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Occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in bovine feces, feed, water, raw milk, pasteurized milk, Minas Frescal cheese and ground beef samples collected in Minas Gerais, Brazil

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Abstract

This study aimed to evaluate the occurrence of Shiga toxin-producing *Escherichia coli* (STEC) in feces samples, water, feed, raw milk, pasteurized milk, Minas Frescal cheese, and ground beef. Seventy samples (40 of feces, 10 of raw milk, 5 of pasteurized milk, 5 of ground beef, 4 of Minas Frescal cheese, 4 of water, and 2 of feed) were collected in two farms of dairy cattle and two farms of beef cattle and in commercial establishments. The samples were processed according to standard methodologies using biochemical methods and molecular and biological assays. Among 209 identified bacterial isolates as *E. coli*, 120, 70, 7, 7, 3, 2, and zero were obtained from samples of feces, water, feed, ground beef, pasteurized milk, Minas Frescal cheese, and raw milk, respectively. An isolate from water sample coming from a beef cattle farm was identified as *E. coli* STEC. This isolate showed cytotoxic effect in Vero cells and the *EhlyA* gene encoding entero-hemolysin. Therefore it is justified to develop studies to limit the spread of this pathogen.

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Introduction

Shiga toxin-producing *Escherichia coli* (STEC) also called verotoxigenic (VTEC) are an important group of emerging enteric pathogens associated with light to severe and bloody diarrhea (Paton and Paton, 1998; Jenkins *et al*., 2003; Bettelheim *et al*., 2005; Cho *et al*., 2006; Jure *et al*., 2010). In some individuals, STEC infection can progress to hemolytic uremic syndrome (HUS), a sequel characterized by renal failure with possible involvement of the central nervous system (Karmali, 2005; Bosilevac and Koohmaraie, 2011).

Cattle are considered the main reservoir for STEC (Kaper *et al*., 2004; Cho *et al*., 2006) with high occurrence in their feces in Brazil, although these strains have also been isolated from other animals such as sheep, goats, dogs, cats, and pigs (Cerqueira *et al*., 1999; Leomil *et al*., 2003, Moreira *et al*., 2003; Vettorato *et al*., 2003; Irino *et al*., 2005; Farah *et al*., 2007; Timm *et al*., 2007; Oliveira *et al*., 2008; Bentancor *et al*., 2010).

In developed countries, O157:H7 serotype is the leading cause of disease in humans. However, the number of reports of non-O157 STEC strains associated with gastrointestinal infections has increased (Silva *et al*., 2001; Yoon and Hovde, 2008). Infections caused by STEC strains are associated with food consumption, contaminated water, and direct contact with the faeces of infected animals (Bergamini *et al*., 2007).

Shiga toxins are the major STEC virulence factors, there are Stx1 and Stx2 that have been associated with the severity of human's infections (Nataro and Kaper, 1998; Stevens *et al*., 2002; Madic *et al*., 2011). Many works with *E. coli* STEC strains have been carried out especially in relation to the various virulence factors (Cergolle-Novella *et al*., 2006; Vaz *et al*., 2006; Cergolle-Novella *et al*., 2007; Aidar-Ugrinovich *et al*., 2007). Thus, this study aimed to determine the occurrence of STEC from feces of dairy and beef cattle, water and feed for animals, milk and dairy products, and ground beef.

Materials and Methods

Samples collection

The 70 samples (40 of feces, 10 of raw milk,

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five of pasteurized milk, five of ground beef, four of Minas Frescal cheese, four of water and two of feed) evaluated in this study were from farms or stores from Rio Pomba, MG, Brazil. They were aseptically collected and transported under refrigeration (4°C) without previous inoculation in culture medium to the Laboratory of Microbiology of the Federal Institute of Education, Science and Technology of Southeast of Minas Gerais. The collection of feces and water samples was performed in two farms of dairy cattle and in two farms of beef cattle, being ten feces samples (200 g) from different healthy animals from each farm and a 100 mL of water sample used in animal feed from each farm collected.

