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Original article

MOLECULAR PROFILING AND ANTIMICROBIAL SUSCEPTIBILITY OF *ESCHERICHIA COLI* O157:H7 ISOLATED IN BULGARIA

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Summary

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The purpose of this study was to detect the presence of shiga-toxin producing *Escherichia coli* (STEC) in faeces of healthy dairy cattle and to determine the sensitivity of isolates to several antimicrobial drugs. A total of 1,104 anal swab samples originating from 28 cattle farms were examined. After the primary identification, 30 strains were found to belong to serogroup O157. By means of conventional multiplex PCR, isolates were screened for presence of resistance genes stx_1, stx_2 and *eae*A. Twenty-nine strains possesses amplicons with a size corresponding to genes stx_2 and *eaeA*, one had amplicons also for the stx_1 gene and one lacked amplicons of all three genes. Twenty-eight strains demonstrated amplicons equivalent to gene *H7*. The results from phenotype analysis of resistance showed preserved sensitivity to ceftriaxone, ceftazidime, cefotaxime, cephalothin, streptomycin, gentamicin, tetracycline, enrofloxacin and combinations sulfamethoxazole/trimethoprim and amoxicillin/clavulanic acid. Sensitivity to ampicillin was relatively preserved, although at a lower extent.

Key words: antibiotic resistance; *Escherichia coli* O157:H7, PCR, shiga toxin

INTRODUCTION

Human diseases caused by shiga-toxin producing *E. coli* (STEC) have been reported from all over the world (Beutin *et al.*, 1993). Unusually virulent enterohaemorrhagic *E. coli* strains, including those belonging to O157:H7 have caused microbiologists, epidemiologists and food quality control specialists to revise regulations pertaining to food safety and hygienic practices for food production and storage. These pathogens turned out to be more relevant than others well acknowledged ones due to the exceptionally serious impact on human health in all age groups of the population. The serotype O157:H7 is known to cause haemorrhagic

colitis, haemolytic-uraemic syndrome and thrombocytic thrombocytopaenic purpura in men (Gyles 2007; Karmali *et al.*, 2010).

According to some researchers, cattle, especially juvenile, are recognised as the main reservoir of *E. coli* O157:H7 (Chapman *et al.*, 1993; Osek *et al.*, 2000).

E. coli O157:H7 has some important epidemiological features e.g. high virulence, low minimum infective dose, exceptional tolerance to acids and association with ruminants.

The numerous potential markers of virulence of *E. coli* O157:H7, in particular the production of shiga-like toxins (stx_1 and stx_2) are at the basis of its pathogenicity.

Shiga-toxin (Stx) is one of the most potent biological poisons. Humans encounter Stx at the time of infection with *Shigella dysenteriae* type 1 or with some *E. coli* serogroups like O157:H7. Together with or instead of stx_1, some *E. coli* strains produce a second type of shiga-toxin (stx_2) whose mechanism of action is the same as that of stx_1, yet it is antigenically different. Every toxin has subtypes, and the prototype toxin for each group is designated as stx_{1a} or stx_{2a}. In men infected with STEC, the most serious clinical disease is the haemolytic-uraemic syndrome, which is more commonly associated with stx_{2a}-producing strains (Melton-Celsa, 2014).

Other virulence factors are the specific protein intimin, encoded by the *eae*A gene, which mediates the adherence of the agent to the glycocalyx of small intestinal mucosal enterocytes, haemolysin production, determined by the presence of the *hly* gene (Doyle *et al.*,1997; Mead & Griffin, 1998) and the presence of the *fliCh*7 gene, determining the motility (Fratamico *et al.*, 2000).

The information about the spread of STEC among large ruminants in Bulgaria is scarce and data for the possible contamination of foodstuffs are almost absent. That is why the purpose of this survey was to establish the rate of *E. coli* O157:H7 carriership, to evaluate virulence potential of isolates and their behaviour to some antimicrobial drugs.

MATERIALS AND METHODS

Sampling

The study was performed in 2016–2017. It included 28 intensive cattle farms from 5 administrative regions of the country. The capacity of all farms was up to 1000 animals, including dairy cows, heifers, calves from 3 to 6 months of age, and suckling calves. A total of 1,104 anal swabs were collected. From each of farms, 30–60 swabs were obtained from 3-6-month-old calves depending on the population size.

