DOI: 10.1002/vms3.175

Original Article

Detection and isolation of Shiga Toxin-producing *Escherichia coli* (STEC) strains in caecal samples from pigs at slaughter in Italy

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) strains are food-borne pathogens of public health concern. Despite ruminants are the most important reservoir, STEC human infections have also been attributed to pigs. We examined for the presence of STEC in 234 samples of swine caecal content collected during the year 2015 at Italian abattoirs in the framework of the harmonized monitoring of antimicrobial resistance (Decision 2013/652/EU). The presence of *stx* genes was detected in 122 (52.1%) samples, which were subsequently subjected to STEC isolation and characterization. The analysis of the 66 isolated STEC strains showed that the majority of the isolates (74.2%) possessed the *stx2a* gene subtype, in a few cases (16.7%) in combination with *stx2b* or *stx2c*. Only 25.8% of isolates possessed the *stx2e* subtype, typical of swine-adapted STEC. None of the isolates possessed the intimin-coding *eae* gene and the majority of them did not belong to serogroups commonly associated with human infections. The results of this study suggest that pigs can be considered as potential reservoir of certain STEC types.

Keywords: STEC prevalence, *stx* subtypes, swine.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are food-borne pathogens of public health concern. STEC infection has been associated with severe clinical diseases in humans, including haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS), which can lead to kidney failure and death (Nataro & Kaper 1998; Karmali *et al*. 2010). The main virulence determinants of STEC are Shiga toxins (Stx), which are divided in two major antigenic forms: Stx1 and Stx2. Large variability in *stx* genes sequences has been described and three subtypes of *stx1* (*stx1a*, *stx1c*, *stx1d*) and seven of *stx2* (*stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f*, and *stx2 g*) have been recognized (Scheutz *et al*. 2012). Although Stx1 has been linked to human illness, STEC that produce Stx2, and particularly subtypes Stx2a, Stx2c and Stx2d are more often associated with the development of the most severe forms of infections (Friedrich *et al*. 2002; Melton-Celsa & O'Brien 2014).

Ruminants, and particularly cattle, are the most important STEC reservoir (Caprioli *et al*. 2005), with colonized animals usually asymptomatic (Gyles 2007). Human infections mainly occur through ingestion of contaminated undercooked meat or by contact with infected animals and contaminated environment (Caprioli *et al*. 2005).

Besides the production of Stx, STEC possess several virulence factors which contribute to the development of the severe forms of the disease, such as HC and HUS. Among these, the locus of enterocyte

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effacement (LEE) harbours genes involved in the attaching-effacing mechanism of intestinal adhesion and represents a common feature of STEC strains associated with HUS (Frankel *et al*. 1998). LEE-negative STEC strains are also isolated from cases of human disease, and for some of these the presence of virulence genes accessory to the genes encoding the Stx has been described. These include the *subAB* operon, coding for the Subtilase cytotoxin (Michelacci *et al*. 2013), the STEC autoagglutinating adhesin-coding gene *saa,* and *tia*, a gene whose expression produces an invasion determinant (Paton *et al*. 2004; Tozzoli *et al*. 2010). LEE-negative strains isolated from human illness belong to a restricted number of serogroups such as O113, O91, O146 and O128, which have been included in the top-20 STEC serogroups associated with human infections in the EU (EFSA and ECDC, 2017).

It has been described that healthy swine may shed STEC in their faeces (Tseng *et al*. 2014) and cases of human STEC infections, including outbreaks, have been attributed to pork products (Conedera *et al*. 2007; Baranzoni *et al*. 2016; Honish *et al*. 2017), although in these reports it could not be excluded that the contamination of pork-derived food may have occurred during the processing or by cross contamination (Tseng *et al*. 2014). Some STEC strains cause oedema disease in pigs, a peracute often-fatal enterotoxemia affecting primarily healthy animals after weaning (Cornick *et al*. 1999). Clinically, the disease is characterized by swelling of the eyelids, typical squeal or snoring sound, neurologic signs and subcutaneous and submucosal oedema in various tissues (Casanova *et al*. 2018). Occurrence of the disease depends on several variables, such as diet, environmental factors, stress factors, immunity and genetic resistance. Lethality rate is high and range from 50% to 90% with frequent recurrences (Casanova *et al*. 2018).

