Romanian Biotechnological Letters *Vol. , No. x,*

ORIGINAL PAPER

Prevalence and Genotypes of Shiga Toxin (Verotoxin)-Producing *Escherichia coli* in Romanian Food

Received for publication, April, 16, 2018
Accepted, June, 15, 2018

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Abstract
In the background of the severe hemolytic-uremic syndrome outbreak occurred in Romania at the beginning of 2016, 1484 different milk and meat food units were investigated for Shiga-toxin producing Escherichia coli (STEC) contamination. Prevalence of the virulence genes was calculated and the isolated STEC strains were pheno-genotypic characterized by chromogenic media technique, PCR and DNA sequencing. Sixty-nine STEC isolates were recovered and, of the all food matrices, ovine carcass swabs were the most contaminated (37/1484; 2.49%), followed by minced meat and meat (22/1484; 1.48%) and milk products (7/1484; 0.047%). Most prevalent Shiga-toxin encoding gene was stx2 (53/69; 76.81%), followed by stx1 (44/69; 63.76%); the eae - intimin encoding gene represented 5.79% (4/69). Among these isolates, one O157 STEC and two non-O157 serotypes, O26 and O113 were identified. Sanger sequencing showed single nucleotide polymorphism for stx genes and more than 10% differences in nucleotide and amino acids structure were encountered on virulence genes and/or eae and serogroup-associated genes. Our findings underlines the importance of the pheno-genotyping characterization of STECs in food relating to public safety and the continuous surveillance for non-O157 STEC emerging serotypes that represents a real support for the surveillance of new STEC infections in humans.

Keywords: Shiga-like toxin producing *Escherichia coli* (STEC)/Verotoxin producing *Escherichia coli* (VTEC); PCR, DNA sequencing.

1. Introduction
Even though it is usually harmless and beneficial inhabitant of the intestinal tract, *Escherichia coli (E. coli)* is increasingly encountered as pathogenic with remarkable versatility in causing diseases in humans and animals. Pathogenic *E. coli* is becoming more incriminated in foodborne outbreaks and, among pathotypes, we can encounter Verocytotoxigenic *E. coli* (VTEC), Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Enteroaggregative *E. coli* (EAggEC) and Diffusely adherent *E. coli* (DAEC) (J.P. NATARO & al.[1]). In the midst of these pathotypes, STEC is a major cause of gastroenteritis that may be complicated by hemorrhagic colitis or HUS (hemolytic-uremic syndrome), which is the cause of fatal acute renal failure in small babies, because STEC produce toxins in the gastrointestinal lumens whereas the gut microflora is poorly developed (N. P. BANATVALA & al. [2], W. T. A. TODD & al. [3], S. RUBINO [4]).

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Since its identification in 1982, STEC O157:H7 has been the cause of a series of outbreaks in Europe, Japan and North America (P. I. TARR & al. [5]). The natural principal reservoir implicated in most of the reported outbreaks were the domestic ruminants, especially cows, sheep and goats (C. GERMINARIO & al. [6], M. BLANCO & al. [7], M. BLANCO & al. [8], M. A. Karmali [9]). The outbreak vehicle can be represented by undercooked meat, unpasteurized dairy products, vegetables or water contaminated by the feces of animals and humans. Person-to-person transmission has been also documented (J. C. PATON & al. [10], CDC & al. [11], A.W. PATON & al. [12]).

STEC elaborates two phage-encoded cytotoxins Stx1 and Stx2, encoded by two genes *stx1* and *stx2*. These toxins can be combined or not with another virulence factor called intimin, which is a protein encoded by the *eae* gene. The intimin is responsible for the intimate attachment of STEC to the intestinal epithelial cells causing attaching-and- effacing lesions in the intestinal mucosa (M. BLANCO & al. [8], M. A. Karmali [9]). Sometimes, it has been demonstrated the loss and transfer of *stx* genes by pathogenic STEC during human infections due to *stx* carrying phage as mobile elements. This hypothetical loss of virulence genes should be always investigated, because this can lead to a false consideration of a safe food (A.W. PATON & al. [12]).

