Detection of virulence factors and antimicrobial resistance patterns in shiga toxin-producing *Escherichia coli* isolates from sheep[1]

Marcos R.A. Ferreira[2], Talícia dos S. Silva[3], Ariel E. Stella[4], Fabricio R. Conceição[2], Edésio F. dos Reis[5] and Cecília N. Moreira[4]*

ABSTRACT.- Ferreira M.R.A., Silva T.S., Stella A.E., Conceição F.R., Reis E.F. & Moreira C.N. 2015. **Detection of virulence factors and antimicrobial resistance patterns in Shiga toxin-producing *Escherichia coli* isolates from sheep.** *Pesquisa Veterinária Brasileira 35(9):775-780.* Departamento de Medicina Veterinária, Universidade Federal de Goiás, Regional Jataí, Rodovia BR-364 Km 192 nº3.800, Parque Industrial, Cx. Postal 3, Jataí GO 75801-615, Brazil. E-mail: cissanm@yahoo.com.br

In order to detect virulence factors in Shiga toxin-producing *Escherichia coli* (STEC) isolates and investigate the antimicrobial resistance profile, rectal swabs were collected from healthy sheep of the races Santa Inês and Dorper. Of the 115 *E. coli* isolates obtained, 78.3% (90/115) were characterized as STEC, of which 52.2% (47/90) carried *stx1* gene, 33.3% (30/90) *stx2* and 14.5% (13/90) both genes. In search of virulence factors, 47.7% and 32.2% of the isolates carried the genes *saa* and *cnf1*. According to the analysis of the antimicrobial resistance profile, 83.3% (75/90) were resistant to at least one of the antibiotics tested. In phylogenetic classification grouped 24.4% (22/90) in group D (pathogenic), 32.2% (29/90) in group B1 (commensal) and 43.3% (39/90) in group A (commensal). The presence of several virulence factors as well as the high number of multiresistant isolates found in this study support the statement that sheep are potential carriers of pathogens threatening public health.

INDEX TERMS: *Escherichia coli,* STEC, multiresistant isolates, molecular characterization, phylogenetic.

RESUMO.- [**Detecção de fatores de virulência e de resistência antimicrobiana em isolados produtores de toxina Shiga de ovinos.**] A fim de detectar os fatores de virulência em isolados de *E. coli* produtoras de toxina Shiga (STEC) e investigar o perfil de resistência aos antimicrobianos, *swabs* retais foram coletados em ovelhas saudáveis das raças Santa Inês e Dorper. Dos 115 isolados de *E. coli* obtidos, 78,3% (90/115) foram caracterizados como STEC, dos quais 52,2% (47/90) possuíam o gene *stx1*, 33,3% (30/90) *stx2* e 14,5% (13/90) ambos os genes. Em busca de fatores de virulência, 47,7% e 32,2% dos isolados apresentaram genes *saa* e *cnf1*. De acordo com a análise do perfil de resistência a antimicrobianos, 83,3% (75/90) eram resistentes a pelo menos um dos antibióticos testados. Na classificação filogenética, os isolados foram agrupados 24,4% (22/90) no grupo D (patogênico), 32,2% (29/90) no grupo B1 (comensal) e 43,3% (39/90) no grupo A (comensal). A presença de vários fatores de virulência, bem como o elevado número de isolados multirresistentes encontrados neste estudo apoia a afirmação de que as ovelhas são portadoras potenciais de patógenos que ameaçam a saúde pública.

TERMOS DE INDEXAÇÃO: *Escherichia coli*, STEC, isolados multirresistentes, caracterização molecular, filogenético.

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) microorganisms cause severe human diseases, e.g., hemorrhagic co-

litis (HC) and hemolytic uremic syndrome (HUS) (Nataro & Kaper 1998). The HUS-causing STEC isolates are called enterohemorrhagic *E. coli* (EHEC) (Mainil 1999). They colonize the gastrointestinal tract of cattle and adult sheep without causing illness, but can induce serious diarrheagenic diseases in animals after weaning (Gyles et al. 2010, Ferens & Hovde 2011, Kumar et al. 2012). The high prevalence of STEC isolates in these young animals represents a threat to public health for being highly pathogenic for humans (Gyles et al. 2010).

Healthy cattle are the main STEC reservoir, although some studies suggest that sheep also contribute significantly to the spread of these pathogens and consequently to the risk of human infection (Ferens & Hovde 2011). The EHEC serotype O157:H7 is the most relevant for humans and has been isolated worldwide from animal (meat and dairy) products and from feces of healthy sheep (Kumar et al. 2012, Momtaz et al. 2012).

