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ONE-YEAR FOLLOW UP STUDY FOR THE DETECTION OF STEC IN FOOD OF ANIMAL ORIGIN-THE PRESENCE OF THE MAIN VIRULENCE GENES, PRESENT AND FUTURE POTENTIAL RISK FOR CONSUMERS

Laurenţiu Mihai CIUPESCU, Isabela Madalina NICORESCU, Iuliana MIHAI, Rodica DUMITRACHE, Rodica TANASUICA

Institute of Hygiene and Veterinary Public Health, 5 Campul Mosilor Street, District 2, 021201, Bucharest, Romania, Phone: +4021.252.46.51, Fax: + 4021.252.00.61, Email: ciupescu_laurentiu@yahoo.com, isabela_nicorescu@yahoo.com, iuliana.mihai@iispv.ro, rdumitrache@yahoo.com, rodica.tanasuica@iispv.ro

Corresponding author email: ciupescu_laurentiu@yahoo.com

Abstract

STEC are very important as emergent food-borne pathogens, being implicated in large outbreaks as well as in sporadic cases of hemorrhagic colitis and haemolytic uraemic syndrome (HUS). Following the largest HUS outbreak reported in Europe in May 2011 (3255 confirmed cases and 33 deaths), in Romania was performed during 2012 a study for detection and identification of Shiga toxin/Verotoxin-producing Escherichia coli (STEC/VTEC) from food of animal origin. According ISO 13136/2011, an E coli strain is considered STEC if its genome contains simultaneously both stx (stx1 and/or stx2) and eae genes.

STEC are widespread in animals but ruminants are thought to be their natural reservoir. Taking into account the high prevalence of STEC in the gastrointestinal tract of ruminants and the possibility to contaminate the meat during the slaughter process, there were investigated carcass swabs and meat subsequently processed. The techniques used included both molecular and microbiological methods. The molecular methods applied was based on the detection by real- time PCR of the major virulence genes of STEC, stx1, stx2 and eae, and as well of the serogroup associated genes O157, O145, O111, O103 and O26 from bacterial enrichment broths and subsequent isolated colonies. From 445 samples tested in this study, the STEC isolation was not achieved, but single and combinations of the target virulence genes stx1, stx2 and eae were detected in 128 samples. The presence of the major toxigenic genes in 28.76 % of the samples infer that the probable origin of target genes that we detected by PCR could be the free-Stx phages from outside bacteria cells that can be present in food samples. Knowing from literature that the Stx phages can propagate in E. coli becoming potentially able to transduce stx genes indicates that STEC food-borne outbreaks can occur anytime.

Key words*: Shiga toxin-producing Escherichia coli (STEC), real time PCR, Romania, stx.*

INTRODUCTION

Escherichia coli is a Gram negative rod (bacillus) from the Enterobacteriaceae family. Most *E. coli* are normal commensals found in the human and animal intestine. Pathogenic strains of this organism are distinguished from normal flora by their possession of virulence factors such as exotoxins. Verocytotoxigenic (or verotoxigenic) *E. coli* (VTEC) produce a toxin that is lethal for continous Vero cell line, but not to some other cultured cell types. There are two major families of verocytotoxins, Vt1 and Vt2. A VTEC isolate may produce one or both toxins. Because verocytotoxin is homologous to the shiga toxins of *Shigella dysenteriae*, VTEC are also called shiga toxin-producing *E. coli* (STEC). STEC bacteria are in an increasing extent recognized as important food-borne pathogens worldwide with more than 265,000 illnesses each year in the United States and 13545 confirmed STEC infections and 777 HUS cases reported in the EU between 2007 and 2010 (EFSA, 2013, Da Silva Felicio et al., 2014). Most important effects of STEC infections are bloody diarrhea or hemolytic uremic syndrome (HUS), with renal failure, hemolytic anemia, and thrombocytopenia that can often leads to a fatal outcome or to haemolytic chronic renal insufficiency, chronic arthritis, irritable bowel syndrome and Guillain-Barré syndrome (Silvestro et al., 2004, European Centre for Disease Prevention and Control and European Food Safety Authority, 2011; **Askar, 2011**). Food-borne outbreaks are often caused by eating animal products undercooked or unpasteurized (STEC O157:H7 can survive for at least nine months in ground beef stored at -20°C) or contaminated vegetables (STEC surface contamination or

