Distribution of the *stx1* and *stx2* genes in *Escherichia coli* isolated from milk cattle according to season, age, and production scale in southwestern region of Goiás, Brazil

[*Distribuição dos genes* stx1 *e* stx2 *em* Escherichia coli *isoladas de bovinos de leite de acordo com a estação, idade e escala de produção na região sudoeste de Goiás, Brasil*]

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ABSTRACT

This study determined the distribution of *stx1* and *stx2* genes in *Escherichia coli* isolated from dairy herds with regard to animal age, season, and farm production-scale, and analyzed the phylogenetic distribution of the groups A, B1, B2, and D of 276 isolates of bovine feces Shiga toxin-producing *E. coli* (STEC). The *stx1* profile was the most common, detected in 20.4% (202/990) of the isolates, followed by *stx2* (4.54%, 45/990) and *stx1+stx2* (2.92%, 29/990). The *stx1* gene was detected more frequently in calves than in adult animals. In the dry season (winter), the presence of *stx1+stx2* profile in cattle feces was higher than in the rainy season (summer), while no significant changes were observed between seasons for the *stx1* and *stx2* profiles. The most predominant phylogenetic groups in adult animals were B1, A, and D, while groups A and B1 prevailed in calves. Our data highlight the importance of identifying STEC reservoirs, since 7.5% of the tested isolates were positive for *stx2*, the main profile responsible for the hemolytic-uremic syndrome (HUS). Moreover, these microorganisms are adapted to survive even in hostile environments and can contaminate the food production chain, posing a significant risk to consumers of animal products.

Keywords: Shiga toxin-producing *Escherichia coli* (STEC), phylogenetic STEC groups, STEC reservoirs; Stx1, Stx2

RESUMO

Esse estudo determinou a distribuição dos genes stx1 *e* stx2 *em* Escherichia coli *isolados de rebanhos leiteiros em relação a idade, estação e produção, e analisaram a distribuição filogenética dos grupos A, B1, B2 e D de 276* E. coli *produtoras de toxina Shiga (STEC). O perfil* stx1 *foi mais comum, detectado em 20,4% (202/990) dos isolados, seguido de* stx2 *(4,54%, 45/990) e* stx1+stx2 *(2,92%, 29/990). O gene* stx1 *foi detectado mais frequentemente em bezerros que animais adultos. No período de seca (inverno), a presença do perfil* stx1+stx2 *nas fezes dos bovinos foi mais prevalente que no período chuvoso (verão), apesar de não haver diferença significativa entre estações para os perfis* stx1 *e* stx2. *Os grupos filogenéticos mais predominantes em animais adultos foram B1, A e D, enquanto grupos A e B2 prevaleceram em bezerros. Nossos dados enfatizam a importância de se detectar reservatórios de STEC já que 7,5% dos isolados testados foram positivos para* stx2, *o perfil mais prevalente em casos de síndrome hemolítica-urêmica. Ademais, esses microorganismos são adaptados à sobreviver em ambientes hostis e contaminam a cadeia alimentar, levando a risco significativo para consumidores de alimentos de origem animal.*

Palavras-chave: Escherichia coli *produtora de toxina shiga (STEC), grupos STEC filogenéticos, reservatórios STEC, Stx1, Stx2*

INTRODUCTION

Enteric disease-inducing *Escherichia coli* have been divided into pathotypes, based on their virulence factors and disease-causing mechanisms (Farrokh *et al.*, 2013). Despite of this fact, the potent cytotoxic produced strains known as Shiga toxin (Stx) are defined as STEC (Shiga toxin-producing *Escherichia coli*) (Nataro and Kaper, 1998). STEC strains can cause hemorrhagic colitis (HC) and the hemolytic-uremic syndrome (HUS), which have been related to the consumption of different types of foods, especially of bovine products. Healthy beef or dairy cattle are the largest reservoirs of several serotypes of STEC, including the serotypes O157 and non-O157 serotypes, and can be transmitted through contaminated water or food and by direct contact with feces of animal carriers (Chiang *et al.*, 2011).

In cattle, STEC colonize the intestinal epithelium, especially in the mucosa adjacent to the anorectal junction, where they are present as commensal microbiota without causing damage to the host (Bolton, 2011). Apart from other factors, such as animal age, sex, and nutrition, the main factor repeatedly indicated as influencing the occurrence of STEC in feces is seasonality (Fernández *et al.*, 2009; Jeon *et al.*, 2013; Ferreira *et al.*, 2014). The elimination in feces decreases dramatically in fall and winter, increases in spring, and peaks in the summer months (Van Donkersgoed *et al.*, 1999; Arthur *et al.*, 2009).