Shiga toxins (stx) 1 and 2, encoded by the genes *stx1* and *stx2*, respectively, correspond to the major virulence factor of STEC and are responsible for blocking protein synthesis in eukaryotic cells (Gyles et al. 2010). The stx1 toxins have an amino acid sequence that is similar to that of the cytotoxin produced by *Shigella dysenteriae* serotype 1, though antigenically different (Beutin et al. 1997, Djordjevic et al. 2001). Epidemiological data indicate that stx2 is more often associated with severe diseases and HUS development than stx1 (Orth et al. 2007, Kawano et al. 2008). Although the production of stx1 and/or stx2 and variants represents the main virulence factor of STEC, the ability of adhesion to the intestinal epithelium is the primary pathogenicity factor. The *eae* gene, which encodes intimin, is a 94-kDa membrane protein involved in intestinal cell adhesion that triggers attaching and effacing injury (AE) (Jerse et al. 1990, McDaniel et al. 1995). Another important adhesin in STEC that do not encode the locus of enterocyte effacement (LEE-negative) is the STEC autoagglutinating adhesin (SAA) responsible for the adhesion and colonization of the bovine and ovine intestinal epithelium (Jenkins et al. 2003, Zweifel et al. 2005, Kumar et al. 2012).

Cytotoxic-necrotizing factors (CNFs) can be separated in two types of cytotoxins, CNF1 and CNF2 (Blanco et al. 1992). These cytotoxic-necrotizing factors are responsible for diarrheagenic diseases and other clinical signs in young lambs, but data in the literature on the distribution in healthy sheep and human are rather limited. CNF1-positive isolates (NTEC) have been widely reported in extra-intestinal infections (urinary tract infections - UTIs) (Dozois et al. 1997).

The indiscriminate use of antimicrobials in animal production paves the way for the development of resistance in microorganisms, both in commensal and potentially zoonotic pathogenic isolates (Zhang et al. 2009). In the last few years, the number of pathogenic isolates with antimicrobial multiresistance has increased, which narrows the options of antibiotics to treat human infections (Saei et al. 2012).

By evolutionary phenomena, several differentiated bacterial groups have emerged within microbial species. The classification of isolates based on the distribution analysis of pathogenicity markers in phylogenetic groups allowed the understanding of how pathogenic isolates acquired virulence genes (Johnson et al. 2001). In a pioneering study, Clermont et al. (2000) used the amplification of the genes *chuA*, *yjaA* and *TspE4.C2* to classify *E. coli* isolates in four major phylogenetic groups (A, B1, B2 and D).

Currently, a sheep herd with a total of approximately 191,000 animals is maintained, primarily for meat production, in the state of Goiás, Brazil (IBGE 2014). In view of the importance of sheep in the State economy and also in view of the potential spread of public health-threatening pathogens, the aim of this study was to characterize the virulence factors of STEC isolates obtained from rectal swabs of healthy sheep in this State, evaluating the prevalence of O157:H7 and non-O157:H7 isolates by molecular characterization, as well as determine the profile of antimicrobial resistance and phylogenetic distribution of these isolates.

MATERIALS AND METHODS

Sampling and *Escherichia coli* isolation. Rectal swabs of 23 healthy Santa Inês and Dorper sheep on a farm in the municipality of Jataí, GO, were collected, 17 of which were adult Santa Inês females, one an adult Dorper male, and five Santa Inês x Dorper crossbred lambs, of which three females and two males, totaling 18 adults and five young animals. The swabs were inoculated in Stuart medium tubes (Difco Laboratories, Detroit, MI, USA), ice-cooled, and analyzed within 24 h. The fecal samples were streaked onto Levine BEM agar (Difco, Detroit, MI, USA) and incubated at 37°C for 24 h. At least five suspect individual *E. coli* colonies (dark with a greenish metallic sheen) per animal were chosen, and their identity was confirmed by biochemical tests, using citrate and the production of indole, acetoin, and methyl red reactive compounds (Koneman et al. 2008).

Molecular characterization. DNA samples were extracted from the isolates (n=115) according to (Keskimäki et al. 2001). Initially, these samples were analyzed by PCR for the presence of *16SrRNA* (internal control), *stx1* and *stx2*, for STEC characterization. DNA samples from 90 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 STEC positive isolates was analyzed by PCR for the presence of the *eae, ehxA, saa* and *cnf1* virulence factor genes. The amplification protocol was carried out in a MJ Research thermocycler using the PCR test conditions and primers 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). The phylogenetic classification of STEC was performed by PCR as described by Clermont et al. (2000) using the *chuA, yjaA* and *TSPE4*.C2 genes. The 232/96 isolate, kindly provided by the Laboratory of Bacteriology in Department of Preventive Veterinary Medicine at the Federal University of Santa Maria, was used as positive control. The primers sequences for molecular characterization of virulence factors of isolates, as well as primers for determination of phylogenetic groups are presented in Table 1. *E. coli* O157:H7 (ATCC EDL 933) and *Klebsiella pneumoniae* (ATCC BAA 1705) DNA were used as positive and negative controls, respectively.

