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Original article

ISOLATION AND PRIMARY IDENTIFICATION OF SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* O157 IN DAIRY CATTLE

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Summary

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During the last years, the significance of diseases associated etiologically to Shiga-toxin producing *Escherichia coli* (STEC) is continuously increasing at a global scale, while the O157 serotype is considered as one of the most important pathogens of animal origin. Large ruminants play a key role in the epidemiology of *E. coli* diseases among men. Bovine faeces are a primary source of contamination of the environment and foods with this agent. The purpose of this study was to test a specific, microbiological algorithm for primary identification of STEC isolates from bovine faeces using sorbitol McConkey agar supplemented with cefixime and tellurite. The attempts were focused not only on increasing the sensitivity and specificity of serotype identification, but also on optimisation of labour and analysis costs. From May 2013 to October 2014, a total number of 1104 faecal swab samples from calves 3 to 6 months of age were collected from 19 farms in different administrative and geographical regions of Bulgaria. Thirty six sorbitol-negative *E. coli* isolates (3.26%) were detected as belonging to the O157 serotype after slide agglutination test.

Key words: cattle, *Escherichia coli* O157, faeces, isolation

INTRODUCTION

Escherichia coli (E. coli) is an ubiquitous intestinal bacterial species in both animals and humans. Colibacteria are the predominant facultative anaerobe commensal organisms in human large intestines (Konowalchuk *et al*.,1977; Bell *et al.,*1994).

Several pathogenic biovars of *E. coli* are able to induce a broad spectrum of diseases in men and animals (Konowalchuk *et al*., 1977; Riley *et al*., 1983; Schmidt *et al*.,1995). During the last three decades, a group of *E. coli* with unique virulence attributes was formed, which colonises the large intestines of large ruminants without clinical expression of disease, but which causes severe illness in men manifested by haemorrhagic diarrhoea and kidney failure, also known as

haemolytic-uraemic syndrome (HUS), often with lethal outcome (Kaper *et al*., 2004). This group is known as shiga-toxin producing *E. coli* (STEC).

From a historical perspective, shiga-toxin producing *E. coli* (STEC) was described for the first time in Canada by the end of the 1970s by Konowalchuk *et al*. (1977). Various research teams have contributed to causal agent detection; O'Brien and LaVeck were the first to prove that *E. coli* strains could produce shiga-like toxin (O'Brien & LaVeck, 1983). The same year, an outbreak of food-borne intoxication after consumption of hamburgers was reported in the USA in patients with haemorrhagic colitis which was associated by Riley and his team with the rare serotype *E. coli* O157: H7 isolated from faecal samples, and later, the production of Shiga toxin (STX) was proved (Riley *et al.,* 1983). Again in 1983, it was demonstrated that the haemolytic-uraemic syndrome (HUS) could be provoked by STEC O157:H7, as well as by other STEC serotypes (Karmali *et al*., 1983a; 1983b).

Shiga-toxin producing *E. coli* O157:H7 (STEC O157:H7) are important human pathogens capable of being transmitted via several alternative routes. The infection of men could be asymptomatic (without clinical signs) or could be accompanied by a variable clinical expression varying from mild watery diarrhoea through bloody diarrhoea, haemorrhagic colitis affecting the large intestinal mucosa (HC), to haemolytic-uraemic syndrome (HUS) and thrombocytic thrombocytopaenic purpura (TTP). Often, the outcome of these life-threatening states is fatal (Mead & Griffin, 1998).

The recommendation of the expert group of the European Food Safety Authority (EFSA) engaged in biohazard evaluation affirms that the monitoring of animals and foods in the EC should be initially concentrated on STEC O157:H7. It is incriminated as the serotype most commonly associated with severe human infections and HUS, but that it should be extended to other serotypes as O26, O103, O104, O91, O145 and O111, which, after O157 are identified as frequent causes of infections of people in Europe. Therefore, the significance of these serotypes should not be underestimated (Anonymous, 2007).

The purpose of this study was to test a specific, microbiological algorithm for primary identification of STEC isolates from bovine faeces using sorbitol McConkey agar supplemented with cefixime and tellurite.