Stx1 is a similar cytotoxin to that produced by *Shigella dysenteriae* serotype 1, but antigenically distinct (O'Brien and Holmes, 1987). The Stx2 cytotoxin has little similarity to Stx1 and Shiga toxin (Stx) from *S. dysenteriae* type 1. Also, Stx2 cannot be recognized and neutralized by the antibodies against the latter two cytotoxin (Scheutz *et al.*, 2012). Stx1 and Stx2 are also known as verotoxins (VT), and in this study, cytotoxicity was tested in Vero cells derived from African green monkey kidney (*Cercopithecus aethiops;* Konowalchuk *et al.*, 1977). The most important STEC subgroup contains the called strains enterohemorrhagic *E. coli* (EHEC) (Nataro and Kaper, 1998). EHEC subgroup is able to colonize the human intestinal mucosa and produce an effacing lesion by a type III secretion system (McDaniel *et al.*, 1995;

Kaper *et al.*, 2004). This subgroup is an important etiologic agent of diseases in humans, including diarrhea, HC, HUS and renal insufficiency (Repetto, 20005; Karch *et al.*, 2005).

The state of Goiás plays an important role in dairy farming in Brazil and is currently ranked as the sixth largest milk-producing state of the country (IBGE, 2017). Since cattle is the main reservoir of STEC, it is important to understand the potential pathogenic risk of STEC isolates and their impact on public health in the state supply chain. Previously, other studies described the prevalence of STEC isolated from healthy cattle in 34 dairy farms localized in the southwestern region of Goiás state, Brazil. It was also evaluated the main risk factors for STEC infection (Freitas-Filho *et al.*, 2014; Ferreira *et al.*, 2014). The aim of the present study was to evaluate the distribution of *stx1* and *stx2* genes in *E. coli* isolated from healthy cattle, taking into account the season, animal age, and farm production scale, as well as to analyze the pathogenic potential of isolated STEC by the phylogenetic classification (Clermont *et al.*, 2000).

MATERIAL AND METHODS

Nine hundred and ninety *E. coli* isolates used in this study were obtained from stool samples of healthy cattle (198 animals) from 34 dairy farms located in the municipality of Jataí, Goiás, Brazil. The 34 farms were divided into three production-scale groups: Low output (100L/milk/day); medium output (100 to 400L/milk/day); and high output (overt to 400L/milk/day). Rectal swab samples were collected and placed in Stuart transport medium (Mumbai, India). The samples were inoculated on MacConkey agar and incubated for 24h at 37°C. Colonies with growth characteristics of *E. coli* (five per plate) were subcultured and identified by biochemical tests of lactose fermentation, indole production, methyl-red and Voges-Proskauer reactions, citrate utilization, urease production, and by the production of hydrogen sulphide (H_2S).

Whole-cell DNA was extracted as previously described (Keskimaki *et al.*, 2001). The PCR reactions were performed in a total volume of 25µL mixture with 2.5µL of the extracted DNA,

10pmol of each primer: universal primer *16SrRNA* (internal control) forward (F) (5'-CCCCCTGGACGAAGACTGAC-3') and reverse (R) (5'-ACCGCTGGCAACAAAGGATA-3'); *stx1*-F (5'-TCTCAGTGGGCGTTCTTATG-3') and *stx1*-R (5'-TACCCCCTCAACTGCTAATA-3') (Wang *et al.*, 2002); *stx2*-F (5'-GGCACTGTCTGAAACTGCTCC-3') and *stx2*-R (5'-GGCACTGTCTGAAACTGCTCC-3') (Paton and Paton, 1998); and GoTaq® DNA polymerase following manufacturer's instructions (Promega, Madison, Wisconsin, USA). *E. coli* O157:H7 EDL933 and *Klebsiella pneumoniae* ATCC BAA 1705 were used as positive and negative controls, respectively.

