ORIGINAL ARTICLE

Phenogenotypic Characterization of *Escherichia coli* O157:H7 Strains Isolated from Cattle at Slaughter

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Abstract

Shiga toxin-producing *Escherichia coli* (STEC) O157:H7 was isolated from 30 (4%) of 744 cattle hide swab samples collected at Estonian slaughterhouses within a 3-year monitoring program of zoonotic pathogens. The isolates were characterized by determining the presence of STEC main virulence factors, the antimicrobial resistance profiles, and the genetic relatedness by pulsed-field gel electrophoresis (PFGE). Thirteen strains carried the *stx2* gene alone and 17 both the *stx1* and *stx2* genes. The most frequently detected *stx* subtype was *stx2c*, occurring alone ($n = 12$) or in combination with subtype *stx1a* ($n = 13$). All isolates harbored the intimin-coding *eae* gene and produced enterohemolysin. Twelve isolates (40%) showed resistance to at least one of the 14 antimicrobials and the isolates were predominantly resistant to streptomycin, sulfamethoxazole, and ampicillin. No extended-spectrum beta-lactamase-producing isolates were detected. PFGE characterization of the isolates showed an overall similarity higher than 75%, and four clusters based on 100% similarity were revealed.

Keywords: cattle, *Escherichia coli* O157:H7, slaughterhouse, zoonotic agents

Introduction

*E*SCHERICHIA COLI IS PART of the normal gastrointestinal microbial flora of humans and animals, but some strains are pathogenic and may cause a variety of infections in humans (Kaper et al. 2004). Shiga toxin-producing *E. coli* (STEC) are a group of *E. coli* that are characterized by the ability to produce toxins closely related with the Shiga toxin (Stx) produced by *Shigella dysenteriae* (EFSA Panel on Biological Hazards 2013). In addition to the common feature to produce Stx1 and Stx2 proteins, pathogenic STEC strains usually possess other virulence factors, such as intimin, a protein encoded by the *eae* gene, and enterohemolysin, encoded by the *ehxA* gene (Paton and Paton 1998).

A considerable number of STEC serotypes have been recognized as causative agents of human disease, but severe infections and outbreaks have been mostly associated with the O157 serogroup (Scallan et al. 2011, EFSA Panel on Biological Hazards 2013). *E. coli* O157:H7 infections are often associated with consumption of raw or undercooked beef because ruminants, particularly cattle, have been deemed as the main reservoir of this pathogen (Ferens and Hovde

2011). Cattle and other ruminants can be linked also with STEC transmission through direct contact with animals and farm environment or consumption of water and fresh produce contaminated with cattle feces (Caprioli et al. 2005).

The incidence rate of STEC infection in humans in Estonia has ranged from 0.2 to 0.6 per 100,000 inhabitants in the period of 2010–2014 (Terviseamet 2015). In most cases, the serogroup of the infecting STEC strains was not identified.

At the slaughterhouse level, cattle hides have been considered major vehicles of carcass contamination by *E. coli* O157 and other STEC serogroups (Keen and Elder 2002, O'Brien et al. 2005). Microbial sampling of hides can give an indication of the STEC load at the slaughterhouse level. The results reflect the possibility of further contamination of carcasses and meat and represent a tool to evaluate the efficacy of good hygiene practices as a control measure of carcass contamination (EFSA 2009).

The aim of the present study was to evaluate the prevalence of STEC O157:H7 in cattle at slaughter in Estonia by investigating the contamination of cattle hides, which is an important source of these zoonotic pathogens in the first steps of beef processing. STEC O157:H7 isolates were analyzed

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for the presence of virulence genes, the subtypes of the Stx-coding genes, the antimicrobial susceptibility spectrum, and the pulsed-field gel electrophoresis (PFGE) patterns.

Materials and Methods

Sample collection

A total of 744 samples were collected and analyzed between January 2011 and December 2013 in the frame of national monitoring program. Hide swabs were taken from the 400-cm^2 brisket area of the animals after exsanguination and before dehiding at slaughterhouse using a sterile abrasive sponge kit (3M^{TM} hydrated sponges). The sponges were premoistened with 10 mL buffered peptone water. Immediately after sampling, the sponges were cooled to $5 \pm 3°C$ and sent to the National Reference Laboratory.

