

ORIGINAL ARTICLE

Virulence markers and serotypes of Shiga toxin-producing *Escherichia coli*, isolated from cattle in Rio Grande do Sul, Brazil

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Abstract

Aims: To determine the prevalence of Shiga toxin-producing *Escherichia coli* (STEC) and serotypes and virulence markers of the STEC isolates from beef and dairy cattle in Rio Grande do Sul, Brazil.

Methods and Results: Faecal samples from beef cattle were collected at slaughterhouses. The isolates were submitted to colony hybridization assay with specific DNA probes for *stx1*, *stx2* and *eae* genes, and serotyped for the identification of O and H antigens. Thirty-nine per cent of beef cattle surveyed harboured at least one STEC strain. Among the distinct serotypes identified, 10 were shared by both beef and dairy cattle. Most of the strains isolated harboured *stx2*. Genotypic and phenotypic profiles allowed the identification of 34 and 31 STEC strains, isolated from beef and dairy cattle, respectively. Serotypes O10:H14, O15:H21, O96:H21, O119:H4, O124:H11, O128:H21, O137:H-, O141:H19, O159:H42, O160:H2 and O177:H11, identified in this study, have not been previously reported as STEC isolated from cattle.

Conclusions: Cattle are an important reservoir of STEC strains associated with human diseases in South America.

Significance and Impact of the Study: Determining the prevalence, genotypic profile and serotypes of STEC strains isolated from cattle enables the prediction of possible risk for public health.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are associated with a variety of human diseases, including diarrhoea, haemorrhagic colitis and haemolytic uremic syndrome (HUS) (Karmali 1989; Paton and Paton 1998).

Cattle have been implicated as the principal reservoir of STEC (Neill 1997; Kaper *et al.* 2004). Foods of cattle origin, especially undercooked meat and raw milk, are among those most frequently implicated in foodborne outbreaks of STEC (Neill 1997; Blanco *et al.* 2004a).

The major virulence factor of STEC is a potent cytotoxin named Shiga toxin, of which there are two immunologically non-cross-reactive groups called Stx1 and Stx2. Several other additional markers that contribute to the pathogenicity have been described. These virulence markers include intimin, the product of the *eae* (for *Escherichia coli* attachment and effacing) gene, an outer membrane protein involved in the intimate attachment of bacteria to the enterocytes (Nataro and Kaper 1998).

STEC, especially the O157:H7 serotype, are important and emergent pathogens in industrialized nations, where

several foodborne outbreaks have been reported (Neill 1997). In Brazil, sporadic cases of diarrhoea and HUS have been associated with O157:H7 and non-O157 STEC strains (Cantarelli *et al.* 2000; Guth *et al.* 2002; Irino *et al.* 2002; Vaz *et al.* 2004; Nishimura *et al.* 2005). STEC occurrence in meat and faeces of healthy cattle has also been reported in our country (Cerqueira *et al.* 1997, 1999; Leomil *et al.* 2003; Moreira *et al.* 2003; Irino *et al.* 2005).

In Rio Grande do Sul (RS), an important European cattle growing state located in the extreme south of Brazil, there are only a few studies on STEC occurrence in cattle and foods and on its implication on human clinical cases. Nevertheless, the results from a recent survey in this region that found STEC in 49% of the healthy dairy cattle faeces samples tested (Moreira *et al.* 2003), together with the high incidence of HUS in the neighbouring Argentina (López *et al.* 1997), stress the necessity of studies not only on STEC occurrence, but also on the pathogenic potential of the isolates.

In the present study, we report on the rate of isolation of STEC and on the serotypes and virulence markers of the STEC isolates from beef and dairy cattle in RS, Brazil.

Materials and methods

Escherichia coli isolates

Swabs obtained from the rectum of 100 beef cattle from 14 farms, situated in different regions of the state, were collected immediately after slaughter at two slaughterhouses in the period from January through July of 2005. The swabs were placed in Stuart's transport medium (Difco Laboratories, Detroit, MI, USA), and taken to the laboratory for analysis.

