Shiga toxin-producing Escherichia coli in slaughtered pigs and pork products

Lia Bardasi, Roberta Taddei, Ilaria Fiocchi, Maria Francesca Pelliconi, Mattia Ramini, Elena Toschi, Giuseppe Meraldi
Institute for Experimental Veterinary Medicine of Lombardy and Emilia Romagna, Bologna, Italy

Abstract
During the years 2015-2016, 83 faecal samples were collected at slaughter from pigs reared in farms located in Central-Northern Italy. During the years 2014-2016 a total of 562 pork products [465 not-ready-to-eat (NRTE) and 97 ready-to-eat (RTE) products] were collected from retail outlets, large retailers and processing plants. The samples were analysed according to ISO TS 13136:2012. Out of 83 swine faecal samples, 77 (92.8%) resulted stx-positive by real time polymerase chain reaction (PCR), 5 stx2+ and 1 stx1+. Shiga toxin-producing Escherichia coli (STEC) strains were isolated. Among the 465 NRTE samples, 65 (14.0%) resulted stx-positive by real time PCR and 7 stx2+ STEC strains were isolated. The stx2 gene was detected more frequently than the stx1 gene both in faecal samples (90.4 vs 8.4%) and in NRTE pork products (13.3 vs 1.3%). All the RTE samples included in the analysis resulted stx-negative. Among the samples resulted positive for stx and eae genes, serogroup-associated genes were detected at high frequency: O26 resulted the most frequent in faecal samples (81.3%) and O145 in pork products (88.1%). The O157 serogroup resulted positive in 83.3 and 78.1% of pork products and faecal samples, respectively. Despite the frequent detection by real time PCR of genes indicating the possible presence of STEC strains belonging to the six serogroups, the bacteriological step did not confirm the isolation of any such strains.

Introduction
Shiga toxin-producing Escherichia coli (STEC) are a group of highly pathogenic foodborne zoonotic pathogens, causing diarrhea, hemorrhagic colitis (HC), and hemolytic uremic syndrome (HUS) in humans. The common feature and main virulence factors of STEC are two phase-encoded cytoxins, called Shiga toxins (stx1 and stx2), which are directly correlated with human pathogenicity (Lindgren et al., 1993). Adherence factors are also critical features of STEC pathogenicity: human pathogenic STEC known to cause severe intestinal disease can attach to intestinal epithelial cells and form attaching and effacing lesions through an outer membrane protein called intimin, which is encoded by the eae gene. Furthermore, as shown by the enterohaemagglutinin E. coli (EAEC) O104:H4 strain that caused the large outbreak in Germany in 2011, other means of attachment such as the factors coded by the aggR regulatory plasmid gene and the chromosomal aaiC gene, when coupled with the production of stx2, can have severe consequences (Beutin and Martin, 2012). More than 200 virulent STEC serotypes have been isolated from human infections (Coombes et al., 2008). Although E. coli O157: H7 is the serotype that has been linked to most outbreaks of food-borne diseases and brought the largest number of cases of HUS, in recent years a growing number of non-O157 STEC strains have been isolated from human clinical cases and outbreaks (Caprioli et al., 2005). The lower intestinal tract of ruminants is considered to be the main natural reservoir of STEC. In most human infections, transmission occurs primarily by ingestion of contaminated food of bovine origin, though few outbreaks have been associated to the consumption of other food products, including pork products contaminated by O157 (CDC, 1995; Williams et al., 2000; MacDonald et al., 2004; Conedera et al., 2007; Trotz-Williams et al., 2012; Honish et al., 2017) and by O111 (Paton et al., 1996). However, the epidemiology and virulence characteristics of STEC carried by on-farm pigs remain largely unknown. The hypothesis that swine-derived STEC strains are similar to human-derived STEC strains and have the potential to contribute to human infections needs to be further investigated (Tseng et al., 2014a).

Unfortunately, epidemiological data on STEC prevalence in swine, the increasing evidence of pork food STEC contamination, and the rising role of non-O157 STEC in human outbreaks, the aim of this study is to investigate on STEC occurrence both in caecal content samples collected from pigs to the slaughterhouse and from pork food products.

Materials and Methods
During the years 2015 and 2016, 83 swine individual faecal samples were collected from the rectum of animals at slaughter in Emilia Romagna Region. Swine were reared in 18 different farms located in Piedmont, Emilia Romagna, Tuscany and Lombardy, 1 to 7 swine from each farm were included.