E. coli strains associated with oedema disease commonly produce Stx2e, which is regarded as the key virulence factor involved in the pathogenesis of the disease, although Stx2e-producing strains are normally isolated also from healthy swine (Cornick *et al*. 1999; Fratamico *et al*. 2004; Meng *et al*. 2014; Tseng *et al*. 2015; Zweifel et al 2006). As far as the public health significance of these STEC strains is concerned, Stx2e-producing *E. coli* are not considered of particular concern (Friedrich *et al*. 2002; Tseng *et al*. 2014), being only sporadically isolated from human cases of mild diarrhoea or from asymptomatic persons (Beutin et al. 2004; Fratamico *et al*. 2004; Meng *et al*. 2014; Tseng *et al*. 2014). To the best of our knowledge, isolation of Stx2e-producing STEC from HUS has been reported only in two cases (Thomas *et al*. 1994; Fasel *et al*. 2014). Nevertheless, the severe outcome of the disease in these reports was probably due to additional causes, as in the first HUS case co-infection with another STEC strain was detected (Thomas *et al*. 1994), while in the second suppression of the patient's immune-system was reported (Fasel *et al*. 2014).

In several countries, cross-sectional studies have been conducted to assess the prevalence of STEC in pigs, showing that this may vary among countries, being sometimes reported as 0% as well as 68.3% in one study from Chile (Tseng *et al*. 2014). Besides Stx2e-producing strains, there is a lack of information on the circulation of STEC strains possessing other Stx subtypes or accessory virulence factors such as the Subtilase cytotoxin, the adhesin Saa and the invasin Tia in swine and the risk of human illness associated with the presence of STEC in this animal species remains uncertain. The objectives of the present study were to investigate on the presence of STEC in pigs at slaughter in Italy by applying a sampling scheme producing prevalence data representative of the Italian country and to characterize the isolated STEC strains for their virulence genes asset.

Materials and methods

Sampling strategy

During 2015, a total of 234 samples of caecal content were collected at slaughter from fattening pigs carcasses in the framework of the sampling scheme planned for the harmonized monitoring of antimicrobial resistance for the year 2015 (Dec. 2013/652/EU). Samples were collected by the local Veterinary Public Health authorities, kept refrigerated and sent to the Istituto Zooprofilattico Sperimentale del Lazio e della Toscana "M. Aleandri" (IZSLT), where they

were examined within 48 hours from sampling. Samples originated from randomly selected epidemiological units (holdings); only one sample per unit was collected. The sample size was stratified among the Italian regions with highest numbers of slaughtered animals per year (Table 1), representing > 89% of slaughtered pigs nationwide. Within each region, stratification took into account the throughput of slaughterhouses.

Analysis of the swine caecal samples for the presence of STEC virulence genes

Twenty-five grams of each caecal content samples was enriched by incubation in buffered peptone water at 37°C for 18–24 h. Following the incubation, 0.1 mL of each enrichment culture was streaked on MacConkey agar (MC) plates and incubated at 41°C for 18–24 h to select for *Enterobacteriaceae*. A loopful from the confluent growth was plated on Tryptic Soy Agar (TSA) and incubated at 37°C for 18–24 h. The bacterial layer was diluted in 0.5 mL of water, treated at 100°C for 10 min and used as template in a specific PCR for the detection of *stx1*, *stx2* and *eae* genes (Paton & Paton 1998). Bacterial cultures positive for at least one of the three genes were inoculated into soft agar tubes and sent to the Istituto Superiore di Sanità (ISS) for STEC strains isolation and characterization.

