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Colibacillosis in lambs is associated to type I heat-stable enterotoxin in a farm in São Paulo State, Brazil

Colibacilose em carneiros é associada à enterotoxina termo-estável do tipo I em uma propriedade rural do estado de São Paulo, Brasil

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- NOTE -

ABSTRACT

Twenty seven (48.2%) culture supernatants of 56 ***Escherichia coli*** *isolated from diarrheic lamb feces (7 to 10 days old) in São Paulo State, Brazil, presented positive results to suckling mice assay (fluid accumulation) but none caused cytopathic effects on Vero and CHO cells, indicating that these strains did not produced LT or VT toxins. PCR assays showed that these 27* ***E. coli*** *strains harbored estA, that codifies for STa, but not for stx1, stx2 or cnf genes. The positive STa strains were checked for genes that codify for F41, F17 and K99 fimbriae, wich are considered colonization factors in ETEC. Only F17 was detect in two samples (7.4%). Twelve of 27 STa positive carried hlyA gene and presented hemolytic activity in blood Agar. Presence of rotavirus was not detected among the diarrheic feces. These data suggests that STa must be an important diarrheagenic factor to small ruminants in São Paulo State.*

Key words: *lambs, STa, colibacillosis,* ***Escherichia coli****.*

RESUMO

Cinquenta e seis ***Escherichia coli*** *isoladas de fezes diarreicas de carneiros (7 a 10 dias) no Estado de São Paulo, Brasil, foram avaliadas quando ao acúmulo de fluidos no intestino de camundongos recém-nascidos. Vinte e sete (48,2%) das amostras foram positivas para esse ensaio, porém nenhuma das 56 amostras foi capaz de induzir efeitos citopáticos em células Vero e CHO, indicando que não produzem toxinas LT ou VT. Análise por PCR mostrou que estas 27* ***E. coli*** *foram positivas para estA, que codifica a proteína STa, mas não para os genes stx1, stx2 ou cnf. As amostras positivas para STa foram também analisadas quanto à presença dos genes que codificam as fímbrias F41, F17 e K99, fatores de colonização em ETEC. Somente F17 foi detectada em 2 amostras (7,4%). Doze das 27* ***E. coli*** *STa positivas também contêm o gene hlyA e apresentaram atividade hemolítica em Agar sangue. Rotavírus não foi detectado nas fezes desses animais. Em conjunto, esses resultados sugerem que STa é um fator diarreiogênico importante para colibacilose de pequenos ruminantes no Estado de São Paulo.*

Palavras-chave: *carneiro, STa, colibacilose,* ***Escherichia coli.***

Escherichia coli is a commensal microorganism present in mammalian guts and is considered one of the most common etiological agent of diarrhea in both humans and animals. The virulence factors (VF) are responsible for survival and adaptation in a host environment, however there are not conclusive information about the VF involved in colibacillosis pathogenicity. ETEC (enterotoxigenic ***E. coli***) causes diarrhea by means of production of two different toxins: LT (heat-labile enterotoxin) and ST (heat-stable enterotoxin). STa were usually associated with diarrhea in humans, piglets and bovine, while STb were associated with diseases in piglets. SMITH & HUGGIS (1983) were the first to report the involvement of ETEC in lamb diarrhea cases. In Brazil, VETTORATO et al. (2003) detected STEC (Shiga toxin producing ***E. coli***) in healthy sheep feces. Thus, our objective was to evaluate which VF was most important for the lamb colibacillosis in São Paulo State, Brazil.

