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ORIGINAL ARTICLE

Shiga toxin-producing *Escherichia coli* and atypical enteropathogenic *Escherichia coli* strains isolated from healthy sheep of different populations in São Paulo, Brazil

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Abstract

Aims: Sheep are important carriers of Shiga toxin-producing *Escherichia coli* (STEC) in several countries. However, there are a few reports about ovine STEC in American continent.

Methods and Results: About 86 *E. coli* strains previously isolated from 172 healthy sheep from different farms were studied. PCR was used for detection of stx_1, stx_2, *eae, ehxA* and *saa* genes and for the identification of intimin subtypes. Restriction fragment length polymorphism (RFLP)–PCR was performed to investigate the variants of stx_1 and stx_2, and the flagellar antigen (*fli*C) genes in nonmotile isolates. Five isolates were eae^+ and stx^-, and belonged to serotypes O128:H2/β-intimin (2), O145:H2/γ, O153:H7/β and O178:H7/ε. Eighty-one STEC isolates were recovered, and the *stx* genotypes identified were $stx_{1c}stx_{2d\text{-}O118}$ (46·9%), stx_{1c} (27·2%), $stx_{2d\text{-}O118}$ (23·4%), and $stx_{1c}stx_{2dOX3a}$ (2·5%). Pulsed-field gel electrophoresis (PFGE) revealed 27 profiles among 53 STEC and atypical enteropathogenic *Escherichia coli* (EPEC) isolates.

Conclusions: This study demonstrated that healthy sheep in São Paulo, Brazil, can be carriers of potential human pathogenic STEC and atypical EPEC.

Significance and Impact of the Study: As some of the STEC serotypes presently found have been involved with haemolytic uraemic syndrome (HUS) in other countries, the important role of sheep as sources of STEC infection in our settings should not be disregarded.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) have emerged as enteric pathogens that can cause severe diseases in humans, such as haemorrhagic colitis (HC) and haemolytic uraemic syndrome (HUS) (Karmali 1989). Domestic ruminants, especially cattle and sheep, have been implicated as the most important reservoir of STEC (Beutin *et al.* 1993, 1997). STEC elaborate two potent phage-encoded cytotoxins called Shiga toxins (Stx1 and Stx2), and different subtypes of Stx1 and Stx2 have been described (Scheutz *et al.* 2001). Stx1 produced by human STEC isolates has been considered to have minimal sequence variability, and the first subtype described, stx_{1cOX3}, was isolated from a sheep (Paton *et al.* 1995). This stx_1 variant was later designated stx_{1c} (Zhang *et al.* 2002a). Whereas Stx1 shows only little sequence variation, several variants of Stx2 with altered antigenic or biological characteristics have been described. Such toxins have been termed Stx2c, Stx2d, Stx2e, Stx2f, Stx2g, and several reports on other Stx2 variants produced by single strains have been published (Scheutz *et al.* 2001; Beutin *et al.* 2007). Epidemiological studies have revealed that Stx2 is more associated with severe disease in humans than is Stx1 (Boerlin *et al.* 1999; Friedrich *et al.* 2002), and that the stx_{2d} sequences are closely associated with sheep (Koch *et al.* 2001; Ramachandran *et al.* 2001). Studies suggest that sheep may be a natural reservoir of stx_{1c}-positive STEC

strains that enter the human food chain (Koch *et al.* 2001; Brett *et al.* 2003). In addition to Stx, other additional markers that contribute to pathogenicity have been described. These virulence markers include intimin, the product of the *eae* gene, an outer membrane protein involved in the intimate attachment of bacteria to the enterocytes, and enterohaemolysin (Ehx), encoded by the *ehxA* gene (Nataro and Kaper 1998). Paton *et al.* (2001) described an adhesin designated Saa (STEC autoagglutinating adhesin) produced by certain STEC strains. This adhesin encoded by the *saa* gene may be a marker for a subset of *eae*-negative STEC strains found among sheep (Urdahl *et al.* 2003), and that are capable of causing gastrointestinal and systemic diseases in humans.