Table 1. Distribution of caecal samples investigated per Region in Italy in 2015, in relation to the slaughtering throughput of the previous year

Italian Region	Number of slaughtered pigs in 2014 per Region	% of slaughtered pigs in Italy per Region in 2014	Number (%) of caecal samples investigated per Region at slaughterhouse in 2015
Lombardia	4 163 622	40.75	118 (50.4)
Emilia Romagna	3 476 347	34.02	88 (37.6)
Piemonte	630 177	6.17	12 (5.1)
Umbria	338 320	3.31	7 (3.0)
Toscana	229 145	2.24	7 (3.0)
Veneto	282 453	2.76	2 (0.9)
Total	9 120 064	89.25	234 (100)

STEC isolation and *stx2*-gene subtyping

The cultures positive for *stx* genes in the screening were seeded on MacConkey agar plates and single colonies (a total of 10 colonies per sample) were assayed for the presence of both *stx1* and *stx2* genes. DNA was extracted by using InstaGene matrix (BioRad, Carlsbad, CA, USA) and analysed by Real-Time PCR with oligonucleotides and probes specific to *stx* genes (Perelle *et al*. 2004). Isolated colonies positive for the presence of *stx2* gene were also assayed by conventional PCR for the presence of the *eae* gene using primers SK1-SK2 (Schmidt *et al*. 1994), and tested for the presence of *stx2e* subtype with primers FK1/FK2 (Franke *et al*. 1995). The other *stx2* subtypes (*stx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2f*, *stx2 g*), were identified by conventional PCR, using the method described by Scheutz and colleagues (Scheutz *et al*. 2012).

Detection of the presence of *subAB*, *saa* and *tia* genes

The presence of virulence genes associated with LEE-negative STEC was investigated in isolated STEC strains, with the exception of *stx2e*-positive strains. DNA was extracted from single colonies of STEC strains using InstaGene matrix (BioRad, Carlsbad, CA, USA) and assayed by PCR using specific primers targeting genes encoding the Subtilase cytotoxin (*subAB*), the adhesin Saa (*saa*) and the invasin Tia (*tia*), as previously described (Paton *et al*. 2004; Tozzoli *et al*. 2010).

Molecular serotyping of STEC isolates

DNAs extracted from all STEC isolates, with the exception of *stx2e*-positive strains, were assayed by PCR for the identification of O91, O113, O128, O146 and O104 serogroups, characteristic of *eae*-negative STEC strains more frequently isolated from human infections, using the primers and the conditions indicated in the EURL-VTEC method "Identification of VTEC serogroups mainly associated with human infections by conventional PCR amplification of O-associated genes" (EURL VTEC_Method_03_Rev1 http://old.iss.it/binary/vtec/cont/EU_RL_VTEC_Method_03_Rev_1.pdf).

Results

Screening of swine caecal samples for the presence of STEC virulence genes

The presence of *stx* genes was detected in 122 out of the 234 (52.1%; 95% CI 45.5%–58.7%) swine caecal content samples tested (Table 2). Four samples were positive for the *stx1* gene alone, in one case together with the *eae* gene. Two other samples were positive for both *stx1* and *stx2* genes, in one case together with the *eae* gene. The remaining 116 samples were positive for the *stx2* gene, in 74 samples together with the *eae* gene (Table 2). Forty-seven samples were positive for the *eae* gene alone (Table 2).

Isolation and characterization of STEC strains

Sixty-six STEC strains were isolated from 56 out of the 118 *stx2*-positive samples (116 with *stx2* and two with both *stx1* and *stx2*), with two different STEC isolated from 10 of these samples (Table 3). The isolation of STEC strains from the *stx1*-positive samples, alone or in combination with *stx2*, was not successful (Table 3).

STEC isolates were tested for the presence of the *eae* gene, all with negative results.