Table 1. Oligonucleotides used in this study

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	
rfbO157	CGGACATCCATGTGATATGG	259	393–412	JF713072.1
	TTGCCTATGTACAGCTAATCC		631-651	
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	
cnf1	AAGATGGAGTTTCCTATGCAGGAG	498	794-817	NC_00796.1
	CATTCAGAGTCCTGCCCTCATTATT		1267-1291	
ehxA	GGTGCAGCAGAAAAAGTTGTAG	1551	238-259	ES204929.1
	TCTCGCCTGATAGTGTTTGGTA		1767-1788	
saa	CGTGATGAACAGGCTATTGC	119	1477-1496	NC_007365.1
	ATGGACATGCCTGTGGCAAC		1576-1595	

The amplified products were viewed after molecular mass determination by 1% agarose gel electrophoresis at 70 V for 120 min, in 1× TBE buffer (Tris-HCl of 89 mM, boric acid of 89 mM, EDTA of 2.5 mM, pH 8.0). A DNA ladder (φX174/*Hae*III) was used as molecular mass pattern; the gels were stained with ethidium bromide (0.5 µg/mL).

Antimicrobial resistance patterns. For the determination of the antimicrobial resistance profile, the isolates were subcultured in BHI broth for 18 hours at 37°C, until turbidity reached 0.5 on the McFarland scale. After growth, isolates were plated on Mueller-Hinton agar (disk diffusion method), using the following antibiotics: 30 µg ceftiofur (CTF), 30 µg amikacin (AMI), 10 µg gentamicin (GEN), 30 µg tetracycline (TET), 30 µg chloramphenicol (CLO), 23.75/1.25 µg sulfametoxazol + trimethoprim (SXT), 30 µg ceftazidin (CAZ), 30 +30 µg amoxicillin + clavulanic acid (AMC), 10 µg nalidixic acid (NAL), 10 µg streptomycin (EST), and 30 µg cefoxitin (CFO). After 18 hours of incubation at 37°C, the inhibition halo in each test was measured and the s classified as resistant, sensitive or intermediate, according to the (Clinical Laboratory Standards Institute 2008).

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

RESULTS

From the 115 *Escherichia coli* sheep isolates, 78.3% (90/115) were characterized as STEC, of which 52.2% (47/90) contained *stx1* gene, 33.3% (30/90) *stx2* and 14.5% (13/90) both *stx1/stx2* genes. In terms of the distribution of STEC isolates per animal category, 60% (15/25) isolated from lambs, 82.2% (70/85) from adult females and 100% (5/5) from adult males. Distribution of the virulence genes according to the profile Shiga toxin of the isolates STEC are discriminates in Table 2. In current work, were not detected STEC/O157:H7-positives (genes

Table 2. Virulence genes according to the profile Shiga toxin of the isolates STEC

Virulence genes (%)	Shiga Toxin		
	stx1	*stx2*	*stx1/stx2*
saa	72.1 (31/43)	18.6 (8/43)	9.3 (6/43)
cnf1	69 (20/29)	24.1 (7/29)	6.9 (2/29)

Table 3. Patterns of antimicrobial resistance and virulence factors in STEC

Antibiotics	Shiga Toxin			Resistance profile (%)
	stx1	*stx2*	*stx1/stx2*	
AMC 30[a]	9	5	4	20 (18/90)
CTF[b]	1	-	-	1.1 (1/90)
AMI[c]	-	-	1	1.1 (1/90)
CFO[d]	-	-	1	1.1 (1/90)
GEN[e]	1	3	-	4.5 (4/90)
NAL[f]	8	6	4	20 (18/90)
EST[g]	16	7	3	28.8 (26/90)
TET[h]	5	5	1	12.2 (11/90)
CLO[i]	1	3	-	4.5 (4/90)
SXT[j]	1	-	-	1.1 (1/90)
CAZ[l]	1	-	4	5.6 (5/90)

[a] Amoxicillin + Clavulanic Acid, [b] Ceftiofur, [c] Amikacin, [d] Cefoxitin, [e] Gentamicin, [f] Nalidixic acid, [g] Streptomycin, [h] Tetracycline, [i] Chloramphenicol, [j] Sulfamethoxazole + Trimethoprim, [l] Ceftazidin.

rfb0157 and *fliCh7*), as well as were negatives for *ehxA* and *eae* genes.