There is mounting evidence that STEC serotypes that commonly inhabit the gastrointestinal tract of sheep are rarely isolated from other hosts (Beutin *et al.* 1993, 1995, 1997; Djordjevic *et al.* 2001, 2004; Blanco *et al.* 2003; Brett *et al.* 2003; Urdahl *et al.* 2003). For example, STEC strains of serotype O5:H− are predominantly isolated from sheep and rarely from cattle, and show different genetic profiles among these species, suggesting the existence of different clonal populations of O5:H− (McLean *et al.* 2005).

Previous studies conducted in our laboratories have demonstrated the occurrence of distinct strains in sheep from different farms (Vettorato *et al.* 2003). These data suggest that sheep are the reservoir of STEC strains belonging to a limited collection of serotypes, a subset of which can be capable of causing serious human disease. The results of the present research reinforce that STEC occurring in single populations of sheep are epidemiologically related despite their virulence diversity.

Material and methods

STEC and atypical EPEC isolates

A total of 81 STEC isolates were obtained over a time period of 3 years (2003–2006). A previous study (Vettorato *et al.* 2003) revealed the results of a 1-year study carried out with 42 STEC. For this study, 39 additional STEC strains and five atypical EPEC (AEPEC) were identified (Guth *et al.* 2006), resulting in a total of 86 *E. coli* strains (81 STEC and five AEPEC). All the *E. coli* strains analysed in this study were isolated from faeces of 172 healthy sheep living on pasture, ageing 4 months to 3 years old, from flocks located at five cities surrounding the São Paulo district. The localization of the farms is established according to their distances from São Paulo, as follows: (A), 153 km; (B), 235 km; (C), 43 km; (D), 72 km and (E), 336 km. Two or three isolated *E. coli* colonies were selected per animal, based on differences in virulence gene profiles. Thus, for this study, 40 stx_1stx_2 isolates, 22 stx_1, 19 stx_2, and five *stx* negative isolates harbouring the *eae* sequence were analysed.

Detection of *eae*, *ehx* and *saa* gene sequences by PCR

All *E. coli* isolates were subjected to PCR assay for the detection of *ehxA* and *eae* as previously described (Vettorato *et al.* 2003). Primer sequences and PCR conditions for intimin typing and for identification of *saa* were employed as described by Zhang *et al.* (2002b) and Paton *et al.* (2001) respectively.

stx subtyping

The presence of stx_1 and stx_2 sequences was confirmed by PCR with specific primer sequences (Pollard *et al.* 1990). A restriction fragment length polymorphism (RFLP)–PCR assay using specific primer sequences was employed for stx_1 and stx_2 subtyping as described by Cergole-Novella *et al.* (2006).

Serotyping of STEC isolates

Escherichia coli O and H antigens were examined as previously described (Vettorato *et al.* 2003). The flagellar antigen (*fli*C) gene was investigated in nontypeable (H−) isolates by following the PCR–RFLP conditions described by Machado *et al.* (2000), using as controls *E. coli* strains harbouring the following antigens: H2, H8, H14, H16, H19, H21 and H28.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) was used to establish clonal relationships among strains of the same serotype. The PFGE assay was performed following the method described by Gautom (1997), with modifications. The digestion time was 16–18 h, and PFGE was performed on a CHEF-DRIII PFGE apparatus (Bio-Rad). The pulse time was increased from 2·2 s to 35 s over a 21·5-h period. The band patterns were analysed by using the GelCompar II program (Applied Maths, Kortrijk, Belgium), and the similarity between PFGE patterns was evaluated using the Dice similarity coefficient (tolerance, 2%). Strains were considered to have the same PFGE patterns when all bands were identical.

Results

stx subtyping

The most common *stx* subtype observed among the STEC isolates from sheep was $stx_{1c}stx_{2d\text{-}O118}$ (Table 1).