In 2016, 28 Member States reported 6378 human cases and 10 deaths, which represents an increase of 8.3% compared with 2015 (EFSA [13]). Instead of STEC O157, which was the most frequent serotype, for the first time, in 2016, the STEC O26 became the most reported one in HUS human cases in EU. In the USA, by comparison with EU, the O157 serotype is still persistent (K. G. J. POLLOCK & al. [14]). Regarding STEC prevalence in food in EU in 2016, the meat and meat products were the most contaminated matrices (9242), followed by milk and milk products (4119) and fruits/ vegetables (1543) (EFSA [13]). In the past, outbreaks caused by STEC O26 have been associated with unpasteurized milk and dairy products in Austria, Belgium and Italy (C. GERMINARIO & al. [6], K. DE SCHRIJVER & al. [15]). In addition, in Romania a large outbreak occurred in the early of 2016, extended rapidly to other European countries, which involved STEC O26 and other non-O157 STEC strains (EFSA [13], E. PERON & al. [16]).

Notwithstanding the EU Regulation No. 20173/2005, which states a STEC microbiological criteria only for sprouts seeds, this paper summarizes a monitoring STEC program in meat and milk products conducted in Romania in 2016 and the microbiological and molecular investigations on the STEC isolates occurred under this program. In the same time, this study asserts the importance of the STEC surveillance in foods in Romania for non- O157 STEC, which give real support to the surveillance of STEC diseases in humans.

2. Materials and Methods

Samples

Bacteriological and molecular investigations were performed in the Institute for Hygiene and Veterinary Public Health in the frame of a national monitoring program for STEC surveillance in 1484 meat and milk samples as table 2.

STEC detection and isolation

Screening of virulence genes *stx*1, *stx*2, *eae* and serogroup- associated genes O157, O145, O111, O103, O26, O104 were done according ISO 13136:2012 [17] with minor modifications. The PCR screening step consisted in the detection of *stx1* and *stx2* genes from DNA extraction of a one mL enrichment broth, followed, in case of a *stx* positive result, by the *eae* and serogroup-associated genes identification from the same DNA extract. For *stx* positive samples, one loop of enrichment was dispersed on TBX agar (Oxoid, Basingstoke

Hampshire, England) and fifty colonies were isolated. Pools of ten colonies were then tested for *stx* and *eae* by PCR. Finally, the intention was to isolate a single bacterial cell that contains *stx* and/or *eae* genes and/or the serogroup-associated genes. DNA was extracted with InstaGene Matrix® (Bio-Rad, Marnes la coquette, France) from 1 mL sample enrichment broth or from one *E. coli* colony according the manufacturer protocol for Gram-negative bacteria. Real-time PCR amplifications were performed on the Applied Biosystems 7900 HT Fast Real time PCR instrument (Applied Biosystems, Foster City, California 94404, USA) within a total reaction mixture of 20 µL containing 2 µL DNA, 0.5 µM (each) primer, 0.2 µM probe (Invitrogen, Carlsbad, USA), 10 µl of GoTaq® qPCR Master Mix 2.0 (Promega Corporation, Madison, USA) and PCR grade water to final volume. The real-time PCR thermal conditions consisted in a first step of initial denaturation of DNA and Taq polimerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for one minute. Negative control (*E. coli* K12) and positive control strains of STEC received from the European Reference Laboratory for *E. coli*, Italy, were used.

All positive strains for one of the target virulent genes (*stx* and/or *eae*) were analyzed by phenotypic techniques. After overnight incubation at 37°C, it was evaluated the ability to convert tryptophan into indole using tryptone broth (Oxoid) and Kovács reagent (Merck KGaA, Darmstadt, Germany), to ferment the sorbitol on CT-SMAC (Oxoid), the glucoronidase and galactosidase activity on chromID O157:H7 (bioMérieux, Marcy l 'Etoile, France) and the hemolysin activity by culturing on 5% blood agar plates.

The isolated strains were preserved on Criobilles (bioMérieux) in a -80^{0}C freezer until sequencing.

