First molecular detection of shiga toxin-producing Escherichia coli *in dogs from serbia: a potential threat to human health?*

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Summary
Shiga toxin-producing *Escherichia coli* (STEC) are considered one of the most significant *E. coli* pathotypes transmitted by food, causing life-threatening conditions in children and elderly people. The aim of this study was to investigate the presence and determine the prevalence of STEC in dogs in Serbia by conventional PCR method, targeting three major virulence genes (*stx1*, *stx2*, and *eae*). The overall percentage of positive samples was 12.87% (13/101), with the *stx2* gene, the more potent of the two toxins, found in all the positive samples. The finding of *eae* gene in combination with *stx* genes (8/13) within the same genetic pool implies the potential presence of enterohemorrhagic *E. coli* or the potential emergence of these strains, considering an efficient mechanism of horizontal transfer of three major virulence genes. Our results also highlight dogs' lifestyle as a risk factor for STEC colonisation. These *E. coli* strains, according to our results, are more likely to be found in dogs living outdoors than those kept in house. Due to significant prevalence of STEC in dogs determined in this research and due to close contact between dogs and humans, dogs could be considered a source of human infections.

Introduction

Acquisition of pathogenicity genes from other species gave certain *Escherichia coli* strains the capacity to induce serious illnesses in both humans and animals. Of the nine (9) *E. coli* groups defined based on pathogenesis; Shiga toxin-producing *E. coli* (STEC) are considered the most significant group transmitted by food (Castro *et al*. 2017). Infections associated with STEC range from mild watery diarrhoea to life-threatening conditions, being the most common cause of acute renal failure in children and the elderly at the global level (Joseph *et al*. 2020). Although STEC mostly induce clinical diseases in humans and in piglets up to two weeks after weaning they can be isolated from wide range of animals including cattle, sheep, pigs, birds, dogs, rodents and even insects (Croxen *et al*. 2013; Kaper *et al*. 2004). The main characteristic and the major virulence factor of STEC is the production of one or both Shiga toxins, i.e., Stx1 and Stx2 and their subtypes (Marks *et al*. 2011). The possibility of some STEC strains to produce attaching and effacing lesions, and the virulence property derived from enteropathogenic *E. coli* (EPEC) strains, is seen as a virulence enhancer. This pathogenic effect is mediated in part by intimin, a protein encoded by the *eae* gene. Since the first multi-state outbreak in 1982 such strains, known as enterohaemorrhagic *E. coli* (EHEC), have been associated with serious disease outbreaks in developed countries (Beutin 1999; Kaper *et al*. 2004).

Sporadic human cases of haemolytic uremic syndrome (HUS) could be a result of STEC transmission from pets. The results of the research conducted by Bentacor *et al*. support this standpoint (Bentancor *et al*. 2012). The majority of the STEC

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strains isolated from dogs in one study showed high similarity of PFGE profiles (90-100%) with those isolated from meat, cattle and humans thus indicating their high zoonotic potential (Gomes *et al*. 2016).

In the Republic of Serbia, except for a limited number of studies on STEC in humans and domestic animals, there is no available data on this group of *E. coli* in dogs.Therefore, the aim of this study was to investigate the presence and determine the prevalence of STEC in dogs in Serbia by conventional PCR method targeting three major genes.

Materials and methods

Sample size

The sample size was calculated on the basis of anticipated STEC prevalence in dogs (7%), which was determined based on the results of other researchers, and the estimated population of owned and stray dogs in the city of Novi Sad (10.000), Serbia, using OpenEpi software (Dean *et al*. 2013). At 95% confidence level the obtained sample size was 100 individuals. Faecal samples from 101 dogs were collected from March to July 2017. The research included pets and stray dogs of different breed, sex, age, clinical status, and lifestyle. Regarding age, dogs were divided into six categories (puppy, junior, adult, mature, senior, geriatric) which correspond to the six stages of ageing, whilst regarding health status dogs were divided into two categories, those with diarrhoea and those without clinical symptoms (i.e., clinically healthy). When considering lifestyle as an epidemiological factor, three groups of dogs were formed as follows: indoor dogs (pet dogs kept in the house), outdoor dogs (pet dogs kept in the yard) and stray dogs.

