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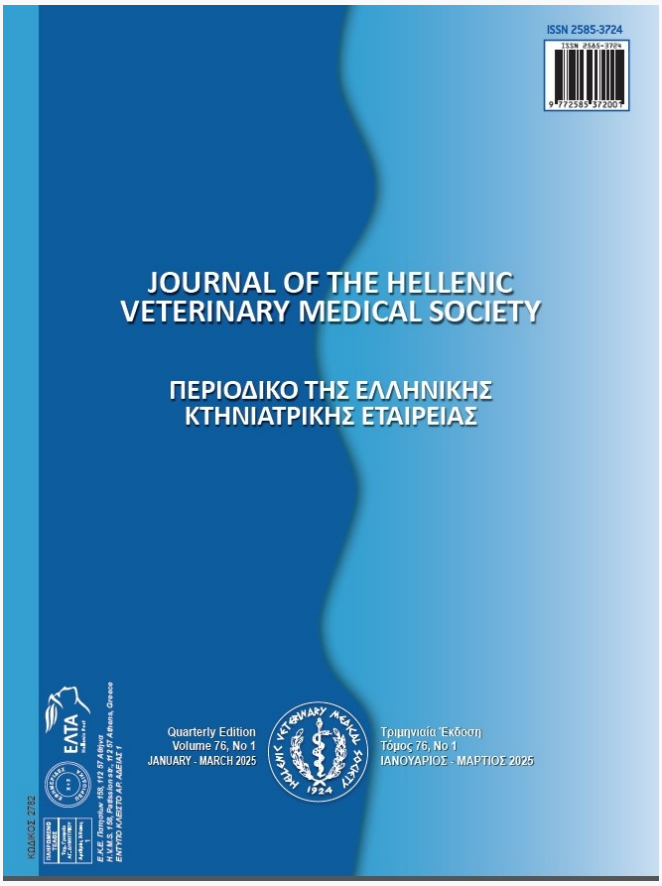

Detection of highly potent Stx2a-encoding gene in pig fecal DNA in Serbia: Could commercial pig farms in Serbia act as source of human STEC infections?

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Research article
Ερευνητικό άρθρο

Detection of highly potent $Stx2_a$-encoding gene in pig fecal DNA in Serbia: Could commercial pig farms in Serbia act as source of human STEC infections?

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ABSTRACT: Shiga toxin-producing Escherichia coli (STEC) strains are major zoonotic foodborne pathogens of public health significance. Although cattle are recognized as the major reservoir for STEC, these strains have also been frequently isolated from the intestinal content of pigs. The Shiga toxin genes were subtyped to assess public health significance of STEC. By subtyping the stx2 gene subtypes in pigs we established the presence of the stx2e gene in 9 out of 82 stx2 positive samples. In addition, the zoonotically significant stx2a gene was detected in 3 samples, which despite its low prevalence is of great public health importance and allows completing he epidemiologic picture of STEC in Serbia.

***Keywords*:** *stx2a*; *stx2e*; subtyping; conventional PCR; pigs; Serbia

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INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) strains, including *E. coli* serotype O157:H7 and non-O157 serogroups, are major foodborne zoonotic pathogens associated with disease outbreaks that are a serious public health concern worldwide (Smith et al., 2014).

Shiga toxin is the main characteristic of STEC and the key virulence factor in STEC causing hemolytic uremic syndrome (HUS). STEC infections in humans are usually manifested as gastrointestinal disorders such as mild or bloody diarrhea and hemorrhagic colitis (HC). Furthermore, they may progress to the life-threatening hemolytic uremic syndrome (Yang et al., 2020). Although STEC-associated symptoms do not usually occur in cattle and other animal species, clinical manifestations may occur in pigs in form of oedema disease, an infectious illness of post-weaning piglets and young finishing-age pigs characterized by vascular necrosis, edema, and neurological signs. Such condition can be fatal, when caused by *E. coli* strains possessing the *stx2e* gene encoding 2e subtype of Shiga toxin (Colello et al., 2016; Ercoli et al., 2016).