The sample size was based on estimates of a predicted prevalence of 12%, with confidence intervals (CIs) of 95% and an accuracy of 4% in the total annual number of slaughtered cattle at 3–24 months of age. All slaughterhouses were ranked by the throughput of interested cattle population, and predetermined numbers of animals to be sampled were distributed according to the proportional throughput from the previous year. Starting with the slaughterhouses of largest throughput, those covering at least 80% of the national throughput were included. The number of slaughterhouses enrolled in the monitoring program varied slightly in three consecutive years, 2011–2013, including 27, 26, and 23, respectively. Animals to be sampled were chosen randomly throughout the year and only one animal per slaughterhouse was sampled per selected day. Furthermore, the request that the animals to be sampled had to originate from different herds was followed.

Isolation of Escherichia coli O157

All samples were analyzed for the presence of *E. coli* O157 using the procedure described in ISO 16654 (ISO 2001). Shortly, 90 mL of prewarmed ($37 \pm 1°C$) modified Tryptone Soya Broth supplemented with novobiocin 20 mg/L (Oxoid) was added to the sponge sample and homogenized in Stomacher blender for 60 s. Following the selective enrichment (6 ± 0.5 h and 21 ± 3 h at $41.5 \pm 0.5°C$), immunomagnetic separation (IMS) was performed using Captivate^{TM} anti-O157 beads (LabM Ltd.) according to the manufacturer's instructions. Fifty microliters of IMS suspension was streaked onto the sorbitol MacConkey agar containing cefixime–tellurite supplement (Oxoid) and CHROMagar^{TM} STEC agar (CHROMagar) plates. Inoculated media were incubated at $37 \pm 1°C$ for 21 ± 3 h. Non sorbitol-fermenting colonies on cefixime tellurite sorbitol MacConkey (CT-SMAC) agar and mauve-colored colonies on chromogenic medium were subcultured and tested for the production of β-glucuronidase and indole by conventional biochemical analyses.

Serological confirmation

Serotyping of the presumptive *E. coli* O157:H7 isolates was performed by conventional agglutination tests using O157 and H7 antisera (Statens Serum Institute) according to the manufacturer's instructions. To exclude false-positive O157 agglutination, the presence of O157 serogroup was confirmed by agglutination of the boiled culture of the strain ($100°C$, 1 h).

Detection of the enterohemolytic phenotype

The enterohemolytic phenotype was detected on blood agar plates containing 10 mM calcium chloride and 5% defibrinated washed sheep erythrocytes as described by Beutin et al. (1989). The plates were examined after 24 h of incubation at $37 \pm 1°C$ for the presence of lysis areas around the bacterial colonies.

Antimicrobial susceptibility testing

The susceptibility to antimicrobials was determined by the minimal inhibitory concentration microdilution method using VetMIC GN panel (version 4) as described by the manufacturer (National Veterinary Institute). The antimicrobial agents tested were as follows: ampicillin, ciprofloxacin, nalidixic acid, gentamicin, streptomycin, tetracycline, florfenicol, colistin, sulfamethoxazole, trimethoprim, chloramphenicol, kanamycin, cefotaxime, and ceftazidime. Based on epidemiological cutoff values, the results were categorized as susceptible or resistant according to the guidelines of European Committee on Antimicrobial Susceptibility Testing and EU Reference Laboratory for Antimicrobial Resistance (EURL-AR 2013).

Detection of stx1, stx2, and eae genes by polymerase chain reaction

Total DNA was extracted from pure bacterial cultures using a commercial DNA extraction kit (RTP$^{®}$ Bacteria DNA Mini Kit; Stratec Molecular GmbH) as described by the manufacturer and used as template in real-time PCR assays.

