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SHIGATOXIGENIC *ESCHERICHIA COLI* SEROGROUPS O157, O111 AND O113 IN FECES, WATER AND MILK SAMPLES FROM DAIRY FARMS

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ABSTRACT

The objective of this study was to determine the prevalence of Shigatoxigenic *Escherichia coli* (STEC) and STEC serogroups O157, O111 and O113 in feces, water and milk sampled in dairy farms in Jaboticabal, SP, Brazil. Feces (n=454), water (n=54) and milk samples (n=30) were collected from 10 herds and assessed for the presence of the virulence genes stx_1, stx_2 and *eae* by polymerase chain reaction (PCR). All *stx* and *eae* positive samples were submitted to a second PCR reaction targeting the sequences *rfb* O157, *rfb* O111 and *rfb* O113. High prevalence of *stx* was detected (59.9%) in fecal samples, whereas the prevalence of sequences *rfb* O157, *rfb* O111 and *rfb* O113 was 18.9%, 3.3% and 30.4%, respectively. All sequences were detected more frequently in calves and heifers. Sequences stx_2 and *eae* were prevalent in the fecal samples. *stx* sequences were detected in 1.9% and 3.3% of water and milk samples, respectively. Low prevalence of *E. coli* O113 was observed in water samples, whereas no *E. coli* O157 or *E. coli* O111 was detected. Furthermore, none of the serogroups were identified in milk samples. STEC was identified in all herds (100%), and serogroups O157, O111 and O113 were observed in 40%, 50% and 90% of the herds, respectively. In conclusion, the high STEC prevalence detected in dairy herds evidences that bovine feces might play an important role as a contamination source in the region of Jaboticabal, Brazil.

Key words: Shigatoxigenic, *Escherichia coli*, milk, water, bovine feces

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* (EHEC) are human pathogens that belong to the Shigatoxigenic *E. coli* (STEC) group. They may cause several diseases, ranging from hemorrhagic colitis to more serious syndromes that may result in death of children and the elderly, such as hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (28,31,47). STEC transmission is often related to the consumption of contaminated food, uncooked beef, water and raw milk (19).

The morbidity and mortality rates associated with many outbreaks of gastrointestinal diseases caused by Shigatoxigenic *Escherichia coli* have highlighted the threat that these organisms pose to public health (35). STEC pathogenicity is mainly attributed to the expression of genes related to the production of cytotoxins (stx_1 and stx_2) and adherence factors (*eae*) (1). Although more than 200 *E. coli* serotypes produce shigatoxins, only a limited but increasing number is considered pathogenic to humans. Serotype O157:H7 is still the predominant serotype among enterohaemorragic *Escherichia coli*, and the serotype most frequently associated with foodborne outbreaks (47).

Dairy cattle have been regarded as the primary host of STEC (3,20,25,42,48). Additionally, calves and heifers have been considered the age group most susceptible to serotype O157:H7 and other STEC (19,20,48). Although isolation have been reported in calves with diarrhea (3,33), these microorganisms

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are frequently recovered from healthy animals (32,48). Since infected animals usually show no clinical signs, the control of disease transmission cannot be easily achieved (16).

STEC strains that are pathogenic to humans have been shown to belong to a broad range of O serogroups. Nevertheless, it seems that especially enterohaemorragic *Escherichia coli* O157 and O111, and more recently also O113, are responsible for the majority of severe cases (27,38).

The present study was carried out to provide additional information about the prevalence and epidemiology of Shigatoxigenic *E. coli* (STEC) in dairy farms.

MATERIALS AND METHODS

A simple random sample was selected by drawing ten dairy herds in the region of Jaboticabal, State of São Paulo, Brazil. Fecal samples from all animals (454), water samples (54) and milk samples (30) were collected between January and March 2002. The prevalence of STEC was evaluated in the 538 samples by polymerase chain reaction (PCR). Afterwards, the prevalence of serogroups O157, O111 and O113 was analyzed by PCR in the STEC-positive samples. The results of fecal samples were analyzed considering three age groups: calves (< 4 months); heifers (from 5 months to 2 years); and cows (> 2 years).

Sampling

Water samples

Samples were collected in six different locations within each dairy farm: water source (shallow well, spring or deep well), drinking water consumed by workers, water used in the dairy stable, drinkers for cows, drinkers for heifers and drinkers for calves.

