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Prevalence and characteristics of verotoxigenic *Escherichia coli* strains isolated from pigs and pork products in Umbria and Marche regions of Italy

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ABSTRACT

In total 1095 samples from 675 pork products, 210 swine colon contents, and 210 swine carcass sponge swabs were collected in Umbria and Marche regions of Italy and examined for the presence of Shiga toxin-producing *Escherichia coli* (STEC), also known as Verotoxin-producing *E. coli* (VTEC). After an enrichment step, each sample was analysed by real-time PCR to detect the *stx1*, *stx2*, and *eae* genes. *stx*-Positive samples were further tested for the "top five" serogroup markers (O157, O26, O103, O111, O145) and cultured onto selective media. The isolates were assigned to *stx* subtypes and tested for the presence of *aaiC* and *aggR* genes. Out of 420 swine samples, 38.6% faecal samples and 13.8% carcass sponge swabs were *stx*-positive. In total, 33 *E. coli* STEC isolates were obtained from 30 samples (4 carcasses and 26 colon contents) indicating a culture-positive rate of 7.1%. A higher culture-positive rate was observed in faecal samples (12.4%) than in carcass sponge swabs (1.9%). Out of 675 pork samples, 19 (2.8%) were *stx*-positive. No STEC strains were isolated from *stx*-positive pork products. We concluded that STEC isolation from foodstuffs remains difficult, despite the application of ISO/TS 13136:2012. Furthermore, in accordance with the results of studies conducted in other countries, we observed that most of swine STEC strains carried stx_{2e} gene and lacked of virulence genes, such as *eae*, *aaiC* and *aggR*, indicative of potential pathogenic characteristics for humans. Although the majority of STEC isolates did not express virulence factors correlating with severe human diseases, the association between swine STEC strains and human illness requires further investigations.

1. Introduction

Escherichia coli is a gram-negative microorganism usually found as a commensal in the gastrointestinal tract of many animal species. However, Shiga toxin-producing *Escherichia coli* (STEC) can cause severe human diseases, such as haemorrhagic colitis (HC) and haemolytic-uraemic syndrome (HUS), due to the production of cytotoxins known as Verotoxins or Shiga toxins (Stx1 and Stx2). Shiga toxins are encoded by the *stx1* and *stx2* genes generally carried by prophages. Several variants of both Shiga toxins have been identified: Stx1 subtypes are designated as Stx1a, Stx1c, and Stx1d, whereas Stx2 comprises seven variants referred to, in alphabetical order, as Stx2a–Stx2g (Scheutz et al., 2012). STEC strains can express a combination of one or more *stx* subtypes (Karve and Weiss, 2014). However, epidemiological studies showed that strains producing Stx2a and Stx2c tend to be more frequently isolated from patients with HUS than those expressing other Stx variants (Caprioli et al., 2005). Many STEC strains that are highly pathogenic to humans and included in the subset of enterohaemorrhagic *E. coli* (EHEC), are often characterised by the production of an outer membrane protein called intimin. This protein mediates the attachment of bacteria to enterocytes and induces cytoskeletal changes accompanied by the accumulation of actin and causing characteristic histopathological lesions defined as "attaching and effacing" (A/E). The intimin-encoding gene (*eae*), which belongs to a large pathogenicity island (PAI) called Locus of Enterocyte Effacement (LEE), is an important virulence factor. However, *eae*-negative strains, such as O91:H21, O113:H21 and, more recently, O104:H4, have been associated with serious diseases, indicating the importance of other virulence factors in the pathogenesis of clinical symptoms (Bouvet et al., 2001, 2002a). Such factors located on mobile genetic elements, like PAI or plasmids, have been identified. Nevertheless, in some cases, their role in the pathogenic process has not been fully elucidated (Caprioli et al., 2005). Furthermore, different *E. coli* strains may belong to more than one pathotype group owing to the expression of different virulence factors. For example, *E. coli* O104:H4, involved in Germany's outbreak

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in 2011, was positive for *stx2* and also carried *aaiC* and *aggR* virulence genes, which are typical of enteroaggregative *E. coli* (EAEC) (Frank et al., 2011).

STEC outbreaks are a serious public health concern owing to their association with severe clinical symptoms (Shen et al., 2015). However, STEC-induced infections are relatively uncommon compared to other foodborne diseases (Caprioli et al., 2005; EFSA and ECDC, 2015). In 2014, 5955 confirmed cases of STEC infections were reported in the EU by the European Food Safety Authority (EFSA) with a notification rate of 1.56 cases per 100,000 individuals, which was 1.9% lower than the notification rate in 2013. The EU notification rate in the 2 years following the large outbreak in 2011 was higher than before the outbreak and remained so in 2014. This is possibly an effect of increased awareness and of more laboratories testing also for other serogroups than O157. Nevertheless, the most commonly reported serogroup in 2014 was O157 followed by O26 (EFSA and ECDC, 2015).