The primers, probe systems, and real-time PCR assay conditions described in ISO technical specification ISO/TS 13136 (ISO 2012) were used for the identification of the virulence genes *stx1*, *stx2*, and *eae*. The primers and probes were obtained from Eurogentec S.A. Real-time PCR amplification for each gene was performed in a final volume of 25 μL containing 12.5 μL of AmpliTaq Gold 360 Master Mix (Applied Biosystems$^{®}$; Thermo Fisher Scientific, Inc.), 5 μL of primer mix (final concentration of each 1 μM), 4 μL of sterile water, 3 μL of template DNA, and 0.5 μL of probe with final concentration 0.2 μM. Each sample was run in duplicate. DNA extracted from the reference *Escherichia coli* O157 strain, EDL 933, was used as positive control, and sterile water was used as negative control. Real-time PCRs were performed using the real-time PCR cycler. The thermal cycling was carried out with the pre-PCR activation at $50°C$ for 120 s and at $95°C$ for 600 s following 40 cycles of denaturation at $95°C$ for 15 s, annealing and extension at $60°C$ for 60 s, and final extension at $72°C$ for 120 s.

Detection of stx1 and stx2 gene subtypes by conventional PCR

Conventional PCR for the detection of *sxt1* and *stx2* gene subtypes was performed as described by Scheutz et al. (2012).

Typing by PFGE

The genetic relationship among the O157:H7 isolated strains was investigated by PFGE in accordance with the standard operating procedure of EFSA external scientific report (Caprioli et al. 2014). The restriction profiles obtained with *Xba*I were analyzed with the BioNumerics software, version

7.1 (Applied Maths). Each PFGE pattern was given a number within the serotype O157:H7, for example, EC01, EC02, and EC03. The patterns were compared using Dice's similarity coefficient with tolerance and optimization values at 1.5%. Strains were deemed to have the same PFGE patterns when all bands were 100% identical. The dendrogram was built using the unweighted pair-group method with arithmetic averages.

Statistical analysis

The prevalence and CI based on probabilities derived from binominal distribution for proportion were calculated using the software VassarStats (Lowry 2014). The statistical significance of the difference in prevalence between different groups was investigated using Fisher's exact test to the respective reference. A p-value <0.05 by Fisher's exact test was considered statistically significant.

Results

E. coli O157 was isolated from 30 (4.0%; CI_{95} 2.8–5.7%) of 744 hide swabs of cattle collected within a 3-year monitoring program. No statistically significant difference was found in terms of *E. coli* O157 prevalence in the three consecutive years (Table 1). Two-third of the positive animals ($n = 19$) were slaughtered at three main slaughterhouses located in different geographical regions of the country. *E. coli* O157-positive cattle originated from 26 farms, located in 12 of the 15 counties in Estonia.

E. coli O157 was isolated more often after the 6-h enrichment step compared with an enrichment time of 18–24 h ($p < 0.0001$). There was only one sample found to be negative in 6-h time point, but positive after 18–24 h. Twenty-nine samples were found to be positive after 6-h enrichment.

All isolates agglutinated with H7 antiserum showed enterohemolytic phenotype and possessed the intimin-coding *eae* gene. Statistically significant difference ($p < 0.0001$) was found between the occurrences of the *stx* gene families, *stx1* and *stx2*, among the 30 isolates: 13 (43.3%) of them carried the *stx2* gene only, whereas the remaining 17 (56.7%) harbored both the *stx1* and *stx2* genes. *Stx2c* was the most frequently detected *stx* subtype, occurring alone ($n = 12$, 40.0%) or in combination with subtype *stx1a* ($n = 13$, 43.3%). Subtype *stx2a* alone was detected in one isolate and in combination with *stx1a* in four isolates. None of the isolates harbored genes of the *stx1c*, *stx1d*, *stx2b*, *stx2d*, *stx2e*, *stx2f*, and *stx2g* subtypes.

Comparison of PFGE profiles showed that the isolates presented an overall similarity of 76.9%. In total, 20 different *Xba*I PFGE patterns were differentiated among the 30 isolates, of which 14 grouped into four clusters based on a genetic

TABLE 1. THE PREVALENCE OF *ESCHERICHIA COLI* O157:H7 FROM THE HIDE SWAB SAMPLES OF CATTLE

Year	No. of samples	No. of positive samples (%)	95% confidence interval (CI95)
2013	254	9 (3.5)	1.9–6.6%
2012	246	13 (5.3)	3.1–8.8%
2011	244	8 (3.3)	1.7–6.3%
Total	744	30 (4.0)	2.8–5.7%

relatedness criterion of 100% (EC14, EC01, EC04, and EC07, Fig. 1). The number of isolates belonging to the same cluster ranged from two to five. Cluster EC14 grouped two isolates from year 2013, whereas the other three clusters included strains isolated in 2011 and 2012. Both strains showing the EC14 pattern were isolated from cattle originating from the same locality, but from different farms and slaughtered in different facilities. Sampling time was different as well. Clusters EC04 and EC07 showed more than 97% similarity to other PFGE patterns of isolates from 2012 originating from geographically dispersed localities.