MATERIALS AND METHODS

The study was performed in 2013–2014. It included 19 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 1104 anal swabs were collected. From all farms, 30–60 swabs were obtained from 3-6-month-old calves depending on the farm population size.

Bacteriological examinations

Initially, selective enrichment of rectal swabs were done in tryptic soy broth (Tryptone Soya Broth Modified, Oxoid) supplemented with novobiocin (Novobiocin Supplement, Oxoid). The aim was to increase the number of target organisms and at the same time, to inhibit the concurrent microflora (*Bacteroides* spp*., Lactobacillus* spp*., Proteus* spp*., Enterococcus* spp*., Clostridium* spp.). The incubation was performed in a thermostat at 37 °C for 4–6 h.

Then the cultured samples were subcultured on selective medium –Sorbitol Mac Conkey Agar (SMAC) (Oxoid), supplemented with cefixime and tellurite (Cefixime, Tellurite, Oxoid). SMAC is considered as the medium of choice for isolation. SMAC containing cefixime and potassium tellurite is the most commonly used nutrient medium for selectively enriched bovine samples with or without immunomagnetic separation (IMS). The incubation was under aerobic conditions, temperature 37 °C for 18–20 h.

After a careful inspection, 5 single, sorbitol-negative colonies were selected and cultured on Kligler's polytrope medium (Kligler agar, Oxoid), poured in tubes standing in upright positions or on agar slants. They were incubated under aerobic conditions, 37 °C for 24 h, and the utilisation of lactose, production of gas from glucose fermentation, hydrogen sulfide production, urease activity were evaluated. The isolates identified as *E. coli* were cultured again on selective medium – Sorbitol Mac Conkey Agar supplemented with 4-methylumbelifery-β-D-glucuronide (MUG supplement, Liofilchem).

On the next stage of the identification protocol, MUG-negative colonies were inoculated onto ordinary agar. After 24-hour aerobic incubation at 37 °C, sorbitol non-fermenting, beta-glucuronidase negative colonies were tested in latex agglutination test (Oxoid *Escherichia coli* O157 Latex test) to detect if the suspected isolate belonged to the O157 serotype, i.e. if it was a potential Shiga-toxin (STX) producer.

RESULTS

From all analysed 1104 anal swabs, 36 of sorbitol-negative *E. coli* isolates (3.26 %), were positive in the agglutination test with *E. coli* O157 antiserum (Table 1).

The table shows that a large percentage of samples, more than 70%, contained sorbitol negative colonies. It should be noted that some of the samples, respectively the plate contained several sorbitol negative colonies, while others were all sorbitol negative. A similar trend in percentage (71.2%) was also observed in the next test step, namely their determination as *Escherichia coli* of Kligler Iron Agar (KIA) – yellow colour of the slant presence of gas and absence of hydrogen sulphide. Only 13.4 percent or 77 of the proven *E. coli* strains showed lack of beta glucuronidase activity from the last less than half (46.7%) belonging to O157 serogroup, through agglutination testing.

DISCUSSION

The calves from which the samples were obtained were not randomly selected but their age was compliant to multiple field studies. According to literature data, cattle from the age group within 3 and 18 months are at the highest risk for shedding STEC O 157:H7. This age range corresponded to the period of life when calves left the individual boxes and formed groups, i.e. about the 70th day of life.

The cultivation of ruminant faeces for isolation of a specific serotype is a real challenge at the background of the existing huge diversity and amount of concurrent microflora. In existing studies, various methods with excellent results are reported. The combination of several techniques and methods of cultivation increases the probability for detection of the target organisms.

Table 1. Biochemical characteristics of tested isolates

Farm No	Number of samples	Sorbitol negative	Positive for *E.coli* in KIA	MUG positive	Seropositive for O157
1	52	45	38	2	
2	44	38	26	9	3
3	36	25	18	–	–
4	50	36	24	6	4
5	50	42	32	5	3
6	60	39	28	13	5
7	58	34	21	3	1
8	40	29	15	2	–
9	64	56	38	3	1
10	60	48	40	5	2
11	60	45	36	–	–
12	26	19	12	2	2
13	60	38	26	4	3
14	25	20	9	–	–
15	56	49	31	6	2
16	112	69	54	5	3
17	69	35	27	2	1
18	83	65	46	3	3
19	97	72	52	7	3
Total	1104	804	573	77	36

The methodology of cultivation changes incessantly and the efforts are concentrated not only on enhancing the sensitivity of serotype identification, but also on reducing the labour and analysis costs.