STEC strains are zoonotic agents that can be transmitted to humans through person-to-person contact, ingestion of food or water contaminated with animal faeces, and by direct contact with animals (Caprioli et al., 2005; Smith et al., 2014). Although STEC strains have been frequently isolated from the intestinal content of a wide range of animal species, ruminants, especially cattle, are recognised as the main natural STEC reservoir. Unlike cattle, that typically do not exhibit any STEC-associated symptoms (Tseng et al., 2014a), swine may present with clinical manifestations due to STEC infections (Tseng et al., 2014b). Oedema disease, an infectious illness that often affects post-weaning piglets and young finishing-age pigs, is caused by *E. coli* strains expressing the stx_{2e} gene, encoding Stx2e. Cross-sectional epidemiological studies conducted in several countries showed that STEC prevalence in clinically healthy swine ranged from 0% to 68.3% (Tseng et al., 2014b). Only in rare cases, pork consumption is associated with severe clinical symptoms caused by highly pathogenic STEC strains. Moreover, in such cases it is usually unknown whether the contamination of food products occurred during pork processing or via cross-contamination from foodstuffs of different origin. The expression of the stx_{2e} gene has rarely been reported in STEC strains that cause HUS (Fratamico et al., 2004). Moreover, Stx2e-producing STEC strains isolated from humans and pigs had different serogroups, distinct virulence profiles, and dissimilar parameters of interaction with intestinal epithelial cells. Typical swine pathogenic *E. coli* strains generally express Stx2e and usually belong to a limited number of serogroups (O8, O108, O138, O139, O141,O147, and O149), which differ from those commonly associated with stx_{2e}-positive human isolates (Sonntag et al., 2005; Schierack et al., 2006). Because of the limited epidemiologic data about STEC prevalence in swine and an increasing role of non-O157 STEC in human illness, the association between swine STEC strains and human disease needs to be elucidated (Tseng et al., 2014a).

The epidemiological data on STEC prevalence in pigs and in pork products are sparse and not directly comparable among the European countries (EFSA and ECDC, 2015). In this study, 420 faecal and carcass samples were collected, in an attempt to study the prevalence of STEC carriage in healthy pigs. In addition, 675 pork samples were collected from the main processing plants in Umbria and Marche regions of Italy. Since in this area pork meat is the basic ingredient of a large amount of typical food products, the prevalence of STEC in pigs and pork products is of particular interest.

2. Material and methods

2.1. Study design and sampling

In the period from March 2013 to March 2014, 420 swine specimens collected during the slaughter process were analysed for the presence of STEC. In particular, 210 colon-content specimens and 210 carcass sponge swabs were collected during 21 sampling days from 210 randomly selected pigs. Swine samples were obtained from eleven different facilities in Umbria and Marche regions of Italy in numbers roughly proportional to the slaughter capacity of each abattoir. In order to obtain a statistically significant sample size, the number of tested animals was calculated based on the number of annually slaughtered pigs and on STEC prevalence rates reported in literature (5% prevalence, 3% standard error and 95% confidence level) (EFSA and ECDC, 2012; Caprioli et al., 1993). Faeces were sampled directly from the colon of each animal after evisceration. Carcass samples were collected with sterile pre-moistened sponges rubbed over the half-carcass surface, before chilling. Samples were transported to the laboratory in thermal boxes, stored at 4 ± 1 °C and processed within 24 h.

In the period from May 2012 to March 2014, 675 pork samples, which included fresh meat and dried pork products, were tested for the presence of STEC. The sampling procedure was performed according to regional food safety monitoring plans. In total, 135 food specimens, each consisting of 5 sample units, were collected directly from processing plants.

2.2. Bacterial culture and DNA extraction

Carcass sponge swabs were suspended in 90 mL of Buffered Peptone Water (BPW; Oxoid, Basingstoke, UK). After incubation at 37 ± 1 °C for 16–22 h, DNA was extracted from 300 μL of broth using the boiling method. The broths were heated at 100 °C for 5 min and then cooled in ice for 5 min. After centrifugation at 13,000 ×*g* for 5 min, the supernatant (200 μL) was collected and used as template for PCR assays.

For faecal sample analysis, 5 g of colon content from each animal were incubated in 45 mL of modified Tryptone-Soy Broth (mTSB; Oxoid) at 37 °C ± 1 for 16–22 h. DNA was extracted from 1 mL of broth culture using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany).

Pork products were analysed in accordance to ISO/TS 13136:2012(E) (ISO (International Organization for Standardization), 2012). Briefly, 25 g of each sample was suspended in 225 mL of mTSB supplemented with 16 mg/L of novobiocin (mTSB + N) and incubated at 37 °C ± 1 for 18–24 h. DNA was extracted from 1 mL of each enrichment broth specimen using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions for gram-negative bacteria.