A total of 562 pork products were collected during January 2014 to August 2016 in Emilia Romagna Region from retail outlets, large retailers and processing plants. Food samples comprised 465 not-ready-to-eat (NRTE) products to be consumed after cooking (62 pork meat, 109 pork minced meat, 294 fresh meat pork sausages and processed meat products) and 97 ready-to-eat (RTE) samples (65 salami, 26 dry-cured ham, 2 mortadella, 2 pancetta, 2 coppa). The samples were analysed according to ISO TS 13136:2012 (ISO, 2012); 25 g of each food sample were diluted ten-fold (w/v) in modified Tryptone Soya Broth (mTSB) supplemented with 16 mg/L of...
novobiocin (mTBS+N) and incubated at 37±1°C for 21±3 h. Five grams of each faecal sample was diluted ten-fold (w/v) in Tryptone Soya Broth and incubated at 37±1°C for 21±3 h. Bacterial DNA was extracted from 1 mL of enriched broth using Gen elute™ bacterial genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA) as described by the manufacturer. All primers and probes used in this study are reported in ISO 13136:2012 and published previously (Perelle et al., 2004; Nielsen and Andersen, 2003; ISO, 2012). Multiplex real time polymerase chain reaction (PCR) targeting the virulence genes eae, stx1 and stx2 was conducted in a 25 µL reaction volume using the following reaction mixture: 1 X Taqman®Universal PCR Master mix (Applied Biosystems, Foster City, CA, USA), 450nM each of the forward and reverse primers, 100 nM of each labeled probe and 4 µL DNA template. A commercially available TaqMan® Exogenous Internal Positive Control (Applied Biosystems) was included in each PCR reaction. Real time-PCR thermal cycling was conducted using a StepOne Plus system (Applied Biosystems). The cycling parameters were: 95°C hold for 10 min for initial denaturation of the DNA and activation of the hot-start Taq polymerase, followed by 40 cycles of amplification of 95°C for 15s, and 60°C for 60s. Samples resulted positive for the presence of stx1 and/or stx2 gene were tested for E. coli O104 serogroup-associated genes. Sample positive for the presence of stx1 and/or stx2 in association with eae gene were tested for the detection of E. coli O103, O111, O145, O157, O26, serogroup-associated genes. Serogroup specific PCR reactions were conducted in a 25 µL reaction volume using the following reaction mixture: 1 X Taqman®Universal PCR Master mix (Applied Biosystems), 900 nM each of the forward and reverse primers, 250 nM of the labeled probe and 4 µL DNA template. The PCR instrument and program were the same used for the previous reaction. When stx1 and/or stx2 genes were detected, the isolation of the strain from the enrichment sample broth was attempted. Enriched samples were plated on Tryptone Bile X-Glucoronide (TBX) agar and incubated for 18-24 h at 37±1°C. Up to 50 colonies with E. coli morphology were picked up and point-inoculated on Nutrient agar (NA). Pools of 10 colonies were tested by real time PCR for the presence of virulence genes eae, stx1 and stx2, afterward colonies from positive pools were tested singularly in order to identify STEC strain.

STEC stains without eae gene were tested for the presence of addC and aggR genes 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).

**Results**

Out of a total of 83 swine faecal samples tested by real time PCR for the presence of stx1, stx2 and eae, 92.8 % (77/83) resulted stx-positive with stx2 gene more frequently detected than the stx1 gene (75/83 equals to 90.4% vs 7/83 equals to 8.4%). Two out of 83 (2.4%) samples tested positive for stx1 associated with eae gene, 13/83 samples (15.7%) resulted positive only for the stx2 gene, 5/83 (68.7%) tested positive for the stx2 gene in association with eae gene and 5/83 samples (6.0%) resulted positive for stx1, stx2 and eae (Table 1). Serogroup O26, O103, O104, O111, O145 and O157 and associated genes were detected respectively in 32/46 (72.2%), 19/42 (45.2%), 5/65 (7.7%), 8/42 (19.0%), 37/42 (88.1%) and 35/42 (83.3%) NRTE pork samples.

Among the samples tested for O-group associated genes, 87.5% (56/64) faecal samples and 90.5% (38/42) food samples resulted positive to more than one serogroup. Percentages of samples tested simultaneously positive to 1 up to 6 serogroups are reported in Table 2.