Microbiological analyses

Initially, selective enrichment of rectal swabs were done in tryptic soy broth (Tryptone Soya Broth Modified, Oxoid) supplemented with novobiocin (Novobiocin Supplement, Oxoid). MacConkey Agar (Oxoid) plates containing Cefixime-tellurite Supplement (Oxoid). After 24 h incubation at 42 °C, sorbitol negative colonies were tested for the O157 antigen by latex agglutination (Oxoid) and up to six agglutination positive colonies were taken for PCR analysis. The detailed analysis protocol is described in Koev *et al.* (2018).

DNA extraction

A total of 30 *E. coli* O157:H7 strains isolated from a previous study of ours (Koev

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

Gene	Primer sequence	Reference
eae	< F-5`-TCAATGCAGTTCCGTTATCAGTT-3 ’; R-5’-GTAAAGTCCGTTACCCCAACCTG-3’>	Kim & Bhunia, 2008
*stx*1	< F-5’-CAGTTAATGTGGTGGCGAAGG-3’; R-5’-CACCAGACAATGTAACCGCTG-3’>	Kim & Bhunia, 2008
*stx*2	< F-5’-ATCCTATTCCCGGGAGTTTACG -3’; R-5’-GCGTCATCGTATACACAGGAGC-3 ’>	Kim & Bhunia, 2008
fliCh7	< F-5’-ATCCTATTCCCGGGAGTTTACG-3’; R-5’-GCGTCATCGTATACACAGGAGC-3 ’>	Gannon *et al.,* 1997

et al., 2018) were used. Pure culture of each of strains was inoculated on tryptic soy agar (CASO) and incubated at 37 ºC for 18 h under aerobic conditions. From the bacterial culture, suspension for genomic DNA extraction was prepared. One mL sterile TE buffer was poured in sterile Eppendorf microtubes. Several single colonies were collected via sterile cotton swab from the agar plate and suspended in the buffer. The turbidity resulting bacterial suspension was adjusted to McFarland standard 0.5.

Bacterial suspension was submitted to cell membrane destruction by heating the suspension to 99.5 ºC for 10 min. As a result from cell membranes disruption after heating, free DNA remained dissolved in the TE buffer. For sedimentation of cell elements, microtubes were centrifuged at 10,000 rpm for 10 min at 4 ºC.

Aliquots (500 µL) of dissolved bacterial genomic DNA in the supernatant were transferred in new sterile tubes for storage in a freezer at –20 ºC until PCR amplification. Extracted DNA concentration and protein contamination were evaluated by measurement of absorption at 160 and 280 nm and calculation of the A260/280 coefficient in Nanodrop. DNA yield should be adjusted to supernatant concentrations of 200–400 ng/µL with purity 1.8–2.0 A260/280.

PCR amplification

Genomic DNA segments corresponding to eaeA, stx_1, stx_2, H7 of *E. coli* were amplified using primers described in Table 1.

PCR reaction mixture included 1×PCR buffer, 1.5 mM $MgCl_2$; 2 µM dNTP, 0.5 µM primer; 1.0 U Taq polymerase and 1 µL bacterial DNA. In the thermo cycler, all samples were initially incubated at 94°C for 3 min, then followed 35 cycles of 94°C for 60 s, 60°C for 90 s and annealing at 72 °C for 90 s. Final extension was done at 72 °C for 7 min. Genomic DNA(IRMM-449, No 0242) of STEC was used as positive control while a PCR mix with deionised water served as for negative control.

PCR amplicons were separated by horizontal 2% agar gel electrophoresis. DNA ladder in the first lane was from 100 to 1100 bp with a step of 100 bp. Amplicon sizes are presented in Table 2.

Table 2. Amplified segment size

Amplicon	Size
*eae*A	482 bp
stx_1	348 bp
stx_2	584 bp
*fliCh*7	625 bp

Antimicrobial susceptibility testing

Phenotypic analysis of isolates' behaviour to antimicrobial drugs included 12 chemotherapeuticals from 5 different classes. The disk diffusion method was used according to EUCAST requirements (Matuschek *et al.*, 2013). The disks (Oxoid, England) were loaded with the following drugs: ampicillin (10 µg), amoxicillin/clavulanic acid (20/10 µg), cephalothin (30 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), tetracycline (30 µg), enrofloxacin (5 µg), gentamicin (10 µg), streptomycin (10 µg), sulfamethoxazole/trimethoprim (25 µg).