The swabs were streaked onto MacConkey agar (Oxoid, Basingstoke, Hampshire, UK) plates that were incubated overnight at 37°C. Five individual lactose-positive colonies from each plate were confirmed as *E. coli* isolates, based on biochemical tests, including utilization of citrate and production of indole, acetoin and methyl-red reactive compounds (Hitchins *et al.* 1995). The colonies confirmed were subcultured in brain heart infusion (BHI; Merck, Darmstadt, Germany), and incubated overnight at 37°C. The BHI cultures were mixed with one volume of glycerol in 80% phosphate-buffered saline (PBS, 0.01 mol l⁻¹, pH 7.4) and stored at -70°C. The isolates were grown in BHI at 37°C when needed.

A total of 68 *E. coli* strains, isolated from dairy cattle and previously screened as STEC by Vero cell assay (Moreira *et al.* 2003), were also included in this study for virulence marker and serotype determination.

DNA probing of isolates

The isolates were tested by colony hybridization, as described by Maas (1983). The following specific gene probes were used: Stx1 (Shiga toxin gene 1), a 1142-bp *Bam*HI fragment of plasmid pJN37-19 (Newland and Neil 1998); Stx2 (Shiga toxin gene 2), an 842-bp *Sma*I-*Pst*II fragment from plasmid pNN111-19 (Newland and Neil 1998) and EAE (*E. coli* attaching and effacing gene, *eaeA*), a 1-kb *Sal*I-*Kpn*I fragment from plasmid pCVD434 (Jerse *et al.* 1990). The DNA probe fragments were labelled with [α -³²P]dATP by nick translation (Rigby *et al.* 1977).

Serotyping

The identification of O and H antigens was carried out by following the standard procedures (Ewing 1986; Schetz *et al.* 2004), using currently available O (O1-O181) and H (H1-H56) antisera, prepared at Instituto Adolfo Lutz, with reference strains obtained from the *E. coli* and *Klebsiella* International Reference Centre, Copenhagen, Denmark. O antigens were determined by tube agglutination test using heated bacterial suspensions. H antigens were also determined by tube agglutination test using formalinized (0.5%) suspensions of actively motile bacteria obtained after several passages in semi-solid agar (0.2% agar).

Results

Beef cattle

Thirty-nine per cent out of 100 beef cattle surveyed, harboured at least one STEC strain (Table 1). Among the 14 farms from which the animals came from, only one did not have STEC carriers. The average percentage of carrier animals per farm was 40.75%.

Nineteen animals harboured strains carrying *stx1* and *stx2* genes, 18 presented strains with *stx2* gene alone, and eight with *stx1* alone. The *eae* gene was found in four strains isolated from four animals, all without *stx* genes.

Twenty-nine different STEC serotypes were identified among the isolates. The most frequent were ONT:H21, ONT:H7, ONT:H19, O22:H8 and ONT:H14, which were found in 8, 5, 4, 3 and 3 animals, respectively.

The genotypic and phenotypic profiles allowed the identification of 34 strains, as five serotypes presented more than one genotypic profile (Table 1). Eleven strains carried *stx1* and *stx2* genes, 16 carried *stx2* gene alone and seven *stx1* alone.

The strains most frequently isolated from beef cattle were ONT:H21 *stx1*+ *stx2*+ and ONT:H7 *stx1*+ *stx2*+, found respectively in six and four animals, and ONT:H21

Table 1 Farm prevalence, genotypic profile and serotypes of Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from beef cattle in Rio Grande do Sul, Brazil