There are two types of Shiga toxins, Stx1 and Stx2, which differ in antigenicity, degree of cytotoxicity and host specificity despite their 56% similarity in shared amino acid sequence . Based on phylogeny of Stx holotoxin sequences, Scheutz et al. (2012) proposed three subtypes of Stx1 (Stx1a, Stx1b, Stx1d) and seven subtypes Stx2 (Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f, Stx2g) . In recent years, both subsets of Shiga toxins have been expanded with description of a few novel variants of Stx1 and Stx2, Stx1e and Stx2h to Stx2o, respectively, found in diverse animal hosts and food products in variable prevalence rates .

The primary target of Stx are microvascular endothelial cells, but other cell types such as neurons are also susceptible and there is not known antidote for the toxin neutralization . The damage produced by the action of cytotoxins Stx1 and Stx2 is an important cause of acute kidney injury, whereby Stx2 and its subtypes are more frequently associated with the development of HUS, leading to acquired chronic kidney disease predominantly in children and the elderly (Colello et al., 2016).

Although they are mainly carried by lambdoid phages and predominantly present in *Escherichia* and *Shigella* species, genes encoding Stxs could be also found in a variety of bacteria such as *Citrobacter freundii*, *Enterobacter cloacae*, *Acinetobacter haemolyticus*, *Aeromonas* sp., and even the distantly related genus *Enterococcus*. Such findings may be due to the ability of lambdoid phages to infect other bacterial hosts within the family *Enterobacteriaceae*, However, the presence of *stx* genes in these atypical hosts waned after repeated subcultures, suggesting that the phages may not propagate efficiently within them or that the *stx* genes themselves are unstable .

Although ruminants, especially cattle, are recognized as the main natural reservoir of STEC strains, attention has been given to several reports which indicate that swine shed STEC at a similar rate as cattle, posing a direct risk to humans by introducing these strains into the food chain, or indirectly by soil and water contamination (Tseng et al., 2014; Ercoli et al., 2016; Cha et al., 2018).

Due to the limited epidemiological data on STEC prevalence in swine in Serbia and an increasing role of non-O157 STEC in human illness, the aim of this research was to determine the presence of the zoonotically significant *stx2* gene subtypes in the population of pigs in Serbia.

MATERIALS AND METHODS

In this study 374 samples of total DNA from pig feces were used, which were collected for the purposes of the previous research conducted in 2016. All the samples were collected from 7 pig farms located in the seven administrative districts of the Autonomous Province of Vojvodina (North Backa, West Backa, South Backa, North Banat, Central Banat, South Banat and Srem District). Pigs of all categories were proportionally included in the investigated population, and sampling was done by the method of random selection, directly from the rectum of the selected animals. Genomic DNA extraction was performed with the QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions.

The previous research included the amplification of virulence factor genes (*stx1*, *stx2*, *eae*), which was performed by multiplex PCR on a TC-412 Thermal Cycler (Techne, Stone, UK) under the conditions and primers previously described by Fadel et al. (Fadel et al., 2017).

For the subtyping of *stx2*, the conventional simplex PCR method previously described by Sheutz et al. (2012) was used for each of the seven subtypes

using primers shown in Table 1, whereas the primer annealing temperature of 65°C was chosen, as it gave the best results in multiple testing (Scheutz et al., 2012). The reaction mixture with a total volume of 20 µl contained 5 µl of the tested DNA extract, 10 µl of Hot Start Taq 2x Master Mix (BioLabs, New England, USA), 1.25 µl of each primer (5 µmol) and 1.25 µl of sterile ddH2O. After separation of the PCR products and a 100 bp marker by electrophoresis, PCR products were stained using ethidium bromide. The expected size of the PCR products, shown in Table 1, was documented in a Serva BlueCube 300 (SERVA Electrophoresis GmbH, Heidelberg, Germany) visualization system.