Fecal samples

Rectal swabs were collected from every animal with or without diarrhea, and transported in Cary-Blair transport medium (Copan, Italy).

Milk samples

Samples were collected directly from the refrigerated milk tank through the faucet. Sampling was performed at the beginning, in the middle and at the end of the milking process. Samples were taken to the laboratory under refrigeration and processed immediately.

Polymerase Chain Reaction (PCR)

Sample preparation

Feces: Samples were streaked onto cystine lactose electrolyte deficient agar (CLED - Difco, France) and incubated at 37ºC for 18-24h to form a bacterial lawn (confluent growth). Polymicrobial growth was collected in approximately 2 mL of phosphate buffered saline (PBS; pH 7.4) and 100 µL were diluted using sterile distilled water (1:10). Total DNA was extracted from the diluted bacterial suspension by boiling for 10 min. Bacterial glycerol stocks were prepared using two aliquots of the bacterial suspension (0.5 mL) diluted with 0.5 mL of 2X tryptic soy broth (TSB - Oxoid, Basingstone) containing 20% glycerol and stored at -70ºC (10).

Water: Sources, human consumption and dairy stable – 100-mL aliquots of the samples were filtered through a sterile membrane (0.45 µm, Millipore) and poured into flasks containing 50 mL TSB. After incubation at 37ºC for 18-24h, the samples were streaked onto cystine lactose electrolyte deficient agar. Further procedures were similar to those used with fecal samples. Animal drinkers - Thirty milliliters of each water sample were transferred to a sterilized flask containing 30 mL of 2X TSB, homogenized and incubated overnight at 37ºC (22). Samples were streaked onto cystine lactose electrolyte deficient agar and further procedures were similar to those described for fecal samples.

Milk: Samples (30 mL) were transferred to a sterile flask containing 30 mL of 2X TSB, homogenized and incubated overnight at 37ºC. Aliquots (1 mL) were taken and centrifuged (12). The supernatant was discarded and the pellet was streaked onto cystine lactose electrolyte deficient agar. Further steps were as described above for fecal samples.

Multiplex PCR for stx_1, stx_2 and eae

The detection of stx_1, stx_2 and *eae* was performed by a multiplex PCR previously described (11). The following primer sets were used to detect *eae*, stx_1 and stx_2 sequences of shigatoxigenic *E. coli*: ep1 and ep2, stx1R and stx1F, stx2R and stx2F (BioSynthesis, United States):

ep1 - 5' AGG CTT CGT CAC AGT TG 3'
ep2 - 5' CCA TCG TCA CCA GAG GA 3'
stx1R - 5' AGA GCG ATG TTA CGG TTTG 3'
stx1F - 5' TTG CCC CCA GAG TGG ATG 3'
stx2R - 5' TGG GTT TTT CTT CGG TATC 3'
stx2F - 5' GAC ATT CTG GTT GAC TCT CTT 3'

The PCR mixture (20µL) was prepared using 2.0 µL of 10X PCR buffer (Gibco BRL), 0.6 µL of 1.5 mM $MgCl_2$ (Gibco BRL), 0.4 µL of 2 mM dNTP (Gibco BRL), 0.1 µL (250 mM) of each primer, 0.2 µL of Taq DNA polymerase - (1U Gibco BRL), 11.2 µL of sterile water and 5 µL of template DNA. Amplifications were carried out in a thermal cycler (MJ Reserch, Inc) according to the following conditions: initial denaturation at 94ºC for 5', 30 cycles at 94ºC for 30'', 52ºC for 30'', 72ºC for 1'30''. Controls were included in every batch of samples. A negative control without DNA was used, as well as control reactions including DNA extracted from Stx-negative *E. coli* $DH5_\alpha$ (K12), Stx-positive STEC strain E40705 – stx_1 and *eae* (CPHL, London)

and Stx-positive STEC strain E30138 – stx_2 and *eae*. Amplified products were visualized by electrophoresis in 1.0% agarose gel stained with ethidium bromide.