Farm	Number of animals studied	Number of STEC-positive animals (%)	Animal	Genotypic profile	Serotype*			
A	9	2 (22,2)	F 02	<i>stx2</i>	ONT:H2			
			F 06	<i>stx2</i>	O174:H21			
B	8	4 (50,0)	F 12	<i>stx1/stx2</i>	ONT:H21			
			F 13	<i>stx1/stx2</i>	ONT:H21			
			F 16	<i>stx2</i>	ONT:H21			
			F 17	<i>stx2</i>	O96:H19			
				<i>stx2</i>	O96:H21			
C	11	2 (18,9)	F 19	<i>stx2</i>	O96:H21			
				<i>stx2</i>	O178:H19			
			F 23	<i>stx2</i>	O178:H19			
				<i>stx2</i>	O74:H28			
D	3	0						
E	3	2 (66,7)	F 32	<i>stx2</i>	O159:H42			
			F 33	<i>stx2</i>	ONT:H42			
F	7	2 (28,6)	F 38	<i>stx2</i>	O74:H-			
			F 39	<i>stx2</i>	O22:H8			
			F 43	<i>stx2</i>	O22:H8			
G	9	2 (22,2)	F 49	<i>stx1</i>	ONT:H11			
			F 50	<i>stx2</i>	O22:H8			
H	10	4 (40,0)	F 51	<i>stx1/stx2</i>	OR:H16			
				<i>stx1/stx2</i>	ONT:H19			
				<i>stx1/stx2</i>	ONT:H16			
			F 52	<i>stx1/stx2</i>	ONT:H21			
			F 53	<i>stx1/stx2</i>	ONT:H21			
			F 56	<i>stx1/stx2</i>	ONT:H19			
			I	8	6 (75,0)	F 61	<i>stx1/stx2</i>	ONT:H4
							<i>stx1/stx2</i>	O119:H4
						F 62	<i>stx1</i>	ONT:H19
						F 63	<i>stx1/stx2</i>	ONT:H14
							<i>stx1</i>	O10:H14
							<i>stx1</i>	ONT:H14
							<i>stx1</i>	ONT:H14
J	8	5 (62,5)	F 64	<i>stx1</i>	ONT:H14			
			F 65	<i>stx1</i>	ONT:H14			
			F 68	<i>stx2</i>	O22:H8			
			F 69	<i>stx2</i>	ONT:H8			
				<i>stx1/stx2</i>	ONT:H7			
			F 70	<i>stx1</i>	O124:H11			
			F 72	<i>stx1/stx2</i>	ONT:H7			
				<i>stx1</i>	O124:H11			
			F 73	<i>stx2</i>	ONT:H21			
				<i>stx2</i>	O116:H21			
K	6	2 (33,3)	F 76	<i>stx1/stx2</i>	ONT:H18			
			F 79	<i>stx1/stx2</i>	ONT:H21			
				<i>stx2</i>	ONT:H7			
				<i>stx2</i>	ONT:H21			
L	6	3 (50,0)	F 82	<i>stx1/stx2</i>	ONT:H7			
			F 83	<i>stx1/stx2</i>	ONT:H7			
			F 84	<i>stx1/stx2</i>	ONT:H19			
			F 88	<i>stx1</i>	O15:H21			
M	5	1 (20,0)		<i>stx1</i>	O2:H45			
			F 94	<i>stx1/stx2</i>	OR:H42			
N	7	4 (57,1)		<i>stx2</i>	ONT:H11			
			F 95	<i>stx1/stx2</i>	ONT:H21			
			F 98	<i>stx1/stx2</i>	ONT:H16			
				<i>stx2</i>	O113:H21			
			F 99	<i>stx1/stx2</i>	O82:H8			
Total	100	39 (39,0)	39		29			

*NT, nontypable; R, rough.

stx2+, O22:H8 *stx2+*, ONT:H14 *stx1+* and ONT:H19 *stx1+ stx2+*, each one found in three animals. The strains found in greater number of farms were ONT:H21 *stx1+ stx2+*, O22:H8 *stx2+*, ONT:H21 *stx2+* and ONT:H7 *stx1+ stx2+*, which were isolated from 4, 3, 3 and 3 farms, respectively.

More than one strain was isolated from 13 (38%) animals, 10 of which harboured two different strains and three carried three distinct strains.

Dairy cattle

Among the 68 STEC isolates from dairy cattle investigated, 31 harboured *stx1* and *sxt2* genes, 35 carried *stx2* alone, one *stx1* alone, and one *stx1* and *eae* genes (Table 2). Twenty-three serotypes were identified, the most frequent being ONT:H21, ONT:H7, ONT:H19, O22:H8, and O91:H21, which were found in 13, 8, 7, 5 and 5 isolates, respectively.