RESULTS

From the total of 374 samples, the presence of the *stx2* gene was detected in 82 samples (21.9%), which were further subtyped for the s*tx2a*, *stx2b*, *stx2c*, *stx2d*, *stx2e*, *stx2f* and *stx2g* genes. By this panel of primers *stx2* subtypes were determined in 11 samples. The *stx2a* gene was determined in 3 samples acquired from two piglets in the category of under 20 kg and one piglet from 20-49 kg respectively, all originating from the South Backa District. This result represents 0.8% of the total pig population, i.e., 3.65% of *stx2*-positive samples. The *stx2e* gene was found in 9 samples from 3 different districts, with the prevalence of 2.4% in the total pig population and 10.97% of the *stx2* positive samples. Only one sample was simultaneously positive for the *stx2a* and *stx2e* genes and originated from the South Backa District. Six *stx2e*-positive samples originated from Central Banat, two from Srem, and one from South Backa District, whereas all of them were sampled from piglets in the category of under 20 kg.

All the *stx2*-positive samples failed to give amplicon using stx2-subtypes specific primers for the *stx2b*, *stx2c*, *stx2e*, *stx2f* and *stx2g* genes.

DISCUSSION

STEC strains can express a combination of one or more Stx subtypes (Karve and Weiss, 2014; Ercoli et al., 2016), and the strains which produce Stx2, specifically Stx2a, Stx2c and Stx2d, are more often associated with the development of the most severe forms of the disease in humans (Capps et al., 2021). Furthermore, data obtained by Beutin and Martin (2012), Soborg et al. (2013), and Bielaszewska et al. (2013) also suggest that Stx2a is strongly associated with the induction of HUS in humans (Smith et al., 2014). In addition to this, a food-borne outbreak caused by Stx2a-producing *E. coli* O104:H4 in Germany and other countries affected over 4000 people, whereas almost 23% of cases developed HUS (Beutin and Martin, 2012; Smith et al., 2014).

Studies from around the world have demonstrated that commercial pig populations have a high prevalence of STEC that are clinically relevant for humans. In Italy, Arancia et al. (2019) determined the

Table 1: List of primers used for *stx2* gene subtyping (Sheutz et al., 2012)

Primer	Sequence (5' - 3')	Position	Amplicon size (bp)
stx2			
Subtyping			
stx2a-F2	GCGATACTGRGBACTGTGGCC	754-774	349
stx2a-R3	CCGKCAACCTTCACTGTAAATGTG	1079-1102	347
stx2a-R2	GGCCACCTTCACTGTGAATGTG	1079-1100	
stx2b-F1	AAATATGAAGAAGATATTTGTAGCGGC	968-994	251
stx2b-R1	CAGCAAATCCTGAACCTGACG	1198-1218	
stx2c-F1	GAAAGTCACAGTTTTTATATACAACGGGTACCG	926-955	177
stx2c-R2	GCCACYTTTACTGTGAATGTA	1079-1102	
stx2d-F1	AAARTCACAGTCTTTATATACAACGGGTG	927-955	
stx2d-R1	TTYCCGGCCACTTTTACTGTG	1085-1105	179
stx2d-R2	GCCTGATGCACAGGTACTGGAC	1184-1206	280
stx2e-F1	CGGAGTATCGGGGAGAGGC	695-713	411
stx2e-R2	CTTCCTGACACCTTCACAGTAAAGGT	1080-1105	
stx2f-F1	TGGGCGTCATTCACTGGTTG	451-475	424
stx2f-R1	TAATGGCCGCCCTGTCTCC	856-874	
stx2g-F1	CACCGGGTAGTTATATTTCTGTGGATATC	203-231	573
stx2g-R1	GATGGCAATTCAGAATAACCGCT	771-793	

presence of the *stx2* gene in 50.4% of STEC strains isolated from the contents of the caecum of pigs at slaughter. Most of them possessed the *stx2a* subtype (74.2%), present in combination with *stx2b* and *stx2c* subtypes in 16.7% of the tested samples (Arancia et al., 2019). Similar results were reported by Goma et al. (2019), who found the *stx2a* gene in 75% of *E. coli* O157:H7 strains isolated from pork, pig feces and water samples from Surakarta, Central Java Province, Indonesia. The results of their research showed that the *stx2a* gene was found in *E. coli* O157:H7 isolates i.e., in 87.5% of pork samples, 70% of pig stool samples, and 50% of water samples, respectively (Goma et al., 2019). In concordance with the previous results was the report by Ateba and Mwebe (2011) in South Africa, who found *stx2* in 70.96% (22/31) of *E. coli* O157:H7 isolates from pig fecal samples (Ateba and Mbewe, 2011).

