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The prevalence of Shiga toxin-producing *Escherichia coli* in domestic animals and food in Serbia

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SUMMARY

Faecal samples of 2660 domestic animals from 116 farms and 956 samples of food were examined for the presence of Shiga toxin-producing *Escherichia coli* (STEC). STEC was recovered from 126 (15·3%) cattle, 135 (11·3%) pigs, 135 (66·8%) sheep, 31 (73·8%) goats, 4 (1%) chicken, and 15 (1·6%) food samples. Of all STEC isolates, 21·5, 25·8 and 15% produced enterohaemolysin, α-haemolysin, and aerobactin respectively, 1·6% displayed localized adherence (LA) to HEp-2 cells, 27·6% were sorbitol negative, and 30% were resistant to antibiotics. Only 14 (3·1%) of the STEC isolates belonged to human infection-associated serogroups (O26, O55, O111, O128 and O157), designated as enterohaemorrhagic *E. coli* (EHEC). This study revealed that STEC are prevalent in domestic animals, and to a lesser extent in food of animal origin in Serbia, but the absence of a EHEC phenotypic profile (characteristic serogroup, LA, enterohaemolysin production) in most animal STEC strains may explain the low incidence of human STEC infection in this part of the world.

INTRODUCTION

Shiga toxin (Stx)-producing *Escherichia coli* (STEC), also called verotoxin-producing *E. coli* (VTEC) has been regarded as an emerging pathogen which can cause severe diseases in humans such as haemorrhagic colitis and haemolytic–uraemic syndrome [1, 2]. Many outbreaks and sporadic infections have been reported worldwide, notably in industrialized countries [3].

It is well established that domestic animals are the primary reservoir of STEC. Their faeces serves as the source of this pathogen which is transmitted most commonly by contaminated food of animal origin, but vegetables, water, and contact have also been reported as transmission routes of STEC [3]. Although a large number of STEC variants have been described, the majority of strains associated with outbreaks and sporadic diseases have properties in common which are related to their virulence for humans. These are, besides the production of Shiga toxins and their variants (Stx-I, Stx-II, Stx-IIv), induction of attaching and effacing (A/E) lesions of enterocytes mediated by intimin (outer membrane protein), the production of enterohaemolysin, and the expression of characteristic serogroup O antigens such as O26, O55, O91, O103, O111, O113, O128, O145 and O157. Strains with such features are classified in a subgroup of STEC known as enterohaemorrhagic *E. coli* (EHEC) [1, 2]. The best known of this subgroup are strains of the O157 serogroup, notably of serotype O157:H7, which are a common cause of disease in human beings [1, 3]. Therefore, laboratory

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methods designed for the detection of these strains have been developed [4]. However, in recent years there has been a growing awareness of the role of non-O157 STEC serogroups as a cause of human disease [5].

Whereas STEC infections in humans are a major health problem in the developed world, the importance of these infections in developing countries is not well understood. Several investigations performed so far rarely registered STEC infections in the human population in these regions [3, 6, 7]. Similar to these findings, in a study carried out in Belgrade in 1995, we recovered STEC from only 8 (0·3%) out of 2625 cases with acute diarrhoea [8], and the first documented human infection with EHEC O157 in Serbia was registered in 2001 [9].

The aim of this study was to determine the prevalence of STEC in faecal samples of domestic animals and in food of animal origin and to test the isolated strains for some of the phenotypic traits typical for human isolates of this pathogen, i.e. EHEC. The results obtained would enable us to assess the risk for humans of acquiring STEC infection in this part of the world.

METHODS

Origin and numbers of samples

This investigation was carried out in a semi-urban community of Belgrade, Serbia, from January 1998 to the end of December 2002. Faecal samples of five domestic animal species were collected at no defined periodicity during veterinarian visits. From January 1998 to December 2001 faecal samples from 824 cattle originating from 174 small private farms as well as from one large-scale feedlot farm were analysed for STEC. During the period from January 1999 to December 2001, 1191 pigs from 128 farms were included in the study. Faecal samples of 401 chickens from 92 farms were tested for the presence of STEC during 2001. Faecal samples from 202 sheep originating from six flocks were analysed for this agent on six occasions as well as samples from 42 goats of one flock on one occasion in 2002. In the same year 956 food samples (264 samples of raw minced meat, 649 sample of fresh cheese, and 43 samples of salads and cakes) were obtained from Centre for Food Analysis, Microbiology Laboratory, Belgrade, and tested for the presence of STEC.

