Faecal shedding of verotoxigenic *Escherichia coli* in cattle in the Czech Republic

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ABSTRACT: A survey to estimate the prevalence of verotoxigenic *E. coli* (VTEC) or enterohaemorrhagic *E. coli* (EHEC) in rectal swabs from healthy dairy cattle aged three weeks, three months and one year was conducted in three herds from the Czech Republic. Screening for the presence of the *stx1*, *stx2* and *eaeA* genes in faecal swab cultures was performed by PCR, and in positive samples, isolated colonies were examined. Immunomagnetic separation was used for the isolation of the VTEC serogroup O157 from samples. VTEC were detected in animals from all three herds under study. In the group of 3-week-old calves, VTEC were only detected in samples collected in the summer months. However, in the other age-groups, VTEC were detected in both the summer and winter months. EHEC shedding was observed in 30 to 100% of the total samples collected from cattle aged three months and one year in the summer months, and in 30 to 60% of samples taken in the winter months. EHEC strains of serogroup O157 were detected in two herds. The range of verotoxins shed by VTEC isolates of serogroup O157 differed between herds. Besides serogroup O157, additional EHEC belonging to the antigen groups O26, O103, O128 and O153 have been identified, and in some of them, no somatic antigen was detected.

Keywords: verotoxin; shiga-toxin; VTEC; EHEC; STEC

Verotoxin-producing *Escherichia coli* (VTEC) also known as shiga-toxin-producing *E. coli* (STEC) are food-borne pathogens, causing sporadic cases and outbreaks in humans. Serotype O157:H7 was previously thought to be most important (Riley et al., 1983; Paton and Paton, 1998) but in actual fact over 100 serotypes of VTEC have been isolated from humans (Johnson et al., 1996). The predominant VTEC serotype O157:H7 and other VTEC serotypes are generally regarded as the most important cause of haemorrhagic diarrhoea and haemolytic uremic syndrome (HUS) in the temperate climatic zone and as a significant cause of uncomplicated watery diarrhoea in different geographical areas (Karch et al., 1999). Even though the main emphasis is still put on serotype O157:H7, there are also other commonly implicated VTEC serogroups, including O26, O103, O111 and O145, which have potential as causative agents of HUS disease (Bielaszewska and Karch, 2000). Regarding typical features of the disease, VTEC-causing HUS are termed enterohaemorrhagic *E. coli* (EHEC). Today the EHEC include all *E. coli* strains, from humans and animals, producing Stx and attaching and effacing lesions, or harbouring the genetic information coding for them. However, this definition is not universally accepted as not all of these *E. coli* cause HC (haemorrhagic colitis) in humans. VTEC and STEC are the names for the strains producing only Stx, like the oedema disease associated *E. coli* in piglets (Mainil and Daube, 2005).

Cattle are considered to be the principal natural reservoir not only of VTEC O157:H7, but also of other serotypes (Orskov et al., 1987; Wells et al., 1991; Chapman et al., 1992). However, VTEC have also been isolated from other animal species, particularly from sheep, goats, horses, dogs, red deer, gulls and pigeons. The shedding of VTEC of serotypes other than O157:H7 is usually observed in sheep and goats (Beutin et al., 1993).

The main virulence factors identified for VTEC are verotoxins of groups VT1 and VT2 (Paton and Paton, 1998) and intimin, responsible for the at-

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taching and effacing effect required for intestinal colonization (Knuton, 1994). Intimin is known to be an important virulence factor which together with verotoxins is involved in the pathogenesis of human VTEC infection (Boerlin et al., 1999). Verotoxins are encoded on highly diverse lambdoid phages (O'Brien et al., 1984). The formation of attaching and effacing lesions is mediated by the *eae* gene (2.8 kb) that encodes an outer membrane protein, intimin (94 kDa), in both EPEC (Jerse et al., 1990) and VTEC (Yu and Kaper, 1992; Karmali, 2003), which is associated with the locus for enterocyte effacement (LEE) pathogenicity island (Nataro and Kaper, 1998).

There is a lot of information concerning the rates of VTEC shedding in animal faeces in different countries around the world. The situation in the Czech Republic is not very well known, however. VTEC O157:H7 detection in non-pasteurized goat milk as a source of infection for people was reported by Bielaszewska et al. (1997). Examination of calves on a farm in the South Moravia region revealed the presence of VTEC O157 in 20% of collected samples and, moreover, they were isolated from untreated slurry and trapped rats (Cizek et al., 1999). Data illustrating the occurrence of other VTEC serogroups in the Czech Republic are not available.

This study was undertaken to extend the knowledge regarding the occurrence of VTEC or EHEC in cattle from selected herds in the Czech Republic.