Generally, the PFGE profiles reflected the *stx* gene subtypes, with the exception of profile EC04 (Fig. 1). Three isolates recovered from the same locality harbored the *stx2c* gene, whereas one isolate having the same PFGE pattern and originating from the nonadjoining county carried genes of the *stx1a* and *stx2a* subtype. The determination of toxin subtype genes for the latter isolate was repeated and results confirmed the presence of *stx1a* and *stx2a* genes. The high diversity of PFGE patterns among three *E. coli* O157:H7 strains isolated from cattle of the same farm and harboring both *stx1a* and *stx2a* genes was observed.

Table 2 shows the distributions of the minimal inhibitory concentration (MIC) values for the 30 *E. coli* O157:H7 isolates. All the isolates were susceptible to nalidixic acid, ciprofloxacin, florfenicol, colistin, chloramphenicol, cefotaxime, and ceftazidime. Eighteen isolates (60.0%) were sensitive to all the tested antimicrobials, while resistance to one or more agents was detected in 12 (40.0%) isolates.

Multiple drug resistance, which was defined as resistance to three or more unrelated antimicrobials, was detected in eight (26.7%) isolates. A high proportion of isolates were resistant to streptomycin (33.3%) and sulfamethoxazole (33.3%). Resistance against ampicillin, trimethoprim, kanamycin, tetracycline, and gentamicin was found for five (16.7%), four (13.3%), two (6.7%), one (3.3%), and one (3.3%) isolate(s), respectively. The highest MIC values (Table 2) were obtained for sulfamethoxazole, streptomycin, and ampicillin.

Coincidence between PFGE patterns and antimicrobial susceptibility was found among isolates in profiles EC01 and EC14. Conversely, isolates belonging to the PFGE profiles, EC04 and EC07 (Fig. 1), showed different resistance profiles.

Discussion

Contaminated bovine meat is considered to be a major source of foodborne STEC infections in humans, particularly those sustained by *E. coli* O157:H7 (Farrock et al. 2013). Sampling cattle hides at slaughterhouse for STEC monitoring is advisable since the highest prevalence is expected at this stage of production and hides represent a major source of carcass contamination by *E. coli* O157 (Keen and Elder 2002, O'Brien et al. 2005).