Nucleotide sequencing

The *stx*1, *stx*2, *eae*, *wzx*O157 and *wzy*O113 genes were amplified in a Bio-Rad C1000 PCR instrument (Bio-Rad). PCR reaction consisted in a 25 µL reaction mixture containing 2 µL of 10 to 50 ng extracted DNA with InstaGene Matrix (Bio-Rad), 0.5 µM of each primer (table 1), 1U Maxima Hot Start Taq DNA Polymerase (Thermo Fisher Scientific, Waltham, USA), 1x buffer, 2.5 mM $MgCl_2$, and 0.5 µM of each deoxynucleoside triphosphate (dNTP). PCR was performed with initial denaturation at 95^{0}C for 5 min, followed by 35 cycles of 95^{o}C for 30 sec, 52^{o}C for 50 s and 72^{o}C for 30 sec (*stx*1)/ 58^{o}C for 50 s and 72^{o}C for 30 sec (*stx*2)/ 62.5^{o}C for 30 s and 72^{o}C for 30 sec (*eae*)/ 58^{o}C for 30 s and 72^{o}C for 30 sec (*wzx*O157)/ 60^{o}C for 30 s and 72^{o}C for 1 min (*wzy*O113), and a final extension at 72^{0}C for 7 min. The amplicons were directly sequenced by reverse and forward primer previously used (table 1) in each direction using an ABI BigDye Terminator 3.1 cycle sequencing kit (Life Technologies Corporation, Carlsbad, USA) according the manufacturer protocol.

Table 1. Primers used for DNA sequencing

Primer	*Direction*	*Primer sequence (5'-3')*	*Fragment size (bp)*	*Reference*
*stx*1	Forward	ACACTGGATGATCTCAGTGG	614	(P. K. FAGAN & al. [18])
	Reverse	CTGAATCCCCCTCCATTATG		
*stx*2	Forward	CCATGACAACGGACAGCAGTT	779	
	Reverse	CCTGTCAACTGAGCAGCACTTTG		
*wzx*O157	Forward	GCTGCTTATGCAGATGCTC	133	(S.R. MONDAY & al. [19])
	Reverse	CGACTTCACTACCGAACACTA		
*wzy*O113	Forward	AGCGTTTCTGACATATGGAGTG	593	(A.W. PATON

	Reverse	GTGTTAGTATCAAAAGAGGCTCC		& al. [20])
eae	Forward	GACCCGGCACAAGCATAAGC	384	(A.W. PATON & al. [12])
	Reverse	CCACCTGCAGCAACAAGAGG		

Phylogenetic analysis

Homology searches for *stx*, *eae*, *wyx*O157 and *wyz*O113 partial sequences were performed using BLAST through the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). The nucleotide sequences obtained were aligned using of ClustalW from MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets (S. KUMAR & al. [21]). A phylogenetic tree was constructed by the neighbor-joining method, and genetic distances were calculated by the Kimura two-parameter method (B. EFRON & al. [22]). The resulting tree was drawn with the same MEGA7 software. The Gen Bank strains used for comparison of the isolated STEC strains in this study were KM596779, DQ452096, KU158845, LC151942, KY466168, FR875155, AJ579368, AJ879906, AJ57930 and EU499352.

3. Results and discussion

Of 1484 samples of milk and meat with bovine and ovine origin, 330 samples were detected as positive by screening real-time PCR for one or both verocytotoxin genes and/or *eae* and serogroup- associated genes (samples named as presumptive), out of which STEC was isolated from 69 samples (table 2). Five STEC's were found in milk and milk products, 16 in meat and meat products and 32 in swab carcass, all of them containing only the *stx*2 gene. *stx*1 gene was found in the strains isolated from three milk products, nine meat and meat products and 32 swabs. Twenty-eight *stx*1/*stx*2 mixed genotype strains were found in one milk product, three meat products (one bovine minced meat and two mixed bovine/ovine minced meat) and 24 swabs (two from bovine and 22 from ovine). One *stx*2/*eae* O157 STEC genotype was isolated from a bovine meat and a minced bovine meat contained a *stx*1/*eae* O26 STEC strain. A *stx1* O113 STEC genotype was found in a minced mixed meat of bovine/ovine origin.

Table 2. STEC detection results - The origin and matrix involved

Origin	*Matrix*	*Negative*	*Positive*	*Presumptive*	*Total*
Slaughterhouse	Swab carcass bovine	271	3	32	306
Slaughterhouse	Swab carcass ovine	139	37	19	195
Farm	Milk	51	3	11	65
Row milk dispenser	Milk	93	0	17	110
Milk processing plant	Milk products	16	2	5	23
Markets and fairs	Milk and milk products	29	0	21	50
Market, supermarket, hypermarket	Milk and milk products	214	2	26	242
Market, supermarket, hypermarket	Meat, minced meat, meat product	147	15	70	232
Steakhouse	Meat, minced meat, meat product	99	4	38	141
Butcher shops	Meat, minced meat, meat product	76	2	16	94
Restaurant	Meat, minced meat, meat product	19	1	6	26

Total	*1154*	*69*	*261*	*1484*

Regarding phenotypic characterization, all isolated STEC strains (less strain STEC O157) disclosed fermentation of sorbitol and glucoronidase and galactosidase activity. Only five strains, including STEC O157 and O113, displayed hemolysin activity.