Multiplex PCR for rfb O157, O113 and O111

All *stx*- and *eae*-positive samples were submitted to a second multiplex PCR according to the procedure described by Paton and Paton (36), using the primers described below (BioSynthesis, United States):

O157 F - 5' CGG ACA TCC ATG TGA TAT GG 3'
O157 R - 5' TTG CCT ATG TAC AGC TAA TCC 3'
O113 F - 5' AGC GTT TCT GAC ATA TGG AGTG 3'
O113 R - 5' GTG TTA GTA TCA AAA GAG GCT CC 3'
O111 F - 5' TAG AGA AAT TAT CAA GTT AGT TCC 3'
O111 R. - 5' ATA GTT ATG AAC ATC TTG TTT AGC 3'

PCR mixtures (20µL) were prepared using 2.0 µL of 10X PCR buffer (Gibco BRL), 0.6 µL of 1.5 mM $MgCl_2$ (Gibco BRL), 0.4 µL of 2 mM dNTP, 0.1 µL (250 mM) of each primer, 0.2 µL of Taq DNA polymerase (1U), 11 µL of sterile water and 5 µL of template DNA. Amplification was carried out using a thermal cycler (MJ Research, Inc) under the following conditions: 10 cycles at 95ºC for 1', 65ºC for 2', 72ºC for 1'30'', 15 cycles at 95ºC for 1', 60ºC for 2', 72ºC for 1'30'', 10 cycles at 95ºC for 1', 60ºC for 2', 72ºC for 2'30''. Control reactions were included in every batch of samples: negative control without DNA, Stx-negative *E. coli* $DH5_\alpha$ (K12), Stx-positive STEC strains E40705 or E30138 (O157), *E. coli* O113:H21 (O113) and EPEC strain B171 (O111). Amplified products were visualized by electrophoresis in 1.0% agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

There was a high prevalence (59.9%) of *stx* sequences in feces from dairy cattle (Table 1). STEC prevalence data of previous studies in dairy farms are highly variable. In the USA, Wells *et al.* (48) reported that STEC was detected in 19% of feces from calves and 8% of feces from adult cows. In Canada, a herd prevalence of 45% was reported in 1992 (26). In Argentina, Sanz *et al.* (43) found a prevalence of 44% in cows. Busato *et al.* (7), in Switzerland, detected STEC in 44.3% of the animals and Cerqueira *et al.* (10) found prevalence as high as 82% in some herds of the State of Rio de Janeiro, Brazil

The prevalence of serogroup O157 was 18.9% in feces (Table 1). This value is higher than the findings presented by Hancock *et al*. (21) and Vold *et al.* (47), who reported prevalence of 1.0%. The frequencies of *stx* (59.9%) and *rfb* O157 (18.9%) detected in the present study were relatively high. Nevertheless, data on prevalence of STEC and *E. coli* O157 in cattle are difficult to be compared due to differences in the procedures and detection methods that are used.

Table 1. PCR detection (%) of sequences *stx*, *rfb* O157, *rfb* O111 and *rfb* O113 of *Escherichia coli* in feces, water and milk samples. Jaboticabal, SP, Brazil, 2002

	stx (STEC)	*rfb* O157	*rfb* O111	*rfb* O113
Feces	59.9%	18.9%	3.3%	30.4%
Water	1.9%	0%	0%	1.9%
Milk	3.3%	0%	0%	0%

Only 3.3% of animals were shown to carry *rfb* O111-positive *E. coli* (Table 1). Although this prevalence is relatively low, it should be pointed out that serogroup O111 has been involved in most non-O157 STEC infections (2). Besides, STEC O111 has been reported as responsible for outbreaks (6,8,9,23,37) and also sporadic cases (15,18,19,46).

STEC strains carrying the *eae* gene are considered more virulent for humans than strains lacking *eae* (1). However, it has been reported that *eae*-negative *E. coli* O113 strains are capable of colonizing the human gastrointestinal tract and cause hemolytic uremic syndrome (38). It should be noted that high prevalence (30.4%) of *E. coli* carrying O113 *rfb* sequences was seen in the present study (Fig. 1). Therefore, measures must be taken in order to reduce the risk of environmental contamination and transmission to humans.

It is known that water is an important means of spreading STEC between animals and humans. From 54 analyzed water samples, 1.9% presented *stx* and 1.9% presented the sequence *rfb* O113, but in no samples *rfb* O157 or O111 sequences were detected (Table 1). Although STEC prevalence in water samples was low, the importance of water as a vehicle of disease transmission cannot be disregarded. Many outbreaks of gastrointestinal diseases have been reported and evidences indicate the importance of water in STEC epidemiology (14,17,25,30,45). Moreover, the etiological agent is not recovered in most gastrointestinal disease outbreaks involving water (41). Therefore, any required and possible measures should be taken in order to prevent water contamination.