2.3. PCR screening, isolation and characterization of STEC

The real-time PCR assay targeting the virulence genes *eae*, *stx1*, and *stx2* was performed by using primers and probes described in ISO/TS 13136:2012(E) and reported in Table 1 (Perelle et al., 2004; Nielsen and Andersen, 2003). Real-time PCR analysis was conducted in a 25-μL reaction volume by using the following reaction mix: 1× QuantiFast Probe PCR Master Mix (Qiagen), 25 pmol of each primer, 5 pmol of FAM-labelled probe, and 3 μL of the DNA template. A commercially available Internal Positive Control (TaqMan Exogenous Internal Positive Control Reagent-VIC probe; Thermo Fisher Scientific, Waltham, MA, US) was included in each PCR reaction. Real-time PCR amplification was performed using a Stratagene Mx3005P system (Agilent Technologies, Santa Clara, CA, US) with the following cycling conditions: 95 °C hold for 3 min for initial denaturation of DNA and Taq polymerase activation was followed by 45 cycles of amplification at 95 °C for 3 s and at 55 °C for 30 s. As documented in Table 2, faecal and carcass samples, which tested positive for the presence of *stx1* and/or *stx2*, were further examined for the presence of *E. coli* O26, O103, O145, O111, and O157 serogroup-associated genes using multiplex end-point PCR assay proposed by Monday et al. (2007). Food specimens, which tested positive for the virulence factors during the screening step, were investigated further for the "top five" serogroup-associated genes according to ISO/TS 13136 with primers and probes reported in Table 1 using the amplification protocol described above. When one or both *stx* genes were detected, we attempted to isolate STEC strains from the corresponding enrichment sample broths. One loopful of the *stx*-positive enrichment

Table 1
Primers and probes used for real-time PCR.

Target gene	Sequence (5′ to 3′)[a]	Expected size of the PCR product (bp)	Reference
stx1	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC Probe-CTGGATGATCTCAGTGGGCGTTCTTATGTAA	131	Perelle et al. (2004)
stx2	TTTGTYACTGTSACAGCWGAAGCYTTACG CCCCAGTTCARWGTRAGRTCMACRTC Probe-TCGTCAGGCACTGTCTGAAACTGCTCC	128	Perelle et al. (2004)
eae	CATTGATCAGGATTTTTCTGGTGATA CTCATGCGGAAATAGCCGTTA Probe-ATAGTCTCGCCAGTATTCGCCACCAATACC	102	Nielsen and Andersen (2003)
rfbE (*O157*)	TTTCACACTTATTGGATGGTCTCAA CGATGAGTTTATCTGCAAGGTGAT Probe-AGGACCGCAGAGGAAAGAGAGGAATTAAGG	88	Perelle et al. (2004)
wbdI (*O111*)	CGAGGCAACACATTATATAGTGCTTT TTTTTGAATAGTTATGAACATCTTGTTTAGC Probe-TTGAATCTCCCAGATGATCAACATCGTGAA	146	Perelle et al. (2004)
wzx (*O26*)	CGCGACGGCAGAGAAAATT AGCAGGCTTTTATATTCTCCAACTTT Probe-CCCCGTTAAATCAATACTATTTCACGAGGTTGA	135	Perelle et al. (2004)
ihp1 (*O145*)	CGATAATATTTACCCCACCAGTACAG GCCGCCGCAATGCTT Probe-CCGCCATTCAGAATGCACACAATATCG	132	Perelle et al. (2004)
wzx (*O103*)	CAAGGTGATTACGAAAATGCATGT GAAAAAGCACCCCCGTACTTAT Probe-CATAGCCTGTTGTTTTAT	99	Perelle et al. (2005)
aggR	GAATCGTCAGCATCAGCTACA CCTAAAGGATGCCCTGATGA Probe-CGGACAACTGCAAGCATCTA	102	EU-RL VTEC[b]
aaiC	CATTTCACGCTTTTTCAGGAAT CCTGATTTAGTTGATTCCCTACG Probe-CACATACAAGACCTTCTGGAGAA	160	EU-RL VTEC[b]

[a] Y is (C, T), S is (C, G),W is (A, T), R is (A, G), M is (A, C).
[b] http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_05_Rev_1.pdf.

