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Enterohemorrhagic *Escherichia coli* O157:H7 from healthy dairy cattle in Mid-West Brazil: occurrence and molecular characterization[1]

Edismauro G. Freitas Filho[2], Marcos R.A. Ferreira[3], Jefferson F.N. Pinto[4], Fabrício R. Conceição[5] and Cecília N. Moreira[3]*

ABSTRACT.- Freitas Filho E.G., Ferreira M.R.A., Pinto J.F.N., Conceição F.R. & Moreira C.N. 2014. **Enterohemorrhagic *Escherichia coli* O157:H7 from healthy dairy cattle in Mid-West Brazil: occurrence and molecular characterization.** *Pesquisa Veterinária Brasileira 34(1):24-28*. Departamento de Medicina Veterinária, Centro de Ciências Agrárias e Biológicas, Universidade Federal de Goiás, Rodovia BR-364 Km 192 n° 3.800, Pq. Industrial, Jataí, GO 75801-615, Brazil. E-mail: cissanm@yahoo.com.br

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 represents the major Shiga toxin-producing *E. coli* (STEC) strain related to large outbreaks and severe diseases such as hemorrhagic colitis (HC) and the potentially lethal hemolytic uremic syndrome (HUS). The aim of this study was to report the occurrence and molecular characterization of O157:H7 isolates obtained by rectal swab from 52 healthy dairy cattle belonging to 21 farms in Mid-West of Brazil. Detection of *16SrRNA*, *stx1*, *stx2*, *rfbO157, fliCh7*, *eae*, *ehxA*, *saa*, *cnf1*, *chuA*, *yjaA* and *TSPE4*.C2 genes was performed by PCR. The isolates were further characterized by serotyping. Two hundred and sixty *E. coli* isolates were obtained, of which 126 were characterized as STEC. Two isolates from the same cow were identified as serotype O157:H7. Both isolates presented the *stx2, eae, ehxA, saa* and *cnf1* virulence factor genes and the *chuA* gene in the phylogenetic classification (virulent group D), suggesting that they were clones. The prevalence of O157:H7 was found to be 1.92% (1/52 animals), demonstrating that healthy dairy cattle from farms in the Mid-West of Brazil are an important reservoir for highly pathogenic *E. coli* O157:H7.

INDEX TERMS: Shiga toxin-producing *Escherichia coli,* STEC, hemorrhagic colitis, hemolytic uremic syndrome, reservoir, virulence factors.

RESUMO.- [***Escherichia coli* enterohemorrágica O157:H7 em bovinos leiteiros saudáveis no Centro-Oeste do Brasil: ocorrência e caracterização molecular.**] *Escherichia coli* enterohemorrágica (EHEC) sorotipo O157:H7 representa as principais cepas de *E. coli* produtoras de toxina Shiga (STEC) relatadas em grandes surtos e doenças graves, tais como colite hemorrágica (CH) e síndrome hemolítica urêmica (SHU), potencialmente letais. O objetivo deste estudo foi reportar a ocorrência e caracterização molecular de STEC O157:H7 isoladas por *swab* retal de 52 bovinos saudáveis pertencentes a 21 rebanhos leiteiros do Centro-Oeste do Brasil. A detecção dos genes *16SrRNA*, *stx1*, *stx2*, *rfbO157, fliCh7*, *eae*, *ehxA*, *saa*, *cnf1*, *chuA*, *yjaA* e *TSPE4*.C2 foi realizada por PCR. Os isolados foram ainda caracterizados por sorotipagem. Dos 260 isolados de *E. coli* obtidos, 126 foram caracterizados como STEC. Dois deles, oriundos do mesmo animal, foram caracterizados como pertencentes ao sorotipo O157:H7. Ambos apresentaram os genes de virulência *stx2, eae, ehxA, saa* e *cnf1* e na caracterização filogenética, o gene *chuA* (grupo patogênico D), sugerindo que eles foram clones. A prevalência de O157:H7 foi de

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[2] Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo (USP), Ribeirão Preto, SP 14049-900, Brazil.

