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

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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 phage-encoded cytotoxins, 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 enteroaggregative *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 pigs and in food products made of pork are collected at the European level only by a few countries and they are not always comparable (EFSA and ECDC, 2016). Following *E. coli* O104:H4 outbreak occurred in Germany in May 2011, Emilia Romagna Region instituted in 2012 a testing program for O157, O26, O111, O103, O145 and O104:H4 STEC, both at production and at retail level, for foodstuff considered at risk of contamination. During years 2012-2013 monitoring plan STEC virulence genes were detected at high frequency (19%) in fresh pork sausage and one verocytotoxic *E. coli* O103 strain was isolated from pork meat (Bardasi *et al*., 2015).

Because of the limited epidemiologic 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.

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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 (mTSB+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 10min 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 60 s. Samples resulted positive for the presence of *stx1* and/or *stx2* gene were tested for *E. coli* O104 serogroup-associated gene. Sample positive for the presence of *stx1* and/or *stx*2 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-Glucuronide (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 *aaiC* 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, 57/83 (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 52/64 (81.3%), 51/64 (79.7%), 48/77 (62.3%), 23/64 (35.9%), 51/64 (79.7%) and 50/64 (78.1%) faecal samples. Out of a total of 562 pork products tested, no one of the 97 RTE sample resulted positive for *stx* genes and among the 465 NRTE samples, 65 (14.0%) resulted *stx*-positive. The *stx2* gene was detected more frequently than the *stx1* gene (62/465 equals to 13.3% *vs* 6/465 equals to 1.3%). One sample out of 465 (0.2%) resulted positive only for *stx1* gene, 2/465 (0.4%) samples were positive for *stx1* associated with *eae* gene, 22/465 (4.7%) samples tested positive only for *stx2* gene, 37/465 (8.0 %) were positive for the *stx2* gene in association with *eae* gene and 3/465 (0.7%) samples resulted positive for *stx1*, *stx2* and *eae*. Serogroup O26, O103, O104, O111, O145 and O157 associated genes were detected respectively in 32/42 (76.2%), 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
stx_1	0/83	0/97	1/465 (0.2)
stx_1+*eae*	2/83 (2.4)	0/97	2/465 (0.4)
stx_2	13/83 (15.7)	0/97	22/465 (4.7)
stx_2+*eae*	57/83 (68.7)	0/97	37/465 (8.0)
stx_1+stx_2+*eae*	5/83 (6.0)	0/97	3/465 (0.7)
O26*	52/64 (81.3)	nd	32/42 (76.2)
O103*	51/64 (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	37/42 (88.1)
O157*	50/64 (78.1)	nd	35/42 (83.3)

RTE, ready to eat; NRTE, not ready to eat; nd, not done. *The percentage are calculated *vs* the number of samples subjected to analysis according to ISO/TS13136, *i.e.* the number of samples positive for stx_1 and/or stx_2 genes for O104 serogroup and the number of samples positive for stx_1 and/or stx_2 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	5/64 (7.8)	9/64 (14.1)	4/64 (6.3)	5/64 (7.8)	18/64 (28.1)	20/64 (31.3)
NRTE food samples	5/42 (11.9)	7/42 (16.7)	12/42 (28.6)	15/42 (35.7)	3/42 (7.1)	1/42 (2.4)

NRTE, not ready to eat.

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 *stx1*+ strain and 5 *stx2*+ strains were isolated, two strains *stx2*+ 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 s*tx2*+: 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 one of 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 *stx* and *eae* positive samples) and O145 in pork products (88.1% of the *stx* and *eae* positive samples). The O157 serogroup resulted positive in 78.1% and 83.3% of the faecal samples and pork samples tested for O-group associated genes, respectively. Furthermore, 61 out of the 64 (95.3%) faecal samples and all the 42 pork samples tested for the six serogroups (O26, O103, O104, O111, O145 and O157) resulted contaminated with one or more serogroups, the higher percentage of samples being positive to all six serogroups in faecal samples and to four serogroups in pork products. Nevertheless STEC strains belonging to the six 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 a*ggR* 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 a*ggR* 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 stains 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 pathogenic potential is not yet fully defined.

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