Veterinary Microbiology 175 (2015) 150–156

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Short Communication

Diversity of Shiga toxin-producing *Escherichia coli* in sheep flocks of Paraná State, southern Brazil

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ARTICLE INFO

Article history:
Received 28 August 2014
Received in revised form 31 October 2014
Accepted 2 November 2014

Keywords:
Sheep
Shiga toxin-producing *E. coli*
Serotypes
Virulence factors
Genetic diversity

ABSTRACT

Sheep constitute an important source of zoonotic pathogens as Shiga toxin-producing *Escherichia coli* (STEC). In this study, the prevalence, serotypes and virulence profiles of STEC were investigated among 130 healthy sheep from small and medium farms in southern Brazil. STEC was isolated from 65 (50%) of the tested animals and detected in all flocks. A total of 70 STEC isolates were characterized, and belonged to 23 different O:H serotypes, many of which associated with human disease, including hemolytic-uremic syndrome (HUS). Among the serotypes identified, O76:H19 and O65:H– were the most common, and O75:H14 and O169:H7 have not been previously reported in STEC strains. Most of the STEC isolates harbored only *stx1*, whereas the *Stx2*b subtype was the most common among those carrying *stx2*. Enterohemolysin (*ehxA*) and intimin (*eae*) genes were detected in 61 (87.1%) and four (5.7%) isolates, respectively. Genes encoding putative adhesins (*saa*, *iha*, lpf_{O113}) and toxins (*subAB* and *cdtV*) were also observed. The majority of the isolates displayed virulence features related to pathogenesis of STEC, such as adherence to epithelial cells, high cytotoxicity and enterohemolytic activity. Ovine STEC isolates belonged mostly to phylogenetic group B1. PFGE revealed particular clones distributed in some farms, as well as variations in the degree of genetic similarity within serotypes examined. In conclusion, STEC are widely distributed in southern Brazilian sheep, and belonged mainly to serotypes that are not commonly reported in other regions, such as O76:H19 and O65:H–. A geographical variation in the distribution of STEC serotypes seems to occur in sheep.

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http://dx.doi.org/10.1016/j.vetmic.2014.11.003
0378-1135/

1. Introduction

Sheep farming is an important activity in the animal production sector of Brazil. Currently, this country has both the largest flock (approximately 17 million head) and sheep meat production (85,000 tonnes) of the American continent (FAO, 2012). The increasing consumption of ovine meat has boosted the expansion of Brazilian sheep industry. Consequently, there is an increased risk of exposure to zoonotic pathogens as Shiga toxin-producing *Escherichia coli* (STEC), which can be transmitted by food production chain (Schimmer et al., 2008), as well as contact with these animals and their environments (Ogden et al., 2002). STEC infection can lead to life-threatening complications such as hemorrhagic colitis (HC) and hemolytic-uremic syndrome (HUS).

Although cattle are considered to be the major reservoir of STEC strains, sheep also represent important carriers of these pathogens, and higher prevalence of STEC have been detected in ovine feces (Mora et al., 2011; Amézquita-López et al., 2012) and meat (Momtaz et al., 2013) when compared to samples of bovine origin. Nevertheless, only a few studies have reported the occurrence of STEC in Brazilian sheep populations (Vettorato et al., 2009; Maluta et al., 2014; Martins et al., 2014) and, particularly in the South region where approximately 30% of the national flock is found, these data are unknown so far. Thus, the aim of this study was to investigate the occurrence of STEC in sheep flocks from Paraná State, southern Brazil, to better understand the role played by these animals as reservoir of potentially pathogenic strains.

2. Methods

2.1. Sample collection and isolation of E. coli

A single rectal swab was obtained from 130 randomly selected sheep without diarrhea (one week to eight years old) between April and September 2010. The animals were originated from ten small and medium farms located in Paraná State, southern Brazil, and each locality was visited once. Samples were transported to the laboratory in Cary-Blair medium (Difco, USA), streaked onto MacConkey Agar (Difco, USA) and incubated overnight at 37 °C. At least three lactose-fermenting colonies were selected from each bacterial growth, confirmed as *E. coli* by standard biochemical tests, and then stored in Brain Heart Infusion broth (Difco, USA) with 20% glycerin at −20 °C until use.

2.2. Detection of STEC by multiplex polymerase chain reaction (mPCR)

Bacterial DNA was obtained by boiling method. Briefly, the isolates were cultivated onto Luria-Bertani (LB) Agar (Difco, USA) overnight at 37 °C. A loopful for each bacterial growth was resuspended in 300 μL of sterile ultrapure water, boiled at 100 °C for 10 min and centrifuged to 10,000 × *g* for 5 min. The supernatants were utilized as templates in mPCR for detection of the Shiga toxin (*stx1* and *stx2*) and intimin (*eae*) genes, according to Paton and Paton (1998). Amplification reactions were performed on GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, USA), with mixtures of 25 μL containing 2 μL of bacterial lysates, 200 μM dNTPs, 1X PCR buffer, 2 mM $MgCl_2$, 25 pmol of each primer and 1 U of *Taq* DNA Polymerase (Invitrogen, USA). PCR products were analyzed by 1.5% agarose gel electrophoresis and visualized under ultraviolet light (Vilbert Loumart, France) after staining with ethidium bromide.

2.3. Serotyping

Determination of O and H antigens was carried out as previously described (Guinée et al., 1981), employing all available O (O1–O185) and H (H1–H56) antisera. All antisera were absorbed with the corresponding cross-reacting antigens to remove the nonspecific agglutinins. The O and H antisera were produced in the Laboratorio de Referencia de *E. coli* (USC, Lugo, Spain). Isolates that did not react with O antisera were considered as non-typeable (ONT) and those nonmotile were H–.

2.4. Genotypic characterization of STEC

STEC isolates were investigated by PCR for the presence of genes encoding enterohemolysin (*ehxA*), STEC auto-agglutinating adhesin (*saa*), Irg homologous adhesin (*iha*), long polar fimbriae (lpf_{O113}), subtilase cytotoxin (*subAB*) and cytolethal distending toxin (*cdtV*). The *stx2* subtyping was carried out by PCR using primers for *stx2*a, *stx2*b, *stx2*c and *stx2*d genes. Typing of the *eae* gene was performed by PCR and sequencing. All primers used in this study and corresponding references are listed in Table S1 (Supplementary information).

2.5. Phenotypic assays

The cytotoxicity assay on Vero (African green monkey kidney) cells was carried out according to Beutin et al. (2002) with some modifications. STEC supernatants were prepared as described previously (Rocha and Piazza, 2007), and 1:10 dilutions were tested in duplicates on Vero cells. Supernatants of *E. coli* O157:H7 (EDL933) and K-12 (DH5α) strains were used as positive and negative controls, respectively. After 72 h-exposure to STEC supernatants, cellular metabolic activity was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma–Aldrich, USA) assay, according to manufacturer's instructions. The level of cytotoxicity was calculated using the following formula: (absorbance of the sample − absorbance of the negative control)/(absorbance of the positive control − absorbance of the negative control) × 100. Three independent experiments were performed.

Adherence capacity of STEC isolates to HEp-2 (human laryngeal epithelial carcinoma) cells was tested according to Cravioto et al. (1979) after 6 h of bacteria–cell interaction. Production of hemolysin was determined by the method described by Beutin et al. (1989).

2.6. Phylogenetic grouping

The phylogenetic group (A, B1, B2 and D) was determined using a triplex PCR for *chuA*, *yjaA* genes and