culture was directly streaked onto the MacConkey agar, Tryptone Bile X-Glucuronide (TBX) agar, or Sorbitol-MacConkey agar (SMAC) (Oxoid). The use of TBX agar for isolation of STEC is recommended in ISO/TS 13136, however the choice of other media is allowed. It should be noted that in some studies an atypical colony morphology was observed on differential agar media (Rainbow® Agar O157, CHROMagar STEC™, MacConkey agar modified by Possé et al., 2008). These studies indicate that colony appearance may not provide a reliable tool to identify target STEC in presence of other *E. coli* (Gill et al., 2014; Verhaegen et al., 2015). Therefore, in case of a positive PCR reaction for genes associated with particular serogroups, immunomagnetic separation (IMS) (Dynabeads; Thermo Fisher Scientific) was performed to facilitate isolation of STEC strains. Plates were incubated for 18–24h at 37 ± 1 °C. Up to 50 colonies with *E. coli* morphology were inoculated onto Nutrient Agar (NA) (Oxoid) and pooled in water to a total of 10 per pool. Detection of *stx* genes was performed in each pool. The colonies forming *stx*-positive pools were tested individually for *eae* and *stx* genes by real-time PCR. Isolated STEC strains were subjected to slide agglutination with antisera specific for O157, O103, O26, O111, and O145 serogroups (Statens Serum Institute, Copenhagen, Denmark). The other serogroups were not tested. Subtyping of the *stx* genes was performed by multiplex PCR (Scheutz et al., 2012), whereas *aaiC* and *aggR* genes were detected by real-time PCR assay described by EU Reference Laboratory for *E. coli* (EU-RL VTEC, web site: http://www.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_05_Rev_1.pdf). Primers and probes are reported in Table 1. STEC strains tested negative for examined *stx* subtypes were sent to the EU Reference Laboratory for *E. coli* (EU-RL VTEC) for further analysis.

2.4. Pulsed-field gel electrophoresis (PFGE)

STEC isolates were analysed by PFGE according to the PulseNet protocol for *Escherichia coli* O157:H7 (CDC, web site: http://www.cdc.gov/pulsenet/pathogens/index.html) and EFSA External Scientific Report about molecular typing of verocytotoxin-producing *E. coli* (Caprioli et al., 2014). STEC strains were digested with the *Xba*I restriction enzyme (Promega, Madison, WI, US). Restriction fragments were separated using a CHEF-DR III electrophoresis system (Bio-Rad, Hercules, CA, US). The gels were stained with ethidium bromide and DNA bands were visualized with a UV trans-illuminator (UVItec, Cambridge, UK). PFGE gel images were analysed using the BioNumerics

Table 2
Primers used for end-point multiplex PCR.

Target gene	Sequence (5′ to 3′)	Expected size of the PCR product (bp)	Reference
wzx (*O26*)	ATCCTTGCTTCGCCTGTT CAGCGATACTTTGAACCTTAT	268	Monday et al. (2007)
wzx (*O103*)	TATCCTTTCATAGCCTGTTGTT AATAGTAATAAGCCAGACACCTG	320	
wzx (*O111*)	GTTGCGAGGAATAATTCTTCA CCATAGTATTGCATAAAGGC	829	
wzx (*O145*)	TTGAGCACTTATCACAAGAGATT GATTGAATAGCTGAAGTCATACTAAC	418	
wzx (*O157*)	GCTGCTTATGCAGATGCTC CGACTTCACTACCGAACACTA	133	

software (Applied-Maths, Sint-Martens-Latem, Belgium) to establish genotypic relatedness between the isolates.

3. Results

3.1. STEC prevalence in swine carcasses

Out of 210 swine carcass sponge swabs, 181 (86.2%) tested negative for *stx* genes (Table 3). Of these 181 *stx*-negative samples, 144 (68.6%) tested positive for *eae*. Of the 29 *stx*-positive broths, 26 (12.4%) tested positive for both *stx2* and *eae*, 2 (0.9%) were positive only for *stx2*, and one (0.5%) tested positive for both *stx1* and *eae*. Positive samples were examined further to identify "top five" serogroup-associated genes: 14 out of 29 samples (48.3%) were found positive for the O157 serogroup, 6 samples (20.7%) — for the O26 serogroup, 4 samples (13.8%) — for the O103 serogroup, 1 sample (3.4%) — for the O145 serogroup. No sample contained the O111-associated gene. STEC isolates were recovered from 4 samples: 5 strains were obtained from 4 carcass sponge swabs collected on different sampling days. Two different STEC strains were isolated from the same carcass. All STEC isolates were positive for *stx2*, while *eae* gene was absent. None of the strains belonged to the tested serogroup and none carried *aaiC* and *aggR*. Three STEC strains were positive for stx_{2e} and one strain was positive for stx_{2d}. For one strain, its *stx2* subtype could not be determined with the available assays.

