111 in rectal samples of dairy cattle is very interesting, even though it is at a very low prevalence (0.5 per cent). Our study was focused mainly on the IMS detection of STEC O157 and O26, responsible for most human infections, and other STEC (O111 and OND isolates) were found by culturing *stx*-positive enrichment broths. Nevertheless, despite considerable efforts, STEC strains could be isolated from a minority (35 per cent) of PCR-positive cultures only. This fact should be considered when discussing the prevalence of STEC other than O157 and O26 found in this study, which are probably underestimated.

The O111 serogroup is not common in slaughtered cattle and many studies reported negative findings (Jenkins and others 2003, Pearce and others 2004, 2006, Thomas and others 2012). In Belgium, STEC O111 was detected in male cattle faeces (0.5 per cent), but not in samples from female animals (Joris and others 2011). A recent Swiss study based on the application of a real-time PCR system for the detection of STEC O26, O103, O111, O145 and O157 in cattle faeces found a very low proportion (0.8 per cent) of STEC O111-positive samples (Hofer and others 2012). According to the EFSA and ECDC (2014), in EU countries STEC O111 was reported in 1.9 per cent of serogroup-confirmed human cases only, but in Italy it was responsible for 11.6 per cent of the human cases, being the third most frequently detected serogroup following O26 (39.5 per cent) and O157 (32.6 per cent).

In the USA the prevalence of STEC O157 in cattle faeces ranged between 10 and 28 per cent (Karmali and others 2010). In the EU, the prevalence of STEC was observed mainly in animals at slaughter. Data reported from Austria indicate the highest prevalence, showing 32.1 per cent of the tested cattle, over two years old, and 35.7 per cent of the young cattle (one to two years old), positive for *eae*-negative STEC belonged to the O15, O22, O39, O43, O46, O91, O113, O168, O178 and O179 serogroups. One (1.8 per cent) *eae*-positive STEC O129 was isolated from an adult animal. Among young cattle the prevalence of STEC O157 and STEC O103 in faeces was 1.8 per cent each and the prevalence of STEC O26 was 5.4 per cent. The analytical method used in Austria was able to detect several STEC serogroups, while in contrast the other European member states investigated STEC O157 only. In Denmark, 8.4 per cent of faecal samples were found to be positive for STEC O157; in Sweden, Italy and Finland, prevalence rates of 3.1, 1.8 and 1.7 per cent were reported, respectively. In Estonia, hide samples were used in accordance with EFSA's STEC monitoring specifications (EFSA 2007) and a prevalence of 5.3 per cent of STEC O157 positive samples was recorded (EFSA and ECDC 2014).

Apart from faeces, the occurrence of human pathogenic STEC on cattle hide is a particular food safety concern, as the nature of hide removal at slaughter may facilitate the transfer of the microorganisms to the underlying sterile carcase tissue (Baird and others 2006). In particular, the brisket area is frequently reported as posing the highest risk of contamination

with faecal bacteria during the slaughtering process (Reid and others 2002, EFSA 2007, Thomas and others 2012). Nevertheless, in a recent study the PFGE (pulsed-field gel electrophoresis) profiles of STEC O157 hide and carcase isolates were different from each other, thus demonstrating that the hide might not have been the most important source of carcase contamination at slaughter (Thomas and others 2012).

The hide prevalence observed in the current study (2.5 per cent for STEC O157 and 1.3 per cent for STEC O26) is generally lower than those reported by other authors. As we swabbed 100 cm^2 of the brisket area, the reduced dimension of the tested area could have influenced the final result. For example, in the USA Kalchayanand and others (2009) swabbed approximately 1000 cm^2 of different sites of cattle hides at a processing plant and found prevalence rates ranging from 76.0 per cent (left shoulder area) to 78.7 per cent (belly area). Similarly, the detection and enumeration of *E coli* O157: H7 on beef cattle hides at slaughter was performed by swabbing 1000 cm^2 of the brisket plate area (Brichta-Harhay and others 2007); a very high proportion (46.9 per cent) of *E coli* O157:H7-contaminated hides, in comparison with faecal samples (16.7 per cent), was found. The enumeration of the pathogen detected a median value of 8.0×10 colony forming units (cfu)/100 cm^2 in the hide samples and 1.6×10^3 cfu/g in the faecal samples, with 9.8×10^3 cfu/100 cm^2 and 5.7×10^6 cfu/g as maximum values, respectively. In a previous study, Elder and others (2000) found that 28 per cent of faecal samples and 11 per cent of hide samples (collected by swabbing approximately 450 cm^2 of the ventral brisket) from cattle at slaughter during summer months were contaminated by *E coli* O157:H7.