Table 2 Genotypic profile and serotypes of Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from dairy cattle in Rio Grande do Sul, Brazil

Number of strains	Genotypic profile	Serotype*
7	<i>stx2</i>	ONT:H21
6	<i>stx1/stx2</i>	ONT:H21
5	<i>stx2</i>	O22:H8
5	<i>stx1/stx2</i>	ONT:H19
5	<i>stx1/stx2</i>	ONT:H7
4	<i>stx2</i>	O91:H21
3	<i>stx2</i>	ONT:H7
3	<i>stx1/stx2</i>	O181:H4
2	<i>stx2</i>	ONT:H19
2	<i>stx2</i>	ONT:H16
2	<i>stx1/stx2</i>	O178:H19
2	<i>stx1/stx2</i>	ONT:H18
2	<i>stx2</i>	O116:H21
2	<i>stx1/stx2</i>	ONT:H2
2	<i>stx2</i>	O79:H19
1	<i>stx2</i>	O179:H8
1	<i>stx1/stx2</i>	ONT:H16
1	<i>stx1</i>	O178:H19
1	<i>stx1/stx2</i>	O163:H19
1	<i>stx1/eae</i>	ONT:H2
1	<i>stx2</i>	ONT:H2
1	<i>stx2</i>	O177:H11
1	<i>stx1/stx2</i>	O91:H21
1	<i>stx2</i>	ONT:H8
1	<i>stx1/stx2</i>	OR:H19
1	<i>stx1/stx2</i>	OR:H7
1	<i>stx2</i>	O22:H16
1	<i>stx1/stx2</i>	O141:H19
1	<i>stx2</i>	O156:H7
1	<i>stx2</i>	O112:H2
1	<i>stx2</i>	O160:H2

*NT, nontypable; R, rough.

Thirty-one strains with different genotypic and phenotypic profiles were identified, as seven serotypes presented distinct genotypic profiles. Thirteen of those strains carried *stx1* and *stx2* genes, 16 *stx2* gene alone, one *stx1* alone and one *stx1* and *eae* genes.

The profiles most frequently found were ONT:H21 *stx2+*, ONT:H21 *stx1+ stx2+*, O22:H8 *stx2+*, ONT:H19 *stx1+ stx2+*, and ONT:H7 *stx1+ stx2+*, identified in 7, 6, 5, 5 and 5 strains, respectively.

Discussion

In only one of the 14 beef cattle farms surveyed, no STEC strain could be isolated. The broad range of infected animals (0–75%) found within farms may probably be associated with the extensive production system used, in which the breeding in large areas allows different levels of contamination among the animals.

A high diversity of STEC serotypes was identified in both dairy and beef cattle isolates. Similar result was found in a recent survey made in dairy cattle from São Paulo, Brazil (Iriño *et al.* 2005), although, in other studies from Brazil, such diversity was not found (Cerqueira *et al.* 1999; Leomil *et al.* 2003). Among the distinct serotypes identified, 10 (O22:H8, O116:H21, O178:H19, ONT:H2, ONT:H7, ONT:H8, ONT:H16, ONT:H18, ONT:H19, ONT:H21) were shared by both beef and dairy cattle. Although there are areas where milk or beef cattle predominate, the division is not clear, and there are no exclusive areas for milk and beef production in RS. In fact, it is very common to find the two production systems in the same farm. Thus, the contact between the two types of cattle is usual even within farms, allowing cross-contamination of the animals.

Interestingly, the serotypes of STEC from dairy cattle, identified in the present study, were different from that found in a previous survey (Moreira *et al.* 2003), even though they were part of the same set of isolates. The different results were probably attributed to the antisera used for typing. In the previous work, the authors were able to identify only six serotypes, all nonmotile, using antiserum prepared with isolates obtained from human diarrhoeal illness. In contrast, the serotyping methodology used in this study included a complete set of antisera prepared with reference *E. coli* strains, according to standard procedures.