internalized in the tissues of some plants like lettuce). The reported survival time for STEC O157:H7 in contaminated soil varies from a month to more than 7 months. In marine water can survive for two weeks. In slurry can survive up to three months. Although it's a harmless bacteria, *E. coli* can „borrow, preserve or exchange" toxin's encoding genes from/by Stx phages, becoming a dreaded pathogen (Usein et al., 2008; Muniesa, 2013).

In 2011, a STEC O104:H4 caused a large outbreak with more than 800 HUS and 33 deaths in Germany. In EFSA Jurnal 2013, Romania reported for 2010 2 cases of human STEC, one belonged to O157 serogroup and the other one to O26.

In 2012, in accordance with the Directive 2003/99/EC and EFSA Technical Report-2009 and taking into account that the ruminants are being recognized as main animal reservoir of STEC (Caprioli et al., 2005), the Institute for Hygiene and Veterinary Public Health (IHVPH) has conducted one-year follow up study on detection of VTEC from carcass swab samples collected from cattle slaughterhouses and from beef/mutton and products thereof from retailers. This paper aims to assert the results from the study and to assess the risk of emerging STEC food-borne outbreaks that could occur in Romania in the future.

MATERIALS AND METHODS

The food matrices used in the study were composed of minced beef meat, mixed minced beef with mutton or pork, beef meat preparations alone or mixed with mutton or pork and cattle carcass swabs from slaughterhouses.

According ISO 13136:2011, the method used for STEC detection comprises five sequential steps: microbial enrichment, nucleic acid extraction, detection of virulence genes (*vtx*1, *vtx*2*, eae)*, detection of serogroup-associated genes (*rfbE* (*O157*), *ihp1* (*O145*), *wzx* (*O103*), *wbdl* (*O111*) and *wzx* (*O26*)) and *E. coli* isolation from positive samples followed by target genes PCR detection from isolated colonies. In this study, samples were tested using ISO, but 30 positive samples for *stx*1/2-*eae* combinations genes without bacterial isolation were tested also by PCR from enrichment broth after filtration by using Millex-GV Syringe Filter Unit, 0.22 µm, gamma sterilized from Merck Millipore.

Meat sample preparation was done by homogenisation in modified tryptone soya broth supplemented with 10 mg/l of the antimicrobial novobiocin. Typically 25 g of sample is homogenised with 225 ml of enrichment medium and incubated at 41.5°C for 18 - 24 hours. For carcass swab samples, the enrichment step was done by immersion of the swabs in buffered peptone water followed by incubation at 37°C for 18 – 24 hours.

The PCR screening step consisted at first step by detection of *stx*1 and *stx*2 genes from DNA extraction of 1 ml enrichment broth, followed, in case of a positive result, by the *eae* gene detection from the same extract. If the two conditions were fulfilled, the next step was to detect the five serogroups of the presumptive present VTEC in the enrichment broth. When all results were positive until this step, it was intended to isolate a single bacterial colony which possess the genes previously detected by PCR from the enrichment broth. After 20 - 24 hours of cultivation at 37°C on agar plates with selective Oxoid mediums like TBX agar (Tryptone Bile X-glucuronide Agar) or SMAC agar (sorbitol MacCONKEY), up to fifty colonies have been isolated from every sample, followed by PCR *stx* and *eae* genes detection from pools of ten isolated colonies. Finally it was intended to isolate a single bacterial cell that included both *stx* and *eae* genes amongst those five serogroups, O157, O145, O111, O103 sau O26 (with PCR confirmation). This combination of virulence genes is often associated with the most severe forms of STEC-induced disease. Before streaking on agar plates, an immuno-magnetic separation (IMS) procedure was carried out using Dynal magnetic beads coated with antibodies specific to the five *E. coli* serogroups. The *E. coli* colonies from agar plates were identified by the indole formation assay before making pools of ten colonies for PCR detection. Presumptive colonies confirmation of VTEC O157, O145, O111, O103 and O26 was done serologically using a slide agglutination test from Statens Serum Institut from Denmark, but identification of the toxin-coding genes (*vtx* and *eae*) was done using Real-time PCR method. Amplification of every target gene was performed separately on Applied Biosystems