The phylogenetic groups were determined in simultaneous amplification tests (PCR-triplex) to identify the genes *chuA*, *yjaA* and fragment TspE4.C2. The oligonucleotide pairs used were *chuA*-F (5'-GACGAACCAACGGTCAGGAT-3') and *chuA*-R (5'-TGCCGCCAGTACCAAAGACA-3'), *yjaA*-F (5'-TGAAGTGTCAGGAGACGCTG-3') and *yjaA*-R (5'-ATGGAGAATGCGTTCCTCAAC-3'), and TspE4.C2-F (5'-GAGTAATGTCGGGGCATTCA-3') and TspE4.C2-R (5'-CGCGCCAACAAAGTATTACG-3'). The groups B2 ($chuA^+/yjaA^+$), D ($chuA^+/yjaA^-$), B1 ($chuA$-/TspE4.C2$^+$), and A ($chuA$-/TspE4.C2$^-$) were classified phylogenetically, as proposed by to Clermont *et al.* (2000). Comparative statistical data analysis was performed by Fisher's exact test.

In the analysis of possible risk factors for STEC presence in cattle, all data were analyzed using the Statistical Analysis System ("Statistical…, 2010) v.9.0, at 5% probability. The effects of animal age, season, and farm output scale on STEC occurrence were analyzed by logistic regression with dichotomous response, using the Wald and odds ratio tests, considering all effects in the full model, with subsequent modeling, after eliminating the non-significant effects.

The study procedures were carried out and the animals treated according to international standards and in compliance with the ethical principles of animal experimentation established by the Colégio Brasileiro de Experimentação Animal (COBEA) (protocol: 206/2009/COEP/ PRPPG/UFG).

RESULTS

The genotyping of the 990 *E. coli* isolates showed that 27.9% (276/990) of the isolates carried one or more of the investigated *stx1* and/or *stx2* genes. *stx1* profile was the most common and was presented in 20.4% (202/990) of the isolates, followed by *stx2* (4.54%; 45/990) and *stx1*+*stx2* profile (2.92%; 29/990). The percentage of STEC-carrying animals was 45.4% (90/198). The target profiles were detected more frequently in calves than in adult animals, although the variability was not significant (Table 1). The season had an influence in STEC prevalence. The *stx1*+*stx2* profile demonstrated higher percentage in cattle isolates in the dry season (winter) than in the rainy season (summer), while no significant changes between seasons were observed for *stx1* and *stx2* profiles analysis.

The 276 STEC strains were divided into four groups (B2, D, B1, and A) by phylogenetic characterization and distributed according to the season and animal age (Table 2). The pathogenic group D was most prevalent in the winter season. There was no difference between the profile distribution of phylogenetic groups in cows and calves.

Table 1. Prevalence of *Escherichia coli* isolated from dairy cattle, carrying *stx1*, *stx2* or *stx1*+*stx2* genes, according to the age, season and farm production scale

stx genotype	AGE[a]		Season[a,b]		Farm Production Scale[b]			Total
	Cow	Calf	Winter	Summer	Low	Medium	High	
stx1	60	142	91	111	95 A	91 A	16 B	202
stx2	15	30	24	21	19 A	21 A	5 B	45
stx1+*stx2*	5	24	21 A	8 B	21 A	8 B	0 C	29
Total	80	196	136	140	135	120	21	276

[a]Non-significant (P> 0.01) for all genes according to the animal age, and for *stx1* and *stx2* genes according to the season.
[b]Different letters in a row indicate significantly different (P< 0.01), and same letters in a row statistically non significantly different values.

Table 2. Prevalence of the phylogenetic groups B2, D, B1, and A of STEC strains isolated from dairy cattle, according to the season and animal age

Phylogenetic group	SEASON		AGE		Total
	Summer	Winter	Cow	Calf	
Group B2 ($chuA^+/yjaA^+$)	2	0	1	1	2
Group D ($chuA^+/yjaA^-$)	11	51	18	44	62
Group B1 ($chuA^-/TspE4.C2^+$)	76	54	43	87	130
Group A ($chuA^-/TspE4.C2^-$)	51	31	18	64	82
Total	140	136	80	196	276

DISCUSSION

Some STEC serotypes can induce human diseases, although not all serotypes found in cattle or foods are associated with severe disease in humans. Several STEC outbreaks have been reported, and one fatal occurred in Sakayin, Japan, in July 1996, with 8,000 cases and 6 deaths (Vidovic *et al.*, 2011). In the present study, an extremely high number of STEC-carrying animals (45.4%) was detected. The rates of prevalence in cattle have been determined for decades; however, comparisons between these studies are hampered by the differences in sampling and methodologies (Chase-Topping *et al.*, 2008).

