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

Silvia Arancia*†, Manuela Iurescia†, Serena Lorenzetti‡, Fiorentino Stravino†, Carmela Buccella†, Andrea Caprioli†, Alessia Franco†, Antonio Battisti†, Stefano Morabito* and Rosangela Tozzoli*

*Laboratorio Nazionale di Riferimento per E. coli, Istituto Superiore di Sanità, Rome, Italy and †Centro di Referenza Nazionale per l’Antibioticor Resistenze, Istituto Zooprofilattico Sperimentale del Lazio e della Toscana “M. Aleandri”. Direzione Operativa Diagnostica Generale, Rome, Italy

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.

Correspondence: Silvia Arancia, Istituto Superiore di Sanità, Viale Regina Elena 299, 00161 Rome, Italy. E-mail: silvia.arancia@iss.it

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 stx2g) 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
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.

**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).

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

| 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  |

| 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 SEPAI, 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 cyto-toxin (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.
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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.

References

Baranzoni G.M., Fratamico P.M., Gangiredla J., Patel I., Bagi L.K., Delannoy S. et al. (2016) Characterization of Shiga Toxin Subtypes and Virulence Genes in Porcine Shiga Toxin-Producing Escherichia coli. Frontiers in Microbiology 7, 574.

Bardasi L., Taddei R., Fiocchi I., Pelliconi M.F., Ramini M., Toschi E. & Meriali G. (2017) Shiga Toxin-Producing Escherichia coli in Slaughtered Pigs and Pork Products. Italian Journal of Food Safety 6, 6584.

Beutin L., Krause G., Zimmermann S., Kaulfuss S. & Glier K. (2004) Characterization of Shiga toxin-producing Escherichia coli strains isolated from human patients in Germany over a 3-year period. Journal of Clinical Microbiology 42, 1099–1108.

Caprioli A., Morabito S., Brugere H. & Oswald E. (2005) Enterohaemorrhagic Escherichia coli: emerging issues on virulence and modes of transmission. Veterinary Research 36, 289–311.

Casanova N.A., Redondo L.M., Diallof G.C., Arenas D. & Fernández Miyakawa M.E. (2018) Overview of the role of Shiga toxins in porcine edema disease pathogenesis. Toxicon 15, 149–154.

Cha W., Fratamico P.M., Ruth L.E., Bowman A.S., Nolt- ing J.M., Manning S.D. & Funk J.A. (2018) Prevalence and characteristics of Shiga toxin-producing Escherichia coli in finishing pigs: implications on public health. International Journal of Food Microbiology 264, 8–15.

Conedera G., Mattiazzi E., Russo F., Chiesa E., Scorzato I., Grandesso S. et al. (2007) A family outbreak of Escherichia coli O157 haemorrhagic colitis caused by pork meat salami. Epidemiology and Infection 135, 311–314.

Cornick N.A., Matise I., Samuel J.E., Bosworth B.T. & Moon H.W. (1999) Edema disease as a model for systemic disease induced by Shiga toxin-producing E. coli. Advances in Experimental Medicine and Biology 473, 155–161.

EFSA (2009) Technical specifications for the monitoring and reporting of verotoxigenic Escherichia coli (VTEC) on animals and food (VTEC surveys on animals and food). EFSA Journal 11, 43.

EFSA & ECDC (2016) The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2015. EFSA Journal 14, 231.

EFSA & ECDC (2017). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA Journal 15, 228.

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. *International Journal of Food Microbiology* **232**, 7–14.

Fasel D., Mellmann A., Cernela N., Hachtler H., Fruth A., Khanna N. et al. (2014) Hemolytic uremic syndrome in a 65-Year-old male linked to a very unusual type of stx2e- and eae-harboring O51:H49 shiga toxin-producing *Escherichia coli*. *Journal of Clinical Microbiology* **52**, 1301–1303.

Fleckenstein J.M., Koepecko D.J., Warren R.L. & Elsinghorst E.A. (1996) Molecular characterization of the tia invasion locus from enterotoxigenic *Escherichia coli*. *Infection and Immunity* **64**, 2256–2265.

Franke S., Gunzer F., Wieler L.H., Baljer G. & Karch H. (1996) Construction of recombinant Shiga-like toxin-IIv (SLT-IIv) and its use in monitoring the SLT-IIv antibody status of pigs. *Veterinary Microbiology* **43**, 41–52.

Frankel G., Phillips A.D., Rosshinse L., Dougan G., Kaper J.B. & Knutton S. (1998) Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. *Molecular Microbiology* **30**, 911–921.

Fratamico P.M., Bagi L.K., Bush E.J. & Solow B.T. (2004) Prevalence and characterization of shiga toxin-producing *Escherichia coli* in swine feces recovered in the National Animal Health Monitoring System’s Swine 2000 study. *Applied and Environmental Microbiology* **70**, 7173–7178.