Sample collection and screening for STEC

Fresh faecal samples or rectal swabs were immidiately plated onto MacConkey agar (Difco, Detroit, MI, USA) and transported to the laboratory within 3 h for further processing. After overnight incubation at 37 °C bacterial growth was tested for the presence of STEC by using Vero cell assay according to the 'colony sweep-polymyxin' method [10]. The same procedure was used for screening for the presence of STEC in bacterial growth obtained after incubating food samples in Brilliant Green bile broth and spreading on MacConkey or blood agar. When cytotoxic activity was registered in a colony sweep, individual colonies were picked, restreaked on MacConkey agar, and Vero cell assay performed in order to obtain a single colony that produced Stx. The presence of STEC in a sample was not accepted unless a single Stx-producing colony was obtained.

Vero cell assay

Bacterial growth of colony sweep or individual colony in L broth was pelleted by centrifugation and the pellet resuspended in 1 ml of polymyxin B-sulphate (0·1 mg/ml) (Serva Feinbiochemica, Heidelberg/New York), incubated for 30 min at 37 °C, centrifuged, and the supernatant filter-sterilized using a 0·22 μm filter (Flow Laboratories Ltd, Irvine, Ayrshire, Scotland, UK). The cytotoxic effect of the sterilized supernatant (50 μl) was assayed using semi-confluent growth of Vero cells in 96-well flat-bottomed tissue culture plates. The cells were observed during a period of 72 h and the cytotoxic effect that caused death of at least 50% of Vero cells was registered. Isolated Stx-producing strains were identified by biochemical tests.

Determination of serogroup

Serogroups of isolated STEC were determined by slide and confirmed by tube agglutination test with commercially available sera (Institute of Immunology, Zagreb, Croatia) for EHEC characteristic serogroups (O26, O55, O111, O128 and O157).

Haemolytic activity

Haemolytic activity of the STEC strains was tested by streaking the strains on both blood agar plates with 10 mM $CaCl_2$, with either 5% washed or unwashed sheep erythrocytes. A clear zone of haemolytic activity around colonies on both plates, detectable after

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4 h incubation at 37 °C, was recorded as α-haemolysin production, whereas turbid haemolysis that appeared only on blood agar with washed erythrocytes after overnight incubation indicated the production of enterohaemolysin [11].

Sorbitol fermentation

The ability of the isolated STEC strains to ferment sorbitol was tested by spreading the strains on MacConkey agar base (Difco) (40 g/l of distilled water) to which D-sorbitol (10 g) was added. After overnight incubation at 37 °C sorbitol-negative strains occurred as colourless colonies, whereas sorbitol-fermenting strains yielded bright pink colonies [12].

Adherence test

In vitro adhesive ability of isolated STEC strains was performed with HEp-2 cells. A total of 50 μl of overnight bacterial cultures in L broth were inoculated into 500 μl of Eagle's essential medium with 10% fetal calf serum (Flow Laboratories) and 1% D-mannose in wells with coverslips which had been seeded with HEp-2 cells 48 h earlier. Cultures were incubated for 3 h at 37 °C in a 5% CO_2 atmosphere, the wells were washed three times with PBS, and the cells were incubated for a further 3 h under the same conditions. The coverslips were washed, fixed with methanol, stained with 5% Giemsa solution, and examined by light microscopy. Pattern of adherence was recorded as localized (LA), aggregative (AA) or diffuse (DA) [1].

Aerobactin production

Aerobactin-producing STEC strains were detected by slightly modified bioassay (2,2-dipyridyl, 200 μM, served as an iron chelator instead of EDDA), with *Shigella flexneri* SA 201 and *E. coli* RW 193 as control strains [13].

Antibiotic resistance

The STEC strains were examined for resistance to ampicillin (10 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), cephalothin (30 μg), nalidixic acid (30 μg), cotrimoxazole (25 μg), amikacin (30 μg), gentamicin (10 μg) and ceftriaxone (30 μg), using commercial disks (Institute Torlak, Belgrade, Serbia) by the disk diffusion test.