MATERIAL AND METHODS

The survey was conducted on three dairy farms in the Czech Republic. Three groups of healthy young cattle were included in the study: (1) calves younger than three weeks (n = 62), (2) cattle aged three months (n = 60) and (3) cattle aged 1 year (n = 60). All the animals were kept in a loose housing system. The first sampling was performed in the period between June and October, and the second between November and March. Rectal swabs were taken from animals, placed in Amies medium and transported to the laboratory for analysis. The collected samples were examined immediately after delivery to the laboratory.

Preliminary screening for VTEC in faeces

Swabs were inoculated onto Columbia blood agar and MacConkey agar. After a 16-hour culture at 37 °C, screening was performed for the genes encoding VT1, VT2 and the *eaeA* gene (Alexa et al., 2010). A culture grown on an area of 0.5 cm^2 on Columbia agar was harvested using a bacteriological loop, and suspended in 50 µl water. The suspension was warmed at 100 °C for 10 min and centrifuged. The supernatant was used as template in a PCR reaction. Using a multiplex PCR method as previously described (Paton and Paton, 1998) the presence of the *stx1*, *stx2* and *eaeA* genes was probed in the supernatant. As a positive control, the O157 *stx1, stx2, eaeA* positive *E. coli* strain 11785 was used, and as a negative control, a non-pathogenic *E. coli* strain was used. PCR positive samples were further examined; samples giving negative PCR results were excluded from further analyses. Up to 50 *E. coli* colonies were isolated from cultures of positive samples and multiplex PCR screening for the presence of the *stx1*, *stx2* and *eaeA* genes followed.

Immunomagnetic separation of VTEC O157

Rectal swabs were also cultured in mTSB-Broth with Novobiocin (Merck). After 16 h of culture in mTSB, immunomagnetic separation was performed using anti-*E.coli* O157 Dynabeads (Dynal) (EN ISO 16654:2001), and after washing, the bodies were inoculated onto Mac Conkey agar with sorbitol (SMAC), agar (Merck) and Fluorocult *E. coli* O157:H7-Agar (Merck). Sorbitol-negative suspect colonies were isolated and further analysed.

Identification of *E. coli* isolates by PCR

Isolates of *E. coli* were characterized for the presence of the *stx1*, *stx2* and *eaeA* genes, using the aforementioned multiplex PCR method. An isolated colony was suspended in 50 µl deionized water, warmed at 100 °C for 10 min and briefly centrifuged. For the reaction, Mastermix (Qiagen), primers and a temperature regime according to Paton and Paton (1998) were used. Electophoresis of the PCR product was performed on a 2% agarose gel. The gel was stained with ethidium bromide and visualized under UV illumination.

Serological typing

Somatic O-antigen typing was performed using a U-type microplate agglutination assay (Salajka

et al., 1992). *E. coli* strains were cultured in nutrient broth (Imuna, Slovak Republic). After culture, viability staining of *E. coli* strains was performed, using TTC (triphenyltetrazolium chloride); heating at 120 °C for 1 h followed. Agglutination was performed with a set of 70 types of O-antisera, encompassing the most common O-serogroups.

Detection of verotoxin production by a Vero cell line

E. coli strains were cultured in 5 ml of medium containing 12.5 g acid casein hydrolysate, 12.5 g enzymatic casein hydrolysate and 1 g yeast extract per 1 l medium at pH 7.5 for 16 h at 37 °C. After incubation cultures were lysed by adding polymyxin B (0.1 mg/ml). After 1 h the cultures were centrifuged at 10 000 × g for 15 min and supernatants were collected and used for examination on a Vero cell line.

Vero cells (density 3×10^5 per ml) were grown overnight in 96 well Nunclon plates in 100 µl/well MEM medium (Eagle) containing 10% bovine foetal serum. The above mentioned lysate of each tested *E. coli* strain (30 µl) was added to two wells of the cell monolayer. Plates were then incubated at 37 °C in an atmosphere of 5% CO_2. Results were evaluated after 72 hours. A cytopathic effect shown by more than 50% cells was considered as a positive result for verotoxin production.