3.2. STEC prevalence in swine faecal samples

Of the 210 faecal samples obtained from pigs at slaughter, 129 (61.4%) were negative for Shiga toxin-encoding genes (Table 4). Out of these 129 *stx*-negative samples, 105 (50.0%) tested positive for *eae*. In the remaining 81 *stx*-positive samples, *stx2* and *eae* genes were detected in 75 (35.7%) of enriched broths, while 6 (2.9%) broths were positive for *stx2* only. In contrast to observations in carcasses, *stx1* was not detected in any faecal sample. Genes associated with "top five" serogroups O157, O26, O103, O145, and O111 were detected, respectively, in 70/81 (86.4%), 31/81 (38.3%), 14/81 (17.3%), 8/81 (9.9%), and 1/81 (1.2%) of *stx*-positive samples. STEC strains were isolated in 26 faecal samples: 28 strains were obtained from 26 swine colon contents collected on 13 different sampling days. In two cases, two different STEC strains were isolated from the same faecal sample. Similarly to the isolates from carcasses, all STEC strains tested positive for *stx2* but did not carry *eae*. None of the STEC strains belonged to one of the "top five" serogroups and none carried *aaiC* and *aggR* genes. Out of 28 STEC isolates, 27 tested positive for stx_{2e}, while one isolate was negative for all *stx2*-subtypes tested, similarly to one of the strains isolated from the carcass of the same pig. Isolates that could not be typed were subjected to sequencing at the EU-RL VTEC. We found that these two isolates carried stx_{2e}, but had an insertion sequence, located in the *stx* operon, downstream of the gene stx_2A. This was the reason why in our initial assays subtype-specific genes could not amplify properly. Moreover, stx_{2e} gene expression was inactivated by a single nucleotide deletion (the loss of a G in the stx_2A gene), which caused a shift in the reading frame, during DNA translation.

3.3. STEC prevalence in pork samples

In total, 675 pork samples, including fresh and dried products, were analysed for the presence of STEC strains using molecular and isolation methods. Of these 675 pork samples, 656 (97.2%) were negative for Shiga toxin-encoding genes, while 19 (2.8%) fresh sausage samples were *stx*-positive (Table 5). Of these 19 *stx*-positive enrichment cultures, 10 (1.5%) contained both *eae* and *stx2*, 5 (0.7%) tested positive only for *stx1*, and 4 (0.6%) were found to be positive only for *stx2*. Out of 19 *stx*-positive samples, 18 tested positive for at least one serogroup in real-time PCR. The most prevalent serogroup detected in 18/19 samples was O145 (94.7%). The O103-associated gene was detected in 15/19 (78.9%) samples. Eight out of 19 (42.1%) samples were found positive for the O157 serogroup, 7/19 (36.8%) were positive for the O26 marker, while no sample contained the O111-associated gene. One enrichment culture was negative for all serogroups tested. Unlike carcasses and faecal samples, STEC strains were not isolated from *stx*-positive pork products. This result could be due to the presence of preservative substances added to the minced meat or to the effect of ripening. Also the addition of novobiocin to the enrichment medium, as described in ISO/TS 13136, could inhibit the growth of some non-O157 STEC strain (Kanki et al., 2011).

3.4. PFGE profiles of STEC isolates

All 33 STEC isolates were analysed by PFGE but only 31 of them produced clear bands that allowed characterization of PFGE profiles. Two STEC isolates could not be analysed because of DNA degradation. PFGE analysis produced a number of DNA fragments, which comprised between 13 and 22 bands. The selected 31 STEC isolates showed a similarity of 62.5% or greater. PFGE patterns are shown in Fig. 1. Five clusters of isolates demonstrated identical PFGE profiles. Three of these 5 clusters were isolated from faeces of pigs kept on the same farms and sampled on the same day, while two clusters were obtained from faeces and carcasses of the same animals, suggesting that auto or cross-contamination occurred. In addition, two strains with similarity of 67.5% were recovered from a single carcass. Furthermore, another two STEC strains with similarity of 67.5% were isolated from a single faecal sample. Nonetheless, the small number of STEC isolates that could be analysed by PFGE did not allow making reliable epidemiological extrapolations.

4. Discussion

The prevalence of STEC strains in the swine population and pork products has been reported in some epidemiologic studies conducted in several countries. However, these data often could not be directly compared due to differences in study designs, application of dissimilar sample collection methods, or use of different STEC detection and isolation protocols (Tseng et al., 2014b).

STEC strain occurrence rate in swine faecal samples varies within a wide range of values depending on the study. Out of 210 swine faecal samples collected in this study, 38.6% (81/210) of faecal samples were *stx*-positive. In total, 28 STEC isolates were obtained from 26 faecal samples corresponding to the culture-positive rate of 12.4% (26/210).

Table 3
STEC detection in carcass sponge swabs by molecular analysis.

Virulence factors	Number of positive samples	%	O157[a]	O145[a]	O103[a]	O111[a]	O26[a]
eae + *stx2* + *stx1* −	26	12.4	12	1	3	0	4
eae-stx2 + *stx1* −	2	0.9	1	0	1	0	2
eae + *stx2-stx1* +	1	0.5	1	0	0	0	0
Positive tot.	29	13.8	14	1	4	0	6
Negative tot.	181	86.2					
Total	210	100	48.3%	3.4%	13.8%	0%	20.7%

[a] The same sample could be positive for more than one serogroup.

