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Virulence properties and antimicrobial susceptibility of Shiga toxin-producing *Escherichia coli* strains isolated from healthy cattle from Paraná State, Brazil

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Abstract: The presence of Shiga toxin-producing *Escherichia coli* (STEC) strains in feces samples of cattle was determined using the cytotoxicity assay on Vero cells and a screening PCR system to detect *stx* genes. The STEC isolates were serotyped, tested for antimicrobial susceptibility, and analyzed for virulence genes using multiplex PCR. The verocytotoxin-producing *E. coli* – reverse passive latex agglutination (VTEC–RPLA) assay was also used to detect Shiga toxin production. The frequency of cattle shedding STEC was 36%. The isolates belonged to 33 different serotypes, of which O10:H42, O98:H41, and O159:H21 had not previously been associated with STEC. The most frequent serotypes were ONT:H7 (10%), O22:H8 (7%), O22:H16 (7%), and ONT:H21 (7%). Most of the strains (96%) were susceptible to all antimicrobial agents tested. Shiga toxin was detected by the VTEC–RPLA assay in most (89%) of the STEC strains. The frequency of virulence markers was as follows: stx_1, 10%; stx_2, 43%; stx_1 plus stx_2, 47%; *ehxA*, 44%; *eae*, 1%; and *saa*, 38%. Several strains belong to serotypes associated with human disease, and most of them carried a stx_2-type gene, suggesting that they represent a risk to human health. The screening PCR assay showed fewer false-negative results for STEC than the Vero-cell assay and is suitable for laboratory routine.

Key words: STEC, *stx* genes, virulence genes, Shiga toxin, antimicrobial susceptibility.

Résumé : La présence de souches d'*Escherichia coli* produisant la toxine de Shiga (STEC) dans des échantillons fécaux du veau a été déterminée par un essai de cytotoxicité sur les cellules Vero et par un criblage par PCR afin de détecter les gènes *stx*. Le typage sérologique des isolats de STEC a été réalisé, leur sensibilité antimicrobienne a été testée, et la présence de gènes de virulence a été analysée par PCR multiplex. L'essai VTEC–RPLA a aussi été utilisé pour détecter la production de toxine de Shiga. La fréquence de libération de STEC par le veau était de 36 %. Les isolats appartenaient à 33 types sérologiques, dont les types O10:H42, O98:H41 et O159:H21 qui n'avaient jamais été associés auparavant avec les STEC. Les types sérologiques les plus fréquents étaient ONT:H7 (10 %), O22:H8 (7 %), O22:H16 (7 %) et ONT:H21 (7 %). La plupart des souches (96 %) étaient sensibles à tous les agents antimicrobiens testés. La toxine de Shiga a été dé-

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tectée par l'essai VREC–RPLA dans la plupart des souches STEC (89 %). La fréquence des marqueurs de virulence était la suivante : stx_1, 10 %; stx_2, 43 %; stx_1 et stx_2, 47 %; *ehxA*, 44 %; *eae*, 1 %; et *saa*, 38 %. Plusieurs souches appartiennent à des types sérologiques associés à la maladie chez l'humain et plusieurs d'entre elles comportaient un gène de type stx_2, ce qui suggère qu'elles constituent un risque pour la santé humaine. L'essai de criblage par PCR a présenté moins de résultats faussement négatifs dans la détection des STEC comparativement à l'essai sur les cellules Vero, et est approprié pour des tests de laboratoire de routine.

Mots-clés : STEC, gènes *stx*, gènes de virulence, toxine de Shiga, sensibilité antimicrobienne.

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Shiga toxin-producing *Escherichia coli* (STEC) strains are an important cause of food-borne disease, representing a serious problem to public health in several countries. These organisms are associated with sporadic cases and outbreaks of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (WHO/CSR/APH 1998; Mainil and Daube 2005). More than 400 serotypes of STEC have been described, and over 150 implicated in human disease (WHO/CSR/APH 1998; Zweifel et al. 2005). The main virulence factor of STEC is the Shiga toxin (Stx) of which 2 major types, Stx1 and Stx2, exist; variant forms have also been described. In addition to Stx, STEC may also carry genes such as *eae*, which is encoded in the pathogenicity island LEE (locus of enterocyte effacement), coding for the adhesin intimin, which mediates the production of attaching and effacing lesions on intestinal mucosa; and *ehxA* that codes for enterohemolysin (Mainil and Daube 2005). The Saa protein (STEC auto agglutinating adhesin), coded by the *saa* gene, is also a putative virulence factor in LEE-negative strains (Paton et al. 2001).