In the present study, the prevalence of STEC O157:H7 on cattle hides ranged from 3.3% to 5.3% in three consecutive years. The studies on the prevalence of *E. coli* O157 in cattle at slaughter performed in several countries are not fully comparable because of the differences in sampling site and time, sample type and size, study population, and analytical methods. In Italy, Bonardi et al. (2015) reported a 2.5% prevalence of STEC O157 in cattle hides. However, the samples were taken
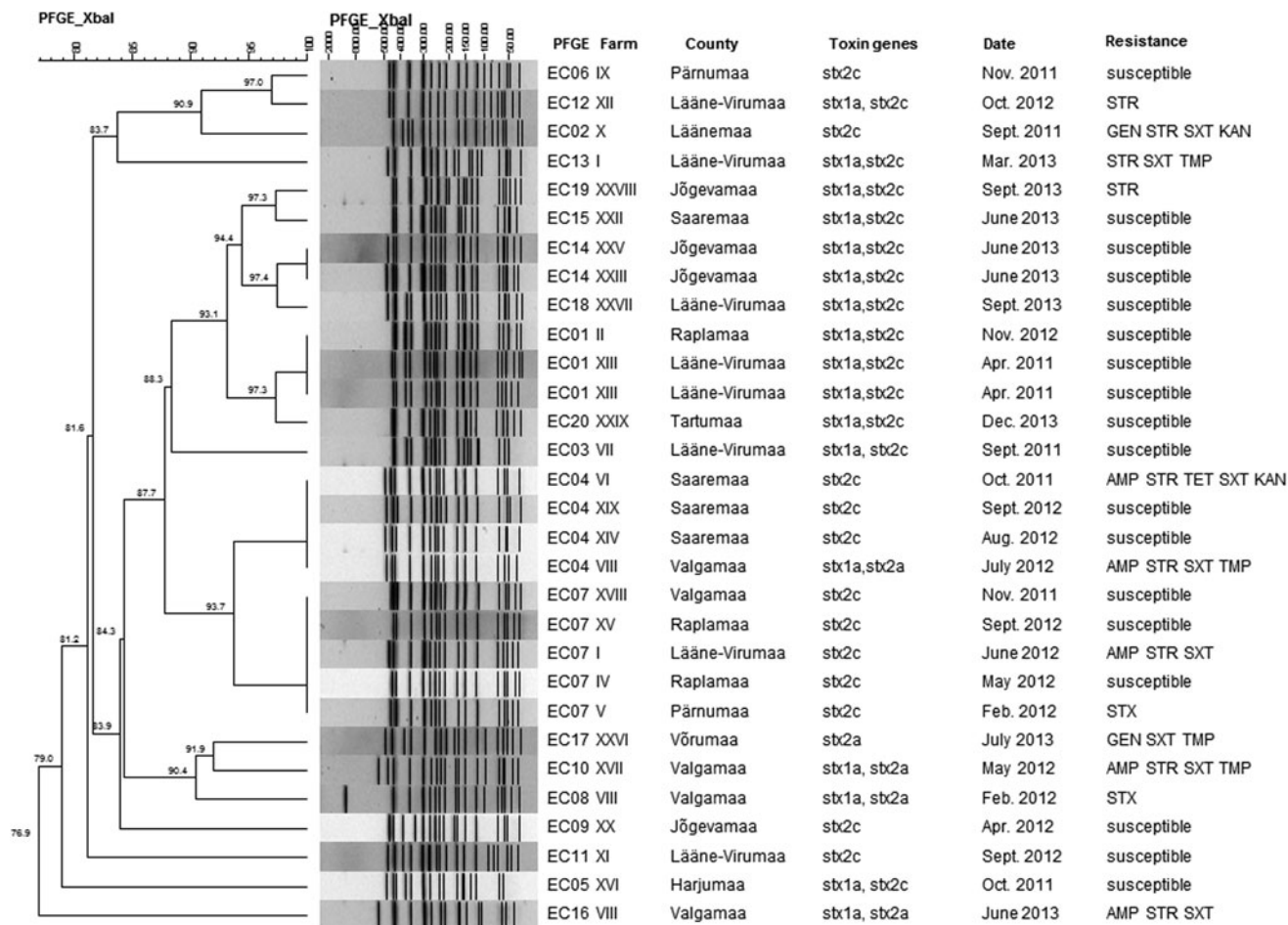
FIG. 1. Dendrogram of the pulsed-field gel electrophoresis profiles obtained after *Xba*I digestion of DNA from *Escherichia coli* O157:H7 isolates from cattle hide samples during the period 2011–2013. The scales at the *top* indicate the similarity indices and molecular sizes in percentages and kilobases, respectively. STR, streptomycin; GEN, gentamicin; SXT, sulfamethoxazole; TMP, trimethoprim; AMP, ampicillin; TET, tetracycline; KAN, kanamycin.