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For this study, one strain was isolate of each feces samples, selected from 60 lambs (7 to 10 days olds) with diarrhea, on a single farm in Araras, São Paulo State, Brazil. These strains were biochemical identified by EPM-MILi-Citrate medium (TOLEDO et al., 1982a,b). Cytotoxicity assays were performed with ***E. coli*** strains cultured in 10ml of TSB with shaking at 150rpm (New Brunswick Scientific Co) for 18h at 37ºC. The culture supernatants were collected after centrifugation and filtered through a 0.22µm filter (Millipore) and applied in cell cultures. Vero (African green monkey kidney) and CHO (Chinese hamster ovary) cells were cultivated in 96-wells plates (TPP) with Eagle´s minimum essential medium (MEM, Nutricell) supplemented with 10% fetal bovine serum, at 10^4 cells per ml. Morphological alterations were observed after 24 and 48 hours, using an inverted microscope (Nikon). Gene amplifications were performed by PCR. The primers used, annealing temperatures and the amplified fragments size are in table 1. The reactions were heated to 94ºC in an automated thermal cycler (Mastercycler Gradient, Eppendorf) for 2min followed by 30 cycles of denaturation, annealing and extension, followed by a final extension step. The amplicons were visualized into a 0.8% agarose gel at UV translluminator after staining with ethidium bromide. The STa positive ***E. coli*** culture supernatants were tested in the suckling mice assays following the methodology described by DEAN et al. (1972). For hemolytic activity, blood Agar plate assay were used at *hlyA* positive strains. The blood Agar plates were prepared with 5% sheep erythrocytes for á-haemolysin detection and, for â-haemolysin, the sheep erythrocytes were washed with PBS three times. The strains were cultivated in TSB and plated in the plates. Observations of hemolytic zones around the colonies were made after incubation for 18h at 37ºC. ***E. coli*** O157:H7 and ***E. coli*** K-12 were used as positive and negative controls, respectively. Furthermore, fecal suspensions were analyzed for Rotavirus presence. Briefly, the nucleic acids were extracted with phenol-chloroform, precipitated with ethanol and analyzed by polyacrylamide gel electrophoresis (PAGE), using 7.5% slab gels, stained with silver nitrate (HERRING et al., 1982).

Among the 60 isolates, 56 were identified as ***E. coli***. The other 4 samples were not identified because they did not belong to the Enterobacteriaceae family. These 56 strains were evaluated by cytotoxicity assays and did not cause any morphological alterations in Vero and CHO cells. This suggests that these 56 ***E. coli*** did not produced LT, neither Shiga toxin. In Spain, BLANCO et al. (1996) analyzed 144 ***E. coli*** and they observed that only 1.3% produced LT and 4.2% produced VT1 but neither produced ST. We also performed PCR for genes encoding VF. PCR demonstrated that 27 (48.2%) isolates were positive to *estA* (Table 1), but not to *estB* (data not

Table 1 - Primers sequence, annealing temperature and predicted lengths of PCR amplification products.

Gene	Sequence (5' 3')	Amplicon (bp)	Anneling temperature	References
estA	TCC GTC AAA CAA CAT GAC GC ATA ACA TCC AGC ACA GGC AC	176	50ºC	BLANCO et al., 1992
stx1	CGC TGA ATG TCA TTC GCT CTG C CGT GGT ATA GCT ACT GTC ACC	304	55ºC	BLANCO et al., 2003
stx2	CTT CGG TATA CCT ATT CCC GC CTG CTG TGA CAG TGA CAA AAC GC	516	55ºC	BLANCO et al., 2003
cnf-s	CTG GAC TCG AGG TGG TGG CTC CTG TCA ACC ACA GCC	533	63ºC	BLANCO et al., 1994
F41	GGC TAT GGA AGA CTG GAG AGG G GGG GTG ACT GAG GTC ATC CC	431	58ºC	FIDOCK et al, 1989
F17	CTG ATA AGC GAT GGT GTA ATT AAC GCA GAA AAT TCA ATT TAT CCT TGG	537	58ºC	BERTIN et al, 1996
K99	TAT CCA CCA TTA GAC GGA GC TGG GAC TAC CAA TGC TTC TG	450	60ºC	ROOSENDAAL et al, 1984
hlyA	AAC AAG GAT AAG CAC TGT TCT GGC T ACC ATA TAA GCG GTC ATT CCC GTC A	1117	63ºC	YAMAMOTO et al., 1995