In Europe, a study recently performed in Ireland detected STEC O157 in 1.7 per cent of cattle faeces and 13.4 per cent of brisket hides, and STEC O26 in 1.5 per cent of faeces and 0.2 per cent of hides. The swabbing area was 100 cm^2 (Thomas and others 2012). A previous Irish study observed a prevalence of 7.3 per cent for STEC O157 on cattle hide (O'Brien and others 2005). In Sweden, a national monitoring study tested faecal and ear hide samples of cattle at slaughter, observing prevalence rates for STEC O157 of 3.4 and 12.0 per cent, respectively. Ear samples showed less variation in prevalence than faecal samples among age groups of cattle and seasons, thus being useful to assess STEC contamination at slaughter (Boqvist and others 2009).

In the current study, the most frequently observed STEC O157 virulence profile included *stx2c*, *eae* and *e-hlyA* (12/14 isolates; 85.7 per cent), followed by *stx1*, *stx2c*, *eae* and *e-hlyA* (2/14; 14.3 per cent). All *stx2*-positive *E coli* O157 isolates of our study corresponded to *stx2*c variant and no one harboured *stx2a* variant. Our data are in accordance with Lefebvre and others (2009), who tested *stx2*-positive bovine isolates in Canada for *stx2a* and *stx2c* subtypes and found they harboured *stx2c* variant only. In a recent study, only *stx2c* and *stx2d* variants were detected among *stx2*-positive *E coli* O157 isolates from cattle in Turkey (Ayaz and others, 2014). In contrast, in Sweden most STEC O157 isolated from cattle and associated with human cases were characterised by the Shiga toxin profile *stx2a+stx2c* (Eriksson and others 2011). Interestingly, in the same country STEC O157 isolated from cattle and not linked to human cases showed a variety of virulence profiles (*stx1*, *stx2a*, *stx2c*, *stx2a+stx2c*, *stx1+stx2c)*, thus suggesting that the presence of the *stx2a* gene in STEC O157 cattle isolates, either alone or in combination with the *stx2c* gene, could be responsible for their more aggressive behaviour in humans than STEC O157, harbouring the *stx2c* gene alone or combined with the *stx1* gene (Aspán and Eriksson 2010).

In German patients affected by HUS and HC, the *stx2a* variant was most frequently associated with HUS cases (54.9 per cent); apart from *stx2a*, the only variant associated with HUS was *stx2c*.The *stx2c* genotype was found in 3.7 per cent of patients with HUS, in 5 per cent of patients affected by diarrhoea without HUS, and in 5.2 per cent of asymptomatic people (Friedrich and others 2002). Virulence of *stx2c*-harbouring STEC O157 is also described by Lefebvre and others (2009), who demonstrated that *stx2c*-positive *E coli* O157 from cattle showed higher toxicity for Vero cells than bovine isolates which possessed *stx1+stx2c* toxins or *stx1* toxin alone. Consequently, as the *stx2c* variant was detected in 100 per cent of STEC O157 in our study, we speculate that they genetically harbour the ability to cause severe human infections. Furthermore, one OND STEC strain possessed both *stx2a* and *stx2c* variants, together with *eae* and *e-hlyA* genes, suggesting its high virulence for humans.

In our study, the virulence profile among STEC O26 included *stx1, eae,* and *e-hlyA*. Although in the study by Friedrich and others (2002), *stx1*-positive STEC were associated mainly with asymptomatic individuals (46.8 per cent) and with patients with diarrhoea (42.4 per cent) rather than with HUS cases (3.7 per cent), their pathogenic potential to humans cannot be excluded.