Since STEC strains have biochemical properties similar to those of *E. coli* from normal enteric flora the detection of Shiga toxins, using the cytotoxicity assay in Vero cells or immunological tests, or the presence of genes coding for these toxins are alternatives to identify such strains (Karch et al. 1999; Bettelheim and Beutin 2003).

Cattle are the major reservoir of STEC, and the fecal contamination of meat during slaughter is the main route by which these pathogens enter the food supply (Meng and Doyle 1998). The dispersion of manure in the environment and its use as fertilizer can also be a source of food contamination (Caprioli et al. 2005). Few epidemiologic studies on the occurrence of STEC have been conducted in Brazil, and most of these report isolates from the São Paulo and Rio de Janeiro states (Cerqueira et al. 1999; Leomil et al. 2003; Irino et al. 2005). Paraná State has a cattle herd composed of approximately 10 million animals (www.agricultura.gov.br), while the herd of the whole of the South region constitutes about 15% (30 million animals) of the Brazilian herd, most of which have been raised in an extensive grazing system, based on free-range pasture.

In this work, the presence of STEC in beef cattle from Paraná State of southern Brazil was determined using a PCR screening system and the cytotoxicity assay in Vero cells. STEC strains were differentiated by serotype; antimicrobial susceptibility; *stx* typing; and the presence of *eae*, *ehxA*, and *saa* genes. Three serotypes (O10:H42, O98:H41, and O159:H21), out of 33 found in the cattle samples, were not previously associated with production of Shiga toxin and may represent a yet unidentified potential hazard for human health.

Swabs of rectal feces from 190 healthy cattle (140 adults and 50 calves less than 2 months old), extensively grazed, from 43 different localities of Paraná State, were collected from slaughterhouses from December 2002 to November 2003. Samples from up to 5 randomly chosen animals from each location were collected. Swabs containing the fecal samples were transported in Cary–Blair medium and within 6 h were cultured on sorbitol–MacConkey agar. For each sample, up to 10 colonies, including sorbitol-negative and sorbitol-positive colonies, were selected and identified using the following biochemical tests: acid from glucose, gas from glucose, H_2S, urease, L-tryptophane deaminase, indole, lysine decarboxylase, and motility. Those confirmed as *E. coli* were tested for Shiga toxin production using the cytotoxicity assay in Vero cells (Gentry and Dalrymple 1980) and for the presence of *stx* genes by using a screening PCR protocol (Lin et al. 1993) with primers LinF (5′ GAACGAAATAATTTATATGT 3′) and LinR (5′ TTTGATTGTTAC(A/C)GTCAT 3′). DNA was extracted by the boiling method (Olsvik and Strockbine 1993). A multiplex PCR protocol was used to detect the stx_1, stx_2, *eae*, *ehxA*, and *saa* genes (Paton and Paton 2002); this assay was used twice for each strain. For strains in which doubt persisted, PCR with a single primer pair was used. STEC O157:H7 (EDL 933) and *E. coli* ATCC 25922 were used as positive and negative controls, respectively. In addition, the VTEC–RPLA Seiken (Denka Seiken Co. Ltd., Tokyo, Japan) was performed according to the manufacturer's instructions, to a maximum extract dilution of 1:16. Titres higher than 1:4 were considered positive for Shiga toxin.

Of 190 stool samples analyzed, 69 (36%) were positive for STEC that were found only in adult cattle. Most of the strains were sorbitol positive (Table 1). Samples from 3 animals had 2 different STEC strains isolated with distinct sorbitol reactions, belonging to different serotypes and carrying distinct sets of virulence markers, producing a total of 72 STEC strains. Animals shedding STEC were found in 27 (63%) of the 43 localities. There was a clear difference in STEC frequency according to the period of collection—summer having the highest frequency (56%) and winter the lowest (26%). The highest frequency of STEC during summer was also described in other studies (Karch et al. 1999). The overall frequency (36%) was similar to that found in Rio de Janeiro (Cerqueira et al. 1999) but higher than that in São Paulo (Leomil et al. 2003; Irino et al. 2005), both in the southeast of Brazil. Studies conducted in

Table 1. Characteristics of Shiga toxin-producing *Escherichia coli* (STEC) strains isolated from Brazilian cattle.