Statistical analysis

GraphPad InStat Version 3.00 (GraphPad Software Inc., La Jolla, CA) was used for determination of 95% confidence limits.

RESULTS

From all examined 1,104 samples, 30 (2.72%) sorbitol-negative strains positive in the agglutination test for *E. coli* O157 were isolated. Their genetic profile was evaluated with regard to toxin production genes stx_1, stx_2, *eae* and flagellar motility gene *fliCH7*.

Genetic determinants specific for shiga-toxin producing *E.coli* O157:H7 were found out in 3 out of the surveyed 28 farms (10.7%). The carriership of stx_1, stx_2, *eae* and *fliCH7* genes was 3.3%, 96.6%, 96.6% and 93.3%, respectively .

Fig. 1 depicts gene identification of the thirty strains isolated from three farms. All strains except for No. 30 showed amplicons with size 584 bp and 482 bp, corresponding to stx_2 and *eaeA* genes. Only strain No 8 carried also the stx_1 gene as evidenced by 348 bp amplicon. The

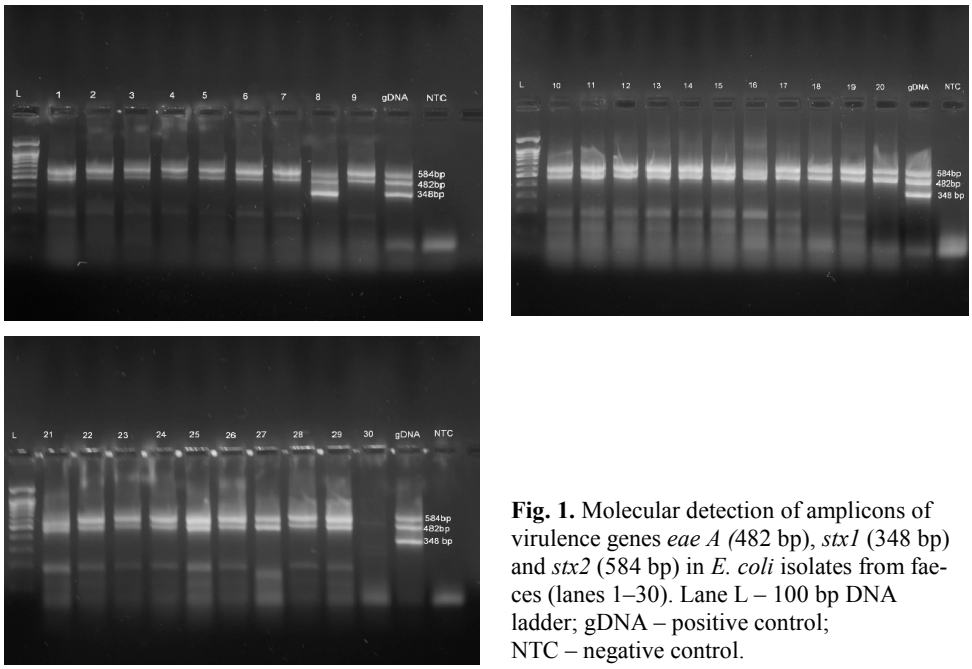

Fig. 1. Molecular detection of amplicons of virulence genes *eae A (*482 bp), *stx1* (348 bp) and *stx2* (584 bp) in *E. coli* isolates from faeces (lanes 1–30). Lane L – 100 bp DNA ladder; gDNA – positive control; NTC – negative control.

