

Molecular Screening and Characterization of Shiga Toxin-Producing *Escherichia coli* By Multiplex PCR Assays for *stx*₁, *stx*₂, *eaeA*, H7 in Raw Milk

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Abstract

The presence of Shiga-toxin producing *Escherichia coli* (STEC) was evaluated in bulk milk from dairy cattle farms in Bulgaria. From 20 regional farms only 4 were selected as a source for milk sampling on the basis of their annual STEC shedding in feces. All the samples were PCR analyzed after selective enrichment. Positive samples were detected in subsequent molecular testing as: two of samples (9.08%) were positive for *stx*₁, one sample (4.54%) for *stx*₂, 10 samples (45.54%) for *eae* and 12 (54.54%) for H7 coding genes.

Keywords: *Escherichia coli* O157: H7, Dairy farms, Bulk milk, Enrichment, PCR

Çiğ Sütte Shiga-Toksin Üreten *Escherichia coli*'nin *stx*₁, *stx*₂, *eaeA* ve H7 Bakımından Multipleks PCR ile Moleküler İncelenmesi ve Karakterizasyonu

Öz

Bulgaristan'da süt sığırları işletmelerinden toplanan çiğ sütlerde Shiga-toksin üreten *Escherichia coli* (STEC) varlığı değerlendirildi. Toplam 20 bölgesel çiftlikten sadece 4'ü yıllık dışkıyla STEC yayma miktarı temel alınarak süt toplama kaynağı olarak seçildi. Tüm örnekler selektif zenginleştirme sonrasında PCR ile analiz edildi. Pozitif örnekler daha sonra moleküler test ile değerlendirildi. İki örnek (%9.08) *stx*₁ için, 1 örnek (%4.54) *stx*₂ için, 10 örnek (%45.54) *eae* için ve 12 örnek (%54.54) H7 kodlayan genler için pozitif bulundu.

Anahtar sözcükler: *Escherichia coli* O157: H7, Süt çiftliği, Çiğ süt, Zenginleştirme, PCR

INTRODUCTION

Shiga-toxin producing *Escherichia coli* O157: H7 is a dangerous food pathogen that could induce a wide range of illnesses in human - from mild transient to haemorrhagic diarrhoeas accompanied with abdominal pain. It could be also the causative agent of the life-threatening haemolytic uremic syndrome (HUS) ^[1]. Other possible sources could be fresh, RTE (Ready to eat) products of plant or animal origin, contaminated with bovine faeces. Large ruminants have no specific receptors for toxins, they do not exhibit clinical disease but are considered asymptomatic carriers ^[2].

Consequently, STEC are commonly encountered as faecal contaminants of foods of animal origin as meat, milk and dairy products. As a result, STECs are often found to be faecal contaminants of foods of animal origin such as meat, milk and dairy products.

It is no surprise that raw milk has been identified as a source for contamination with STEC with potential sequels for public health from its consumption, particularly in line with the recent trend for consuming raw, non-thermally processed milk ^[3].

The transmission of STEC to humans is realized by various



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pathways, e.g. consumption of undercooked meat, vegetables, water contaminated with faeces of carriers, less frequently from man to man or from the contaminated environment. In 2016, Li et al.^[4] have performed a large survey in China on fresh meats and vegetables and reported that out of 35 isolates, 27 had virulence genes for *eae* (77.1%), while 7 *E. coli* O157 strains were positive for *stx*₂. The cytotoxic isolates also carried *eae* in addition to *stx* and belonged to the H7 serotype (11%)^[4].

Dairy farms are a specific source of *E. coli* (STEC), *Listeria*, *Staphylococcus aureus*, *Bacillus cereus*, *Campylobacter* and *Salmonella*^[5]. The risk of human infection with STEC originating from dairy farms could emerge after consumption of raw milk, dairy products whose technology does not imply thermal processing of milk or contaminated meat from dairy cattle^[6].

The severity of disease, high lethality rates and the lack of sufficiently efficient treatment requires protection of consumers from the risk of exposure to occasional low-level contamination of foods with *E. coli* O157. This further entails compliance to high standards of control in production enterprises in order to prevent cross-contamination^[7].