7900 HT Fast Real time PCR intrument (Figure 1) within a mixture of 20 µl containing 2 µl of the prepared DNA sample extracted with Instagene Matrix from Biorad-USA (from 1 ml enrichment broth or from one colony), 0.5 µM (each) primer, 0.20 µM probe (Life Technologies, USA), 10 µl of TaqMan Environmental Master Mix 2.0 (Applied Biosystems) and PCR grade water to final volume. The real-time PCR conditions consisted 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&extension at 60°C for 1 min. Negative control *E. coli* K12 and positive control strains of STEC O157 (*stx*1+, *stx*2+,*eae*+), O26 (*stx*2+,*eae*+), O111 (*stx*1+, *eae*+), O103 (*stx*1+,*eae*+) and O145 (*stx*2+,*eae*+) were received from the European Reference Laboratory from ISS Rome, Italy.

Figure 1. ABI 7900 HT Fast Real time PCR System

RESULTS AND DISCUSSIONS

According ISO 13136:2011, a STEC is a single *E. coli* bacterial cell that contains simultaneously *vtx*1 or *vtx*2 genes in combination with the intimin-coding *eae* gene (this gene is responsible for the attaching and effacing mechanism of adhesion of the bacteria to enterocytes). The results of real-time PCR for 445 food samples collected from different counties of Romania are given in Figures 2, 3 and 4.

Over 28% of all *samples* tested contain *virulence* genes on PCR screening step. In meat samples, the proportion of all target genes was 37 %, whereas the proportion of *vtx-eae* genes combination (relevant for STEC) was 12 %.

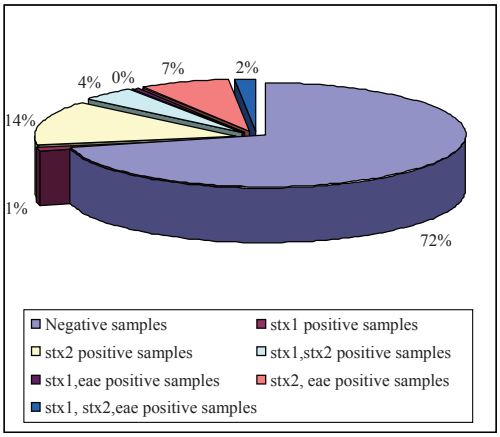

Figure 2. Virulence genes proportion in all analysed samples

Regarding the target genes presence per type of matrix, all matrices have approximately the same level of *vtx-eae* genes (~12%).

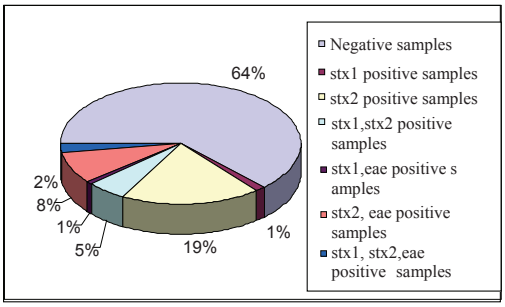

Figure 3. Virulence genes proportion in meat samples

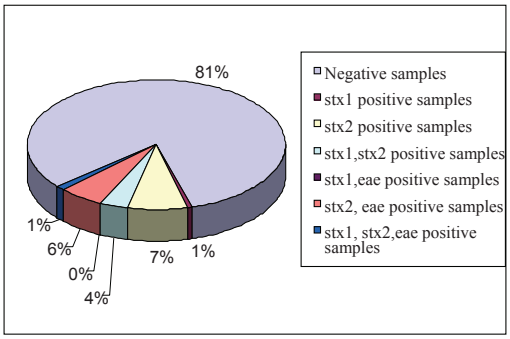

Figure 4. Virulence genes proportion in carcass swab samples

Regarding carcass swabs, all target genes were detected in 19% of samples, whereas the *vtx-eae* genes combination was found in 7%.