Table 1 Serotypes and virulence profiles of ovine Shiga toxin-producing *Escherichia coli* (STEC) and atypical EPEC (AEPEC) isolates in State of São Paulo, Brazil

Serotype (no. isolates)	Farm	*stx* genotype	*eae*/intimin	*ehxA*	*Saa*
ONT*:H–	E	stx_{1c}, stx_{2dO118}	–	+	–
ONT:H8(4)	A	stx_{1c}, stx_{2dO118}	–	–	–
ONT:H8(3)	A	stx_{1c}, stx_{2dO118}	–	+	+
ONT:H8(3)	A	stx_{1c}	–	–	–
ONT:H8	A	stx_{1c}	–	+	–
ONT:H8	A	stx_{1c}, stx_{2dO118}	–	–	–
ONT:H8	A	stx_{1c}, stx_{2dO118}	–	–	–
ONT:H14	C	stx_{1c}	–	+	+
ONT:H14	C	stx_{1c}	–	+	+
ONT:H16(3)	B	stx_{1c}	–	+	+
ONT:H16(2)	A	stx_{2dO118}	–	–	–
ONT:H16(2)	B	stx_{1c}, stx_{2dO118}	–	+	+
ONT:H16	B	stx_{1c}	–	–	+
ONT:H16	A	stx_{1c}, stx_{2dO118}	–	–	–
ONT:H16	B	stx_{1c}, stx_{2dO118}	–	+	+
ONT:H19(2)	B	stx_{1c}	–	+	+
ONT:H19	D	stx_{1c}, stx_{2dO118}	–	+	+
ONT:H19	D	stx_{1c}, stx_{2dO118}	–	+	+
ONT:H19	A	stx_{2dO118}	–	–	–
O5:H– (2)	D	stx_{1c}, stx_{2dO118}	–	+	+
O5:H–	D	stx_{1c}, stx_{2dO118}	–	+	+
O5:H19	B	stx_{1c}	–	+	+
O5:H19	D	stx_{1c}, stx_{2dO118}	–	+	+
O16:H– (2)	B	stx_{1c}, stx_{2dO118}	–	+	+
O16:H19	B	stx_{1c}	–	+	+
O75:H8 (6)	B,C	stx_{1c}, stx_{2dO118}	–	+	+
O75:H8	B	stx_{1c}	–	+	+
O75:H8	D	stx_{1c}, stx_{2dO118}	–	+	+
O75:H8	E	stx_{2dO118}	–	+	+
O87:H16(13)	A,E	stx_{2dO118}	–	–	–
O91:H14	B	stx_{1c}, stx_{2dO118}	–	+	+
O112:H2(3)	C	stx_{1c}	–	+	+
O128:H2(2)	C	stx_{1c}	–	+	+
O128:H2	C	–	+/β	+	
O128:H2	B	–	+/β	+	
O145:H2	C	–	+/γ	+	
O146:H21(2)	B	stx_{1c}, stx_{2dO118}	–	+	+
O146:H21	D	stx_{1c}, stx_{2dOX3a}	–	+	+
O149:H2	D	stx_{1c}	–	+	+
O153:H7	D	–	+/β	–	
O172:H–	A	stx_{2dO118}	–	–	–
O174:H8(5)	D	stx_{1c}, stx_{2dO118}	–	+	+
O174:H8	D	stx_{1c}, stx_{2dOX3a}	–	+	+
O178:H7	D	–	+/ε	–	
OR:H19	B	stx_{1c}	–	–	+
OR:H19	B	stx_{1c}, stx_{2dO118}	–	+	+

ONT*nontypeable; H–, nonmotile; OR, Rough.

Specifically, 38 of 81 (46·9%) isolates carried the $stx_{1c}stx_{2d\text{-}O118}$ sequence. Twenty-two out of 81 (27·2%) isolates possessed the stx_{1c} sequence, and $stx_{2d\text{-}O118}$ and $stx_{1c}stx_{2\text{-}OX3a}$ were found in 19 (23·4%) and two (2·5%) isolates respectively. All frequencies were well distributed among the localities, except for one farm (E), where the $stx_{2d\text{-}O118}$ genotype prevailed in 14 out of 15 isolates.