In the period from 2008 to 2012, at least six more cases of haemolytic anaemia, HUS and renal failure which required dialysis and life-saving kidney transplantation were reported again in the USA in people aged 2-27 years^[8].

In our country, there are no in-depth research data on the carriership rates in calves, cattle, milk, meat, fodders etc. as well as on the virulence profile, Shiga-toxin production and seasonal shedding variations. That is why, the present study was designed to investigate the genetic virulence profile STEC and presence of amplicons for the *eae* gene coding for intimin production in bulked raw milk.

MATERIAL and METHODS

Sampling Conditions

A total of 48 dairy farms from 20 of 28 administrative districts of Bulgaria were previously investigated by our team in the period 2014-2016 for the presence of *E. coli* O157:H7, and only in 4 of these farms have been found Shiga toxin-producing *E. coli* in fecal samples of cows^[9]. These 4 farms have been selected as the subject of this study. Each of the selected farms has over 100 dairy cows and at least 2 tanks for bulk milk storage after milking. A total of 56 samples of raw milk in a volume of 50 mL were collected during the summer months (July, August, September) in 2017. Milk samples are collected once a week, from each storage tank for raw bulk milk, after morning and after evening milking at the farm. The samples are chilled to a temperature of 6-8°C and transported under refrigerated conditions to the Laboratory of the

Department Contagious Diseases at the Trakia University.

Enrichment of *E. coli* O157:H7 in Raw Milk

In a laboratory conditions, each milk sample was homogenized by shaking and 10 mL was transferred to a sterile Falcon test tube with a cap. Each milk test tubes were centrifuged for 10 min at 4000 rpm⁻¹ for cell structure sedimentation. Then the formed milk cream was carefully removed by sterile cotton swab and liquid milk was discarded. The sediment was suspended in 5 mL sterile physiological saline and centrifuged once again under the same conditions. The supernatant was discarded and the pellet was resuspended by vortexing in 9 mL sterile selective enrichment broth (Tryptone Soya Broth Modified (mTSB) supplemented with Novobiocin, Oxoid, UK (SR01181) Enrichment procedure was similar to enrichment described in ISO 16654:2001 for STEC O157 in all food types (ISO, 2001).

All the test tubes were incubated aerobically at 37°C for 10-12 h for selective STEC *E. coli* enrichment. After incubation the samples were centrifuged (Rotofix 32A, D-78532) again at 4000 rpm⁻¹ for 10 min and the pellet containing bacterial cells was double washed in 1 mL sterile physiological saline. In the final centrifugation the bacterial cells were suspended in 500 µL deionized MilliQ DNA free pure water (GenPure Ultrapure Water System) and transferred in sterile 1.5 mL microtubes.

Bacterial DNA Extraction and Identification of STEC by Multiplex PCR

Bacterial DNA was extracted by boiling method as each 1.5 mL microtube with bacterial cell suspension were capped with parafilm and heated in boiling water for 10 min over sealing rack. Then followed by a centrifugation at 14000 rpm⁻¹ for 10 min at room temperature and the supernatant was separated from the pellet by pipetting 250 µL in sterile 1.5 mL lock caps microtube. DNA concentration was measured by (GeneQuant 1300, GE) and only samples with DNA yield between 200 and 500 ng/µL were considered as positive enriched and this samples were further evaluated by multiplex PCR.

PCR Amplification

PCR was performed in a volume of 20 µL with primer sequences (Eurofins Genomics) for virulence genes encoding *eae*; shiga-like toxin1 (*stx*₁), shiga-like toxin 2 (*stx*₂) and *fliCh7* of pathogenic *E. coli*. PCR reaction mixture contains 1xPCR buffer, 1.5 mM MgCl₂; 200 µM each dNTP, 0.5 µM each primer; 2.0 U Taq polymerase (Fermentas, Lithuania) and 1 µL bacterial DNA. Thermocycler (Quanta Biotech QB-96 thermocycler) was programmed for: initial incubation step of 3 min at 94°C; 35 cycles: 60 sec at 94°C, 90 sec at 60°C, and 90 sec at 72°C and a final extension step of 7 min at 72°C followed by final 4°C. Genomic DNA (IRMM-449, No 0242) of STEC *Escherichia coli* was used as positive control

and PCR mix with deionized water for negative control. *Table 1* presents primers used for target genes of *E. coli* are synthesized in the following sequence.