---

**Table 1. Detection of virulence genes and O-group associated genes in swine faecal samples and in food pork samples.**

| No of positive samples/No of tested samples (%) | Faecal samples | Food samples |
|-----------------------------------------------|----------------|-------------|
|                                               | RTE            | NRTE        |
| stx1                                          | 0/83           | 0/97        |
| stx1+eae                                      | 2/83 (2.4%)    | 0/97        | 2/465 (0.4%) |
| stx2                                          | 13/83 (15.7%)  | 0/97        | 22/465 (4.7%) |
| stx2+eae                                      | 57/83 (68.7%)  | 0/97        | 37/465 (8.0%) |
| o26                                          | 52/64 (81.3%)  | nd          | 3/465 (0.7%) |
| o103                                          | 5/64 (79.7%)   | nd          | 19/42 (45.2%) |
| o104                                          | 48/77 (62.3%)  | nd          | 5/65 (7.7%) |
| o111                                          | 23/64 (35.9%)  | nd          | 8/42 (19.0%) |
| o145                                          | 51/64 (79.7%)  | nd          | 37/465 (88.1%) |
| o157                                          | 50/64 (78.1%)  | nd          | 35/465 (83.3%) |

RTE, ready to eat; NRTE, not ready to eat; nd, not done. *The percentage are calculated on the number of samples subjected to analysis according to ISO/TS13136, i.e. the number of samples positive for stx1 and/or stx2 genes for O104 serogroup and the number of samples positive for stx1 and/or stx2 genes in association with eae gene for the remaining serogroups.

**Table 2. Not ready to eat food products and faecal samples positive to one or more serogroups.**

| No of detected serogroups | 1 | 2 | 3 | 4 | 5 | 6 |
|----------------------------|---|---|---|---|---|---|
| No of positive samples/No of tested samples (%) |   |   |   |   |   |   |
| Faecal samples            |   |   |   |   |   |   |
| NRTE food samples         | 0/97 | 2/465 (0.4%) | 22/465 (4.7%) | 37/465 (8.0%) | 3/465 (0.7%) | 19/42 (45.2%) |
|                            | 5/64 (7.7%) | 5/65 (7.7%) | 8/42 (19.0%) | 37/465 (88.1%) | 35/465 (83.3%) | 1/465 (0.2%) |

NRTE, not ready to eat.

[page 80] [Italian Journal of Food Safety 2017; 6:6584]
Six STEC strains were isolated from faecal samples giving a culture positive STEC rate of 7.8% (6/77) for stx-positive samples and 7.2% (6/83) for all samples: one stx\(^{+}\) strain and 5 stx\(^{2+}\) strains were isolated, two stx\(^{2+}\) being isolated from swine reared in the same farm.

Seven STEC strains were isolated from NRTE food samples giving a culture positive STEC rate of 10.8% (7/65) for stx-positive samples and 1.5% (7/465) for all samples. The strains were all stx\(^{2+}\): 4 were isolated from minced meat and 3 from fresh sausages. None of the isolated STEC strains belonged to one of the Top five or O104 serogroups. None of the isolated strains carried aggR and aaiC genes.

**Discussion**

Epidemiological studies performed in European countries have reported a STEC contamination rates in faecal samples of slaughtered pigs estimates by PCR for stx genes of 22% out of 630 samples analysed in Switzerland (Kaufmann et al., 2006), 23.8% (24/101) in Belgium (Botteldoorn et al., 2002), 31% (56/182) in France (Bouvet et al., 2002) and 38.6% (81/210) in Italy, Umbria and Marche regions (Ercoli et al., 2016) with a STEC isolation rate ranging from 7.9% (8/101) (Botteldoorn et al., 2002) to 12.4% (26/210) (Ercoli et al., 2016). In Germany and Italy, swine population has been investigated resulting in 10.1% of 475 animals and none of 102 animals positive for STEC isolation, respectively (EFSA and ECDC, 2016). In our study, 92.8% (77/83) swine faecal samples resulted stx-positive, and a STEC isolate was recovered in 7.2% (6/83) of samples. STEC contamination rate assessed by real time PCR for stx genes resulted higher while the isolation rate resulted comparable to that reported in other European countries. However, it is very difficult to make comparisons among different studies since sampling collection method, sample size, geographic location and different protocols applied for STEC PCR detection and isolation can significantly influence the results (Fratamico et al., 2004; Tseng et al., 2014b). As reported by EFSA and ECDC (2016), 859 samples of pork meat have been tested in EU during 2015 by ISO13136:2012 with a STEC isolation rate of 2.56% (22/859). In Italy STEC contamination rate in fresh pork sausages assessed by PCR for stx genes varied from 16% (20/126) (Villani et al., 2005) to 19% (41/213) (Bardasi et al., 2015) and the analysis of 675 samples including both fresh and dried products revealed 19 (2.8%) stx-positive fresh sausage samples (Ercoli et al., 2016). The STEC isolation rate ranged from 0% (Ercoli et al., 2016) to 10% (13/126) (Villani et al., 2005). In this study, no of one 97 RTE samples and 65 (14.0%) of 465 NRTE samples resulted stx positive by real time PCR with an isolation rate of 1.5% (7/465) among NRTE samples.