Sample collection

Individual faecal samples were collected in sterile plastic containers, with sterile plastic spoon, immediately after defecation, avoiding environmental contamination. As the sampling method caused no discomfort nor stress, according to national legislation, ethical approval for this research was not needed. The owners' consent was obtained from all dog owners included in the study, while samples from stray dogs were collected with the permission of the management of the city dog shelter.

DNA extraction

Genomic DNA was extracted from canine faeces by using the commercial GeneMATRIX Stool DNA Purification Kit (EURx, Poland) according to the manufacturer's protocol.

Amplification of the virulence genes (i.e., *sxt1*, *stx2,* and *eae*), was done by conventional multiplex PCR on TC-412 Thermal Cycler (Techne, Stone, UK), following the conditions and with primers (Microsynth AG, Balgach, Switzerland) previously described by Fadel *et al*. (Fadel *et al*. 2017). PCR reaction mix of 25 µl volume contained 12.5 µl of Hot Start Taq 2x Master Mix (New England Biolabs, Beverly, MA, USA), 0.5 µl of each primer (20 µmol), 4.5 µl of sterile water for PCR work (Fisher Scientific, New Jersey, USA) and 5 µl of DNA template. Two non-template controls and one positive control containing DNA extract from *E. coli* O157:H7 strain ATCC 35150 were used. PCR products were separated on 1.5% agarose gels during 45 min on 100 V in 1x Trisborate-EDTA (TBE) buffer and visualised and recorded using ethidium bromide staining on Serva BlueCube 300 (SERVA Electrophoresis GmbH, Heidelberg, Germany).

The chi-square test or Fischer test and odds ratio, both with 95% confidence interval were calculated, when possible, in order to estimate the statistical significance of different epidemiological factors in regard to STEC harbouring. A p-value less than 0.05 (≤ 0.05) was considered statistically significant. Taking into account the uneven distribution of individuals within different dog breeds, it has not been determined if breed as a factor influenced STEC prevalence.

Results

Overall, 13 of the 101 faecal samples were positive for one or both Stx encoding genes, thereby making the STEC prevalence of 12.87% in the tested dog population. Of these 13 samples, 11 (10.89%) were positive for *stx2* only and 2 (1.98%) for both *stx1* and *stx2*. Furthermore, besides the presence of *stx* genes, 8 (7.92%) samples tested positive for the *eae* gene, indicating the possible presence of EHEC in these dogs (Table I).

Table I. *Virulence gene patterns of the STEC positive samples*

Sample number	Virulence genes		
	stx1	stx2	eae
24		+	+
40		+	+
48		+	+
52		+	
53		+	
54		+	
57		+	+
58		+	+
79	+	+	+
84		+	+
87		+	
88	+	+	
97		+	+
Σ	2	13	8

The prevalence of EPEC in the studied dog population was 31.68% as 32 samples were found to be positive for the gene only.

The summary of dogs' epidemiological data regarding the carrier status of *stx* genes is shown in Table II. No statistical significance was found between STEC harbouring and clinical stat\us, sex or age as *p*-values were 0.478, 0.483 and 0.648, respectively. However, the analysis showed that STEC are significantly more prevalent in dogs living outdoors (stray dogs and dogs kept in the yard) compared to dogs living indoors with a *p*-value of 0.015.

Table II. *Epidemiological data of dogs harboring stx genes*

Epidemiological factors	Clinical status		Sex		Age categories						Life style		
	healthy	diarrheic	♂	♀	I	II	III	IV	V	VI	house	yard	stray
Positive/Total	11/91	2/10	5/48	8/53	1/11	1/14	2/18	6/27	2/15	1/16	0/36	8/39	5/26

I = puppy; II = junior; III = adult; IV = mature; V = senior; VI = geriatric

Discussion

Several studies had in their focus the investigation of STEC presence in dogs, but due to differences in examined populations, aimed STEC strains, and sensitivity and specificity of the methods used in these studies it is difficult to compare obtained prevalence. In general, STEC prevalence ranges from 0% in three studies from Chile, Brazil and Argentina, up to 68.8% in dogs with diarrhoea, from Iraq (Galarce *et al*. 2019; Hasan *et al*. 2016; Nakazato *et al*. 2004; Zotta *et al*. 2015).