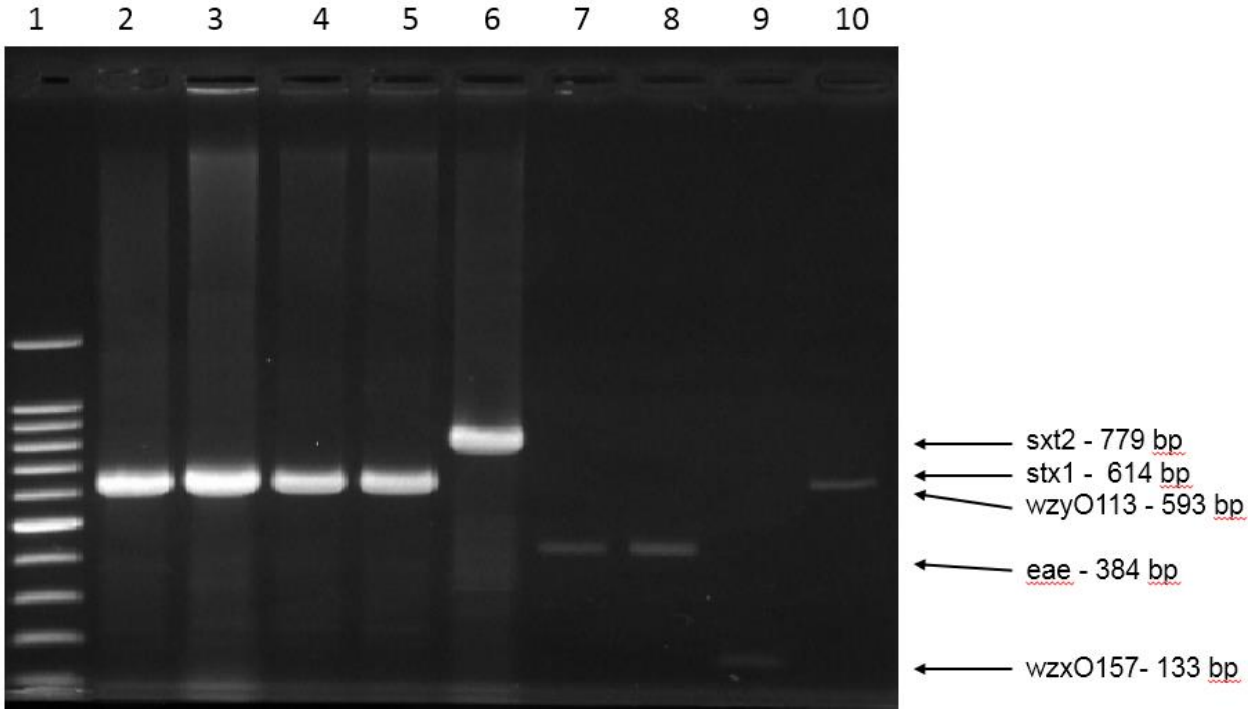

Figure 1. Conventional PCR. Lane 1- 100 bp DNA Ladder; lane 2- strain 215 (genotype *stx*1/O113 positive); lane 3- strain 315 (genotype *stx*1/*eae* positive); lane 4- strain 415 (genotype *stx*1 positive); lane 5- strain 515 (genotype *stx*1 positive); lane 6- strain 725 (genotype *stx*2/*eae*/O157 positive); lane 7- strain 315 (*eae* positive); lane 8- strain 725 (*eae* positive); lane 9- strain 725 (*wzx*O157 positive); lane 10- strain 215 (*wzy*O113 positive).

Based on the similarity of genes between the isolates and the hemolysin activity, only five strains were chosen and directed to DNA sequencing, strain 215 (genotype *stx*1 /O113 positive), strain 315 (genotype *stx*1/*eae* positive), strain 415 (genotype *stx*1 positive), strain 515 (genotype *stx*1 positive) and strain 725 (genotype *stx*2/*eae*/O157 positive). The sequences were blasted and compared with other STEC strains at nucleotides level, obtaining a phylogenetic tree as figure 2.