PCR for *Stx* genes

A total of 105 STEC strains isolated from cattle were tested for the presence of *stx* genes by the PCR method. DNA was prepared from 1 ml of overnight broth culture of each strain, as previously described [14]. PCR was performed in separate reactions with *stx1*-specific primers designated *stx1c* and *stx1d* [15], *stx2*-specific primers designated *stx2c* and *stx2d*, and *stx2* variant-specific primers designated *stx2v-1* and *stx2v-2* [16]. Prepared samples (5 μl) were amplified in a 50-μl reaction mixture containing 1 × reaction buffer [50 mM KCl, 10 ml Tris–HCl (pH 8·3), 0·1% Triton X-100], 1·5 mM $MgCl_2$, 200 μl of each dNTP, 1 μl of each of the oligonucleotide primers (0·1 μM) and 2 U of *Taq* DNA polymerase (Boehringer–Mannheim GmbH, Wien Biochemica). Reactions were overlaid with 50 μl of sterile mineral oil. The PCR was performed using a DNA thermal cycler (Hybaid Limited, Middlesex, UK). The cycling profile comprised an initial step at 94 °C for 5 min followed by 35 cycles consisting of denaturation at 94 °C for 2 min, annealing of primers at 55 °C for 2 min and primer extension at 72 °C for 1 min. After the last cycle, the PCR tubes were incubated for 7 min at 72 °C. The amplified product (10 μl) was visualized by standard submarine gel electrophoresis on a 2% agarose gel stained with ethidium bromide (1 μg/ml). A reagent blank in which the prepared sample supernatant was substituted with sterile distilled water, was included in each amplification run.

Statistical analysis

Results were evaluated using χ^2 test with or without Yates' correction or Fisher's exact test (Epi-Info, version 5; CDC, Atlanta, GA, USA).

RESULTS

A total of 3616 samples from animals and food were investigated for STEC, and the overall prevalence of this pathogen was 446 (12·3%). Table 1 summarizes the number of animals, farms, and food specimens tested by Vero cell assay with the rates of STEC isolation.

Among cattle this agent was recovered from faecal samples of 15·3% of animals at 40% of the investigated farms. The recovery rate was significantly higher in heifers (22·7%), and adult cows (16·7%) compared to calves (10·7%) ($P<0{\cdot}009$, and $P<0{\cdot}05$

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Table 1. *Isolation rates of Shiga toxin-producing* Escherichia coli *(STEC) in domestic animals and food in Serbia*

Source of isolation	No. of samples from		No. (%) of STEC isolates from	
	Animals	Farms	Animals	Farms
Cattle	824	175	126 (15·3)	70 (40)
Calves (<4 months)	233		25 (10·7)	
Heifers (4–12 months)	75		17 (22·7)	
Cows (12 months)	516		84 (16·3)	
Pigs	1191	128	135 (11·3)	35 (27·3)
<2 months	823		84 (10·2)	
>2 months	368		51 (13·9)	
Sheep	202	6	135 (66·8)	6 (100)
Goats	42	1	31 (73·8)	1 (100)
Chicken	401	92	4 (1)	4 (4·3)
Total	2660	402	431 (16·2)	116 (28·9)
Food	956		15 (1·6)	

respectively). At the time of sample collection the animals appeared healthy, except for 15 calves in which diarrhoea was registered. STEC was isolated from four (26·7%) of these 15 calves. Stool samples from 235 animals originating from the large-scale feedlot cattle farm yielded only 17 (7·2%) Stx-producing strains compared to 109 (18·5%) out of 589 animals from small farms ($P<0·000$). STEC was even found in 1-day-old calves; of 49 animals tested, this pathogen was isolated from three (6·1%). The frequency of STEC isolation showed significant seasonal variation; of 446 faecal samples collected during the cold season (October–March), STEC was recovered from 89 (19·9%) compared to 37 (9·8%) positive samples out of 378 obtained during the warm season (April–September) ($P<0·000$).