It is worth highlighting that all the 97 RTE samples included in the analysis resulted stx-negative. Various production processes were comprised in the panel of the RTE tested samples: heat-curing (mortadella), dry-curing (ham, pancetta and coppa), curing of fermented and air-dried meat (salami). Our data indicate that these manufacturing processes may have rendered the tested samples safe with respect to contamination by STEC.

As reported by EFSA and ECDC (2016), among the 859 samples of pork meat tested in EU in 2015 one O157 STEC strain (0.12%) and no O26, O103, O145, O111 STEC strains were isolated. Ercoli et al. (2016), reported high percentage of detection of Top five serogroup associated genes both in pork samples (O157: 42.1%, O145: 94.7%, O103: 78.9%, O26: 36.8%) and in swine faecal samples (O157: 86.4%, O145: 9.9%, O103: 17.3%, O26: 38.3%, O111: 1.2%), but no STEC strains belonging to these serogroups has been isolated.

Verocytotoxin-producing E. coli O157 has been isolated from swine faecal samples at low frequencies in Europe, ranging from 0% (0/630) in Switzerland (Kaufmann et al., 2006), France (0/182) (Bouvet et al., 2002), Belgium (0/101) (Botteldoorn et al., 2003) and United Kingdom (0/1000) (Chapman et al., 1997), 0.08% (2/2446) in Sweden (Eriksson et al., 2003), 0.1% (2/1976) in Norway (Johnsen et al., 2001) and 0.7% (1/150) in Italy (Bonardi et al., 2003).

The genes associated with the top five and O104 serogroups were detected at high frequency in this study, in particular O26, O145 and O157 serogroups resulted positive with high percentage in both faecal and food samples. O26 resulted the most frequent serogroup in faecal samples (81.3% of the four serogroups have not been isolated in this study. Additionally, neither strains harboring both stx and eae genes, nor strains harboring stx gene in association with aaiC and aggR genes, have been isolated. The characteristics of the isolated strains indicate that the virulence and the serogroups-associated genes detected at high frequencies by real time PCR in the enriched samples, are presumably located on different bacterial strains. This hypothesis is also supported by the isolation from faecal samples of 19 strains belonging to O26, O103, O145, O111 and O104 serogroups (5 belonging to serogroup O26, 3 to O103, 2 to O104, 1 to O111, 8 to O145, none to serogroup O157) but not characterised by the presence of the stx and/or eae genes and of two E. coli strains only harboring the eae gene. An extensive study carried out in U.S.A. on 181 STEC strains recovered from swine faecal samples reported comparable results: none of the STEC strains characterised by molecular methods carried eae gene or belonged to O26, O103, O111, O157 and O104 serogroups, only one strain being positive to O145 serogroup-associated gene (Baranzoni et al., 2016).

According to the molecular classification scheme proposed by EFSA (2013) STEC can be categorised according to potential risk for consumers health as group I (high potential risk) through to group III (unknown risk). An isolate of STEC serogroups O157, O26, O103, O145, O111, O104 in combination with stx and eae or aaiC and aggR genes (group I) should be considered as presenting a potentially high risk for diarrhoea and HUS. For any other serogroups in combination with the same genes (group II), the potential risk is regarded as high for diarrhoea, but currently unknown for HUS. In the absence of these genes (group III), there is uncertainty whether or not they are able to cause disease and a scientific judgement based on current knowledge of virulence characteristics cannot be done (EFSA, 2013). Remarkably, the STEC strains isolated in this study all belong to group III. Actually, swine are not viewed as an important STEC reservoir given the rare incidence of cases of severe human illness associated with STEC of swine origin (Tseng et al., 2014b).

**Conclusions**

In conclusion, in this study STEC strains have been isolated both in swine feces at slaughter and in NRTE pork products, while all RTE samples resulted stx-negative. The
isolated strains are not the ones correlated with high risk for diarrhoea and HUS; nevertheless their human pathogenetic potential is not yet fully defined.

References

Baranzoni GM, Fratamico PM, Gangiredda J, Patel I, Bagi LK, Delannoy S, Fach P, Boccia F, Anastasio A, Pepe T, 2016. Characterization of shiga toxin subtypes and virulence genes in porcine shiga toxin-producing Escherichia coli. Front Microbiol 21:574.