Although *E. coli* O157:H7 was not isolated in this survey, other serotypes related with human disease, including HUS, such as O113:H21, O22:H8, O91:H21, O112:H2, O163:H19, O174:H21 and ONT:H7, were identified. The O22:H8 and ONT:H7 serotypes were among the STEC most frequently isolated from beef cattle. The latter two were among the serotypes occurring most frequently in

dairy cattle, together with O91:H21, which has already been isolated from children with diarrhoea in RS State (Cantarelli *et al.* 2000). The occurrence of the serotypes O113:H21, O178:H19, O22:H8 and O91:H21 in dairy cattle was reported in a recent study carried out in São Paulo (Iriño *et al.* 2005), and serotypes O113:H21 and O91:H21 were isolated from beef cattle in Argentina (Meichtri *et al.* 2004), a country with a high incidence of HUS. Besides, other authors have also isolated STEC O113:H21 from cattle and raw beef in Brazil and Argentina (Cerqueira *et al.* 1997; Gomez *et al.* 2002; Guth *et al.* 2003; Leomil *et al.* 2003). These results point out the fact that many serotypes isolated in RS also occur in other regions of Brazil and Argentina. It is possible that these serotypes, which can cause severe disease in humans, are disseminated among cattle herds in the south cone of Latin America. To our knowledge, serotypes O10:H14, O15:H21, O96:H21, O119:H4, O124:H11, O128:H21, O137:H-, O141:H19, O159:H42, O160:H2 and O177:H11, identified in this study, have not been previously reported as STEC isolated from cattle (<http://www.lugo.usc/ecoli>).

Most of the strains isolated harboured *stx2*, either alone or with the *stx1* gene. This represents a potential danger to public health, as STEC strains carrying *stx1* have been associated with uncomplicated diarrhoea in healthy individuals, while those carrying *stx2* have been associated with severe disease in humans (Boerlin *et al.* 1999; Brooks *et al.* 2005), including adults with HUS (Bonnet *et al.* 1998). Thirteen serotypes, including O91:H21, O178:H19 and O15:H21, presented more than one genotypic profile, corroborating the high diversity of STEC identified by serotyping.

The *eae* sequence was detected in only one STEC strain from dairy cattle. These results are in accordance with other studies, which show that the majority of the STEC strains, isolated from healthy cattle, usually do not harbour the *eae* gene (Blanco *et al.* 1997, 2004a; Guth *et al.* 2003; Iriño *et al.* 2005). Interestingly, Hurley *et al.* (2001), studying Shiga toxin translocation across intestinal epithelial cells, observed that *eae*-negative strains induced more neutrophil transmigration and interleukin-8 production by enterocytes than *eae*-positive strains, and associated this fact with Shiga toxin translocation. Moreover, several *eae*-negative STEC strains have been associated with HUS (Bonnet *et al.* 1998; Paton and Paton 1998, 1999). Recently, Paton and Paton (2002) described a novel megaplasmid-encoded adhesin (Saa), which may be an important virulence factor of *eae*-negative STEC strains.

The serotypes previously associated with STEC strains that cause HUS, isolated in this study, presented the profiles O22:H8 *stx2*, O91:H21 *stx2*, O91:H21 *stx1 stx2*, O113:H21 *stx2*, O163:H19 *stx1 stx2*, O174:H21 *stx2*, O112:H2 *stx2*, ONT:H7 *stx2* and ONT:H7 *stx1 stx2*. The O91:H21 *stx2*, O112:H2 *stx2*, O113:H21 *stx2*, O174:H21

stx2 and ONT:H7 *stx2* profiles have also been identified in bovine STEC isolates in Argentina (Parma *et al.* 2000; Blanco *et al.* 2004b; Meichtri *et al.* 2004). Strains with profile O113:H21 *stx2* have been previously isolated from cattle in Brazil (Guth *et al.* 2003; Iriño *et al.* 2005). All serotypes associated with HUS, identified in this study, carried the *stx2* gene, suggesting that bovine is an important reservoir of STEC strains associated with human diseases in South America. The reason for the HUS occurrence in Brazil being so low remains unknown. The results of Boerlin *et al.* (1999) suggest that STEC isolates from humans constitute a population different from that found in the bovine reservoir, or that they are only a sub-population of the latter. Further studies are necessary in order to elucidate this question.

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