Table 4
STEC detection in swine faecal samples by molecular analysis.

Virulence factors	Number of positive samples	%	O157[a]	O145[a]	O103[a]	O111[a]	O26[a]
eae + *stx2* + *stx1*−	75	35.7	65	7	12	1	28
eae-stx2 + *stx1*−	6	2.9	5	1	2	0	3
Positive tot.	81	38.6	70	8	14	1	31
Negative tot.	129	61.4					
Total	210	100	86.4%	9.9%	17.3%	1.2%	38.3%

[a] The same sample could be positive for more than one serogroup.

Similar prevalence estimates have been reported in studies conducted in other European countries. In France, about 31% (129/182) of swine faecal samples tested using PCR were *stx*-positive, but among the 129 positive specimens, none contained detectable levels of the *uidA* gene associated with the O157:H7 serotype (Bouvet et al., 2002b). In Belgium, from 177 individual rectal swabs, 56 (31%) were PCR-positive for STEC (Botteldoorn et al., 2002). However, many studies reported substantially different STEC prevalence rates. A study conducted in Switzerland reported a lower incidence of STEC in swine faecal samples: of 630 specimens collected at slaughter, 22% and 7.5% were PCR positive for *stx* and *rfbE* (O157 serogroup-associated gene), respectively (Kaufmann et al., 2006). In another study, carried out in northern Italy in 1993, faecal specimens from 242 slaughtered pigs were tested using the Vero cell assay and the presence of STEC was detected only in 7.8% (19/242) of enriched samples (Caprioli et al., 1993).In contrast, the US National Animal Health Monitoring System Swine 2000 Report stated that out of a total of 687 swine faecal samples tested for the presence of *stx1* and *stx2* using PCR assays, 484 (70%) samples were positive for one or both genes. At least one STEC isolate was recovered from 196 faecal samples (196/687, 28.5%) in that study. It is noteworthy that about 80% of STEC isolates belonged to the stx_{2e} subtype, whereas serogroup O157 strains were not detected (Fratamico et al., 2004; Tseng et al., 2014a). A higher isolation rate was reported in a longitudinal study, in which STEC occurrence in faecal shedding during the finishing period was analysed in 150 pigs. In total, 1200 faecal samples were collected and STEC isolates were recovered in at least one faecal sample from 98 out of 150 pigs (65.3%). Most of the STEC strains (97.9%) harboured stx_{2e}, while *E. coli* O157:H7 was not isolated (Tseng et al., 2014b). Furthermore, relatively high STEC prevalence rates were reported in a study conducted in Chile, where STEC strains were identified by DNA hybridisation in 68.3% (82/120) of faecal samples collected from 120 healthy pigs (Borie et al., 1997). At the same time, other South American studies reported less frequent STEC occurrence. In Brazil, one study reported a rather low prevalence rate of 1.35% (1/74) indicated by isolation of just one *stx2*-positive strain from 74 swine intestinal samples (Martins et al., 2011).

As in the case with faecal samples, STEC prevalence in swine carcasses varied widely depending on the country and methods applied for detection and isolation. In our study, we found that 29 out of 210 (13.8%) carcass sponge swabs were *stx*-positive. In total, 5 STEC isolates were obtained from 4 carcass samples indicating a culture-positive rate of 1.9% (4/210).In Belgium, 5 pig carcasses out of 132 examined (12.8%) were *stx*-positive according to PCR analysis (Botteldoorn et al., 2002). In France, the carcass contamination rate determined by molecular methods varied from 12% to 50% (Bouvet et al., 2001, 2002a). In Canada, 51 out of 1067 carcasses (4.8%) were found to be PCR-positive for *stx* genes (Bohaychuk et al., 2011). Borges et al. (2012) examined the presence of STEC strains in 215 swine carcasses using PCR and isolation techniques in Brazil. Shiga-toxin encoding genes were detected in 12 samples (5.6%) and prevalence of STEC isolates comprised 0.4% (1/215). The only STEC strain isolated in that study was positive for stx_{2e} and did not belong to the O157:H7 serotype.