The lack of a standard protocol for diagnostics and just recent recognition of non-O157 STEC importance to public health left gaps in understanding the relationship between these STEC and human diseases (Taniuchi *et al*. 2012). By now it is clear that those STEC strains carrying *stx2* are linked to more severe diseases than those carrying sole *stx1*, as purified Stx2 is 1,000 times more toxic to human renal endothelial cells than Stx1 (Kaper *et al*. 2004). In our research, the gene encoding Stx2 was found in all the positive samples.

This finding points out the potential role of dogs as a source of human infection. Detection of the *eae* gene in combination with *stx* genes within the same genetic pool of individual dog microbiota implies the potential presence of EHEC or the potential emergence of these strains considering an efficient mechanism of horizontal transfer of these virulence genes (Geue *et al*. 2017).

A small number of studies that involved dogs had an aim to determine the relationship between different epidemiological factors and STEC harbouring. In the present study, such a link was not determined regarding age and sex, which agrees with previous reports (Bentancor *et al*. 2007; Coura *et al*. 2018). The same observation was reported by Younis et. al, but in a study based solely on the presence of the O157:H7 strain (Younis *et al*. 2015).

However, the results of our study suggest that STEC strains were significantly more prevalent in free-ranging dogs (stray dogs and dogs kept in the yard) than pet dogs kept in the house. These findings contrast other published data in which no link between dogs' lifestyle and STEC was noted (Salehi *et al*. 2011; Seepersadsingh and Adesiyun 2009). Interestingly, in a study conducted in the USA, no strains of this *E. coli* pathotype were isolated by Jay Russel *et al*. from the faeces of stray dogs (Jay-Russell *et al*. 2014).A possible explanation for the higher prevalence of STEC in free-ranging dogs, in the present study, lies in the fact that the diet certainly affects the composition of the intestinal microbiota and that these animals have no restriction of movement, making them more likely to contact with contaminated food and water as the main source of STEC.

The link between STEC strains and the occurrence of diarrhoea is well clarified in humans, piglets and calves (Hammermueller *et al*. 1995), however in dogs this question is still controversial.

In our study, we did not find statistically significant differences in STEC prevalence between diarrheic and clinically healthy dogs. It should be mentioned that the proportion of diarrheic samples in the total faecal sample (10/101) was small and there is a possibility it could influence the result. However, our result implies that these *E. coli* strains should not be considered a risk factor for the occurrence of diarrhoea in dogs, which agrees with most of the published results (Bentancor *et al*. 2007; Beutin 1999; Turk *et al*. 1998).

A 3-year epidemiological surveillance of the most significant STEC strains - O157:H7 in dogs in Japan showed that the occurrence of diarrhoea does not increase the probability of isolation of this strain (Kataoka *et al*. 2010). The same conclusion was reached by Ojo *et al*. They substantiated their observation with the results of an oral administration of human O157:H7 strains to dogs, where there was no evidence of intestinal colonisation, but viable bacteria were found in the faeces of these animals a few days after the administration (Kusunoki *et al*. 2004; Ojo *et al*. 2014). On the other hand, a few groups of researchers point out a positive correlation

between STEC and diarrhoea in dogs, and their conclusions are based on slightly higher prevalence rates of STEC in dogs with diarrhoea than in clinically healthy dogs (Hammermueller *et al*. 1995; Salehi *et al*. 2011; Staats *et al.* 2003).

In addition to the fact that certain strains of STEC can cause infections in animals, especially younger ones, this group of pathogenic *E. coli* is found in a higher percentage in asymptomatic individuals (Adamu *et al*. 2016; Daly and Hill 2016).

The presence of STEC in clinically healthy individuals in this study suggests that dogs may be asymptomatic carriers of STEC. Due to significant prevalence, dogs can be marked as a reservoir of STEC but also can serve as a vector for zoonotic transmission of those strains which are not able to colonize a dog's intestine.

Conclusions

This study brings the first molecular confirmation of the presence of STEC in a dog population from the Republic of Serbia. Due to close contact between dogs and humans, and the significant prevalence of STEC in dogs determined in this research, dogs could be considered a potential source of human infections. Furthermore, our results highlight the lifestyle of dogs as a risk factor for STEC colonisation. According to our results, these *E. coli* strains are more likely to be found in dogs living outdoors (yard and stray dogs) than those kept in house. In all the positive samples *stx2* gene was found, the most important of the two toxins. Further studies based on Shiga-toxins subtyping are needed to determine the clinical importance for humans of STEC strains harboured by dogs.

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