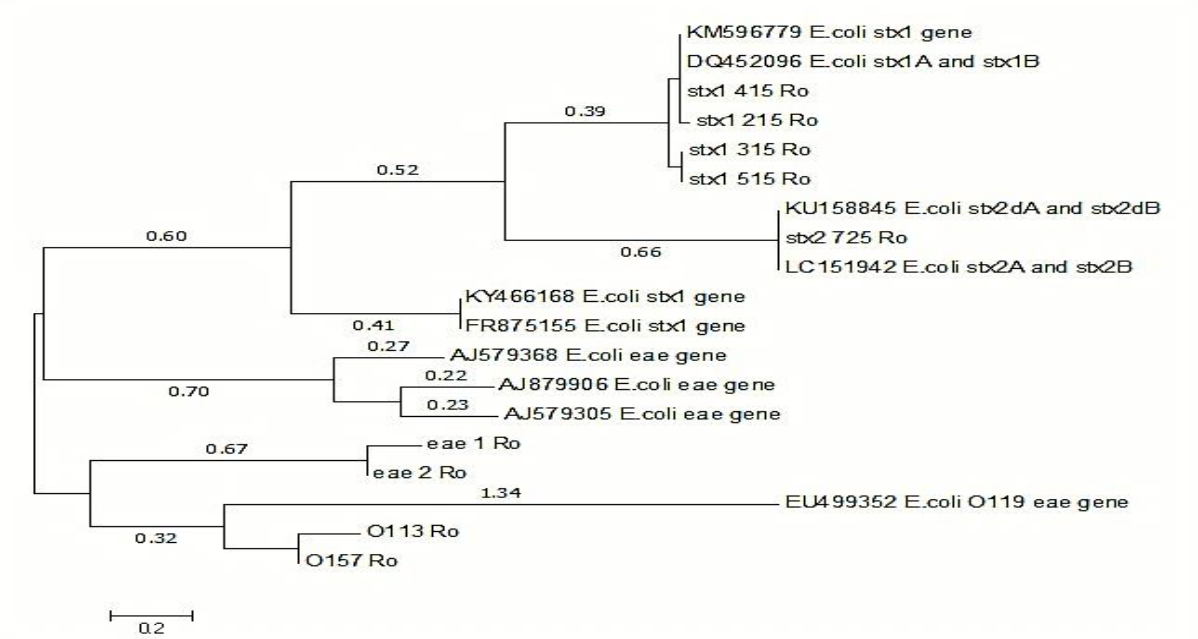

Figure 2. Phylogenetic tree for five Romanian STEC isolates (Ro) based on the neighbor-joining method (M. KIMURA [23], S. F. ALTSCHUL & al. 24]). The branch lengths were indicated by the scale of 0.2, expressed in number of changes in SNPs (single-nucleotide polymorphisms) per strains.

Protein conformation of the translated partial sequence genes was compared to other Gen Bank strains, resulting some differences as figures 3 - 6.