Intimin typing

B-Intimin was identified in three out of the five AEPEC isolates and was distributed among three localities, where serotypes O128:H2 (2) and O153:H7 were related. ε and γ subtypes were identified in an O178:H7 and an O145:H2– isolates respectively (Table 1).

Association between serotype and other virulence markers of STEC and atypical EPEC

A total of 22 different serotypes were identified among the 86 *E. coli* isolates. Ten of these showed the nonmotile H antigen (H–), which were distributed among 35 isolates. Flagellar antigen (*fli*C) genes investigated by the PCR–RFLP revealed that 25 (71·4%) isolates showed a restriction profile identical to one of the following controls: H2, H8, H14, H16, H19, H21 and H28. Four out of 35 (11·4%) isolates did not have a restriction profile identified by this assay. Sixteen out of the 22 *E. coli* serotypes in this study showed an association with one or more genetic virulence markers, such as *ehxA* and *saa* (Table 1). Only STEC serotypes O87:H16 and O172:H–, and all AEPEC serotypes showed no association.

Some serotypes seemed to be associated with one locality, such as ONT:H8, O16:H–, O87:H16, O112:H–, O128:H2 and O174:H8, whereas O5:H–, O75:H– and O146:H21 were found to be distributed on more than one farm (Table 1).

Pulsed-field gel electrophoresis

A high diversity of PFGE profiles was observed (Fig. 1). Twenty-seven profiles were identified among 53 isolates analysed, and 17 isolates could not be analysed because no banding patterns were defined. Some common serotypes isolated from the same farm showed identical PFGE profiles, such as O87:H16 (farm E), ONT:H16 (B), ONT:H14, and five out of seven ONT:H8 (A) (Fig. 1). Identical PFGE patterns were also observed among isolates of serotypes O128:H2 and O146:H21 recovered from different farms. The serotypes 0174:H8 and 0112:H2 showed 90% and 95% genetic similarity respectively; three different genetic profiles were displayed by isolates belonging to serotypes 075:H8 isolated from three different localities.