RESULTS

VTEC were isolated from all PCR-positive faecal cultures with the exception of 3-month old animals in herds B and C. VTEC were isolated only from 57% and 63% of PCR-positive faecal cultures in this group, respectively. All isolates carrying a gene for verotoxin production were found to be verotoxigenic

Table 1. VTEC in PCR-positive rectal swabs collected from cattle in summer

Herd	Age of animals	Type of isolated *E. coli*				Type of isolates
		VTEC	EHEC	VTEC + EHEC	VT–, *eae*+	
		number of positive animals/tested				
A	3 weeks	3/10	3/10			O? VT1+, *eae*+
	3 months	9/10	3/10	1/10	4/10	O26 VT1+, *eae*– O? VT1+, *eae* + O54 VT2+, *eae*– O? VT1+, *eae*– O? VT–, *eae*+
	1 year	10/10	10/10			O157 VT2+, *eae*+ O103 VT1+, *eae*+ O? VT2+, *eae*+
B	3 weeks	1/10	1/10		1/10	O? VT2+, *eae*+ O? VT–, *eae*+
	3 months	4/10	3/10	1/10	5/10	O103 VT1+, *eae*+ O115 VT2+, *eae*+ O153 VT2+, *eae*– O? VT2+, *eae*– O115 VT–, *eae*+
	1 year	8/10	8/10			O157 VT1,VT2+, *eae*+ O? VT1+, VT2+, *eae*+
C	3 weeks	3/11	1/11			O? VT2+, *eae*+ O? VT1+, *eae*– O? VT2+, *eae*–
	3 months	5/10	5/10			O26 VT2+, *eae*+
	1 year	7/10	7/10			O? VT2+, *eae*+ O? VT1+, VT2+, *eae*+

Table 2. VTEC in PCR-positive rectal swabs collected from cattle in winter

Herd	Age of animals	Type of isolated *E. coli*				Type of isolates
		VTEC	EHEC	VTEC+ EHEC	VT–, *eae*+	
		number of positive animals/tested				
A	3 weeks	0/10				
	3 months	4/10	3/10		2/10	O103 VT1+, *eae*+ O128 VT1+, *eae*+ O26 VT–, *eae*+ O? VT–, *eae*+
	1 year	6/10	3/10		2/10	O157 VT2+, *eae*+ O? VT2+, *eae*+ O153 VT2+, *eae*- O? VT2+, *eae*- O156 VT–, *eae*+ O? VT–, *eae*+
B	3 weeks	0/11				
	3 months	7/10	5/10	2/10	1/10	O103 VT1+, *eae*+ O? VT1+, *eae*+ O125 VT2+, *eae*- O? VT–, *eae*+
	1 year	6/10	6/10		1/10	O103 VT1+, *eae*+ O128 VT1+, *eae*+ O? VT1+, *eae*+ O?

(VTEC) on Vero cells. Only isolates harbouring both a gene for verotoxin production and a gene for intimin (*eae*) production were designated as EHEC.

The detection rates of VTEC in rectal swabs from the animals examined in summer are shown in Table 1. The lowest detection rate of VTEC was obtained in the group of calves under three weeks old. All VTEC isolates from calves were defined as EHEC in herds A and B. Three calves were VTEC-positive in herd C, but only one of them was EHEC-positive. Among 3-month cattle EHEC were detected in 30% of animals in herd A, in 30% in herd B and in 50% in herd C. VTEC shedding by 1-year-old animals was observed in all herds and all the isolates were characterized as EHEC. EHEC shedding was observed in 70 to 100% of animals.

The results of examination of rectal swabs collected from animals in winter are presented in Table 2. No VTEC were detected in any group of calves under three weeks old. EHEC were isolated from all PCR-positive samples of faecal cultures from herd A, with the exception of one sample collected from a yearling. EHEC were identified in 30% of animals aged three months and one year. In herd B, EHEC were found in 50 and 60% of animals aged three months and one year, respectively.

EHEC serogroup O157 was isolated only from herds A and B in the summer and winter months, respectively, namely in groups of 1-year-old animals. Strains of this serogroup differed in the variety of produced verotoxins. Both the range of serogroups of the isolated VTEC and the range of isolates characterized as EHEC were wide. In herd A, isolates of serogroup O157, O103, O128 and O54, were detected in 58.8%, 17.6%, 17.6% and 11.8% of EHEC, respectively. In herd B, isolates of serogroup O103 and O157 were detected in 46.7% and 40.0% of EHEC, respectively. In herd C, 38.5% of EHEC were identified as O26 and the remaining isolates were of serogroups outside the range of the used antisera.

All O157 and O103 isolates were both *stx* and *eae* positive. Out of the serogroup O26 isolates, 71.4% were *stx* and *eae* positive.

DISCUSSION

The results obtained in this study provide evidence of a rather high prevalence of EHEC in cattle faeces, particularly from yearlings. VTEC were isolated from the majority of PCR-positive samples as detected in faecal cultures, with the exception

of 3-year-old cattle in herds B and C. Analysis of primary cultures aided in the identification of suspect faecal samples, but it was necessary to subject the isolates to further examination. The positivity of some samples was caused by a mixture of (1) *E. coli* strains, carrying the genes for verotoxin production, except for the *eae* gene, and (2) *E. coli* strains carrying the *eae* gene, but not genes for verotoxin production. Our data showed that the rates of VTEC shedding in faeces were lowest in the group of youngest animals, i.e., less than three weeks old. According to some authors, lower VTEC shedding appears to be a charactersitic of young calves in contrast to older cattle (Zhao et al., 1995), whilst others have reported the highest shedding in the youngest categories, which gradually declined with increasing age (Shaw et al., 2004). However, in the latter study, animals up to the age of two months were included in the youngest category.