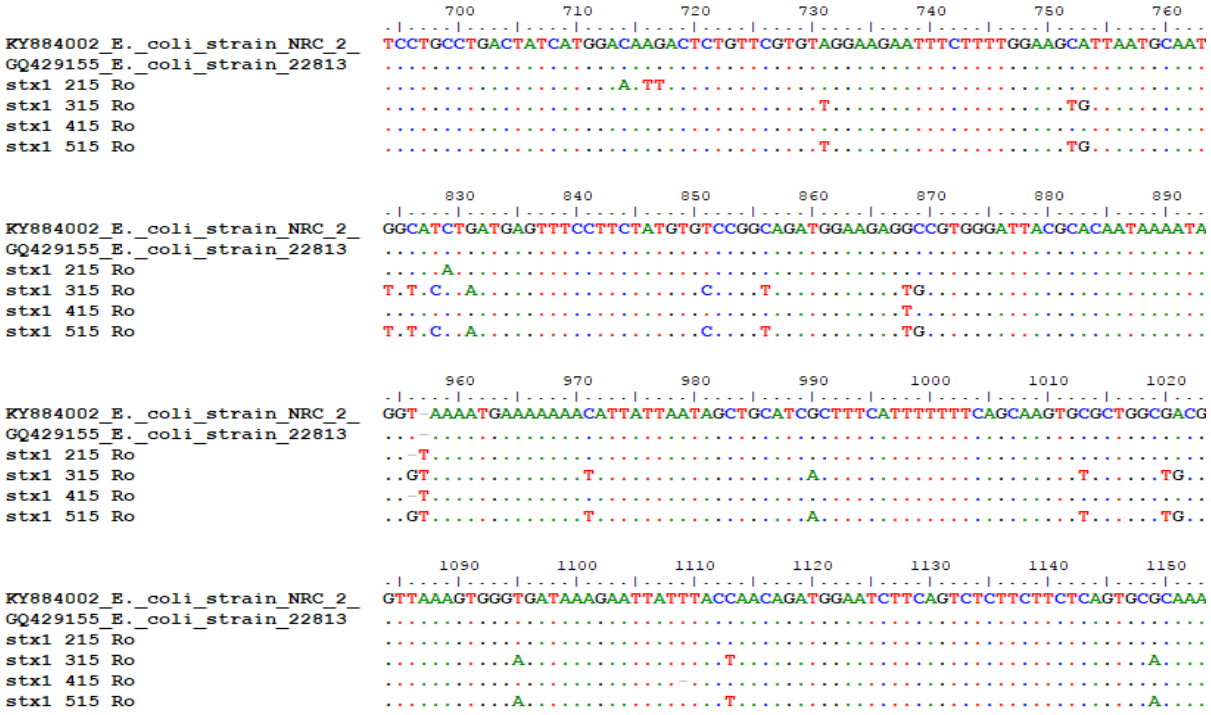

Figure 3. Graphical representation of *stx* nucleotides differences

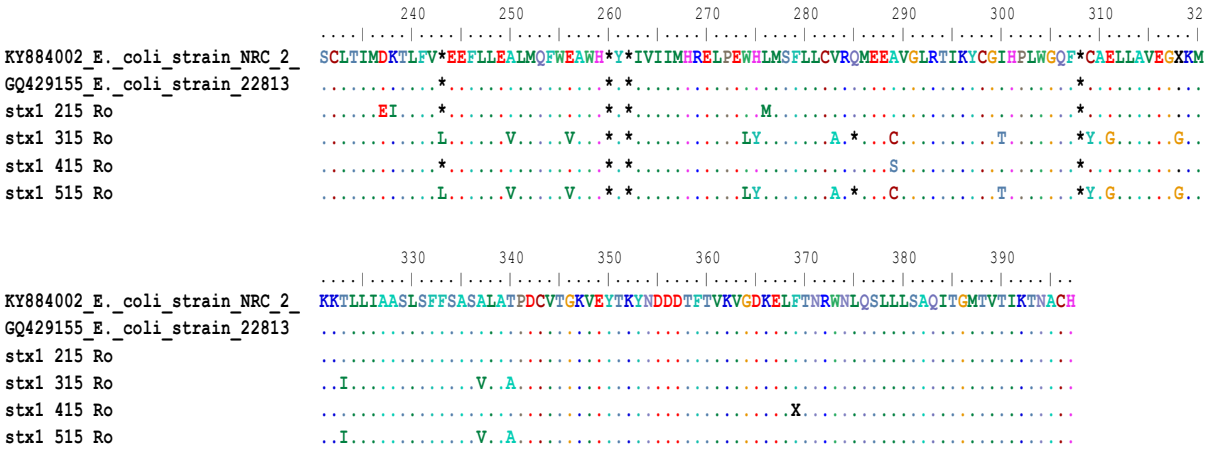

Figure 4. Graphical representation of Stx amino acids differences

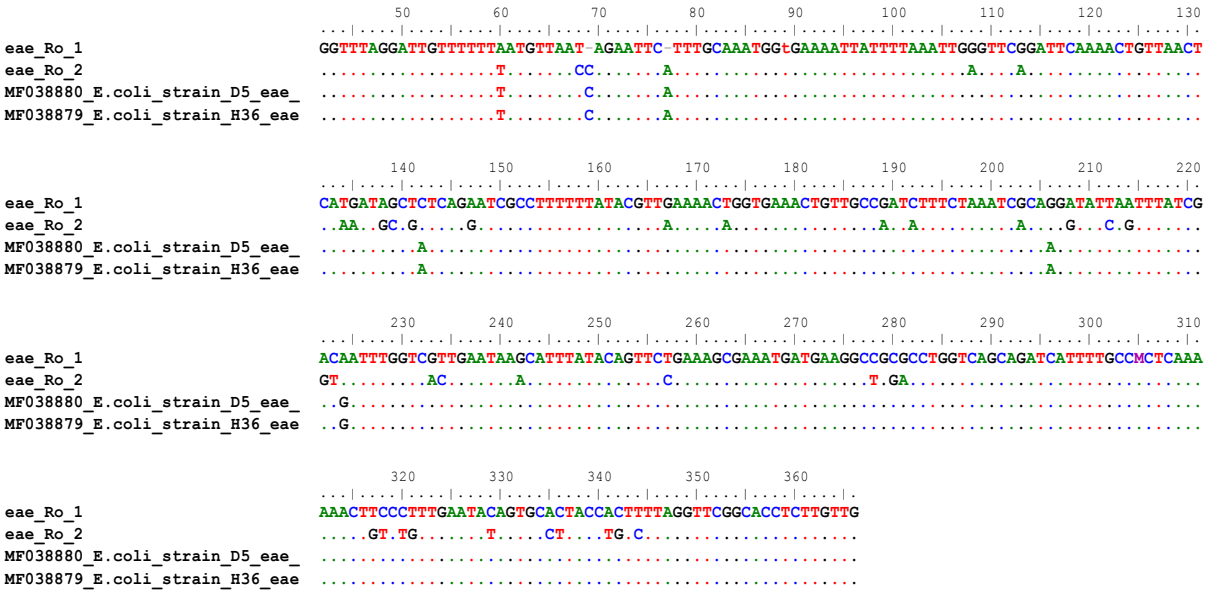

Figure 5. Graphical representation of *eae* nucleotides differences

Figure 6. Graphical representation of intimin amino acids differences

Alignment of the *stx*1 and *stx*2 discussed sequence genes with other Gen Bank strains, revealed 2 to 25 SNPs differences according figure 2 (215 and 415 strains have only 5 and 2 base pairs differences, respectively; strains 315 and 515 encountered 21 nucleotide differences comparing to the Blast sequences KY884002 and GQ429155). The predicted amino acid configuration after translation was identical between strains 315 and 515, but

differed from the others by leucine, valine, tyrosine, alanine, cysteine, threonine, glycine and isoleucine. The 215 strain is different from all the other strains by methionine, glutamate and isoleucine. The 415 strain is different from others only by serine, but contain in the same time an amino acid with an unknown termination (X).

Regarding the alignment of the *eae* gene (fig. 5), the results indicated 39 nucleotide differences between the two Romanian strains and only four differences with the foreign genes. According figure 6 there are significant amino acid differences between the *eae* Romanian strains (tyrosine, glutamine, serine, asparagine, alanine, aspartate, isoleucine, valine, glycine and proline) and only few differences between the strain 725 Ro *eae* gene and the Gen Bank genes (tyrosine, glutamine, serine, proline).