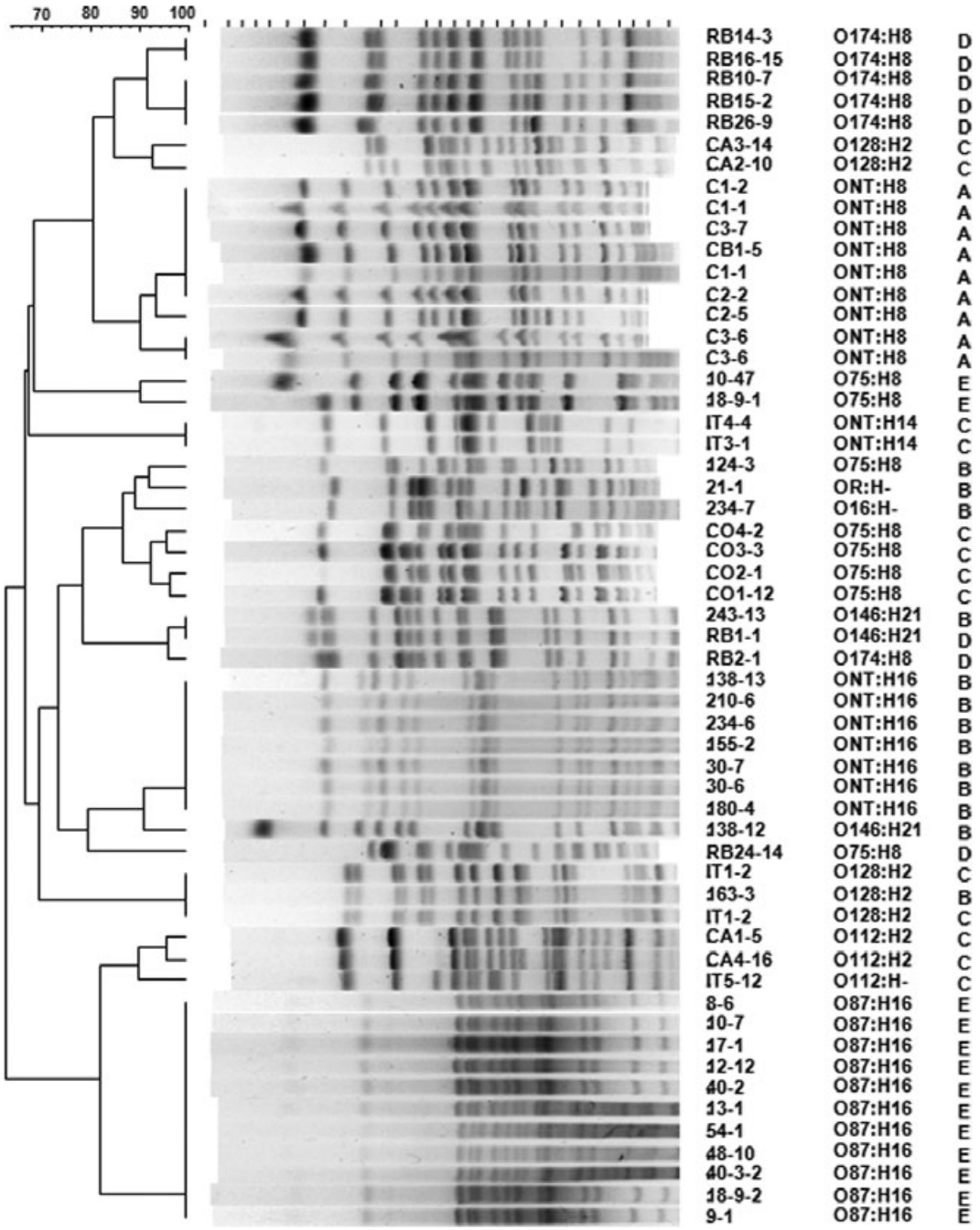

Figure 1 Pulsed-field gel electrophoresis (PFGE) dendrogram of ovine Shiga toxin-producing *Escherichia coli* (STEC) and atypical EPEC (AEPEC) isolates from State of São Paulo, Brazil.

Discussion

As the first detection of Shiga toxin-producing *E. coli*, increasing numbers of genetic variants of the major types of the toxin genes, stx_1 and stx_2, have been described. In this study, *stx* subtypes of 81 sheep isolates were determined. The stx_{1c} variant was found amongst all stx_1 positive isolates. In a study of sheep in Germany, stx_{1c} was detected in 48 stx_1-containing STEC isolates comprising serogroups O5, O125, O128 and O146 (Koch *et al.* 2001). A study in Norway (Urdahl *et al.* 2002) reported stx_{1c} in 71·6% stx_1-positive isolates from sheep faeces. A significant study of stx_1 subtypes in sheep performed by Brett *et al.* (2003) showed the predominance of stx_{1c}

(65·7%) isolated from ovine faeces and the infrequent identification of this subtype in STEC from bovine faeces. The $stx_{1c}stx_{2d\text{-}0118}$ genotype prevailed among our ovine isolates, and was distributed in the five localities surveyed. Moreover, it was found in association with serotypes ONT:H8/H16/H19, O5:H−/H19, O16:H−, O75:H8, O91:H14, O174:H8 and OR:H−. Similar results were obtained in a study carried out by Ramachandran *et al.* (2001) in which the serogroup O91 and serotypes O5:H− and O75:H8 are associated. An O5:H− isolate recovered from an ovine origin and harbouring the $stx_{1c}stx_{2d\text{-}0118}$ sequence was associated with HUS (Starr *et al.* 1998).