During our study, the conditions of transportation to the slaughter plant were recorded. Most cattle (94 per cent) were transported together with others of the same herd (mixed transport) and only 6 per cent (9/160) were transported alone. The single transport may occur if the farm has a reduced number of animals or when the animal's slaughter has not been previously planned by the farmer. The cattle were held at lairage for a relative short time (two to six hours), but during transport and before slaughter the many potential sources of STEC on hides were the animals themselves, the transport trucks and the lairage environment. It has been reported that some animals, termed 'super-shedders', can shed STEC O157 at levels as high as 10^6 cfu/g (Robinson and others 2004). These cattle may easily contaminate many animals in trucks and at

lairage, thus increasing the prevalence of contaminated hides. In the current study, all STEC O157- and O26-positive hide cattle were moved to the slaughterhouse by mixed transport and no hide contamination was observed in STEC O157 and O26 carriers singly transported to the slaughter plant.

At this stage of the meat chain, preventive measures should be enhanced to avoid contamination of meat, by implementing good hygiene practice (GHP) measures and hazard analysis and critical control point (HACCP) plans at slaughter plants. Furthermore, most authors agree that contamination of carcases with pathogens at slaughter comes mainly from hides (Bosilevac and others 2004, Koohmaraie and others 2005, Arthur and others 2007a,b) and therefore different strategies have been proposed to reduce cattle hide contamination before entering the slaughter line. Pre-harvest interventions, including dietary changes, vaccination, use of probiotics and phage sprays, have been proposed to reduce faecal shedding of STEC O157 by cattle (Kudva and others 1999, Potter and others 2004, Van Donkersgoed and others 2005, Younts-Dahl and others 2005, Callaway and others 2009). Post-harvest strategies are widely used in the US beef industry, with increasing attention on hide decontamination and innovative treatments of carcases. They include the use of organic acids (Castillo and others 2002), cetylpyridinium chloride (Cutter and others 2000), acidified sodium chlorite (Yoder and others 2012), activated lactoferrin (Taylor and others 2004), sodium hydroxide, trisodium phosphate, chlorofoam and phosphoric acid (Bosilevac and others 2005a), ozonated water (Bosilevac and others 2005b), and the application of ionising radiation (USDA and FSIS 1999). Trials with antimicrobials have demonstrated that 1 per cent cetylpyridinium chloride, 2 per cent lactic acid and 3 per cent hydrogen peroxide resulted in 1.2- to 1.9-$\log_{10}$ *E coli* cfu/100 cm^2 reduction when applied to clipped hide surfaces of the brisket area (Baird and others 2006). A treatment with hypobromous acid prepared from hydrogen bromide in aqueous solution without subsequent water rinse was tested for reduction of *E coli* O157:H7 on cattle hide, demonstrating a reduction in prevalence of the pathogen from 25.1 to 10.1 per cent at the concentration of 200 parts per million (ppm) (Schmidt and others 2012). Recently, the synergy of lactic acid and sodium dodecyl sulfate to decontaminate *E coli* O157 on cattle hides was investigated (Elramady and others 2013). Most of the interventions were studied for *E coli* O157 inactivation/reduction, but recently Kalchayanand and others (2012) demonstrated that STEC O157 treatments were as effective as for non-O157 STEC.

The recent authorisation in the European Union, under Regulation (EU) No 101/2013, for the use of lactic acid on beef carcases provides the possibility to reduce pathogen load on fresh meat at slaughter plants. This is the only authorised post-harvest intervention in the European countries and it has been demonstrated that, when used by spraying or dipping in lactic acid concentrations between 2 and 5 per cent, is effective on *E coli* O157, reducing the bacterial population of 1–2 $\log_{10}$ cfu/cm^2 (Huffman 2002, King and others 2005). However, according to Duffy and others (2014), the European beef industry does not appear to benefit from the use of decontaminating acid lactic solutions at processing plants, as it is not currently being practised. For this reason, STEC faecal and hide carriage by cattle is of concern to the meat industry in European countries, as pre-harvest and carcase interventions are doubtfully applied.

Consumer habits are of great importance in limiting food-borne disease by STEC, because differences in the way meat is cooked can affect the survival rate of the microorganisms. For example, rare hamburgers are commonly served in the USA and they have been frequently involved in STEC human outbreaks (Kassenborg and others 2004, CDC 2010). Even raw beef preparations, such as steak tartare (Nauta and others 2001) and the Italian 'carpaccio' (raw beef cut into thin slices and served with olive oil, salt and pepper), may pose a risk to consumers. In contrast, well cooked beef is much more safe, reducing the risk of HUS and other STEC-related diseases.

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