The obtained partial sequence genes of the STEC strains *stx*1 215 Ro, *stx*1 315 Ro, *stx*1 415 Ro, *stx*1 515 Ro and *stx*2 725 Ro were loaded in Gen Bank as Romanian *stx* sequences.

According ISO/TS 13136:2012 [17], a sample is positive when the isolation of the STEC is achieved. In the same time, the same method uses the syntagm "presumptive presence of STEC", meaning that the PCR is positive from the sample enrichment, but the isolation step is not achieved (maybe the STEC previously detected by PCR cannot be isolated because it is present in sample in such small amount that does not allow the isolation). For PCR the limit of detection is about two to ten genome copies/1 ml of enrichment broth, but for STEC isolation the limit of detection is more than 100 CFU/25g of sample depending on the existent background flora in the sample (ISO/TS 13136:2012 [25]). Because *stx* genes can be present in non-pathogenic organism like phages, the screening PCR step may have an inaccurate estimation of food safety risk unless a viable strain has been isolated (EFSA [26]). In this study, STEC strains were isolated from 4.65% of food samples, a small percent comparing to other diseases or other countries (K. G. J. POLLOCK & al. [14], K. DE SCHRIJVER& al. [15]). A bigger percent, 17.58% of tested samples were included in the category of STEC presumptions, an older study yielding only such results (L. M. CIUPESCU & al. [27]).