The analysis of the antimicrobial resistance profile showed that 83.3% (75/90) STEC from sheep feces were resistant to at least one antibiotic (mainly to streptomycin, nalidixic acid and amoxicillin 30), 12.2% (11/90) of STEC were resistant to two antibiotics (nalidixic acid + ampicillin 30; nalidixic acid + streptomycin) and 6.7% (6/90) were resistant to more than three antibiotics, mainly to gentamicin, streptomycin and tetracycline. The distributions of the

Table 4. Distribution of STEC isolates with regard to the presence of virulence genes according to the phylogenetic group to which they belong

Phylogenetic group	Virulence gene (%)		
	stx1	stx2	stx1/stx2
A (n=39)	46.2 (17/39)	30.8 (12/39)	23 (10/39)
B1 (n=29)	79.3 (23/29)	17.2 (4/29)	3.5 (2/29)
Commensal A+B1 (n=68)	58.8 (40/68)	23.5 (16/68)	17.7 (12/68)
D (n=22)	31.8 (7/22)	63.6 (14/22)	4.5 (1/22)
B2 (n=0)0 (0)	0 (0)	0 (0)	
Pathogenic D+B2 (n=22)	31.8 (7/22)	63.6 (14/22)	4.5 (1/22)
Total (n=90)	52.2 (47/90)	33.3 (30/90)	14.5 (13/90)

antibiotic resistance profile according to stx production are listed in Table 3.

The phylogenetic classification grouped 24.4% (22/90) in group D (pathogenic), 32.2% (29/90) in group B1 (commensal) and 43.3% (39/90) in group A (commensal). The distribution of STEC isolates with regard to the presence of *stx1* and *stx2* genes according to the phylogenetic group to which they belong is shown in Table 4.

DISCUSSION

Ruminants, in particular healthy cattle and sheep, are the main reservoirs of STEC, by passing the fecal route and acting as a source of contamination of animal products, thus representing a potential pathogenic threat to human health (Martin & Beutin 2011). Studies by Ferreira et al. (2014) and Freitas-Filho et al. (2014) were pioneering in determining the prevalence of STEC and STEC/O157:H7 in healthy cattle in the State of Goiás, Brazil, while our study evaluated the virulence factors and antimicrobial resistance in STEC isolates obtained from healthy sheep in this State. On the contrary, Blanco et al. (2003) found a higher prevalence of non-O157 STEC in sheep compared to cattle, where our study did not reveal the difference between sheep (78.3%) and cattle (72.7%) (Ferreira et al. 2014). However, it would be appropriate evaluation larger number of sheep to determine such difference between those species.

In this study, the prevalence of *stx1* and *stx2* gene in STEC isolates was higher 52.2% (47/90) and 33.3% (30/90) respectively, as similarly found by Ghanbarpour & Kiani (2013) and Blanco et al. (2003) in their studies, where 46.5% (20/43) and 55% (213/384) of the STEC isolates carried *stx1*. On the other hand, Oporto et al. (2008) observed a prevalence of 67.4% of *stx1/stx2*, far above the 17.9% of *stx1* in STEC isolated from sheep in Spain. Experiments with rats indicated that stx2 is 100 times more potent than stx1 (Tesh et al. 1993). In a work carried out in primates, the intravenous inoculation of stx2 produces HUS symptoms, while administration of the same dose of stx1 did not induce the syndrome (Stearns-Kurosawa et al. 2010). Although stx2 is more toxic to humans, stx1 has also been attributed in severe illness such as HUS (Hedican et al. 2009).

In the evaluation of the prevalence of STEC/O157:H7 by multiplex PCR, the results were negative for genes *rfbO157* and *flicH7* in the isolates. Similar results were observed by (Pinaka et al. 2013), who detected no *rfbO157* and *flicH7*

genes in isolates from goats, sheep and cattle, but found *eae* in 54.9% (28/51), which was not confirmed in our study. Also in Spain, the majority of sheep STEC isolates was non--O157, with only 8.7% O157:H7-positive STEC (Oporto et al. 2008). O157 STEC isolates are worldwide involved in different disease outbreaks in humans, while in recent years, outbreaks involving non-O157 isolates have been increasingly reported (Hedican et al. 2009, Scheutz et al. 2011).