The PCR products (10 µL with 2 µL gel loading) from amplification were separated by electrophoresis (APELEX ps304, minipac2) on 2% agarose gel in 1xTBE buffer for 90 min at 120 V, stained with ethidium bromide (0.5 mg/mL) and photographed using a gel documentation system (Image Quant 150, ATIR6D, GE Healthcare). The positive PCR was realized as positive bands in size of 775, 302, 516 and 625 for primers *eae*; *stx*₁, *stx*₂ and *fliCh7*, respectively. DNA ladder 100 bp was used in each agarose gel.

RESULTS

Efforts from our investigation resulted in positive PCR reaction in 28 h after milk samples receiving in a laboratory conditions, which time include sample processing, incubation for enrichment, sedimentation and pellet destruction for DNA extraction and PCR reaction with visualization. The investigation time from milk sampling at farm level to the positive or negative results was nearly 34 h (33-37 h depending the kilometers distances of the farm from the laboratory).

In the milk samples we evaluated more than 39.2% of the samples a positive (22 of 56 milk samples) for *E. coli*, by detection of *eae* genes. Negative samples were 34 of 56 (60.7%). The most common gene in the milk samples was virulence gene H7, which were detected in 12 (54.5%) of positive milk samples after enrichment. One sample was positive for *stx*₂ (4.54%) and another two - to *stx*₁ (9.09%).

Table 1. Primers used for target genes of *E. coli*

Gene	Primer Sequence	Reference
<i>eae</i>	< F-5'-TCAATGCAGTTCCGTTATCAGTT-3'; R-5'-GTAAAGTCCGTTACCCCAACCTG-3'>	[10]
<i>stx</i> ₁	< F-5'-CAGTTAATGTGGTGGCGAAGG-3'; R-5'-CACCAGACAATGTAACCGCTG-3'>	[10]
<i>stx</i> ₂	< F-5'-ATCCTATTCCCGGGAGTTTACG-3'; R-5'-GCGTCATCGTATACACAGGAGC-3'>	[10]
<i>fliCh7</i>	< F-5'-ATCCTATTCCCGGGAGTTTACG-3'; R-5'-GCGTCATCGTATACACAGGAGC-3'>	[11]

Table 2. Virulence factors detected in milk samples after cell sedimentation, washing and selective broth enrichment

Milk Samples and <i>E. coli</i> Virulence Profile	Detected Genes by Primers Pair			
	<i>stx</i> ₁	<i>stx</i> ₂	<i>eae</i>	H7
Type 1 (n=5)	-	-	+	+
Type 2 (n=5)	-	-	+	+
Type 3 (n=5)	-	+	+	+
Type 4 (n=5)	+	-	+	+
Type 5 (n=5)	+	+	-	+
Type 6 (n=34)	-	-	-	-

On the basis of the presence of each of the target genes we classify the milk in 6 different types. Negative samples are type 6 (n=34) in which no of the genes was detected. In type 1 are samples with genes *eae* and H7. In types 3 and 4 are samples with detection of one of the *stx* genes. Most unusual are samples (n=2) in the type 5 where no intimin genes was detected (*Table 2*).

The results on *Fig. 1* present the gene identification in 11 samples (No. 1-11) from two farms.

The data of gene identification demonstrate the presence of amplicons with size 348 bp in strains 4 and 5 and a 482 bp product in milk sample 1, 2, 4 and 7, which corresponded to *stx*₁ and *eae A* genes. The other isolates did not exhibit presence of amplicons coding both genes.

The negative control (NTC) did not detect any amplification product or other carrier of genetic information. *Fig. 2* presents the gene identification of 11 milk samples from three farms.