Among the 59 isolates demonstrating the stx_2 sequence, $stx_{2d\text{-}O118}$ was identified in 57 (96·6%) isolates; whereas two (3·4%) carried the stx_{2dOX3a} variant which was observed only on farm D. None of the *eae*-positive *E. coli* strains in this study produced Stx. Previous studies have described that the production of Stx is not a common characteristic of *E. coli* strains isolated from sheep or goats with diarrhoea (Blanco *et al.* 1996; Cid *et al.* 1996). A study carried out by Djordjevic *et al.* (2004) revealed STEC isolates harbouring intimin sequences which were isolated from young sheep (less than 6 weeks old).

We identified five *eae*-positive isolates that were classified as AEPEC and that were isolated from farms B, C and D. A common serotype O128:H2 found in two isolates, related to farms B and C, was associated with β-intimin, and showed 100% genetic similarity in the PFGE assay, suggesting a clonal dissemination. The presence of AEPEC belonging to classical serotypes among sheep has not been described so far. However, there are several reports that describe the occurrence of sheep isolates that could be classified as AEPEC-like, including serotypes O128:H2, O26:H11 which are common AEPEC serotypes (Blanco *et al.* 2003; Krause *et al.* 2005; Orden *et al.* 2005).

Production of enterohaemolysin is considered an important virulence factor in STEC, and combination of *stx* with *ehxA* is more frequently observed than with the *eae* gene among ovine (Ramachandran *et al.* 2001; Brett *et al.* 2003; Vettorato *et al.* 2003; Barlow *et al.* 2006). A high frequency (53%) of the *ehxA* gene was identified among our ovine isolates; however, heterogeneity in the distribution of this gene among the farms was observed. Three of the farms (B, C and D) concentrated higher levels of *ehxA*, while farms A and E presented lower frequencies (three and two isolates respectively). The *ehxA* gene presented a strong association with the *saa* gene (96%), and this data confirms previous suggestion that these genes are located in the same megaplasmid (Paton *et al.* 2001).

Ovine STEC belonging to over 105 serotypes have been isolated in previous studies carried out by other authors (Blanco *et al.* 2003; Urdahl *et al.* 2003; Djordjevic *et al.* 2004); however, only a small number of serotypes (O5:H−, O91:H−, O128:H2 and O146:H8/21) have been the most frequently and the most commonly found in sheep herds of different countries (Beutin *et al.* 1993; Kudva *et al.* 1997; Djordjevic *et al.* 2001).

In this study, the examination of five sheep farms revealed a considerable diversity of serotypes among the isolates from these farms. Some serotypes such as ONT:H−, ONT:H16, O5:H−, O75:H−, O75:H8, O87:H16, O128:H2, and O146:H21 were observed on more than one farm, whereas others, namely ONT:H8, ONT:H14, ONT:H19, O16:H−, O112:H−, and O174:H8, were restricted to one locality. Although a great variety of PFGE patterns were identified among the STEC strains analysed, some common serotypes belonging to the same farm or even to different farms showed 100% or a high degree of genetic similarity. On the other hand, other serotypes such as O75:H8 and ONT:H8 found on the same farms showed variations in their degree of similarity.

It was interesting to observe that the same serotypes occurring on different farms showed 100% genetic similarity. Although sharing different stx_2 sequences, isolates O146:H21 from farms B and D were grouped together. On the other hand, the two isolates of serotype O128:H2 that were grouped together were atypical EPEC, and displayed the same phenotypic and genotypic characteristics. This study represents for the first time, to best of our knowledge, a detailed analysis of ovine isolates in five localities of one state (São Paulo) obtained in Brazil. Our results suggest that sheep from different localities can harbour the same *E. coli* strains, and also support the hypothesis that some of these animals had belonged to the same flock and had been separated recently.

In conclusion, PFGE patterns showed that substantial genetic heterogeneity exists among common ovine STEC serotypes isolated in the state of São Paulo, Brazil, suggesting the establishment of different clones over time in our settings.

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