Serotype*	*N*	Sorbitol fermentation	Vero cell assay	PCR†	VTEC assay‡	Virulence markers	Susceptibility§
O6:H34	1	+	+	+	Stx2 (1:4)	*stx*$_2$	S
O10:H42	1	+	+	+	NR	*stx*$_1$ *stx*$_2$	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$	S
	1	–	+	+	Stx1 (1:8)	*stx*$_1$ *ehxA saa*	S
O17:H41	1	+	+	+	Stx1 Stx2 (≥1:16/1:8)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
O22:H8	2	+	+	+	Stx2 (1:8)	*stx*$_2$	S
	2	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
	1	+	+	+	Stx2 (1:8)	*stx*$_2$ *saa*	S
O22:H16	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
	1	+	+	+	Stx1 Stx2 (≥1:16/1:8)	*stx*$_1$ *stx*$_2$	S
	1	–	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$	S
	1	–	+	+	Stx1 Stx2 (≥1:16/1:4)	*stx*$_1$ *stx*$_2$ *ehxA*	S
	1	–	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA*	S
O41:H2	1	–	+	+	Stx2 (≥1:16)	*stx*$_2$	S
O74:H42	2	+	+	+	Stx1 Stx2 (≥1:16/1:8)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
O79:H$^-$	1	–	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
O82:H8	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$ *ehxA*	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
O98:H41	1	–	+	+	Stx1 (≥1:16)	*stx*$_1$ *stx*$_2$	S
O110:H2	1	–	+	+	Stx1 (≥1:16)	*stx*$_1$ *stx*$_2$	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
O113:H21	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$ *ehxA saa*	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA*	S
	1	–	+	+	Stx1 (≥1:16)	*stx*$_1$ *stx*$_2$	S
O117:H8	1	–	+	+	NR	*stx*$_1$	S
O124:H11	1	+	+	–	Stx1 (1:4)	*stx*$_1$	S
O159:H21	1	+	–	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
O174:H21	2	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
	1	+	+	+	NR	*stx*$_1$ *stx*$_2$	S
O175:H21	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
O178:H19	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA*	S
O179:H8	1	–	+	+	Stx2 (≥1:16)	*stx*$_2$ *ehxA saa*	S
O181:H4	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$ *saa*	S
ONT:H2	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$ *saa*	S
	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$ *ehxA saa*	S
ONT:H7	2	+	+	+	Stx2 (1:8)	*stx*$_2$	S
	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$ *ehxA saa*	S
	1	+	+	+	NR	*stx*$_2$ *ehxA saa*	S
	1	+	+	+	Stx1 (≥1:16)	*stx*$_1$ *ehxA saa*	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *saa*	S
	1	–	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *saa*	S
ONT:H8	1	+	+	+	NR	*stx*$_1$ *ehxA saa*	S
ONT:H10	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
ONT:H12	1	+	+	+	NR	*stx*$_1$ *stx*$_2$	S
	1	+	+	+	Stx1 Stx2 (≥1:16/1:8)	*stx*$_1$ *stx*$_2$ *ehxA*	S
ONT:H16	1	–	+	+	Stx2 (1:8)	*stx*$_2$	S
	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$	Tc^i
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA*	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
ONT:H21	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
	1	–	+	+	Stx2 (≥1:16)	*stx*$_2$	Tc^i
	1	+	+	+	NR	*stx*$_2$	Tc^i
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$	S
	1	–	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA*	S

Table 1 (*concluded*).

Serotype*	*N*	Sorbitol fermentation	Vero cell assay	PCR†	VTEC assay‡	Virulence markers	Susceptibility§
ONT:H25	1	–	+	+	Stx2 (≥1:16)	*stx*$_2$ *saa*	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$	S
ONT:H38	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S
ONT:H41	1	+	+	+	Stx1 (≥1:16)	*stx*$_1$ *ehxA saa*	S
ONT:H$^-$	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
OR:H10	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$ *ehxA saa*	S
OR:H18	1	+	+	+	Stx2 (≥1:16)	*stx*$_2$	S
ND	1	+	+	+	Stx1 (≥1:16)	*stx*$_1$ *eaeA ehxA*	S
	1	+	+	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA*	S
	1	+	–	+	Stx1 Stx2 (≥1:16)	*stx*$_1$ *stx*$_2$ *ehxA saa*	S

Note: +, positive reaction; –, negative reaction.