Feed (200 g) and raw milk samples (250 mL) were also aseptically collected on each one of two farms of dairy cattle, been collected one feed sample of each farm and five samples of raw milk from different healthy animals in lactation from each farm. In addition, five samples of pasteurized milk (1 L), four samples of Minas Frescal cheese (1 Kg), and five samples of ground beef (500 g) from different sources were collected from stores of Rio Pomba.

Sample preparation and E. coli isolation

The feces samples were subjected to stool culture. For this, 1 g of feces was transferred to 10 mL of modified EC broth (mEC, EMD Millipore, USA) and was incubated at 37°C for 24 hours. The enriched samples were then diluted in 1% buffered peptone water (Difco Laboratories, USA) and 100 µL aliquots of the last three dilutions (10^{-5}, 10^{-6}, and 10^{-7}) were inoculated onto Sorbitol MacConkey Agar (Difco, USA), using the spread plate technique. The plates were incubated at 37°C for 18-24 hours. Five fermenting colonies (pink-red) and five non-fermenting sorbitol (colorless, irregular) were selected and streaked in Methylene Blue Eosin Agar (Difco, USA). The presence of *E. coli* typical colonies, small, dark and shiny metallic green, and non-typical was assessed after 18-24 hours of incubation at 37°C. Five typical and five non-typical colonies from each plate were transferred to tubes containing Standard Plate Agar (PCA, Difco, USA) and to be identified later using IMViC biochemical tests ("I" is for indole test; "M" is for methyl red test; "V" is for Voges-Proskauer test, and "C" is for citrate test) (Kornacki and Johnson, 2001). Those confirmed as *E. coli* were stored at 4°C in semi-solid Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, Hampshire, England).

In order to isolating *E. coli* from water samples, 100 mL of water were used as inoculum so that aliquots of 10 mL were transferred to ten tubes of Lauryl Sulfate Tryptose broth (LST, Difco, USA) in double concentration, which were incubated at 37°C for 48 hours. Positive tubes were transferred to modified EC (mEC, EMD Millipore, USA) and the material was incubated at 37°C for 48 hours. From positive tubes, composed streak in Sorbitol MacConkey Agar (Difco, USA) was made and the plates were incubated at 37°C for 18-24 hours. From this step, the previously described methodology for isolation of *E. coli* from feces was followed, and those were confirmed and stored at 4°C in semi-solid BHI.

For *E. coli* isolation from food samples, they were diluted in peptone saline solution (0.85% of NaCl and 0.1% of peptone) to 10^{-4} dilution. Subsequently, aliquots of 1 mL of each dilution were transferred in triplicate to LST broth (Difco, USA) and tubes were incubated at 37°C for 48 hours. From this stage, the same procedure for *E. coli* isolation from water samples described previously was used.

The raw milk samples were diluted up to 10^{-5}. Thereafter, aliquots of 1 mL of each dilution were transferred in triplicate to LST broth (Difco, USA), being the tubes incubated at 37°C for 48 hours and the *E. coli* isolation was carried out as described previously. For samples of pasteurized milk, aliquots of 10 mL, 1 mL, and 0.1 mL were transferred in triplicate to LST broth (Difco, USA). Samples of Minas Frescal cheese and ground beef were diluted up to 10^{-5}. Subsequently, aliquots of 1 mL of each dilution were transferred in triplicate to LST broth (Difco, USA), and tubes were incubated at 37°C for 48 hours and the *E. coli* isolation was carried out as described previously.

Genotypic identification of STEC

Encoding genes of Shiga toxin type were detected by polymerase chain reaction - PCR, being the SRM 128 (CTGATTGTTGAGCGAAATAATTTATATGTG) and SRM 129 (TGATGATGACAATTCAGTATAACTGCCA) oligonucleotides used for *stx1/stx2* genes amplification (Monday *et al*., 2007), with expected amplicon in positive results of approximately 528 bp.