[3] Departamento de Medicina Veterinária, Campus Jataí, Universidade Federal de Goiás (UFG), Rodov. BR-364 Km 192 nº 3.800, Parque Industrial, Cx. Postal 3, Jataí, GO 75801-615, Brazil. *Corresponding author: cissanm@yahoo.com.br

[4] Departamento de Ciências Biológicas, Campus Jataí, UFG, Cx. Postal 3, Jataí, GO 75801-615, Brazil.

[5] Núcleo de Biotecnologia, Centro de Desenvolvimento Tecnológico, Universidade Federal de Pelotas (UFPel), Cx. Postal 354, Pelotas, RS 96010-900, Brazil.

1,92% (1/52 animais), demonstrando que os bovinos leiteiros saudáveis de fazendas do Centro-Oeste do Brasil são importantes reservatórios de *E. coli* O157:H7 altamente patogênicas.

TERMOS DE INDEXAÇÃO: *Escherichia coli* produtoras de toxina Shiga, STEC, colite hemorrágica, síndrome hemolítica urêmica, reservatório, fatores de virulência.

INTRODUCTION

Brazil exports milk to several countries, and Goiás State (GO, Mid-West of Brazil) is the fourth largest producer, being responsible for 10.9% of the total production of 32.1 billion liters a year. Jataí city in GO is the third larger producer nationally (IBGE 2012). The milk yield in the Brazilian Midwest increased yearly by 5.13% from 1990 to 2010, contributing to a national increase of 3.66% (Sousa et al. 2012). Currently, the control standards of Brazilian milk quality have become more stringent in order to avoid health problems for the consumer.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are subsets of Shiga toxin (Stx)-producing *E. coli* (STEC) that are responsible for severe disease in humans. About 200 STEC serotypes have been isolated from animal and food sources, although not all are implicated in illness. However, serotype O157:H7 is the major STEC strain related to large outbreaks and severe diseases such as hemorrhagic colitis (HC) and the potentially lethal hemolytic uremic syndrome (HUS) (Paton & Paton 2002, Pennington 2010, Karmali et al. 2010). However, death has not been associated with many disease outbreaks in South American countries (Rúgeles et al. 2010, Tanaro et al. 2010, Rivero et al. 2011).

Human diseases caused by STEC involve at least one of the Shiga toxins (stx1 and stx2) that function as an N--glycosidase, cleaving a specific adenine from the 28S rRNA, thereby halting protein synthesis (Johnson & Nolan 2009). Other virulence factors involved are: intimin, an adhesin associated with a microscopic lesion, the attaching and effacing lesion, in intestinal epithelial cells, it is characterized by the destruction of host cell microvilli and intimate attachment of the bacteria to cup-like pedestals at the apical cell membrane from the host intestinal mucosa (Wang et al. 2002); Enterohemolysin, a pore-forming RTX toxin cytolysin, which is active on sheep erythrocytes and certain bovine lymphoma cell lines, and only rarely makes adherence possible for *eae* negative strains providing an example of a particularly virulent serotype (Cookson et al. 2007); STEC agglutinating adhesion (Saa), an adherence factor that is more important for attachment in the gut of animals than in humans (Bolton et al. 2011); and CNF-1 (cytotoxic necrotizing factor), which behaves as a virulence factor in urinary or digestive tract infections by stimulating PMNL cytotoxicity as a result of enhanced adherence to epithelial cells as well as the production of radical oxygen products (Blanco et al. 1996).

STEC strains are part of the intestinal microbiota of cattle, making them the primary reservoir for *E. coli* O157:H7. Transmission to humans occurs through consumption of undercooked ground (minced) beef, unpasteurized milk, dairy products and vegetables or water contaminated with cattle feces (Cergole-Novella et al. 2006, Sandrini et al. 2007, Pennington 2010).

The aim of this study was to report the occurrence and molecular characterization of O157:H7 isolates obtained by rectal swab from 52 healthy dairy cattle belonging to 21 farms in Mid-West of Brazil.