The results demonstrated amplicons with size of 584 bp

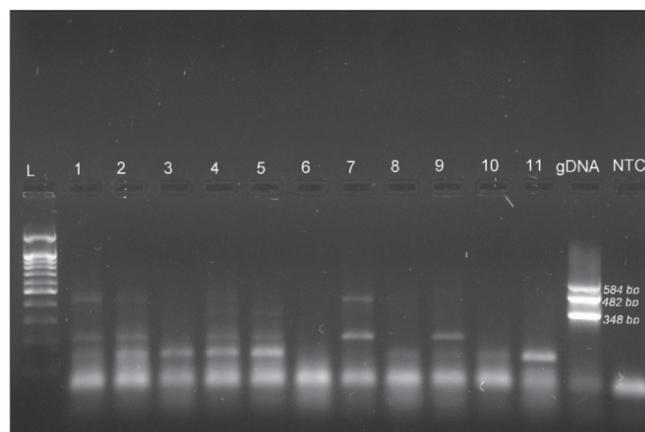


Fig 1. Molecular identification through PCR amplification of virulence genes *eae A*, *stx*₁, *stx*₂, in *E. coli* isolates from milk samples No. 1-11

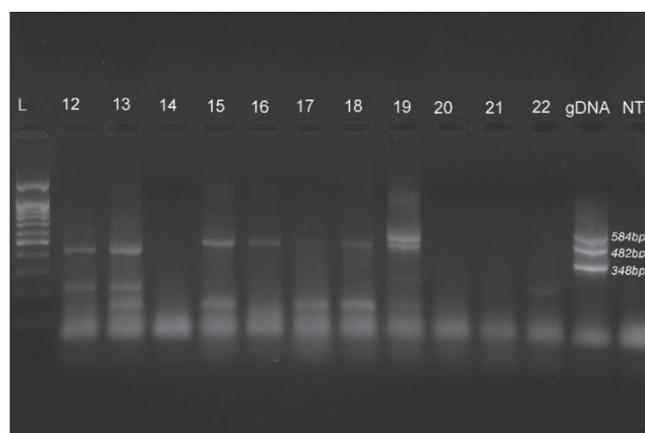


Fig 2. Molecular identification through PCR amplification of virulence genes *eae A*, *stx*₁, *stx*₂, in *E. coli* isolates from milk samples No. 12-22

in strain 19 and size of 482 bp in strains 12, 13, 15, 16, 18, 19, corresponding to *stx*₂ and *eae* A genes, respectively. The 348 bp product corresponding to *stx*₁ was absent in all milk samples.

The negative control (NTC) did not detect any amplification product or other carrier of genetic information.

Fig. 3 presents the genetic identification of 11 strains (No. 1-11). Except for strains 6, 10 and 11, all other tested isolated showed a 625 bp product equivalent to the H7 gene. The fragments' size was read against a 100 bp DNA ladder and through amplification of a positive DNA control from reference strain ATCC (genome DNA) with primer pairs specific for the H7 gene.

The genetic identification of 11 strains (No.No 12-22) depicted on Fig. 4 showed amplicons with size of 625 bp, corresponding to H7 gene in 4 milk samples (isolates 12, 15, 18 and 21). The fragment size was evaluated with the help of a DNA ladder and amplification of positive DNA control from reference strain ATCC (genome DNA) with primer pairs specific for the H7 gene.

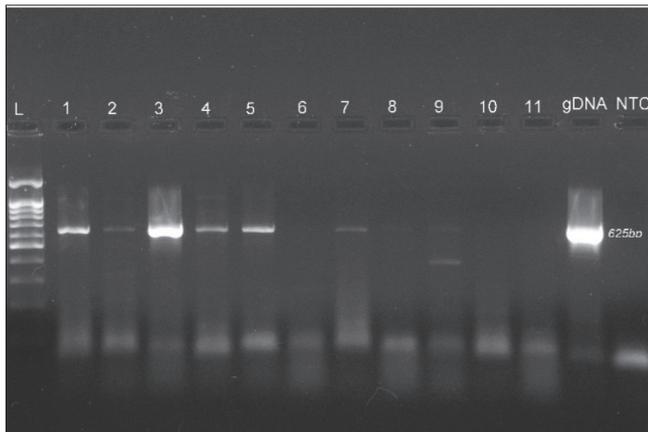


Fig 3. Molecular identification through PCR amplification of virulence gene H7 in *E. coli* isolates from milk samples No. 1-11