For both types of samples (meat and carcass swabs), the proportion of *vtx-eae* genes

combination was about one third from the positive samples for all target genes.
In Figures 5 and 6 it is shown the distribution of the serogroup associated genes detected in PCR screening step in 42 enrichment broths of samples that were firstly positive for *vtx-eae* genes combination.

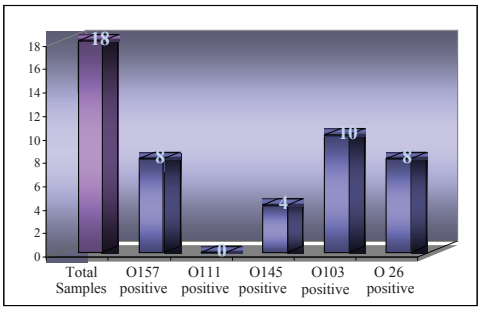

Figure 5. Serogroup associated genes tested by PCR in meat matrix (one sample may contain one or more of the five serogroups associated target genes)

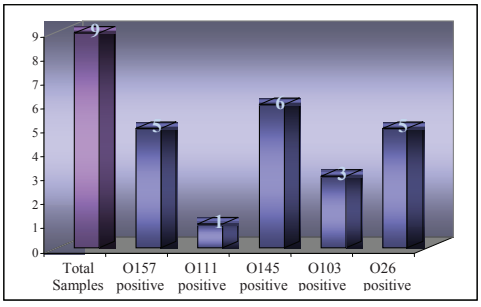

Figure 6. Serogroup associated genes tested by PCR in carcass swabs matrix (one sample may contain one or more of the five serogroups associated target genes)

During the 9th Annual Workshop of the National Reference Laboratories (NRLs) for *E. coli* in the EU (2014), several European countries claimed poor isolation results after they obtain positive results to the virulence genes PCR detection in the screening step. The conclusions were that the isolation should be achieved when the PCR show Ct values (PCR cycle threshold values) under 25. When PCR shows Ct values > 25, denotes that the isolation could not be accomplished. Our results presented at workshop can be seen in table 1. Amongst 445 samples tested only 9 are under Ct 25, but even so, because these samples have the Ct values comprised between 24 and 25 the isolation of the target genes couldn't be accomplished (Table 1 and figures 7 and 8).

Allison HE mentioned in 2007 that the bacteriophages (viruses that infect only bacteria) are the most abundant lifeforms on the globe and, referring to *E. coli*, they can carry the genes encoding Shiga toxin leading to emergence of Shiga toxin-producing pathogens.

Table 1. Detection of VTEC in food: the problem of low isolation rates from samples positive for *vtx* genes at the PCR screening step

Matrix	PCR+ samples with Ct <25 (or = to 25)		PCR+ samples with Ct > 25		Serogroup of the isolated strains
	No. of PCR+ samples	No. (%) with VTEC isolation	No. of PCR+ samples	No. (%) with VTEC isolation	
Beef minced meat (163 samples)	2	No isolation	58	No isolation	No isolation
Beef/ sheep prepared meat (79 samples)	6	No isolation	25	No isolation	No isolation
Carcass hide swabs (203 samples)	1	No isolation	36	No isolation	No isolation