*ONT, not typeable; OR, rough; ND, not determined.

†Detection of *stx* gene by screening PCR.

‡VTEC–RPLA assay, titres are shown in parentheses. NR, not reagent.

§S, susceptible to all antimicrobial agents tested; Tc^i, tetracycline intermediate.

states from southern Brazil reported STEC frequencies of 49% and 39% in Rio Grande do Sul (Moreira et al. 2003; Timm et al. 2007) and 59% in Paraná (Farah et al. 2007). The higher frequency of STEC in cattle of Parana State related by Farah and co-workers could be associated with the period, early autumn, and localities in which the samples were collected. Our studies indicate that there is seasonal variation in the frequency of STEC recovery from cattle, and further work will be necessary to clarify the reasons. Considering that in the present work samples were collected for 1 year, these results could reflect a better estimate of STEC occurrence. It was also observed that the distribution of cattle shedding STEC was not uniform in the different regions of the State—the highest frequencies (68%) were found in cities of the northwest, the lowest (18%) in the southeast. This should be related to differences in the climate and (or) management of the cattle.

STEC strains were serotyped using O (O1 to O181) and H (H1 to H56) antisera prepared at the Adolfo Lutz Institute, São Paulo, Brazil. The strains were classified into 33 distinct groups according to the serotyping reactions (Table 1), of which, O10:H42, O98:H41, and O159:H21 had not previously been associated with STEC. Except for serotypes O79:H$^-$, O110:H2, O117:H8, O124:H11, O181:H4, ONT:H38, and OR:H10, and those not previously associated with STEC, all other serotypes have been associated with human infection (Prager et al. 2005; http://www.microbionet.com.au/vtectable.htm; http://www.lugo.usc.es/ecoli/SEROTIPOSHUM.htm). The most frequent serotypes were ONT:H7 (10%), O22:H8 (7%), O22:H16 (7%), and ONT:H21 (7%). Strains ONT:H7 and O22:H8 were also predominant in the studies by Farah et al. (2007) and Timm et al. (2007) in cattle from the Paraná and Rio Grande do Sul states but not in other states in Brazil; on the other hand, serotype O22:H16 was found to be among the most common serotype isolated in the Rio de Janeiro and São Paulo states (Cerqueira et al. 1999; Aidar-Ugrinovich et al. 2007). This suggests that different serotypes may prevail in different geographic regions of this country. The finding that serotype O111 is the most frequently isolated from human infections (Vaz et al. 2004) and cattle from São Paulo State (Irino et al. 2005; Aidar-Ugrinovich et al. 2007), though it was not found in cattle from other states (Cerqueira et al. 1999; Moreira et al. 2003; Farah et al. 2007; Timm et al. 2007), supports this suggestion. Serotypes ONT:H7, O22:H8, O22:H16, and ONT:H21 were already associated with human disease (http://www.microbionet.com.au/vtectable.htm, http://www.lugo.usc.es/ecoli/SEROTIPOSHUM.htm); however, to our knowledge, human infection in Brazil associated with these serotypes was not reported, although they are commonly found in cattle from this country. One possibility is that the strains prevalent in Brazil have a low level of virulence.

The susceptibility pattern of the STEC strains was determined by the disk diffusion method (National Committee for Clinical Laboratory Standards 2000), using the following antimicrobials: ampicillin, cephalothin, chloramphenicol, ciprofloxacin, gentamicin, imipenem, nalidixic acid, nitrofurantoin, streptomycin, tetracycline, and trimethoprim–sulfamethoxazole. Most of the strains (96%) were susceptible to all antimicrobials tested (Table 1). Three strains showed an intermediate response to tetracycline. None of the strains were resistant to any of the antimicrobials tested, which is in contrast to the resistance level found in STEC isolated from cattle in other countries (Maidhof et al. 2002; Schroeder et al. 2002; Mora et al. 2005). However, a high frequency of susceptibility was also found in STEC strains isolated from cattle from São Paulo State, and among the strains that showed resistance to antimicrobials, all of them were resistant to tetracycline (Cergole-Novella et al. 2006). The low level of resistance found may be associated with the fact that the most common cattle management system is free roaming on grassland, and food supplement containing antibiotics is not a common practice (www.seab.pr.gov.br) possibly due to its high cost in this country.