shown), these samples were confirmed by the suckling mice assay. Enterotoxic activity was maintained after heating at 100ºC for 15min (data not shown). Together, the results suggest that 27 isolates are ETEC strains, because they produced ST toxins. The isolates did not harboring *cnf*, *stx1*, *stx2*, F41, and K99 genes. URDAHL et al. (2002) described that ***E. coli*** isolated in sheep carried *stx*1 and *stx*2, different from our results. THEIL et al. (1996), also comment that an important involvement of K99 piliated ***E. coli*** among other etiologic agents in the lambs diarrhea, however the 27 ***E. coli*** strains in this study did not show adhesive factors associated to ETEC such as K99 and F41. However, CID et al. (1993) studying 627 ***E. coli*** isolated from lambs with diarrhea, observed that neither ***E. coli*** produced adhesins (K99 or F41), but expressed F17 adhesive factor. Interestingly, none of these F17 positive ***E. coli*** strains produced heat-stable enterotoxin (STa). Although, among our STa positive ***E. coli*** strains two (2/27) presented the F17 gene (7.4%) (Table 2). Alpha-hemolysin gene was detected in 44.4% (12/27) of STa positive samples (Table 2) and presented hemolytic activity at sheep erythrocytes (Table 2). Hemolytic activity can be associated with increased virulence by the greater iron availability or causing toxic effets in host defense cells. This VF is commonly associated with ***E. coli*** that cause diarrhea in human and animals (BURGOS & BEUTIN, 2010). Rotavirus can be frequently associated with lamb diarrhea. THEIL et al. (1996) detected rotavirus in 75% of the diarrheagenic feces from neonatal lamb in the United States. To evaluate the presence of rotavirus in diarrheic feces studied in this study, the nucleic acids were extracted from fecal samples and examined by PAGE (HERRING et al. 1982). The results were negative for detection of rotavirus double-stranded RNA genome in PAGE. This data suggests that this virus is not associated with the lamb diarrhea in these farm, so the ***E. coli*** strains isolated in this study were the principal causes of lamb diarrhea.

Our data suggest that STa contribute for the colibacillosis development. This outbreak of diarrhea, in

Table 2 - Detection of *hlyA* and F17 genes and α and β hemolysin in STa positive strains and results by suckling mice assay.

estA$^+$ strains		*hlyA*	F17	----------Hemolytic Activity----------		Suckling mice assay
				α-haemolysin	β-haemolysin	
6		+	-	+	-	+
14		-	-	-	-	+
19		-	-	-	-	+
20		-	-	-	-	+
21		+	-	+	-	+
22		+	-	+	-	+
23		-	-	-	-	+
24		-	-	-	-	+
25		+	-	+	-	+
30		-	-	-	-	+
31		-	-	-	-	+
32		-	-	-	-	+
34		-	-	-	-	+
35		-	-	-	-	+
36		+	+	+	-	+
37		-	-	-	-	+
40		+	-	+	-	+
41		-	+	-	-	+
42		+	-	+	-	+
47		-	-	-	-	+
48		-	-	-	-	+
49		-	-	-	-	+
50		+	-	+	-	+
54		+	-	+	-	+
56		+	-	+	-	+
58		+	-	+	-	+
59		+	-	+	-	+
Total	27	12	2	12	0	27

São Paulo State, was provoked by type 1 heat-stable enterotoxin (STa). It is the first report of STa production by ETEC isolated from lambs in Brazil. Further studies are necessary to understand the possible role of ETEC and other pathotypes in lamb diarrhea.

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BIOETHICS AND BIOSSECURITY COMMITTE APPROVAL

The present study was approved by the Ethical Committee on CEUA/UNICAMP, n. 2504-1, and was in accordance with the Ethical Principles in Animal Experimentation (COBEA)

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