MATERIALS AND METHODS

Over a period of 10 months, from February to December 2012, a rectal swab was collected from each of 52 dairy non-diarrheic animals. Each swab was used to inoculate Stuart medium tubes (Difco Laboratories, Detroit, MI, USA), stored in an ice-pack container and analyzed within 24 hours. The farms were localized in different cities of the South West State. The samples were taken from 31 calves (less than 11 months old) and 21 cows (more than 24 months old). The fecal samples were streaked onto Levine BEM agar (Difco, Detroit, MI, USA) and incubated at 37°C for 24 h. At least five individual suspect *Escherichia coli* colonies each animal (dark with a greenish metallic sheen) were chosen and their identity was confirmed by biochemical tests, including the utilization of citrate and the production of indole, acetoin and methyl red reactive compounds (Feng et al. 2009).

DNA samples were extracted from the isolates (n=260) according to Keskimaki et al. (2001). Initially, these samples were analyzed by PCR for the presence of *16SrRNA* (internal control), *stx1* and *stx2*, for STEC characterization*.* All isolates obtained were characterized by serotyping (tube agglutination test) at the Enterobacteria Laboratory at the Oswaldo Cruz Institute (Fiocruz, Rio de Janeiro, Brazil). DNA samples from 126 STEC positive isolates were analyzed by PCR for the presence of the *rfbO157* and *fliCh7* genes, for identification of O157:H7 isolates. DNA from the O157:H7 positive isolates was analyzed by PCR for the presence of the *eae, ehxA, saa* and *cnf1* virulence factor genes. The primers used in this study are shown in Supplementary Table S1. The amplification protocol was carried out in a MJ Research thermocycler using the PCR test conditions as described previously: *stx2* and *rfbO157* (Paton & Paton 1998), *fliCh7* (Gannon et al. 1997), *stx1* and *eae* (Wang et al. 2002), *saa* (Paton & Paton 2002), *ehxA* (Blanco et al. 2004) and *cnf1* (Yamamoto et al. 1995). *E. coli* O157:H7 and *Klebsiella pneumoniae* DNA were used as positive and negative controls, respectively. These control strains, belonging to a collection maintained in the Technology Development Center/ Biotechnology of the Federal University of Pelotas, were characterized by genotypic and phenotypic methods.

Phylogenetic classification of *E. coli* O157:H7 was performed by PCR following Clermont et al. (2000) using the *chuA, yjaA* and *TSPE4*.C2 genes (Supplementary Table 1). The 232/96 strain, kindly provided by the Laboratory of Bacteriology at the Federal University of Santa Maria, was used as a positive control.

RESULTS

Of the 260 colonies from the rectal swabs collected from 52 healthy animals from 21 farms, 126 were STEC positive. Of the STEC positive isolates, two were identified as O157:H7 by PCR and serotyping, and both isolates originated from the same cow, suggesting that they were clones. These isolates were PCR positive for the *16SrRNA* and *stx2* virulence factor genes (Fig.1a). Phylogenetic classification of the isolates characterized the *chuA* gene as belonging to virulent group D (Fig.1b). In addition, the virulence factor genes *fliCh7, rfbO157* and *eae* were detected these isolates (Fig.1c).

Table 1. Primer pairs used in the PCR for identification of the genes encoding virulence factors and phylogenetic classification