The protocols described in the literature use different enrichment media – tryptic soy broth (TSB), modified *E. coli* broth (mEC) and buffered peptone water (BPW) combined with one or more inhibiting antibiotics (novobiocin, cefixime, cefsulodin, vancomycin).

Potassium tellurite is added to slow-down or stop the growth of *E. coli* and *Aeromonas* spp. and to benefit the replication of STEC O157: H7, whose average resistance to inhibitors is higher than that of the concurrent faecal microflora. Thus, the supplementation of SMAC with potassium tellurite is advised for increasing the sensitivity of *E. coli* O157:H7 detection via differential inhibition of non-O157 *E. coli* and other bacteria (Sanderson *et al.*, 1995).

Cefixime is a third-generation cephalosporin from the aminothiazole group targeted against *Proteus* spp. (which are frequently sorbitol-negative) and sorbitol-fermenting microorganisms, thus reducing false positive results.

It should be however stated that sorbitol-fermenting bovine STEC O157 strains could be missed by this method, although this is exceptionally rare.

Unlike many other *E. coli* serotypes, *E. coli* O157: H7 are b-glucuronidase negative. Media containing 4-methyl-umbelifery-β-D-glucuronide (MUG) have been developed to benefit from this fea-

ture (Thompson *et al.*, 1990; Sanderson *et al.*, 1995). The utilisation of appropriate media containing sorbitol and MUG are recommended for primary isolation from the faeces of animals and humans.

Chapman *et al*. (1994) also affirmed that cefixime-ramnose sorbitol McConkey agar and cefixime-tellurite McConkey agar were more efficient than non-modified sorbitol McConkey agar for isolation of microorganisms from human and bovine faeces, respectively. The authors made a detailed comparative analysis of the isolation of *E. coli* O157 from the faeces of dairy cattle using a method with isolation in direct culture and with immunomagnetic separation (IMS). The samples were obtained over a 4-month period with total of 1024 monitored samples. Among them 84 (8.2%) have exhibited affiliation to the O 157 serogroup.

Many researchers have performed epidemiological survey on the prevalence of *E. coli* O157 among cattle and stated that such populations could be considered as the main reservoir of VTEC.

Some studies are aimed at detection of the agent in faeces, others – in milk, meat etc. For instance, a study of Inat *et al.* (2010) on the prevalence of *E. coli* O157 in raw cow milk was comparable to our study as it detected two strains of the agent in 150 milk samples (0.5 %).

Tahamtan *et al*. (2011) conducted a large-scale survey on the prevalence and molecular characteristics of isolates from 872 cattle and sheep, both healthy and affected with diarrhoeic syndrome. The agent was isolated from 9.75% of collected bovine and 7.90% ovine anal swabs.

Another extensive study in Wisconsin (the USA) carried out by Faith *et al.* (1994) among dairy cattle herds for evaluation of the incidence of *E. coli* O157 serotype on more than 70 farms, a herd prevalence rate of 7.1% was established, whereas 10/250 faecal swabs were positive, i.e. animal prevalence was 1.8% in calved younger than 4 months of age, which is fully in compliance with our results.

CONCLUSION

Remembering that our study reflects only the situation over a short period of time and that a small part of cattle farms in North Bulgaria were surveyed, we could make only suggestions instead of relevant conclusions. Anyway, the results from the study provide a background for future more detailed investigations and analyses on this exciting subject which remains still unexplored due to the character and structure of the national dairy cattle farming. The population level monitoring would contribute for further comprehension of the role of this microorganism in the epidemiology of human diseases. Therefore, the number of samples collected from different geographical and administrative regions of the countries is deemed necessary in order to improve the representativeness and statistical relevance of reported data and consequently, to take action for verification and revision of existing molecular biological approaches for classification and identification of STEC isolates.

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