In search of genes for other virulence factors, 32.2% of the isolates carried gene *cnf1*, and none of the isolates *eae* and *ehxA*. Sheep can act as main disseminators of this pathogen in animal-derived food products and water and as direct transmitters to humans, however, studies on the distribution of CNF-1 in STEC isolates from healthy sheep are limited. In this study we found the presence of *cnf1* gene in 32.2% (29/90) of STEC isolates from healthy sheep, which is higher than reported in previous studies (Orden et al. 2007, Aragão et al. 2012). The STEC isolates carrying the *cnf1* gene were widely reported in relation to HUS outbreaks in humans (Dozois et al. 1997), and our study confirmed the importance of this virulence factor of STEC isolates from sheep in the state of Goiás as a potential threat to human health.

Paton & Paton (1998) first described the SAA in non--O157 STEC serotypes. Subsequently, several authors reported the presence of the *saa* gene in bovine and ovine STEC isolates, correlating the intestinal colonization in the host with the presence of this gene (Zweifel et al. 2005, Aidar-Ugrinovich et al. 2007). This study was the first to determine the presence of the *saa* gene in ovine STEC in the state of Goiás, reporting a prevalence of 47.7% (43/90). The *saa* gene is potential pathogenic marker in *eae*-negatives STEC isolates, and its product can cause a systemic and gastrointestinal disease in humans (Rey et al. 2003). Jenkins et al. (2003) reported *saa*-positives STEC in 13% (6/46) of human patients with HUS and 7% (2/29) of STEC isolates of patients with diarrhea or bloody diarrhea.

The resistance profile showed that 83.3% (75/90) of the antimicrobials were resistant to at least one of the antibiotics tested. The results indicate greater resistance to streptomycin (25.5%), amoxicillin + clavulanic acid (22.2%), and nalidixic acid (18.9%). Antibiotics with greatest sensitivity were ceftriaxone (100%), amikacin (98%), cefoxitin (98%), sulfametazol + trimethoprim (98%), and cefotaxime (98%). Ghanbarpour & Kiani (2013) found 192 ovine STEC isolates to be resistant to at least one of eight tested antibiotics, the antibiotics to which resistance was highest were penicillin (98.4%), cephalexin (94.8%) and tetracycline (91.2%), resistance was lowest to sulfamethoxazole + trimethoprim (51%) and ciprofloxacin (62%), and 28.6% isolates were resistant to all antibiotics tested.

Relating the virulence factors with resistance to the tested antibiotics, the *stx1*-positive STEC isolates were most resistant; 50.7% (38/75) isolates were resistant to at least one antibiotic, followed by 30.7% (23/75) *stx2*-positive STEC isolates and 18.7% (14/75) *stx1/stx2* isolates. In Spain, O157:H7 STEC isolated from sheep, cattle and humans were detected, with a resistance profile of 20% (1/5) of ovine and 53% (42/82) of cattle; in bovine STEC, Mora

et al. (2004) observed greatest resistance to streptomycin (19%), in agreement with our results. Also similarly to our study, Saei et al. (2012) reported low resistance to the antibiotics tetracycline (10%) and gentamicin (4%).

According to the phylogenetic classification, 24.4% (22/90) were from group D (pathogenic), 32.2% (29/90) from group B1 (commensal) and 43.3% (39/90) from group A (commensal). Ghanbarpour & Kiani (2013) also found three distinct phylogenetic groups: A (42.7%), B1 (48.4 %) and D (8.8%) of the 192 ovine STEC isolates, with no isolates of the phylogenetic group B2 either. The group B1 is considered prevalent among phylogenetic groups in STEC isolated in the feces of domestic ruminants (Carlos et al. 2010).

When relating the phylogenetic results with virulence factors, it was shown that the *stx1*-positive STEC were primarily of the pathogenic phylogenetic group D, while the isolates *stx2* and *stx1/stx2* were mostly commensal (A or B1). However, regardless of the phylogenetic classification, the virulence factors of the isolates make them capable of causing disease. This can most likely be explained by the high ability to transfer genetic material between commensal and pathogenic *Eschrichia coli* (Hao et al. 2012).

STEC isolates are highly pathogenic for humans, mainly for being involved in diarrheagenic diseases, hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) in humans. Several studies investigated the prevalence of STEC in ruminants, particularly in cattle and sheep, however studies for this purpose are scarce for certain regions of Brazil, as for the State of Goiás. The high number of STEC-positive isolates in sheep and the high pathogenicity, confirmed by the presence of virulence factors as well as the large number of multiresistants detected in this study, support the statement that small ruminants are potential carriers of these pathogens and a potential threat to public health.

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