The gene encoding enterohemolysin, EHEC-hlyA, was also detected by PCR, using HlyA-F (GCATCATCAAGCGTACGTTCC) and HlyA-R (AATGAGCCAAGCTGGTTAAGCT) oligonucleotides (Osek, 2003) under the recommended conditions and the expected amplicon of positive results was approximately 534 bp.

As positive controls, strains of EDL 933 *E. coli* and C3888 *E. coli* were used. As negative control, strain of K12 *E. coli* was used from the culture collection of Industrial Microbiology Laboratory of

Microbiology Department from Federal University of Viçosa, Minas Gerais State, Brazil.

Amplification reactions of *stx1/stx2* and *EHEC-hlyA* genes were performed in microtubes of 200 µL containing 5 µL 1x buffer (Promega, Madison, Wis.), 2,5 µL of BSA at 2.5 µM and 0.25 µL of *Taq* polymerase (Promega, Madison, Wis.) unit 1.25, 2 µL of dNTPs at 2.5 mM, 1.5 µL of $MgCl_2$ at 25 mM, 0.5 µL of each specific oligonucleotides at a concentration of 10 µM, 5 µL of DNA previously extracted by boiling method as described by Keskimaki *et al.* (2001) and MilliQ water to a final volume of 25 µL. The amplifications occurred in a TC 512 Téchiné thermal cycler (Barloworlo Scientific, Staffordshire - England) under the conditions of initial denaturation at 94°C for 10 minutes, followed by 40 cycles at 94°C for 30 seconds, 53°C for 1.5 minute and 72°C for 1 minute followed by a final extension step at 72°C for 7 minutes. The amplified samples were analyzed by electrophoresis on 1.2% agarose gel containing ethidium bromide at 0.2 µg/ml concentration. The running time was approximately one hour at 85 volts. The images were captured in Eagle Eye (Stratagene, LaJolla, CA, USA) under ultraviolet light.

Cytotoxic potential of the E. coli STEC strain

To increase the release of cytotoxin in the culture medium, an isolate of *E. coli* potentially shiga-toxigenic was cultivated in 5 mL of BHI for 24 hours at 37°C. After incubation for five hours, mitomycin C was added (1 µg/mL). Subsequently, the culture was centrifuged at 10,000 g for 10 minutes at 4°C and the supernatant was filtered through a membrane with pores of 0.22 µm and the filtrate was kept at -20°C.

Lineages cells from African Green Monkey Kidney, Vero cells, were propagated in bottles of 25 cm^2 containing Minimum Essential Medium (MEM), penicillin (1.6 mg/L), streptomycin (0.4 mg/L) supplemented with 10% of fetal calf serum. In cytotoxicity test, confluent cell monolayers were trypsinized using standard methods for later being added in microplates with 24 compartments and were incubated at 37°C and atmosphere with 5% of CO_2. After twenty-four hours of culture, beyond the filtrate without dilution, 50 µL of filtrate prepared in three dilutions, 1/20, 1/40, and 1/80, were added in triplicate in each compartment, containing 0.5 mL of medium. Cell morphological changes, such as loss of monolayer, granulation, lengthening and narrowing of extensions, and cell borders darkening indicating the cytotoxic effect of Shiga toxin were observed by phase contrast microscope after incubation for twenty-four and forty-eight hours (Gentry and Dalrymple, 1980).

Results and Discussion

Two hundred and nine isolates were identified as *E. coli*, being one hundred and twenty obtained from feces samples, seventy from water, seven from feed, seven from ground beef, three from pasteurized milk and two from Minas Frescal cheese. None *E. coli* was isolated from raw milk samples. Sixty-four *E. coli* isolates were obtained from dairy cattle farms, being thirty-one, twenty-six, and seven coming from feces samples, water, and foods, respectively. One hundred and thirty-three isolates were obtained from beef cattle farms being eighty-nine originated from faecal samples and forty-four from water.