Target gene	Primer Sequence	Amplicon (bp)	Location within gene	GenBank accession
16SrRNA	CCCCCTGGACGAAGACTGAC	401	1682–1701	AB035924
	ACCGCTGGCAACAAAGGATA		2063–2082	
stx1	TCTCAGTGGGCGTTCTTATG	338	777-796	M17358
	TACCCCCTCAACTGCTAATA		1095-1114	
stx2	GGCACTGTCTGAAACTGCTCC	255	603-623	NC_004914
	TCGCCAGTTATCTGACATTCTG		837-857	
eae	ATGCTTAGTGCTGGTTTAGG	248	132-151	Z11541.1
	GCCTTCATCATTTCGCTTTC		360-379	
saa	CGTGATGAACAGGCTATTGC	119	1423-1442	NC_007365.1
	ATGGACATGCCTGTGGCAAC		1522-1541	
ehxA	GGTGCAGCAGAAAAAGTTGTAG	1.551	238–259	ES204929.1
	TCTCGCCTGATAGTGTTTGGTA		1767-1788	
rfbO157	CGGACATCCATGTGATATGG	259	393–412	JF713072.1
	TTGCCTATGTACAGCTAATCC		631-651	
cnf1	AAGATGGAGTTTCCTATGCAGCAG	498	794-817	NC_00796.1
	CATTCAGAGTCCTGCCCTCATTATT		1267-1291	
chuA	GACGAACCAACGGTCAGGAT	279	245-264	AF280396.1
	TGCCGCCAGTACCAAAGACA		504-523	
yjaA	TGAAGTGTCAGGAGACGCTG	211	66-84	NC_007779.1
	ATGGAGAATGCGTTCCTCAAC		257-276	
TSPE4.C2	GAGTAATGTCGGGGCATTCA	152	421-440	AE014075.1
	CGCGCCAACAAAGTATTACG		553-572	
fliCh7	GCGCTGTCGAGTTCTATCGAGC	625	69–91	AB781292.1
	CAACGGTGACTTTATCGCCATTCC		671–694	

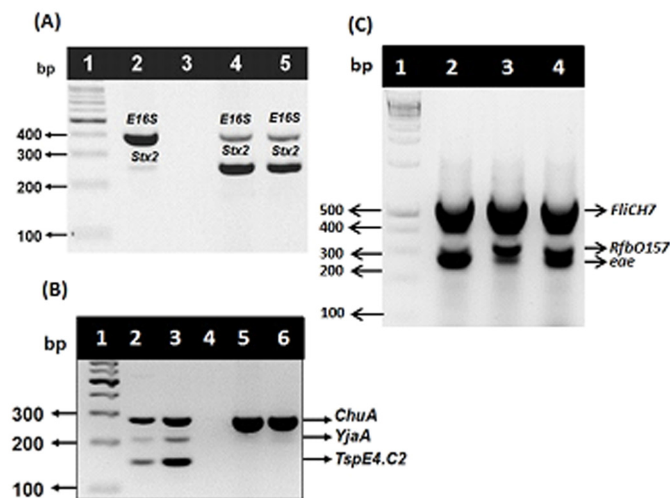

Fig.1. (**A**) Analysis of the PCR products in a 1.5% agarose gel for the presence of virulence genes in the two O157:H7 isolates. Lane 1: molecular mass marker (fragment size 900 to 100 bp), lane 2: positive control, lane 3: negative control, lane 4: O157:H7 isolate 1 and lane 5: O157:H7 isolate 2. Both isolates were positive for *16SrRNA* (401 bp), *stx2* (255 bp). (**B**) Analysis of PCR products in a 1.5% agarose gel, showing the phylogenetic classification. Lane 1: molecular mass marker (fragment size 700 to 100 bp), lanes 2 and 3: positive control for group B2 containing the three genes: *chuA* (279 bp), *yjaA* (211 bp) and *TspE4.C2* (152 bp), lane 4: negative control, lanes 5 and 6: the two O157:H7 isolates positive for *chuA* only, characterizing them as group D. (**C**) Analysis of the PCR products in a 1.5% agarose gel for the presence of virulence genes in the two O157:H7 isolates. Lane 1: molecular mass marker (fragment size 900 to 100 bp), lane 2: positive control, lane 3: O157:H7 isolate 1, lane 4: O157:H7 isolate 2. Both isolates were positive for *fliCh7* (625 bp), *rfbO157* (259 bp) and *eae* (248 bp).

The prevalence of O157:H7 in cows was 4.76% (1/21), 0% (0/31) for calves and 1.92% (1/52) for all the animals studied. The farms prevalence was 4.76% (1/21).

DISCUSSION

EHEC O157:H7 is not considered pathogenic for domestic animals and its occurrence in animals with enteric disease is considered incidental. However, it is a relatively common gut commensal, particularly in cattle, therefore livestock can act as a reservoir for human infection, with transmission either via either consumption of contaminated food (Reinstein et al. 2009) or through direct or indirect contact with animals or their feces (Rivero et al. 2011). Moreover, Bolton et al. (2011) showed that, although O157:H7 strains do not occur frequently in pasture soils, they can persist in such an environment for several months, increasing the risk of infection for cattle. To date, no information has been published on the O157:H7 status of dairy cattle in GO. This information is relevant due to the high milk production in the region.