Bardasi L, Taddei R, Nocera L, Ricchi M, Meriali G, 2015. Shiga toxin-producing Escherichia coli in meat and vegetable products in Emilia Romagna Region, years 2012-2013. Ital J Food Safety 4:4511.

Beutin L, Martin A, 2012. Outbreak of Shiga toxin producing Escherichia coli (STEC) O104:H4 infection in Germany causes a paradigm shift with regard to human pathogenicity of STEC strains. J Food Protect 75:408-18.

Bonardi S, Brindani F, Pizzin G, Lucidi L, D’Incau M, Liebana E, Morabito S, 2003. Detection of Salmonella spp., Yersinia enterocolitica and verocytotoxin producing Escherichia coli O157 in pigs at slaughter in Italy. Int J Food Microbiol 85:101-10.

Botteldoorn N, Heyndrickx M, Roux A, Botteldoorn N, Roux A, Borch A, Gunnarsson A, 2003. Verocytotoxin-producing Escherichia coli O157:H7 in the Swedish pig population. Vet Rec 152:712-7.

Fratamico PM, Bagi LK, Bush EJ, Solow BT, 2016. Prevalence and characterization of verotoxigenic Escherichia coli strains isolated from pigs and pork products in Umbria and Marche regions of Italy. Int J Food Microbiol 232:7-14.

Eriksson E, Nenbrink E, Borch E, Aspa A, Gunarrsson A, 2003. Verocytotoxin-producing Escherichia coli O157:H7 in the Swedish pig population. Vet Rec 152:712-7.

EFSA, ECDC, 2016. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. EFSA J 14:4634.

EFSA. 2013. Scientific opinion on VTEC-serotypepath and scientific criteria regarding pathogenicity assessment. EFSA J 11:1-106.

EFSA, ECDC, 2016. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. EFSA J 14:4634.

Ercoli L, Farneti S, Zicavo A, Mencaroni G, Blasi G, Striano G, Scuota S, 2016. Prevalence and characteristics of verotoxigenic Escherichia coli strains isolated from pigs and pork products in Umbria and Marche regions of Italy. Int J Food Microbiol 232:7-14.

ISO, 2012. International Organization for Standardization. Microbiology of food and animal feed. Real-time polymerase chain reaction (PCR)-based method for the detection of food-borne pathogens. Horizontal method for the detection of Shiga toxin-producing Escherichia coli (STEC) and the determination of O157, O111, O26, O103 and O145 and O157:H7, associated with the world’s most frequent clinical cases. Mol Cell Probes 18:185-92.

MacDonald DM, Fyfe M, Paccagnella A, Trinidad A, Louie K, Patrick D, 2004. Escherichia coli O157:H7 outbreak linked to salami, British Columbia, Canada, 1999. Epidemiol Infect 132:283-9.

Nielsen EM, Andersen MT, 2003. Detection and characterization of verocytotoxin producing Escherichia coli by automated 5’ nuclease PCR assay. J Clin Microbiol 41:2884-93.

Paton AW, Ratcliff RM, Doyle RM, Seymour-Murray J, Davos D, Lanser JA, Paton JC, 1996. Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing Escherichia coli. J Clin Microbiol 34:1622-7.

Pereille S, Dilasser F, Grout J, Fach P, 2004. Detection by 5’-nuclease PCR of Shiga-toxin producing Escherichia coli O26, O55, O91, O103, O111, O113, O145 and O157:H7, associated with the world’s most frequent clinical cases. Mol Cell Probes 18:185-92.

Trotz-Williams LA, Mercer NJ, Walters JM, Maki AM, Johnson RP, 2012. Pork implicated in a Shiga-toxin-producing Escherichia coli O157:H7 outbreak in Ontario, Canada. Can J Public Health 103:e322-6.

Tseng M, Fratamico PM, Bagi L, Delannoy S, Fach P, Manning SD, Funk JA, 2014a. Diverse virulence gene content of Shiga toxin-producing Escherichia coli from finishing swine. Appl Environ Microbiol 80:6395-402.

Tseng M, Fratamico PM, Bagi L, Muzinger D, Funk JA, 2014b. Shiga toxin-producing Escherichia coli in fresh Italian pork sausages, and preparation and use of an antibiotic-resistant strain for challenge studies. Meat Sci 70:181-8.

Williams RC, Isaacs S, Decou ML, Richardson EA, Buffett MC, Slinger RW, Brodsky MH, Ciebin BW, Ellis A, Hockin J, 2000. Illness outbreak associated with Escherichia coli O157:H7 in Genoa salami. Can Med Assoc J 162:1409-13.