Most contaminated matrix analyzed in our study was the carcass swab (40/69; 57.97%), followed by meat (22/60; 31.88%) and milk (7/69; 10.14%). The positive milk and milk products mostly originated in bovines (6/7; 85.71%) as well as the meat and meat products (14/22; 63.63%). The ovine swabs were the most contaminated samples (37/40; 92.5%), being in the same trend as other European countries, where a higher percent of STEC isolates was detected in 2016 in sheep and goats (18,5%) than cows (EFSA [13]).

Worldwide, meat and milk products may have 10% to 300% STEC (V. M. BOHAYCHUK & al. [28], J. BLANCO & al. [29], C. R. USEIN & al. [30]). It is highly recommended to use very sharp HACCP procedures at slaughterhouse level to prevent contamination during evisceration and skinning of ruminants. In addition, the hygiene of food preparation meat in the kitchen should be very rigorous. If we are dealing with food that will be consumed in a raw state, then testing them for STEC should be compulsory because it is not enough to go only for assessing the hygiene microbiological criteria (tests that detects the *E. coli* glucuronidase positive or counts *E. coli* by the presumptive test). Even if the carcass swabs and meat were the most contaminated matrices, meat is consumed only after thermal treatment. Regarding milk and milk products, there are some cheese items that are mostly eaten in row state (only fermentation), giving the possibility to produce STEC disease to consumers [K. DE SCHRIJVER & al. [15], C. R. USEIN & al. [30], ECDC [31].

Other authors found *stx*2/*eae* STEC genotype strains (altogether with or without *stx*1) in human cases and food. These strains may lead to HUS (*stx*2/*eae* mainly) or diarrhea diseases (*stx*1/*eae*) (C. R. USEIN & al. [30]). The same authors claimed that STEC O26 was involved

in the 2016 outbreak when milk products were incriminated mostly. In-depth investigations on seeking links between food and human strains should further be conducted, because, in this study, in food were discovered 53 strains carrying *stx*2 gene and 44 strains carrying *stx*1 gene (of these, 28 were mixed *stx*1/*stx*2 genotype strains) and only one O26 STEC strain harboring *stx*1 and *eae* genes, which was actually isolated in meat. No STEC O26 strain was isolated in milk and milk products in this study.

Regarding phenotypic characteristics, the results showed that STEC strains isolated in this study do not have significant differences than other reported STECs (all the *E. coli*, less serogroup O157, ferments sorbitol and have glucuronidase and galactosidase activity). Only five strains exhibited the property to excrete hemolysin, a feature that can determine the HUS syndrome in children (P. K. FAGAN & al. [18]).

By Sanger DNA sequencing, we analyzed, five STECs isolated from food. The obtained *stx* and *eae* partial sequence genes were confirmed to be variants of related Gen Bank genes because their sequences differed less than 10% from the previously reported variants. Less variability was encountered in case of *stx* genes, even though the origin of this gene is in lambda phages, entities that can determine more genetic variabilities than other mobile elements (S. A. MAURO & al. [32]). Greater variability was observed in the case of *eae* genes but this is not something out of the ordinary because other authors have come to the same conclusions (E. A. MCGRAW & al. [33], W. L. ZHANG & al. [34]). Overall, these results indicate common origin of Romanian isolates comparing to homologous Gen Bank isolates related to *stx* and *eae* genes sequences. The newly *stx* sequences assigned in Gen Bank can contribute to earlier investigations in case of identifying slaughtering ruminant and milk processing plants as a source of STEC O157 and non- O157.

4. Conclusion

In this study, *stx*2 STEC genotypes were the most frequently in the monitored food and the non-O157 genotypes found here could be involved in the future in new emerging outbreaks in Romania as O26 in 2016. Bovine meat and meat products were the most contaminated matrices - this is similar to other Europe regions. In Romania, other STEC sources should be investigated in the future, like ruminant farms in other EU countries. Based on the pheno-genotypic profiles, the isolated STEC strains in this study are commonly found worldwide and continuous monitoring programs should be conducted in the future for earlier identifying the sources of STEC infections in humans.

5. Acknowledgements

This work was supported by funds from the National Sanitary Veterinary and Food Safety Agency in the frame of the 2016 national STEC monitoring program. R.N.T. is the recipient of a doctoral fellowship (No. 241/2016) financed by the University of Agronomic Sciences and Veterinary Medicine of Bucharest.

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