Fratamico P.M., Bhagwat A.A., Injain L. & Fedorka-Cray P.J. (2008) Characterization of Shiga toxin-producing *Escherichia coli* strains isolated from swine feces. *Foodborne Pathogens and Disease* **5**, 827–838.

Friedrich A.W., Bielaszewska M., Zhang W.L., Pulz M., Kuczus T., Ammon A. & Karch H. (2002) *Escherichia coli* harboring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. *The Journal of Infectious Diseases* **185**, 74–84.

Gyles C.L. (2007) Shiga toxin-producing *Escherichia coli*: an overview. *Journal of Animal Science* **85**, E45–E62.

Honish L., Punja N., Nunn S., Nelson D., Hislop N., Gosselin G. et al. (2017) *Escherichia coli* O157:H7 Infections Associated with Contaminated Pork Products - Alberta, Canada, July-October 2014. *MMWR Morbidity and Mortality Weekly Report* **65**, 1477–1481.

Karmali M.A., Gannon V. & Sargeant J.M. (2010) Vero-cytotoxin-producing *Escherichia coli* (VTEC). *Veterinary Microbiology* **140**, 360–370.

MacDonald D.M., Fyne M., Paccagnella A., Trinidad A., Louie K. & Patrick D. (2004) *Escherichia coli* O157:H7 outbreak linked to salami, British Columbia, Canada, 1999. *Epidemiology and Infection* **132**, 283–289.

Melton-Celsa A.R. & O’Brien A.D. (2014) *Therapeutic Developments against Shiga Toxin-Producing Escherichia coli*. *Microbiology spectrum*, 2. New.

Meng Q., Bai X., Zhao A., Lan R., Du H., Wang T. et al. (2014) Characterization of Shiga toxin-producing *Escherichia coli* isolated from healthy pigs in China. *BMC Microbiology* **14**, 5.

Michelacci V., Tozzi R., Caprioli A., Martinez R., Scheutz F., Grande L. et al. (2013) A new pathogenicity island carrying an allelic variant of the Subtilase cytotoxin is common among Shiga toxin producing *Escherichia coli* of human and ovine origin. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* **19**, E149–E156.

Nataro J.P. & Kaper J.B. (1998) Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews* **11**, 142–201.

Paton A.W. & Paton J.C. (1998) Detection and characterization of Shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli hlyA, rfbO111, and rfbO157. *Journal of Clinical Microbiology* **36**, 598–602.

Paton A.W., Srimanote P., Talbot U.M., Wang H. & Paton J.C. (2004) A new family of potent AB(5) cytotoxins produced by Shiga toxigenic *Escherichia coli*. *The Journal of Experimental Medicine* **200**, 35–46.

Perelle 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. *Molecular and Cellular Probes* **18**, 185–192.

Scheutz F., Teel L.D., Beutin L., Pierard D., Buvens G., Karch H. et al. (2012) Multicenter evaluation of a sequence-based protocol for subtyping Shiga toxins and standardizing Stx nomenclature. *Journal of Clinical Microbiology* **50**, 2951–2963.

Schmidt H., Dresselhaus T., Buck F. & Heinz E. (1994) Purification and PCR-based cDNA cloning of a plastidial n-6 desaturase. *Plant Molecular Biology* **26**, 631–642.

Thomas A., Cheasty T., Chart H. & Rowe B. (1994) Isolation of Vero cytotoxin-producing *Escherichia coli* serotypes O9ab: H- and O101: H-carrying VT2 variant gene sequences from a patient with haemolytic uraemic syndrome. *European Journal of Clinical Microbiology & Infectious Diseases: Official Publication of the European Society of Clinical Microbiology* **13**, 1074–1076.

Tozzi R., Caprioli A., Cappannella S., Michelacci V., Marziano M.L. & Morabito S. (2010) Production of the subtilase AB5 cytotoxin by Shiga toxin-negative *Escherichia coli*. *Journal of Clinical Microbiology* **48**, 178–183.

Trotz-Williams L.A., Mercer N.J., Walters J.M., Maki A.M. & Johnson R.P. (2012) Pork implicated in a Shiga toxin-producing *Escherichia coli* O157:H7 outbreak in Ontario. *Canada. Canadian Journal of Public Health = Revue Canadienne de Sante Publique* **103**, e322–e326.
Tseng M., Fratamico P.M., Manning S.D. & Funk J.A. (2014) Shiga toxin-producing Escherichia coli in swine: the public health perspective. Animal Health Research Reviews 15, 63–75.
Tseng M., Fratamico P.M., Bagi L., Manzinger D. & Funk J.A. (2015) Shiga toxin-producing E. coli (STEC) in swine: prevalence over the finishing period and characteristics of the STEC isolates. Epidemiology and Infection 143, 505–514.
Zweifel C., Schumacher S., Beutin L., Blanco J. & Stephan R. (2006) Virulence profiles of Shiga toxin 2e-producing Escherichia coli isolated from healthy pig at slaughter. Veterinary Microbiology 117, 328–332.