From two hundred and nine *E. coli* isolates, only one isolate (0.48%) coming from the water sample of a farm beef cattle was identified as *E. coli* potentially STEC by detection of amplicon with 528 bp. This isolate showed cytotoxic effect in Vero cells and the *EhlyA* gene encodes entero-hemolysin. The low occurrence of STEC isolates may be related to the low number of samples evaluated. This fact is relevant because a large sampling could increase the incidence of this pathogen isolation.

The presence of *E. coli* pathogenic strains in environment represents a potential source for food and water contamination (Maldonado *et al*., 2005). Moreover, such strains are potential reservoir of virulence genes that may give rise to new pathogenic strains by horizontal gene transfer (Kuhnert *et al.*, 2000; Donnenberg and Whittam, 2001).

The presence or absence of a particular gene is insufficient to predict the virulence potential of a STEC strain (Maldonado *et al.*, 2005) because *stx* genes expression, i.e., the toxin production, does not always occur. Thus, there may be variability in the cytotoxic responses of positive strains for *stx* gene, therefore, a means of assessing whether a gene is expressed is to evaluate the cytotoxic effect on Vero cells (O'Loughlin and Robin-Browne, 2001). In this study, we found that *E. coli* STEC isolate obtained from the used water in feeding beef cattle on a farm of Rio Pomba City, MG, expresses the stx gene because it presents cytotoxic effect on Vero cells, which represents a public health risk due to the real possibility of animals contamination and transmission this pathogen to population.

As cattle are the main reservoir of O157 and non-O157 STEC, the microbiota in their gut can contaminate the environment, water, and foods. Thus, STEC strains are mainly transmitted to humans by consumption of undercooked meat, unpasteurized milk, and other feeds contaminated with the cattle feces (Meng and Doyle, 1998; Bosilevac and

Koohmaraie, 2011). However, transmission through water also has been documented (Nataro and Kaper, 1998). Thus, water is an important source of bacterial contamination and several authors have demonstrated the occurrence of *E. coli* STEC strains in water samples (Song *et al*., 2005; Vidal *et al.*, 2005; Dumke *et al.*, 2006; Shelton *et al.*, 2006; Souza *et al.*, 2007).

Also, recently, an outbreak occurred involving some countries of European community, where the infectious agent is a new strain and a possible new pathotype: an entreroaggregative *E. coli* strains that produces the Shiga toxin with several cases and deaths reported. There are suspicions that its origin is from contaminated legumes and vegetables (WHO, 2011).

The pathogenic potential was evaluated among isolated STEC strains from animals and foods in Argentina and Brazil (Guth *et al.*, 2003). *EhlyA* gene was found in 85.7% of isolates and production of entero-hemolysin (*EHEC-Hly*) was observed in all O157 strains and in 85.7% of non-O157 strains (Guth *et al*., 2003). In this study, it was found that the *E. coli* STEC isolate also has *EhlyA* gene, being potentially entero-hemolysin-producing.

Despite the implementation of good agricultural practices to minimize the STEC presence, actually it is not possible to eradicate it from the environment due to asymptomatic colonization of animals and their ability to survive for long periods in cattle faeces, in the pasture, and water (Wang *et al.*, 1996; Caprioli *et al*., 2005). Thus, from a bacteriological standpoint, procedures should be put in place that allows us to discriminate the pathogens that represent high risk to the population health, so that preventive and therapeutic measures can be taken (Tschäpe and Fruth, 2001).

Conclusion

Once one isolate coming from the water sample of farm beef cattle was identified as *E. coli* STEC and showed cytotoxic effect in Vero cells and the *EhlyA* gene encodes entero-hemolysin, it is justified to develop studies to limit the spread of this pathogen in Minas Gerais state, Brazil.

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