The virulence markers were found in 11 different combinations (Table 1), the most frequent being *stx*$_2$ (27%) and then *stx*$_1$ *stx*$_2$ and *stx*$_1$ *stx*$_2$ *ehxA saa* (16% each). The high frequency of the *stx*$_2$ gene (89%) suggests a potential hazard for human health, since *stx*$_2$ is related to higher virulence (Boerlin et al. 1999; Beutin et al. 2004). The *eae* gene was found in only one strain and in association with *stx*$_1$ and

ehxA. The presence of stx_2 plus *eae* is associated with a more severe form of the human disease (Boerlin et al. 1999; Beutin et al. 2004). Although certain serotypes of STEC lacking the *eae* gene may also cause severe disease, this result suggests that the low prevalence of the stx_2 *eae* genotype may be related to the rare occurrence of hemolytic uremic syndrome in Brazil (Guth et al. 2002). The *saa* gene was found only in 28 (38%) strains, so other types of adhesins may be involved in the adhesion mechanisms of these strains. The *ehxA* gene was found in 32 (44%) strains, 22 of which tested positive for the *saa* gene, indicating a significant association between these genes of plasmid origin (Paton et al. 2001).

Some of the strains belonging to the same serotype carried different complements of virulence genes (Table 1), indicating genetic and biochemical diversity of the isolates which may differ in the ability to cause disease. Thus serotyping is not enough to define virulence and virulence genes should be identified in the epidemiologic surveys to better evaluate the risk that STEC strains represent to human health.

Divergent results between the cytotoxicity assay in Vero cells and the PCR screening assay for *stx* genes were observed in 3 strains (Table 1). The multiplex PCR assay revealed that 2 of these strains contained copies of stx_1 plus stx_2, but by the cytotoxicity assay they were false negatives, although Stx expression was detected by the VTEC–RPLA assay (Table 1). Only one false negative in the PCR screening was latter shown to have the stx_1 gene by multiplex PCR, and a weak reaction for Stx1 was observed in the immunoassay. This suggests the presence of a stx_1 allele not recognized by the primers used in the screening PCR. Although there was no significant difference when the results of the Vero cell and PCR screening assays were compared by the χ^2 test ($\chi^2 = 0.04$, $P = 0.83$), the latter offers clear advantages for routine use, since it is easier to perform, less expensive, and allows faster detection of *stx*-containing bacteria.

Sixty-five strains (90%) reacted positively in the VTEC–RPLA assay. Three of the strains identified as producing Stx1 by the VTEC–RPLA assay were found to contain stx_1 plus stx_2 genes by the multiplex PCR. The 7 STEC strains for which the VTEC–RPLA assay was negative (Table 1) were shown to carry stx_1 plus stx_2, stx_2, or stx_1; all these strains were positive in the Vero cell assay, indicating that they produce active Shiga toxin and that the negative result observed in the VTEC–RPLA is probably due to a Stx variant not recognized by the antibodies used in the assay or to a low level of expression. Negative results for strains harboring variants of stx_2 were already observed for VTEC–RPLA assay (Beutin et al. 2004), and substantial differences in the amount of Stx produced by strains with various *stx* alleles were found by Orth et al. (2007).

In conclusion, this work showed the presence of serotypes of STEC associated with human disease in cattle from Paraná State. All strains belonged to non-O157 serogroups, which may explain the prevalence of non-O157 serogroups in human infections by STEC in Brazil (Vaz et al. 2004; Cergole-Novella et al. 2006), and most of them were susceptible to all the antimicrobials tested. Although most of the strains presented a stx_2-type gene, they may be less virulent, since they do not contain the *eae* gene. The low level of virulence of these strains could explain why, in Brazil, STEC are associated with uncomplicated diarrhea, and why cases of severe disease such as hemorrhagic colite and hemolytic uremic syndrome are so rare. However, lateral transfer of virulence genes may give rise to new STEC strains with unpredictable behavior. Thus, although some of these strains have not been associated with severe disease in humans, they can represent a potential hazard for human health.

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