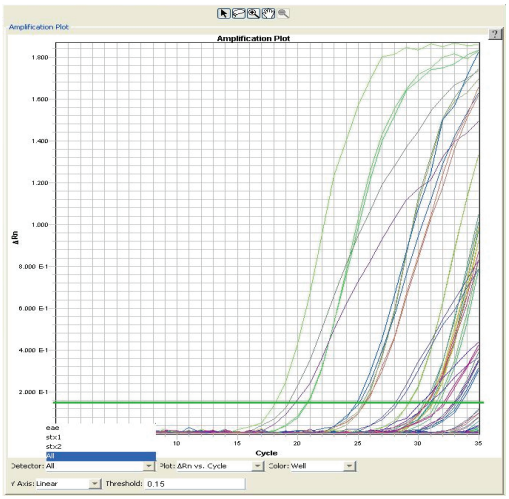

Figure 7. Virulent *stx/eae* target genes PCR detection from enrichment broths. Most of the samples gave Ct values over 25. The controls Ct values are under 25 from suspensions with 100 cfu/ ml

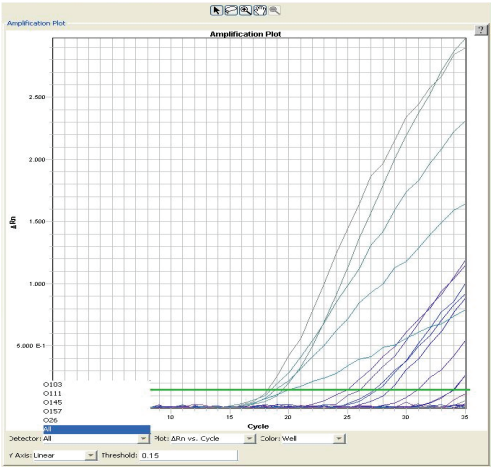

Figure 8. Serogroup - associated genes PCR detection from enrichment broths. Most of the samples gave Ct values over 25. The controls Ct values are under 25 from suspensions with 100 cfu/ ml

Taking into account this information, we performed PCR on enrichment broths filtered from samples which gave positive results to PCR in ISO screening step. After filtration through 0.22 µm, almost of 73 % of meat samples and 58 % of carcass swab samples still remain positive to PCR *vtx* and *eae* genes detection (the tests were done after 2 to 4 days from the first PCR, meanwhile the samples were kept refrigerated). This means that the PCR screening gave these results because of the existence of phages in the samples tested. This theory is also advanced by other researchers who reported that the detection of *stx* genes is not always an indicative of STEC because *stx* can be located in the genome of bacteriophages found in the samples as free particles. This explains the numerous reports of positive *stx* detection without successful STEC isolation (Quirós et al., 2015).

The existence of the Stx-phages in environment, humans, animals, food and water causes variability in STEC by *transduction* (horizontal transmission of the gene), phenomena demonstrated in the lab (Schmidt et al., 1999), in the intestinal tract (Toth et al., 2003), in biofilm (Solheim et al., 2013) and in food and water (Muniesa, 2013). Emergence of new STEC with different virulent genes configurations can occure anytime because Shiga toxin-converting bacteriophages (Stx phages) can carry the *stx* gene and convert nonpathogenic bacterial strains into Shiga toxin-producing bacteria (Brabban et al., 2005).

CONCLUSIONS

From 445 samples tested in this study, the STEC isolation was not achieved, but one, two or all of the target genes *stx*1, *stx*2 and *eae* were detected in 128 samples.

The presence of the major toxigenic genes in more than 28% of the samples point out that the probable origin of target genes that we detected by PCR could be free-Stx phages from outside bacteria cells that can be present in food samples.

Genetic exchanges between phages in the same STEC genome indicate that are not two identical phages after passing through a new host (Muniesa, 2013). The criteria regarding pathogenicity assessment by molecular techniques approach (at least for *stx*1, *stx*2 and the *eae* intimin-coding gene) must be included, because only the classical serotyping with specific antisera is just not enough to know that an isolated *E. coli* is pathogenic or not, rendering unreal the classification of STEC serotypes into seropathotypes by Karmali in 2003.

Even though the prevalence of the virulence genes in tested food samples was relatively low and knowing from literature that the Stx phages can propagate in *E. coli*, becoming able to transduce *stx* genes, indicates that STEC food-borne outbreaks can occur anytime.

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