In several other countries, the prevalence of STEC strains in pig farms, finishing pigs, slaughter and pork products has been reported to range from 0% to 68.3% (Tseng et al., 2015; Haque et al., 2022). However, it is often difficult to directly compare these data due to the differences in study designs, sample collection methods, sample processing or use of different STEC detection and isolation protocols (Tseng et al., 2015; Colello et al., 2016; Ercoli et al., 2016).

To our knowledge, this study is the first report of the prevalence and characterization of STEC strains isolated from pigs in Serbia. The first study in which the presence of STEC in Serbia was established involved human fecal samples, and the prevalence of STEC using the Vero cell assay (VCA) was determined to be 0.8% (Cobeljic et al., 1995). In another study carried out in Serbia, the production of Stx was also determined using the VCA test, in which the production of Stx was recorded in 446 (12.33%) out of 3616 examined food samples, as well as, samples originating from feces obtained from several animal species, including 11.6% of pig fecal samples (Cobeljic et al., 2005). However, since the VCA lacks specificity, the results for some VCA positive samples may occur due to non-Stx cytotoxicity (Rahn et al., 1996). Our research represents the first molecular typing and identification of highly potent Stx2 subtype in pigs in Serbia.

The STEC contamination of pigs takes place at farms, and during the slaughter process STEC are transferred to carcasses, resulting in the contamination of pork products. Thus, the entrance of these strains into the food chain implies a risk to consumers because of severity of the illness they can cause. Furthermore, STEC strains isolated from pork products have been associated with human infections such as diarrhea and HUS, including strains harboring the s*tx2e* subtype. However, it is unknown if the contamination of pork occurred during the processing or by cross contamination (Tseng et al., 2014; Colello et al., 2016).

Both Tseng et al. (2015) and Cha et al. (2018) conducted longitudinal cohort studies on STEC carriage in swine which resulted in the identification of swine as a significant reservoir of STEC in the U.S. The results of Tseng et al. (2015) showed that the majority of the STEC isolates from finishing pigs (97.9%, 279/285) carried the *stx2e* gene. Similarly, Cha et al. (2018) estimated that 68.3% pigs shed STEC at least once, whereas 44.2% (397/898) of the fecal samples tested positive for at least one *stx* gene marker. Even if the majority of these markers, such as *stx2e*, was not linked to the human diseases, the clinically important O157:H7 (*stx2c*, *eae*) and O26:H11 (*stx1a*, *eae*), were recovered at a similar frequency, indicating that commercial pigs may serve as a source of human STEC infections (Cha et al., 2018). However, Cha et al. (2018) found that the majority of the isolates carried *stx2e* only (288/302), while the *stx2a* gene was observed only in 2 isolates (0.66%), which is lower than the prevalence observed in our study (3.65%).

In another study conducted in the United States (U.S.) by Nastasijević et al. (2020), pig carcasses (n=1536) at two pork processors were examined for the presence of *stx* genes using PCR, followed by culture isolation of STEC strains, and the most commonly present Stx subtypes in the isolates were *stx1a*, *stx2a*, *stx2e*, and/or *stx2c* (Nastasijević et al., 2020). This study is in line with a previous retrospective U.S. study by Baranzoni et al. (2016), which demonstrated that swine might carry Stx1a-, Stx2d-, or Stx2e-producing *E. coli* with virulence gene profiles linked to human infections.

On the other hand, Remfry et al. (2021) tested fecal samples (n=598) of healthy pigs from 10 pig farms located in the top swine-producing states in the United States and reported that 152 (85.4%) *stx2*-positive strains were isolated out of 178 STEC isolates. However, none of them possessed the *stx2a* gene, yet all of the 152 stx2-positive isolates carried the *stx2e* subtype (Remfry et al., 2021). Similar result emerged in a study by Meng et al. (2014), which showed a high

prevalence of STEC in healthy pigs (25.42%), where all STEC isolates carried the *stx2e* subtype. Contrary to these findings, Arancia et al. (2019) found that only 25.8% of STEC strains isolated from the ceacal content of pigs possessed the *stx2e* subtype. Baldo et al. (2020) also observed a lower prevalence of the *stx2e* subtype in fecal samples from both diseased (11.16%) and healthy pigs (1.75%), similar to the prevalence of *stx2e* detected in our study (10.97%).