Highly variable rates of *stx*-positive strain incidence have also been observed in pork products collected in many countries. Out of 675 pork samples analysed in our study, 19 (2.8%) were *stx*-positive, but no STEC strains were isolated. At the same time, many studies reported higher prevalence rates of *stx*-positive strains in pork products. In Italy, out of 126 fresh meat samples screened using PCR, 20 (15.9%) were positive for the presence of *stx* genes. Furthermore, 50% of the *stx*-positive pork specimens (10/20) were contaminated with *E. coli* O157, as indicated by positive results of testing for the presence of *rfbE*. Moreover, 24 *stx*-positive strains were isolated from 13 *stx*-positive samples (10.3%, 13/126) and 15 of them were shown to have the O157 serotype (Villani et al., 2005). Bardasi et al. (2015) reported data from a two-year STEC monitoring plan carried out in the Emilia Romagna region of Italy. STEC virulence genes were detected according to ISO/TS 13136 in 41 out of 213 (19%) fresh sausages made from pork meat. In addition, one *E. coli* O103 strain positive for *eae* and *stx1* was isolated (Bardasi et al., 2015). In Britain, testing of pork sausages by DNA hybridisation showed the presence of STEC strains in 46 (25%) of 184 samples (Smith et al., 1991). In the United States, a study was published in which 231 ground pork samples were collected in the Washington D.C. area. Out of 231 samples, 31 (13.4%) were PCR-positive for *stx* genes. Positive specimens were further analysed using colony hybridisation and STEC strains were isolated from 13 (5.2%) pork samples. None of the STEC isolates belonged to O157 serogroup (Ju et al., 2012).

These studies show that the incidence of STEC strains in swine populations and pork products varied in different regions of the world. The epidemiologic data about STEC contamination of pork products suggest that swine may represent an important source of STEC strains in the food chain. The observed differences in STEC strain prevalence rates could be due to variances in husbandry practices and predominant climatic conditions of different geographical areas (Rajkhowa and Sarma, 2014). On the other hand, data about STEC strain prevalence rates in the swine production chain are sparse and discrepancies are potentially attributable to multiple factors, such as different sampling protocols. The variation in methods used for detection and isolation of STEC strains may be yet another factor contributing to differences in prevalence rate estimates (Fratamico et al., 2004; Tseng et al., 2014a).

Table 5
STEC detection in pork samples by molecular analysis.

Virulence factors	Number of positive samples	%	O157[a]	O145[a]	O103[a]	O111[a]	O26[a]
eae + *stx2* + *stx1*−	10	1.5	7	10	8	0	4
eae-stx2 + *stx1*−	4	0.6	1	3	2	0	3
eae-stx2-stx1+	5	0.7	0	5	5	0	0
Positive tot.	19	2.8	8	18	15	0	7
Negative tot.	656	97.2					
Total	675	100	42.1%	94.7%	78.9%	0%	36.8%

[a] The same sample could be positive for more than one serogroup.