Of 1191 tested pigs, STEC was documented in 135 (11·3%). Positive animals represented 42 (26·2%) out of 160 investigated herds from 35 (27·3%) out of 128 farms enrolled in this study. Unlike cattle, the difference in the rates of STEC isolation was not significant among different age groups of pigs (Table 1). Of the total number of investigated pigs, 892 (74·9%) were healthy animals, and 299 (25·1%) were with clinical signs of diarrhoea, or oedema disease. The rate of STEC recovery was significantly higher in diseased animals (27·1%) compared to healthy ones (6·1%) ($P<0·000$). Table 2 provides a summary of the isolation rates in healthy and diseased pigs of different age groups. Among diseased pigs, the highest STEC recovery rate (66·7%) was registered in pigs with oedema disease older than 2 months. Seasonal variation of STEC isolation in pigs was similar, although not so marked as in cattle; 93 (12·7%) out of 733 samples collected during the cold season were positive for STEC, whereas 42 (9·2%) out of 458 samples obtained in the warm season yielded this pathogen, but this difference did not reach statistical significance ($P>0·05$).

Table 2. *Frequency of Shiga toxin-producing* Escherichia coli *(STEC) isolation in healthy and diseased pigs in Serbia*

Clinical status	No. of animals	No. (%) of STEC isolation	*P* value*
Pigs			
Healthy	856	51 (6)	
Diarrhoea	198	41 (20·7)	<0·000
Oedema disease	100	40 (40)	<0·000
Pigs (<2 months)			
Healthy	588	33 (5·6)	
Diarrhoea	165	31 (18·8)	<0·000
Oedema disease	70	20 (28·6)	<0·000
Pigs (>2 months)			
Healthy	304	21 (6·9)	
Diarrhoea	34	10 (29·4)	<0·000
Oedema disease	30	20 (66·7)	<0·000

* Compared to healthy animals.

Testing faecal samples of 202 sheep revealed the presence of STEC in 135 (66·8%) animals from all of the six farms examined.

Of all animal species involved in this study, the highest recovery rate was registered among goats; sampling on one occasion yielded 31 (73·8%) out of

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Table 3. *Phenotypic characteristics of Shiga toxin-producing* Escherichia coli (*STEC*) *isolated from domestic animals and food in Serbia*

Origin of STEC strains (no.)	Haemolysin production*		Adherence to HEp-2 cells	Sorbitol fermentation	Resistance to antibiotics	Aerobactin production
	Ent	Alpha				
Cattle (126)	93 (73·8)†	1 (0·8)	9 (7·1)	88 (69·8)	10 (7·9)	6 (4·8)
Pigs (135)	2 (1·5)	106 (78·5)	10 (7·4)	76 (56·3)	64 (47·4)	8 (5·9)
Sheep (135)	0	6 (4·4)	17 (12·6)	119 (88·1)	29 (21·5)	39 (28·9)
Goats (31)	0	0	7 (22·6)	28 (90·3)	28 (90·3)	9 (29)
Chicken (4)	0	1 (25)	0	0	1 (25)	2 (50)
Food (15)	1 (6·7)	1 (6·7)	4 (26·6)	12 (80)	2 (13·3)	3 (20)
Total (446)	96 (21·5)	115 (25·8)	47 (10·5)‡	323 (72·4)	134 (30)	67 (15)

* Ent, Enterohaemolysin; Alpha, α-haemolysin production.

† No. (%) of strains positive for indicated phenotypic characteristic.

‡ A total of 37, 7 and 3 strains displayed aggregative, localized, and diffuse adherence respectively.

42 animals from one herd that were positive for this pathogen.

STEC was found in only four (1%) chickens from four (4·3%) of the 92 examined farms.

The recovery rate of STEC in food was 1·6% for all samples tested, but this pathogen was significantly more frequently detected in raw minced meat – 4·2% (11/264) than in fresh cheese – 0·6% (4/649) ($P<0{\cdot}000$).