PFGE	Farm	County	Toxin genes	Date	Resistance
EC06	IX	Pärnumaa	stx2c	Nov. 2011	susceptible
EC12	XII	Lääne-Virumaa	stx1a, stx2c	Oct. 2012	STR
EC02	X	Läänemaa	stx2c	Sept. 2011	GEN STR SXT KAN
EC13	I	Lääne-Virumaa	stx1a,stx2c	Mar. 2013	STR SXT TMP
EC19	XXVIII	Jõgevamaa	stx1a,stx2c	Sept. 2013	STR
EC15	XXII	Saaremaa	stx1a,stx2c	June 2013	susceptible
EC14	XXV	Jõgevamaa	stx1a,stx2c	June 2013	susceptible
EC14	XXIII	Jõgevamaa	stx1a,stx2c	June 2013	susceptible
EC18	XXVII	Lääne-Virumaa	stx1a,stx2c	Sept. 2013	susceptible
EC01	II	Raplamaa	stx1a,stx2c	Nov. 2012	susceptible
EC01	XIII	Lääne-Virumaa	stx1a,stx2c	Apr. 2011	susceptible
EC01	XIII	Lääne-Virumaa	stx1a,stx2c	Apr. 2011	susceptible
EC20	XXIX	Tartumaa	stx1a,stx2c	Dec. 2013	susceptible
EC03	VII	Lääne-Virumaa	stx1a, stx2c	Sept. 2011	susceptible
EC04	VI	Saaremaa	stx2c	Oct. 2011	AMP STR TET SXT KAN
EC04	XIX	Saaremaa	stx2c	Sept. 2012	susceptible
EC04	XIV	Saaremaa	stx2c	Aug. 2012	susceptible
EC04	VIII	Valgamaa	stx1a,stx2a	July 2012	AMP STR SXT TMP
EC07	XVIII	Valgamaa	stx2c	Nov. 2011	susceptible
EC07	XV	Raplamaa	stx2c	Sept. 2012	susceptible
EC07	I	Lääne-Virumaa	stx2c	June 2012	AMP STR SXT
EC07	IV	Raplamaa	stx2c	May 2012	susceptible
EC07	V	Pärnumaa	stx2c	Feb. 2012	STX
EC17	XXVI	Võrumaa	stx2a	July 2013	GEN SXT TMP
EC10	XVII	Valgamaa	stx1a, stx2a	May 2012	AMP STR SXT TMP
EC08	VIII	Valgamaa	stx1a, stx2a	Feb. 2012	STX
EC09	XX	Jõgevamaa	stx2c	Apr. 2012	susceptible
EC11	XI	Lääne-Virumaa	stx2c	Sept. 2012	susceptible
EC05	XVI	Harjumaa	stx1a, stx2c	Oct. 2011	susceptible
EC16	VIII	Valgamaa	stx1a, stx2a	June 2013	AMP STR SXT

TABLE 2. MINIMAL INHIBITORY CONCENTRATIONS FOR *ESCHERICHIA COLI* O157:H7 ISOLATES ($N=30$) ORIGINATING FROM SLAUGHTERHOUSE LEVEL IN ESTONIA

			No. of isolates with MIC value (µg/mL) of																		
AA	%[a]	≤[b]	0.008	0.016	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	>[c]
AMP	16.7	0								0	19	6	**0**	0	0	0	0				5
CIP	0.0	0	0	2	23	**5**	0	0	0	0											0
NAL	0.0	0								1	3	24	2	**0**	0	0	0				0
GEN	3.3	8				0		0	0	18	**3**	0	0	0							1
STR	33.3	0								0	2	**18**	2	0	2	2	4				0
TET	3.3	2							0	21	6	**0**	0	0	1	0					0
FFC	0.0	10									0	19	**1**	0							0
CST	0.0	23						0	3	**4**	0										0
SXT	33.3	6										0	10	4	**0**	0	1	1	0		8
TMP	13.3	9					0	14	3	0	**0**	0	0	0							4
CHL	0.0	0									0	10	20	**0**	0	0					0
KAN	6.7	28											**0**	0							2
CTX	0.0	0			0	0	8	22	**0**	0	0	0									0
CAZ	0.0	15						0	**15**	0	0	0	0	0							0

The MIC values for the isolates were evaluated according to the instructions given by the manufacturer (National Veterinary Institute, Uppsala, Sweden).

[a]Percentage of resistant isolates. *Bold* values indicate epidemiological cutoff values between sensitive and resistant isolates.

[b]The number of isolates with MIC values less than or equal to VetMIC GN-panel minimum concentration range.

[c]The number of isolates with MIC values more than VetMIC GN-panel maximum concentration range.