Some factors that contribute to the presence and spread of STEC in a herd are the management practices, stress, diet, population density, geographic region, and season (Fernández *et al.*, 2009; Jeon *et al.*, 2013; Ferreira *et al.*, 2014). The seasonal pattern with peaks in the summer leads to a similar variation in the human disease associated with the agent (Ostroff *et al.*, 1989). However, in the present study, a greater isolation of STEC was detected in the winter (dry season), contrasting with Dunn *et al.* (2004), whom reported a higher STEC frequency in the summer and lower frequency in the winter. The greater presence of isolates carrying both *stx1* and *stx2* genes in the cold and dry months can be related to the pathogenicity, once better adapted strains to cold and drought stress could be more adapted to survive in hostile environments (Allen *et al.*, 2008), e.g., in refrigerated foods of animal origin. Foods of animal origin (milk and meat) were identified as the most important source of STEC entry into the food chain (Martin and Beutin, 2011). In cattle stool samples analyzed at slaughter in an abattoir, Fukushima and Seki (2004) determined a prevalence of 37.5% of STEC isolates.

The present study demonstrated that young animals could be major source of STEC excretion in the environment. The same conclusion was also demonstrated by Werber *et al.* (2007), who stated that young animals have a greater prevalence of colonization with *stx* profiles of STEC than adult animals, and the prevalence in calves older than two months is higher than in younger calves. The results of 70.3% *stx1*-positive isolates and 29.7% *stx2*-positive isolates found in the present study confirmed the findings demonstrated by Andrade *et al.* (2012) and Vargas-Jr *et al.* (2017) in healthy calves in, respectively, Minas Gerais and Rio Grande do Sul states, Brazil.

Our data identified a significantly higher percentage (92.4%; 255/276) of all investigated STEC strains in cattle excretion on the medium and small-scale farms. These results demonstrate that the inspection of products derived from these farms needs to be intensified, despite of the increased contamination pressure of milk as well as of meat (Martin and Beutin, 2011). It was also reported that STEC prevalence is greater in food from organic than from conventional farms. This is a matter of concern, because STEC, as much as non-pathogenic *E. coli,* are components of the normal intestinal flora of cattle and the effect of any treatment to control STEC excretion would be limited (Gill and Gill, 2010).

Studies have shown that extraintestinal pathogenic *E. coli* normally belong to groups B2 and D, whereas pathogenic isolates are classified as groups A, B1 and D (Pupo *et al.,* 1997; Picard *et al.,* 1999). The data of our study show that group B1 was predominant (54.3%) in the summer, followed by groups A, D and B2 (with 36.4%, 7.8% and 1.4%, respectively). In winter, there was similarity between B1 (39.7%) and D (37.5%), with lower prevalence of group A (22.8%), while no B2 isolates were found. Previously, studies done by Escobar-Páramo *et*

al. (2004) showed that the prevalence of group B2 was greater in the temperate regions of France than in tropical countries such as Benin, French Guyana and Colombia. The higher prevalence of B1 groups in both periods confirmed the findings demonstrated by Unno *et al.* (2009), whom reported a higher prevalence of this group in their study. However, our study contradicts the hypothesis that *E. coli* isolates from tropical areas belong mostly to group A (Escobar-Páramo *et al.*, 2004), since in the tropical southeastern region of Brazil, the prevalence of group B1 was higher in both summer and winter, and the prevalence of group A increased in the colder season.

Correlating the phylogenetic groups with the animal age, the prevalence of isolates from adult animals was highest for group B1 (53.8%), followed by group A (22.5%), and the prevalence of group D isolates was similar in both age categories (22.5%). In calves as well as in adult animals, there was a higher prevalence of group B1 (44.4%), followed by group A (32.6%). In phylogenetic studies of STEC/EHEC/*eae*⁺ isolates, Tramuta *et al.* (2008) also observed a higher prevalence of group B1 (38.7%), followed by group A (35.5%) in calves. Similar data were obtained in the study carried from Minas Gerais state, Brazil. Coura *et al.* (2017) demonstrated that STEC and EHEC isolates from healthy and diarrheic calf feces belonged mainly to phylogenetic groups B1 and A.

Our data highlight the importance of pathogenic *E. coli* reservoirs, since 7.5% of the tested isolates were positive for *stx2*, which is mainly responsible for HUS. Moreover, these microorganisms, adapted to survive in hostile environments, with significant levels of pathogenic strains excreted in the colder and dry months, can reach the food production chain, representing a significant risk to consumers of animal products.

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