Haemolytic activity was registered in 211 (47·3%) of the 446 isolated STEC strains. Ninety-six (21·5%) of them produced enterohaemolysin and 115 (25·8%) were α-haemolysin producers. Most of these strains (95·7%) originated from cattle and swine faecal samples, but the frequency of detection of the two haemolysins was strikingly different between these two groups of strains; 93 (73·8%) out of 126 cattle-deriving strains exhibited enterohaemolysin activity, whereas 106 (78·5%) out of 135 swine strains were α-haemolysin producers. On the other hand, only one strain (0·8%) isolated from cattle produced α-haemolysin and only two (1·5%) swine strains were enterohaemolysin producers (Table 3). When comparing α-haemolysin production between STEC strains isolated from healthy and diseased pigs, this phenotypic trait was encountered significantly more often among strains isolated from diseased animals (92·6%) than among strains from healthy animals (57·4%) ($P<0{\cdot}000$). Of 40 STEC strains isolated from pigs with oedema disease, 39 (97·5%) produced α-haemolysin.

Adherence to HEp-2 tissue culture cells exhibited only 47 (10·5%) of the total number of isolated STEC strains. Among them, 37 displayed AA, seven expressed LA, and three exhibited DA. The rates of adherent strains varied from 0% (chicken isolates) to 26·7% (food isolates). Of all isolated STEC strains, 27·6% were sorbitol negative.

The total number of STEC strains resistant to one or more antimicrobial agents was 134 (30%); 103 (23·1%) isolates were resistant to one and 31 (6·9%) to two or more antibiotics. The most commonly encountered resistance among isolated STEC was to cephalosporin (13·2%), streptomycin (10·3%), and tetracycline (9·6%), less frequently to ampicillin and cotrimoxazole (8·3% for each), chloramphenicol (6·2%) and ciprofloxacin (0·5%), whereas resistance to gentamicin, amikacin and ceftriaxone was not registered.

Aerobactin production was detected in 67 (15%) of isolated STEC strains. This phenotypic trait was most commonly found among strains originating from goats and sheep (Table 3). Only five strains simultaneously expressed both systems for iron acquisition (aerobactin and one of haemolysins), of which two produced enterohaemolysin (one was isolated from cattle and one from food) and three produced α-haemolysin (all isolated from pigs).

PCR was carried out on 105 Stx-producing *E. coli* strains isolated from cattle, and revealed that all of them possessed one or more *stx* genes. This method demonstrated the presence of both *stx1* and *stx2v* genes in 31 (24·6%) strains, *stx2* and *stx2v* in 18 (14·3%), *stx1* in 15 (11·9%), *stx2* in 14 (11·1%), *stx1*, *stx2* and *stx2v* in 12 (9·5%), *stx1* and *stx2v* in 10 (7·9%), and *stx2v* in five (4%) strains.

With the available antisera for some of the typical EHEC serogroups (O26, O55, O111, O128 and O157)

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the serogroup of only 14 (3·1%) out of 446 isolated STEC strains was determined. Seven of them belonged to the O128 serogroup (six sheep strains, one goat strain), five to O157 and one each to the O55 (sheep strain) and O26 (cattle strain) serogroup.

All five O157 STEC strains were isolated from cattle and all possessed genes for Stx-I, Stx-II and Stx-IIv. Four of them produced enterohaemolysin, the same number for sorbitol negative, two were resistant to one antibiotic (streptomycin), and none adhered to HEp-2 cells, or produced aerobactin.

DISCUSSION

The results of this study indicate that STEC occurrence is widespread among domestic animals in this part of the world. STEC was found at 116 (28·9%) out of 402 farms and in 431 (16·2%) out of 2660 animals tested. Concerning ruminants as the most important reservoir of STEC, this pathogen was documented at 77 (42·3%) out of 182 farms and in 292 (27·3%) out of 1068 animals investigated, similar to the findings in Germany [17, 18]. Among cattle the prevalence was higher in older (heifers and cows) than in younger (calves) animals, both in this and a Japanese study [19]. On the other hand, some authors reported that calves were more frequently infected with STEC than adult cows [3, 20, 21]. The isolation of STEC in 6·1% of 1-day-old calves in this study and in 3·1% of calves with diarrhoea aged 1–7 days in a study in Spain [22] shows that infection with this pathogen may occur soon after birth. Marked difference in the recovery rates between cattle from the large-scale feedlot farm and from the small pasture farms observed in this study suggests a greater chance for grazing animals to be infected with STEC. In contrast to the seasonal peak of STEC isolation rate registered in the summer months [23], we found this pathogen in cattle more frequently during the colder period of the year.