AA, antimicrobial agents; AMP, ampicillin; CIP, ciprofloxacin; NAL, nalidixic acid; GEN, gentamicin; STR, streptomycin; TET, tetracycline; FFC, florfenicol; CST, colistin; SXT, sulfamethoxazole; TMP, trimethoprim; CHL, chloramphenicol; KAN, kanamycin; CTX, cefotaxime; CAZ, ceftazidime; MIC, minimal inhibitory concentration.

from 100-cm^2 brisket area instead of 400 cm^2. The studies performed by O'Brien et al. (2005) and Thomas et al. (2012) on Irish cattle hides in various years showed a prevalence of 7.3% and 13.4%, respectively. In a monitoring program carried out in Sweden, Boqvist et al. (2009) reported an *E. coli* O157 prevalence of 12%. However, in this study, swabs were taken from ears instead of the brisket area.

All the *E. coli* O157:H7 isolates obtained in the present study possessed the intimin-encoding *eae* gene. The *stx2c* gene, alone or in combination with *stx1a*, was the most frequently detected *stx* subtype, similar to what was reported in studies by Lefebre et al. (2009) and Bonardi et al. (2015). The *stx1a*, *stx2a*, *stx2c*, and *stx2d* subtypes were similarly reported in STEC strains from cattle and beef meat products (Martin and Beutin 2011), and the first three have also been detected in the present study. The same subtypes have been reported in STEC O157 strains from Swedish cattle, and potential links to human infections have been described by Aspan and Eriksson (2010). It is noteworthy that STEC strains possessing *stx2a* and *stx2c* subtypes have been associated with hemorrhagic colitis and HUS in humans (Persson et al. 2007).

The *E. coli* O157:H7 isolates obtained from the monitoring program were characterized by PFGE, a broadly used method for molecular typing of *E. coli* O157 (Caprioli et al. 2014) and, more generally, for investigating bacterial transmission throughout the food production chain and for identifying clusters of STEC strains linked to outbreaks (Louie et al. 1999, Thomas et al. 2012). The results of the present study showed that cattle originating from geographically dispersed farms can carry STEC O157:H7 strains with an indistinguishable PFGE pattern. This could be explained by the presence of STEC O157:H7 clones circulating in a small area of the country and finite cattle population. On other hand, we cannot exclude that cross-contamination may have occurred during subsequent stages, such as at slaughter level. Significant cross-contaminations between animals during transportation and housing in a lairage at slaughterhouse have been demonstrated by Mather et al. (2008).

Further characterization of the isolates was obtained by determining their antimicrobial susceptibility pattern. Resistance to streptomycin, sulfamethoxazole, and tetracycline has been frequently found among different STEC strains from animal and human origin (Morabito et al. 2002, Ziebell et al. 2011). The use of antibiotics for prophylactic purposes or in therapy can select resistant bacteria, and multiresistant strains might reflect the history of antimicrobial usage (Smith et al. 2007). This phenomenon might partly explain the high proportion of *E. coli* O157:H7 isolates (33.3%) that were resistant against streptomycin and sulfamethoxazole in the present study. High resistance was also found against ampicillin (16.7%) and trimethoprim (13.3%). On the contrary, no fluoroquinolone resistance and extended-spectrum beta-lactamase were detected. Multiple drug resistance was observed in 26.7% of our *E. coli* O157:H7 isolates, the most frequent resistance pattern being streptomycin, sulfamethoxazole, and ampicillin. These results seem to be consistent with those of previous studies reporting multiple drug resistance in STEC O157 isolates from cattle origin (Mora et al. 2005, Scott et al. 2009).

In conclusion, the current study provides a first description of the *E. coli* O157:H7 strains circulating among Estonian cattle, including their virulence gene profiles and *stx* gene subtypes. STEC O157:H7 isolates with indistinguishable PFGE pattern

were found from cattle originating from different localities and in different years, suggesting that some persistent clones are present in Estonian beef production chain. The high proportion of strains showing multiple drug resistance may indicate problems caused by an inappropriate use of antimicrobials in the Estonian cattle herds. Up to now, STEC state monitoring in Estonia has been focused only on *E. coli* O157, but other STEC serogroups are also important in STEC epidemiology and should be included into the national monitoring program.

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Author Disclosure Statement

No competing financial interests exist.

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