The *stx2* gene subtyping showed that 17 of the 66 STEC isolates (25.8%) harboured the *stx2e* gene subtype. The remaining 49 isolates (74.2%) possessed *stx2a* gene, in some cases together with *stx2b* (3 strains, 4.5%) or *stx2c* (8 strains, 12%) (Table 3).

Table 2. Detection of STEC virulence genes in the 234 samples of swine caecal content collected at slaughter in Italy

Virulence profile	Number of samples	%
eae stx1 stx2	1	0.43
eae stx2	74	31.62
eae stx1	1	0.43
stx1 stx2	1	0.43
stx1	3	1.28
stx2	42	17.95
eae	47	20.08
Negative	65	27.78
Total	234	100

Molecular serotyping showed that two strains, both possessing the *stx2a* gene subtype, belonged to O128 serogroup, whereas the remaining were negative to all the tested serogroups.

All STEC strains assayed resulted negative for the presence of *subAB* and *saa* genes, whereas six STEC isolates, including four strains possessing *stx2a* gene, one possessing *stx2a* and *stx2b* and one harbouring *stx2a* and *stx2c* genes, possessed the *tia* gene.

Discussion

Swine harbour STEC strains that can be transferred along the food-chain posing a risk for public health (Baranzoni *et al*. 2016). As a matter of fact, STEC have been isolated from pigs and pork products, in some cases associated with episodes of HC and HUS in humans (MacDonald *et al*. 2004; Conedera *et al*. 2007; Fratamico *et al*. 2008; Trotz-Williams *et al*. 2012; Baranzoni *et al*. 2016; Honish *et al*. 2017).

Our study aimed at determining the prevalence of STEC in Italian swine, using a sampling strategy focused on providing representativeness, at the population level, in one of the most relevant pig-producing country in Europe. In Italy this is the first study on the prevalence of STEC in pigs carried out using such a comprehensive approach.

Moreover, we adopted an analytical methodology consisting in the molecular screening of the test samples followed by an isolation step, a procedure in line with the recommendations of the European Food Safety Authority for the monitoring of STEC

Table 3. Number of samples with successful STEC isolation, and *stx2*-subtype profile of the isolated strains

Number of samples	Number of obtained isolates	*stx2* subtype
29	29	*stx2a*
3	3	*stx2a stx2b*
7	7	*stx2a stx2c*
9	18	*stx2e* (9 isolates)*stx2a* (9 isolates)
1	2	*stx2e* (1 isolate)*stx2a stx2c* (1 isolate)
7	7	*stx2e*
Total: 56	Total: 66	

(EFSA, 2009), and a subsequent characterization of the isolates in terms of *stx* subtypes and accessory virulence genes, including virulence factors previously reported in *eae*-negative STEC.

The results obtained in our study showed a high prevalence of STEC (52.1%) in the caecal content of slaughtered pigs in Italy. Previous studies conducted in Italy on the presence of STEC in pigs have produced a wide range of prevalence rates. The analysis of caecal samples from slaughtered pigs in the Emilia Romagna region showed the presence of *stx* genes in 92.8% of the specimens (Bardasi *et al*. 2017), while in another study carried out in the Umbria and Marche regions the presence of *stx* genes was identified in 38.6% of the samples (Ercoli *et al*. 2016). These discrepancies may be due to differences in either the sampling strategies used or the methods applied. For instance, in the above-mentioned studies, screening for *stx* genes was performed directly from the enrichment broth by Real Time PCR (Ercoli *et al*. 2016; Bardasi *et al*. 2017). The EFSA/ECDC EU Summary report on food-borne zoonoses for the year 2015, when the samples assayed in our study were collected, highlighted a STEC prevalence of only 8.3% in pigs (EFSA & ECDC 2016). However, the data reported to EFSA were provided by two Member States only, with the majority of the tests performed in one of them.

In our study, STEC strains were isolated from 56 out of the 122 *stx*-positive samples, indicating an isolation rate of 45.9%. As described in other studies (Meng *et al*. 2014; Ercoli *et al*. 2016), two different STEC strains were isolated from 10 of the positive samples (Table 3).