The discrepancies in reported prevalences may be due to different health conditions of pigs, detection methods as well as anatomic sites of sampling. In this regard, the isolation rate of STEC from fecal samples is notably lower that isolation rates from the small intestine or colon, however fecal samples are commonly used (Meng et al., 2014; Baldo et al., 2020).

Despite the limited number of extensive studies on swine harboring STEC, there is scientific evidence which implies that swine may serve as a reservoir for STEC strains potentially pathogenic to humans, including those expressing *stx2e* (Tseng et al., 2014; Remfry et al., 2021; Haque et al., 2022).

In a study conducted by Gill et al. (2022), previously unreported variants of established stx2 subtypes were found and characterized using multiple sequencing technologies to minimize the generation of artifacts and to ensure accurate sequencing of *stx* sequences. Since STEC strains often carry multiple copies of the *stx* gene, including different alleles, single atypical *stx* sequences may be present with partial subunits of other *stx* subtypes (Gill et al., 2022).

The significance of the unidentified Stx2 subtypes in this study upon the virulence of the isolates remains to be further investigated. The method used in this study, described by Scheutz et al. (2012), is intended for application on isolated STEC strains. In this study, we used total DNA extracted from fecal samples of swine. Since other species of bacteria besides *E. coli* can possess *stx* genes, the positive PCR signal may not have originated from the STEC (Bosilevac and Koohmaraie, 2011). However, detection of *stx* genes in the genetic pool of enteric microbiota is significant considering the possibility of horizontal gene transfer in microbial community. Our approach for targeting genes in the total DNA from feces raises the chances of detection of important *stx* gene subtypes in the samples compared to a limited search in a few randomly picked colonies from the plate. False negative PCR results can be caused by substances in the sample that inhibit DNA polymerase (Altwegg, 1995). Another possible cause of the negative results in this research may be the low sensitivity and/or specificity of the used stx PCR primers due to the existence of different gene variants of *stx*, all of which might not be necessarily detected by various PCR assays (Feng et al., 2011; Gill et al., 2022). The diversity of STEC strains implies constant potential for mutation of *stx* phages, therefore many established PCR-based methods exclude novel subtypes other than the distinguished three Stx1 (a, c, and d) and seven Stx2 (a, b, c, d, e, f, and g) subtypes . Therefore, it is possible that the unidentified Stx2 subtypes in this study either belong to novel subtypes which are undetectable by the PCR method proposed by Scheutz et al. (2012), or that only a partial subunit of distinguished subtypes was present, which the primers could not detect.

Although with a low prevalence, the presence of the zoonotically significant *stx2a* gene is of great importance for public health, considering that among STEC strains, those producing Stx2a cause more severe diseases. Precisely, emergence of EHEC O157 as a life-threatening zoonosis is associated with the presence of Stx2a subtype that, in cytotoxicity assays, exhibits 1000 times more toxic effect than Stx1 to human renal endothelial cells (Fitzgerald et al., 2019).

CONCLUSIONS

In this study, we detected the existence of gene encoding highly potent Stx subtype 2a in pig fecal DNA on commercial pig farms in Serbia. The prevalence of 0.8% in the examined pig population indicates that pigs can be a reservoir of zoonotic STEC strains, which raises concern in One Health perspective. Further research involving a larger number of samples and the determination of other Stx subtypes is necessary in order to establish the role of pigs in the epidemiology of STEC infections, as well as to understand the impact on public health of different strains circulating in the pig population in Serbia. Due to the finding of *stx2a*, associated with the development of the most severe forms of the disease in humans, special attention should be paid to establish effective control measures from farm-to-fork. This includes reducing STEC carriage in pigs, preventing contamination during slaughter and preventing contamination of carcasses and meat products and their introduction into the food chain.

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