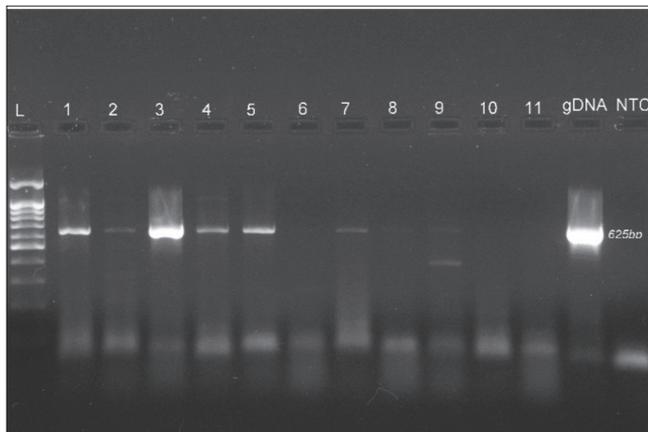


Fig 4. Molecular identification through PCR amplification of virulence gene H7 in *E. coli* isolates from milk samples No. 12-22

DISCUSSION

Unlike the most *E. coli* O157:H7 screening studies, our experimental design was different, as we investigated the presence of genes coding for toxin production directly in milk samples from bulk milk tanks. Dairy farms used as a sampling sources where preliminarily confirmed as positive presence of *E. coli* O157: H7 in milking cows. From the study results we were targeting shorter time for STEC bacterial detection and reliable and faster identification and virulence fingerprinting in the same time of the positive milk samples.

Hlavsa et al.^[12] demonstrated that the direct consumption of whole milk obtained under non-hygienic conditions and its use as a source in the production of cheeses increases the risk for infections along the food chain and hence, the risk from a severe disease or death caused by *E. coli* O157: H7^[12].

Our results showing low prevalence of STEC are similar to those reported by Caro et al.^[11] from screening sheep milk samples: out of seven cases suspicious for STEC only three *E. coli* O157: H7 isolates have been confirmed. Genetic profiles of the three isolates showed that all were *stx*₂-positive, and one was *stx*₁-positive. Furthermore, all possessed the *eae* virulence gene coding for intimin synthesis. It should be noted that the medium used for selective enrichments at the time of the initial identification of milk samples was modified tryptone soya broth supplemented with novobiocin in line with our research^[11].

McKee et al.^[13] screened 420 milk samples from two dairy enterprises in North Ireland and found out that 9 (2.14%) STEC-positive samples, four among which carried only the *stx*₂ gene, four carrying both *stx*₂ and *eae* genes, one sample positive for both *stx*₁ and *eae* genes - a profile, very similar to that in the present study^[13].

A similar experiment with 130 milk samples was investigated by Brenjchi et al.^[14]. After selective enrichment, 8 sorbitol-non-fermenting isolates were identified, and using biochemical markers, only one was confirmed as *E. coli*. The latter was identified as *E. coli* O157: H7 by means of PCR and virulence gene profiling showed presence of the gene coding from *stx*₂^[14].

In a similar research in Libya^[15], 108 cow, camel and goat raw milk and soft cheese samples were analysed. Three *E. coli* O157 isolates were confirmed in raw milk - one from cow milk and 2 from goat milk.

Comparable results have also been found in Rahimi et al.^[16] in Iran the highest prevalence of *E. coli* O157 was found in samples of water buffalo milk (5.5%), followed by cattle (3.6%) and all 3 isolates from *E. coli* O157: H7 were positive for *stx*₁, *stx*₂, as well as genes^[16].

This study emphasised the important of STEC for milk

and dairy products by revealing the potential risk from contamination of the milk with *E. coli* O157. The role of dairy cows in the epidemiology of STEC-induced human pathology and especially the role of serotype O157:H7 was confirmed. This necessitates strict biosecurity measures and good hygiene practices on farms to prevent the risk from contamination of milk at the time of its production and storage. Additionally, measures aimed at reduction of the possibility for transfer of strains among the farms are also needed.

CONFLICT OF INTERESTS

None of the authors had any conflict of interests in the writing of this paper.

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