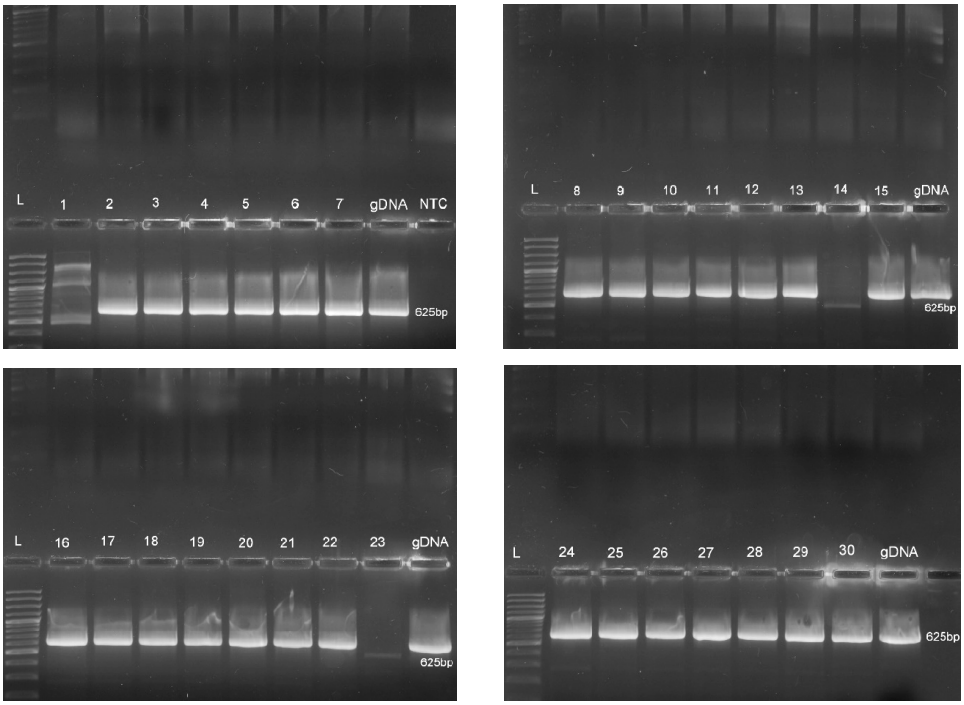

Fig. 2. Molecular detection of virulence gene *H7* through PCR amplification in *E. coli* isolates from faeces (lanes 1–30). Lane L – 100 bp DNA ladder; gDNA – positive control; NTC – negative control.

Table 3. Antimicrobial drug behaviour of resistant *E. coli* isolates from calves (n=30)

Antimicrobial drugs	Sensitive (%)	Resistant + intermediate (%)	Confidence limits
Ampicillin (AMP)	80	20	7.8÷35.9
Cefalothin (KF)	96.6	3.4	0÷12.7
Amoxicillin/clavulanic acid (AMC)	93.3	6.7	0.5÷17.1
Ceftazidime (CAZ)	100	–	–
Ceftriaxone (CRO)	100	–	–
Cefotaxime (CTX)	96.1	3.9	0.04÷13.6
Gentamicin (GEN)	100	–	–
Streptomycin (S)	90	10	2.0÷23
Tetracycline (TE)	96.6	3.4	0÷12.7
Enrofloxacin (EX)	100	–	–
Trimethoprim/sulfamethoxazole (STX)	100	–	–

strain No 30 lacked amplicons for all 3 genes.

Fig. 2 presents the virulence profiles of isolates in association with the *fliCH7* gene. All tested strains except for No.1, 14 and 23 exhibited 625 bp amplicons corresponding to the gene.

The antimicrobial drug behaviour of the tested 30 *E. coli* O157:H7 isolates (Table 3) showed preserved sensitivity to all third-generation cephalosporins. The sensitivity to aminoglycosides (streptomycin and gentamicin) and tetracycline, enrofloxacin, sulfamethoxazole/trimethoprim was also preserved. Relatively high sensitivity was exhibited to ampicillin and ampicillin/clavulanic acid.

DISCUSSION

Recently, several outbreaks in men caused by enterohaemorrhagic *E. coli* were reported. It is affirmed that most of them resulted from consumption of EHEC-contaminated foods. Information for the spread of these pathogens in animal reservoirs, cattle in particular is presented (Beutin *et al.*, 1993; Holland *et al.*, 1999; Singh *et al.*, 2015; Badouei *et al.*, 2016; Schneider *et al.*, 2018).

The knowledge on genetic features of bovine *E. coli* isolates is important to determine the extent at which these strains could be tranferred to men e.g. their pathogenic potential. In this study, a low prevalence of EHEC O157 in dairy cattle has been determined: 2.72%. In only 3 from the surveyed 28 cattle farms, a total of 30 sorbitol-negative strains positive in the *E. coli* O157 agglutination test were isolated. Similar results were reported by Sánchez *et al.* (2010) with STEC O157:H7 isolation rate of 2.6% among 268 cattle.