Of the investigated domestic animals, the highest isolation rates of STEC in this study was documented in goats and sheep. These results are broadly similar to the findings in Germany [17, 18, 24], and Spain [25], and support the observation that STEC is better adapted to persist in the alimentary tract of sheep than other pathogenic *E. coli* [26].

Pigs were also noted as an important STEC reservoir for human infections [27]. In previous reports the prevalence of STEC was between 7·5% and 14% [18, 28], and the result of this study (11·3%) is within the reported rates. Similar to the results of other authors [28, 29], we detected this pathogen significantly more frequently in pigs with diarrhoea and with oedema disease than in healthy pigs (Table 2).

Judging by the results of this investigation, that are in accordance with the results obtained in Germany [18], chickens rarely excrete STEC.

Testing food of animal origin, we found STEC most frequently in raw minced meat, with the isolation rate similar to the result obtained in India [6]. The prevalence of this pathogen in dairy products was relatively low, both in this, and a study in France [23].

Another objective of this study was to characterize animal STEC for some of their virulence markers characteristic for human pathogenic strains (EHEC). In line with the results of others [17, 19, 30, 31] we found over 70% enterohaemolysin-producing bovine STEC isolates. On the other hand, this phenotypic trait was rarely registered among sheep and goat strains, in contrast to the findings in Germany [17], Spain [25], and Australia [32]. α-Haemolysin-producing STEC predominated among pig isolates, whereas enterohaemolysin production was rarely encountered in these strains. This finding is in accord with the results obtained in Germany [18], and Denmark [33]. Significantly more frequent finding of α-haemolytic strains in diseased animals support the observation that enhanced virulence of *E. coli* isolates from pigs with oedema disease is attributable to α-haemolysin [34].

Aerobactin production as an iron acquisition system was detected in 15% of STEC, mainly among sheep and goats strains (Table 3). Most of aerobactin-positive STEC strains in this study did not express haemolytic activity, and vice versa. Therefore, the finding that aerobactin and haemolysin are reciprocally found in human blood strains of *E. coli*, and that they are alternative mechanisms of iron acqusition [35] could be extended to STEC.

Adhesiveness *in vitro* was not a common feature among STEC strains in this study. The pattern of adherence most frequently found was AA, whereas LA was rarely encountered among isolated strains. This finding may be explained by the results of some authors that animal STEC strains rarely possess bundle-forming pili (BFP), and EPEC adherence factor (EAF) plasmid that mediate LA [19, 30], but others have described 81·1% of LA-positive strains islolated from calves with diarrhoea which were negative for *bfp* genes [36]. On the other hand, the

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study in Germany revealed that all adherent STEC strains expressed DA [30].

A substantial proportion (27·6%) of STEC strains in this, as well as in a study in Australia [32], were unable to ferment sorbitol, although most of them were not of the O157 serogroup for which this feature is characteristic.

Thirty per cent of STEC strains in this study were resistant to one or more antibiotics. In India, 49·2% of STEC strains were resistant to these drugs [6], and the study in France reported 4% of cattle isolates, 33% of food isolates, and 40% of child isolates resistant to antimicrobials [23].

Detection of genes characteristic of STEC in bovine strains by PCR using specific primers demonstrated that half of them possessed both *stx1* and *stx2* genes (including *stx2v*), 35·2% *stx2*, and 14·3% *stx1* genes. Broadly, similar findings were obtained in Germany [17], India [37], and Hong Kong [38], whereas in the studies in Germany [36] and Spain [22] cattle STEC strains which harboured only *stx1* genes predominated. Another group of investigators in Spain and Germany found strains possessing the *stx2* gene the most prevalent [20, 24, 39]. Finally, serogroups characteristic for EHEC strains, including O157, were rarely found among animal STEC isolates, in this, as well as in other studies [6, 17, 19–23, 30].

It may be concluded that domestic animals frequently harbour STEC, and that this pathogen is present in food of animal origin, notably in raw meat products, in this part of the world. On the other hand, the rarity of phenotypic traits typical for EHEC among animal and food isolates may explain the apparently low incidence of human STEC-associated disease in Serbia.

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