Dairy cattle are considered natural hosts of STEC O157 strains that are pathogenic for humans (24). Milk and meat products, which are both produced by dairy herds, are responsible for the majority of the foodborne outbreaks caused by these microorganisms (44). Therefore, although *rfb* O157, O111 or O113 sequences have not been detected in milk samples in the present study, the detection of *E. coli* carrying *stx* sequences in 3.3% of milk samples may pose a risk to public health (Table 1).

Higher prevalence of *stx*, *rfb* O157 and *rfb* O111 was observed in calves and heifers compared to cows (Table 2). These results are in agreement with findings described by Wells *et al.* (48), Wilson *et al*. (50), Zhao *et al*. (51), Rahn *et al*. (40)

and Busato *et al*. (7). The reasons for such difference in prevalence between age groups are still unknown, but the variations might reflect differences in ruminal development, diet and resistance to infections, among other factors (48). Notwithstanding, it is also necessary to control STEC infection in older animals, since most ground beef and dairy products are derived from adult animals (13).

Table 2. PCR detection (%) of sequences *stx*, *rfb* O157, *rfb* O111 and *rfb* O113 of *Escherichia coli* in fecal samples according to age. Jaboticabal, SP, Brazil, 2002.

Age Group	*stx* (STEC)	*rfb* O157	*rfb* O111	*rfb* O113
Calves (n=100)	57.0%	39.0%	9.0%	4.0%
Heifers (n=209)	69.9%	21.5%	1.4%	37.8%
Cows (n=145)	47.6%	1.4%	2.0%	13.8%

In the present study, the prevalence of *eae* and stx_2 sequences was higher in fecal samples, 45.8% and 31.5%, respectively (Fig. 1). These findings agree with data reported by Blanco *et al*. (5), Miyao *et al*. (31), Sanz *et al.* (43), Vold *et al.* (47) and Cerqueira *et al*. (10). The importance of these data lies on the fact that *eae*-positive STEC strains are considered more virulent for humans than *eae*-negative STEC strains, as well as strains carrying only stx_2 genes (1,34,49). According to Blanco *et al.* (4), Stx_2-producing strains of STEC are part of the normal intestinal flora in cattle, which is probably the reason why stx_2 sequence was detected more frequently.

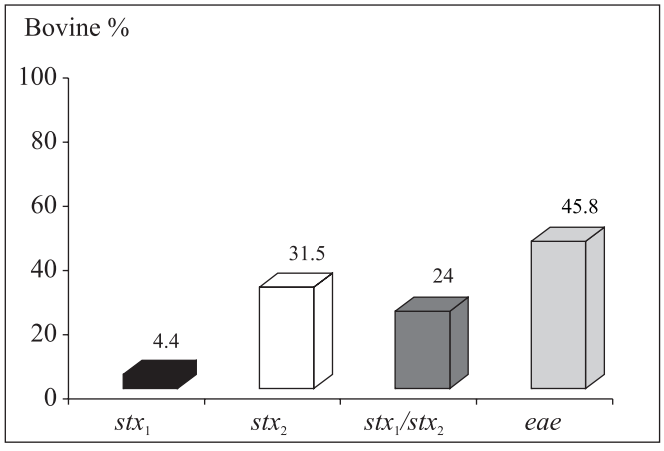

Figure 1. Percentage of animals that presented stx_1, stx_2, stx_1/stx_2 and *eae* sequences of *Escherichia coli* in fecal samples. Jaboticabal, SP, Brazil, 2002.

Moreover, stx_1 and stx_2 sequences are located on mobile genetic elements and might have different mobility patterns. Therefore, the transference of stx_2 from non-pathogenic to pathogenic serotypes may happen more frequently (47).

The percentage of dairy farms in which *stx* and *rfb* O113 sequences of *E. coli* were detected in fecal samples was very high, 100% and 90%, respectively. The sequences *rfb* O157 and O111 were also observed in reasonably high percentages, 40% and 50% (Fig. 2). These results give evidences that the four groups of bacteria are widely spread among animals in dairy farms in Jaboticabal, SP.