A wide range of VTEC serotypes were detected in the herds under investigation. The serotypes differed between respective herds and between different age categories of cattle. The question arises as to what is the role of different serogroups in food contamination. Serougroups O26, O91, O103, O111, O113, O121, O145 and O157 are known to cause HUS disease in humans (Karmali et al., 2010). Among these, serogroup O157 is predominant, particularly serotype O157:H7. EHEC isolates of this serogroup are mostly sorbitol-negative and can be identified by culture in sorbitol-Mac Conkey agar (March and Ratnam, 1986), primarily after immunomagnetic separation (Wray et al., 1993). Due to this fact, most data are available for serogroup O157. The other EHEC serogroups, but also some O157, are sorbitol-positive. Their identification, particularly in animal faeces, requires high numbers of isolates.

The prevalence of VTEC O157 in cattle was represented by 10.2% of the total faecal samples collected in up to 96% of feedlots (Sargeant et al., 2003), and 13% in rectal swabs from beef bulls (Low et al., 2005) in North America. Zhao et al. (1995) reported the finding of VTEC on 50% of dairy cattle farms, in 5.3% of cattle aged two to four months and in 2.9% of animals under the age of two months. In Australia, Fegan et al. (2004) detected O157 in 10% of faecal samples from grazing cattle and in 15% of faecal samples from feedlot cattle. In The Netherlands, 0–61% of dairy cattle carried O157 VTEC. The highest shedding prevalence was found among cattle aged three to 12 months (Heuvelink et al., 1998). In Scotland, 6.5% of cattle aged 12 to 30 months had O157 in faeces, and 22.7% of farms were positive for O157 (Gunn et al., 2007). In Italy, VTEC O157 were detected in faeces from 42% (Albonetti et al., 2004) and 24% of slaughtered cattle (Alonso et al., 2007). Oporto et al. (2008) detected VTEC O157:H7 and non-O157 in 3.8% and 35.9% of cattle herds, respectively. Data from Norway showed the finding of VTEC O157 in up to 75% of all animals examined (Urdahl et al., 2003). Cizek et al. (2008) isolated VTEC O157 from 2% of milk filters on 192 dairy farms in the Czech Republic. Even though VTEC were detected in all herds examined in the present study, VTEC O157 predominated in herd A only. In herd C, no VTEC O157 were detected. However, in herds B and C, VTEC O103 and O26 prevailed, respectively. Pearce et al. (2006) investigated the prevalence of serogroups O26, O103, O111 and O145 in cattle from Scotland and reported the isolation of O26, O103 and O145 on 23%, 22% and 10% of farms, respectively. In non-O157 isolates, genes for VT production, together with *eae* (EHEC) were found in only 4.4% of isolates. Out of serogroup O26, 28.9% of isolates were positive for both *stx* and *eae*, in serogroups O103 and O145, *stx* and *eae* positivities were very low. In contrast to this data, positivity for *eae* in VTEC serogroups O103 and O26 in our isolates was high (100 and 71.4%, respectively).

Our study revealed a high prevalence of virulence factor genes in the serogroup O157 strains, all isolates of which were positive for both *stx* and *aea* genes. Karama et al. (2008) isolated VTEC from 33% of cattle. These belonged to 24 serotypes, apart from O157:H7. However, only two (O153:H19 and O103:H2) of these isolates harboured the *eae* gene. Zweifel et al. (2004) screened for VTEC serotypes in sheep from Switzerland. Most of the sorbitol positive non-O157 isolates belonged to the serogroups O87, O91, O103, O128 and O176. Shaw et al. (2004) found VTEC O26 (particularly *stx1*, *eae*) in very young calves only, and their rates decreased with advancing age. In our study, VTEC O26 was only detected in one herd of 3-month-old cattle. In Poland, Osek (2004) identified VTEC serogroup O157 in 7.6% of isolates from calves aged 3 to 5-months; 46.7% of the isolates carried the *eae* gene.

Demonstrating the prevalence of EHEC in cattle faeces during a survey of feedlot cattle in the summer and winter seasons, the present study provides evidence of the serious and topical nature of this issue in the Czech Republic. The results show the importance of EHEC monitoring in accordance with the guidelines (EFSA 2009).

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