Only STEC strains possessing *stx2*-coding genes could be isolated. They displayed different *stx2* subtypes, including *stx2a*, alone or in combination with *stx2c* or *stx2b*. Interestingly, the strains possessing *stx2* subtypes other than *stx2e* accounted for 74.2 % of the total STEC isolates (49 out of 66), while other studies reported a lower frequency of *stx2* subtypes different from *stx2e* in STEC isolated from swine (Fratamico *et al*. 2008; Baranzoni *et al*. 2016; Cha *et al*. 2018). In this study, the majority of the strains possessed the *Stx2a*-coding gene, a *Stx* subtype frequently found in STEC isolated from HC and HUS. Indeed, the presence of the *stx2a* subtype in swine STEC isolates has been previously reported in a study performed in the US (Cha *et al*. 2018), but with a much lower frequency (two isolates out of 352 strains investigated) than that observed in this study. However, none of the strains isolated in the present study possessed the *eae* gene, coding the adhesion factor intimin, considered a hallmark of STEC strains causing severe human disease (Caprioli *et al*. 2005), and only two strains belonged to a serogroup, O128, included in the "top 20" list of STEC serogroups causing human disease in the years 2014-2016 (EFSA & ECDC, 2017).The investigation of the presence of a panel of virulence-associated genes described in *eae*-negative STEC strains allowed to identify the presence of the gene encoding the invasion-associated protein Tia (Fleckenstein *et al*. 1996) in six strains. The product of this gene has been described for the first time as an invasion determinant in enterotoxigenic *E. coli* (ETEC) (Fleckenstein *et al*. 1996) and has been reported as part of the SE-PAI, pathogenicity island, frequently found in *eae*-negative STEC strains associated with human diarrhoea (Michelacci *et al*. 2013). Interestingly, in those isolates the *tia* gene was always associated with the presence of an operon encoding the Subtilase cytotoxin (SubAB) (Paton *et al*. 2004; Michelacci *et al*. 2013), while in the isolates characterized in this study, the *subAB* operon was not identified.

The results presented in this study showed a high prevalence of STEC in the caecal content of slaughtered pigs in Italy indicating that swine can be considered reservoir of certain STEC types. The public health significance of this finding has yet to be ascertained, as the STEC identified were all negative for the presence of the intimin-coding *eae* gene as well as of other virulence-associated determinants, such as the *subAB* and *saa*. Moreover, with the exception of two O128 isolates, the majority of the STEC isolates did not belong to serogroups commonly associated with cases of human disease.

Further studies should be carried out to ascertain the role of swine in the epidemiology of STEC infection as well as to understand the implication in terms of animal health of the different STEC strains/types circulating in the swine population.

Acknowledgements

The authors would like to thank Paola Chiani for the technical advise for STEC isolation. The authors sadly report that one of the co-authors, Serena Lorenzetti, died during the revision process of this paper.

Source of funding

The study was supported by intramural funds of the Istituto Superiore di Sanità.

Conflict of interests

The authors declare that they have no conflict of interests.

Ethics Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The US National Research Council's guidelines for Cara and Use of Laboratory Animals were followed.

Contributions

Silvia Arancia performed the experiments for isolation and characterization of STEC and drafted the manuscript. Manuela Iurescia carried out the screening of the swine caecal samples for the presence of STEC and prepared the cultures to be further analysed at ISS, with experimental support provided by Serena Lorenzetti, Fiorentino Stravino and Carmela Buccella. Andrea Caprioli, Alessia Franco and Antonio Battisti designed the sampling scheme and contributed to draft and revise the manuscript. Stefano Morabito contributed to the critical revision and finalization of the manuscript and supported in the experimental design of the study. Rosangela Tozzoli conceived the experimental design, coordinated the scientific activities and largely contributed in the manuscript drafting and revision. Finally, all the authors participated in the data analysis and approved the manuscript to be published.

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