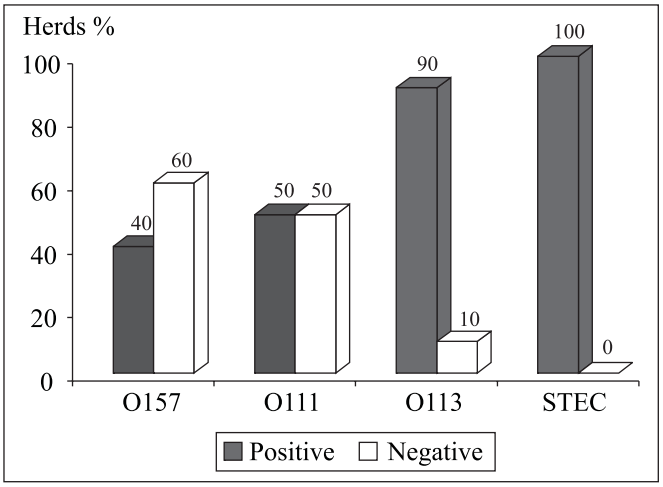

Figure 2. Percentage of herds (%) in which *Escherichia coli* carrying the sequences *stx* (STEC) and *rfb* O157, O111 and O113 were detected in fecal samples. Jaboticabal, SP, Brazil, 2002.

Efforts should be focused on infection control in order to reduce the prevalence of such microorganisms (40). According to Kudva *et al*. (28), *Escherichia coli* O157 may survive at least six weeks in feces and possibly multiply in this material. Serotype O157:H7 was isolated more frequently from environmental samples collected next to manure deposits (39). Furthermore, higher STEC and O157 prevalence may be seen in herds that graze in paddocks treated with manure (20, 29). Therefore, adequate manure management is one of the methods that prevent bovine feces from becoming a source of environmental contamination with shigatoxigenic *Escherichia coli*.

Besides appropriate manure management, there are other measures to be taken in order to prevent the spread of STEC. These include stress prevention, controlling food and water quality, as well as feedlot conditions and contact between adult and young animals (40).

In conclusion, this study showed high prevalence of Shigatoxigenic *E. coli* in dairy cattle, especially in calves and

heifers. Besides, STEC is widely spread in dairy farms in Jaboticabal, SP, Brazil. It is suggested that dairy cattle are important reservoirs of *E. coli* serogroups O157, O111, O113 and other STEC, and are an important source of environmental contamination, through microorganism shedding in the feces.

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RESUMO

Escherichia coli Shigatoxigênicas sorogrupos O157, O111 e O113 detectadas em fezes de bovinos, água e leite de propriedades leiteiras

Este trabalho teve como objetivo determinar a prevalência de *Escherichia coli* Shigatoxigênicas dos sorogrupos O157, O111 e O113 em bovinos leiteiros, água e leite de propriedades rurais do Município de Jaboticabal/SP. Para isso, foram colhidas 454 amostras de fezes, 54 amostras de água e 30 amostras de leite e pesquisada a presença de seqüências *stx*$_1$, *stx*$_2$ e *eae* pela reação em cadeia da polimerase (PCR). Todas as amostras *stx* e/ *eae* positivas foram submetidas a uma nova reação de PCR para detecção das seqüências *rfb* O157, *rfb* O111 e *rfb* O113. Uma alta ocorrência (59,9%) de *stx* foi detectada nas fezes dos bovinos. Os coeficientes de prevalência das seqüências *rfb* O157, O111 e O113 nas fezes dos bovinos foram, respectivamente, 18,9%, 3,3% e 30,4%. Todas as seqüências foram encontradas com maior freqüência em bezerros e novilhas. As seqüências *eae* e *stx*$_2$ foram as mais detectadas entre as amostras de fezes. Foram detectados 1,9% e 3,3% de seqüências *stx* na água e no leite, respectivamente. Nas amostras de água observou-se uma baixa prevalência de O113 e não foram detectadas O157 e O111. Nenhum dos sorogrupos pesquisados foi encontrado nas amostras de leite. STEC foram identificadas em todos os rebanhos (100%) e os sorogrupos O157, O111 e O113 foram observados em 40,0%, 50,0% e 90,0% dos rebanhos. Conclui-se que a prevalência de STEC em fezes de bovino, no Município de Jaboticabal/SP é alta, caracterizando-a como importante via de contaminação ambiental para esses microrganismos.

Palavras-chave: Shigatoxigênica, *Escherichia coli*, leite, água, fezes de bovinos

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