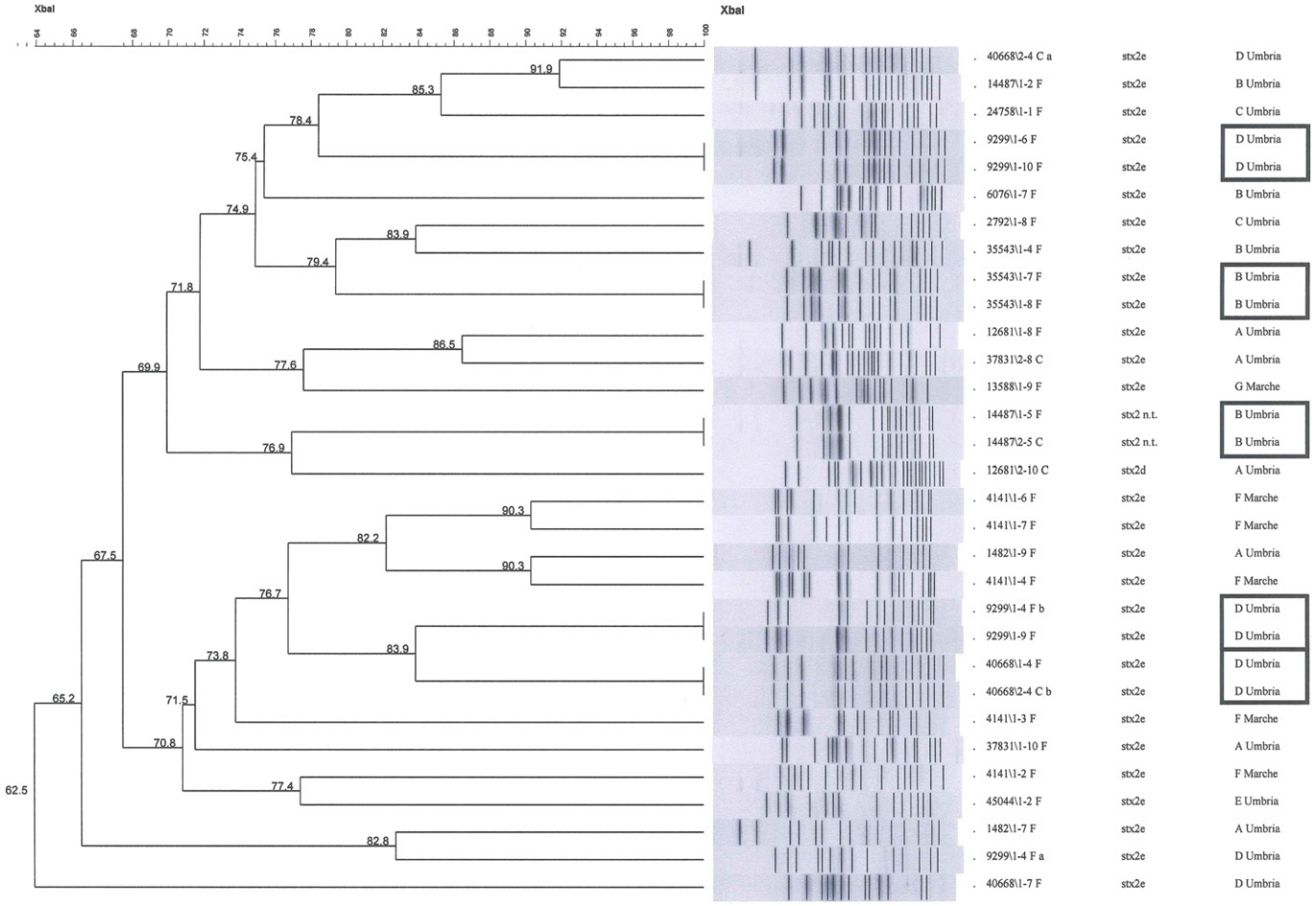

Fig. 1. Dendrogram of STEC isolates from faecal samples (strain codes including F letter) and carcasses (strain codes including C letter). Five clusters were identified (100% of identity): three clusters originated from slaughterhouse "D Umbria" and two from "B Umbria". Two strains (40668\2-4Ca and 40668\2-4Cb) were recovered from a single carcass and two strains (9299\1-4Fa and 9299\1-4Fb) were isolated from a single faecal sample.

Although pigs are a potential reservoir of STEC strains, distinct genetic profiles have been described in STEC strains of swine and human origin (Sonntag et al., 2005). Out of 33 STEC isolates obtained in our study, 32 (97%) were *stx*$_{2e}$-positive and none belonged to any of the "top five" serogroups. Only a very small number of *stx*$_{2e}$-positive strains have been detected in humans accounting only for 0.9%–1.7% of all human STEC isolates (Sonntag et al., 2005; Beutin et al., 2008). Stx2e-producing STEC strains have been usually found in patients showing mild diarrhoea or in asymptomatic carriers. However, severe clinical symptoms, such as HUS, have also been associated with such strains (Thomas et al., 1994; Fasel et al., 2014). In these human cases, no particular source of infection has been identified (Tseng et al., 2014a). In fact, it is important to note that only in a few instances pork products have been proven as a vehicle involved in STEC infection outbreaks (Fratamico et al., 2004; Conedera et al., 2007). Although a small number of STEC strain outbreaks implicating pork as the likely source of infection has been reported, the results of these investigations emphasised the importance of considering meat products other than beef as potential vehicles of STEC strain transmission (Troz-Williams et al., 2012; Tseng et al., 2014a).

The results of our study indicate that swine may represent a potential reservoir of pathogenic STEC strains. Since the majority of STEC strains isolated from faeces and carcasses did not express virulence factors able to cause severe human disease, the role of pork products as a potential source of foodborne infections needs to be further investigated. In fact, many challenges related to the isolation of non-O157 STEC strains remain, since these represent a heterogeneous group of pathogens with different phenotypic features (Smith et al., 2014). Our study has also revealed the difficulty of obtaining STEC isolates from *stx*-positive enrichment cultures, in particular from food samples, despite the application of culturing methods outlined in ISO/TS 13136:2012. This result could be due to the ripening or to the addition of preservatives to the ground meat. Failure to isolate STEC from *stx*-positive specimens may be attributable also to the addition of novobiocin to the enrichment medium, during the analysis, to the presence of viable but non-culturable (VBNC) bacterial cells, to the loss of Stx prophages during subculturing, to the high levels of background microflora, or to low levels of target bacteria in the sample (Ju et al., 2012; Farrokh et al., 2013; Meng et al., 2014).

Implementation of monitoring programmes, good hygiene practices, and efficient validated Hazard Analysis Critical Control Point procedures in the whole food production chain is an important instrument to control public health risks associated with STEC strain infections (EFSA, 2007). Prevention and control of foodborne diseases caused by STEC strains also require continuous improvement of analytical tools to understand the virulence, origins, and epidemiology of these bacteria, in order to devise strategies that lessen the risk of foodstuff contamination and eventually anticipate the emergence and the spread of new forms of these pathogens (Franz et al., 2014).

Conflict of interest

The authors declare no potential conflict of interest.

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