In Bulgaria, Urumova *et al.* (2015) have analysed 1,094 anal swab samples and detected 36 *E. coli* strains (3.3%) from the O157 serogroup in the agglutination test. Therefore, it could be concluded that at present, the serotype was only relatively poorly spread among dairy cattle at a national scale. Their possible occurrence in milk through faecal contamination and compromised pasteurisation however poses an epidemiological risk.

An even lower prevalence was reported by Singh *et al.* (2015), detecting only one strain in 100 tested samples. They however collected samples from cows, where the carriership is less frequent than in calves. Slightly higher values were established by Schneider *et al.* (2018) – in 5% of tested calves. This could be attributed to the fact that cited study was performed in beef cow-calf herds.

Apart the affiliation to a certain serotype, acknowledged to be part of the STEC group, the confirmation of the pathogenic potential of isolates requires detection of the presence of specific genes encoding toxin production. The studies reporting information about virulence factors and carriership of genes responsible for toxin production have recently increased. Similar to our data were presented by Tahamtan *et al.* (2011) in a large-scale survey on the prevalence and molecular characteristics of STEC isolates from healthy and diarrhoeic cattle and sheep. Among collected 872 anal swabs, *E. coli* O157:H7 was present in 9.75% of bovine and 7.90% ovine samples. According to authors, the stx_2 gene in cattle was more commonly detected that stx_1 (54.02% vs 26.43%), a fact confirmed by our study as well.

Erol *et al.* (2016) also proved a higher occurrence of the gene encoding stx_2 in

the virulence profile of *E. coli* O157:H7. The authors reported a prevalence of 3.9%, with all isolates positive for at least one *stx* gene: 12 (29.3%) and one (2.4%) isolates being carriers of stx_2 and stx_1 genes, respectively.

Gonzalez *et al.* (2016) investigated markers of virulence among STEC isolates from faeces of healthy cattle in Rio de Janeiro, Brazil in a study with very similar experimental design to ours. A total of 105 STEC strains were isolated, virulence genes were tested by means of PCR. Prevailing serotype was O157:H7 (12.4%), all carrying the gene encoding stx_2, in line with our results.

In agreement with our data, Sánchez *et al.* (2010) isolated 7 STEC O157:H7 from 268 cattle samples, all positive for genes stx_2, *eae, ehx*A and *fliCh*7.

The results reported by Osek & Gallien (2002) were comparable to ours. The authors investigated 14 *E. coli* O157 isolates from cattle and pigs in Poland and Germany, and detected only 2 strains positive for *fliC* (H7), 9 isolates with toxin production genes – stx_1 (1 isolate), stx_2 (4 strains) or both (4 strains) whereas *eae*A gene encoding intimin was found out in 9 isolates: 8 bovine and one porcine.

Having tested 540 bovine rectal swabs obtained immediately after slaughtering, Kalender (2013) reported that all STEC O157 isolates contained *hlyA* and *eae* genes, in agreement with the results in the present report. The author has tested also the antimicrobial drug behaviour of isolates and demonstrated that all STEC O157 were sensitive to ceftiofur (a third-generation cephalosporin), enrofloxacin and trimethoprim. Although in this study included cephalosporins from the same generation were ceftazidime and cefotaxime, obtained results were similar, including results for sensitivity to fluoroquinolones and antifolate agents.

The study of Urumova *et al.* (2015) reported that 100% of isolates were sensitive to cephalosporins, gentamicin and enrofloxacin and resistant to ampicillin (19.4%) in complete agreement to present data (100% sensitivity to third-generation cephalosporins, gentamicin and enrofloxacin and 20% resistance to ampicillin.

The antimicrobial sensitivity of 11 bovine and 14 ovine *E. coli* O157: H7 isolates examined by Goncuoglu *et al.* (2010) showed sensitivity to cefazolin, gentamicin, ciprofloxacin, imipenem, chloramphenicol, ceftiofur, trimethoprim/sulfamethoxazole, e.g. a behaviour very similar to this established in the present study.

In conclusion, the results from the present study confirmed once again the role of large ruminants as a primary carrier of *E. coli* O157:H7 – pathogens potentially dangerous for human health. The eradication or reduction of STEC excretion at the farm level would decrease their spread and circulation with the final goal to reduce the risk from infection of people.

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