In this study, one animal from 52 healthy dairy cattle was positive for the presence of O157:H7 in a rectal swab. This is equivalent to a prevalence of 1.92%. The reported prevalence of O157:H7 in dairy cattle is highly variable. In the USA, Reinstein et al. (2009) reported a prevalence of 6.5% (2/322). In Argentina, Tanaro et al. (2010) found that 11 out of the 288 (3.8%) fecal samples were O157 positive. In Brazil, Vicente et al. (2005) found a prevalence as high as 18.9% (86/454) for serogroup O157 in some herds in São Paulo State. However, Sandrini et al. (2007) reported a prevalence of less than 0.3% (3/1127) in dairy cattle from Rio Grande do Sul State and Cerqueira et al. (1999) observed a prevalence of 1.5% (3/197) for O157:H7 in healthy dairy cattle from Rio de Janeiro State.

The presence of EHEC virulence markers in *E. coli* isolates represents a potential risk to human health and all O157:H7 isolates possess a common combination of virulence factors: *stx2*, *eae* and *rfbO157* (Pennington 2010). The two isolates identified in the current study included these virulent factors. Based on the epidemiological and experimental data, the frequency of the severe complications that can occur in bloody diarrhea is dependent on the toxin produced (Persson et al. 2007). The *stx2* toxin may be a more significant factor for the development of HUS than *stx1*. Furthermore, the presence of *stx2* has been associated with a more virulent infection, partly due to its increased expression (Chattaway et al. 2011).

The enterohaemorrhagic haemolysin EhxA makes adherence possible for *eaeA* negative strains (Mainil & Daube 2005). When both the *ehxA* and the *eae* genes are present this is an indicator for increased pathogenicity (Clermont et al. 2000). Both of the O157:H7 isolates characterized in this study contained the *ehxA* and *eae* genes. EhxA is required for infection in humans and is common in ruminant STEC, providing further evidence of the link between bovine STEC and human disease (Bolton 2011). In Argentina, Padola et al. (2004) and Tanaro et al. (2010) showed that all O157:H7 isolates were positive for the *eae* and *ehxA* genes.

The *saa* gene, responsible for producing autoagglutinating adhesin, has been involved in development of HUS (Paton et al. 2001). Several studies have showed that STEC isolated from cattle presenting *saa* are *eae*-negative (Paton et al. 2001, Toma et al. 2004, Cergole-Novella et al. 2006). Controversially, this study revealed the presence of both genes (*saa* and *eae*) in both O157:H7 isolates, highlighting the pathogenic potential of them.

Pathogenicity markers in *E. coli* have been used in studies for phylogenetic classification to understand the evolution of microorganisms and they can be classified into four main groups: A, B1, B2 and D (Chao & Dreyfus 1997). The virulent strains belong to groups B2 are characterized by the presence of *chuA*, *yjaA* and TSPE4.C2. The *chuA* gene is necessary for heme transport in EHEC, the function of the *yjaA* gene remains unknown and the TSPE4.C2 fragment is situated within a gene encoding a putative lipase esterase (Gordon et al. 2008). Strains classified into group D contain only the *chuA* gene. In this study, both of the O157:H7 isolates were PCR positive for the presence of the *chuA* gene, indicating that they belonged to group D, suggesting that they had the potential to be highly pathogenic.

CONCLUSIONS

Both of the O157:H7 isolates were considered highly pathogenic, as they were positive for the presence of the *stx2, eae, ehxA, saa*, *cnf1* and *chuA* virulence factor genes, thereby representing a potential risk for humans.

The results of the current study suggest that healthy dairy cattle from Mid-West of Brazil may be an important reservoir of highly pathogenic O157:H7 and that their farms are potential sources of environmental contamination through shedding of microorganisms in cattle feces.

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