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Scientific Research

Molecular Identification of Shiga-Toxin Producing and Enteropathogenic *Escherichia coli* (STEC and EPEC) in Diarrheic and Healthy Young Alpacas

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Abstract

Isolation and biochemical and molecular identification of 303 strains of *Escherichia coli* obtained from diarrheic and healthy young alpacas of Puno-Peru, were realized. PCR amplification for 7 virulence factor genes associated with STEC, STEC O157:H7, EPEC: *sxt*1, *sxt*2, *rfbO*157, *fliCH*7, *hlyA*, *eae y bfp* were determined. A total of 39 strains (12.88%) showed amplification for one or more of these genes. Twenty three strains (59%) were classified as STEC and 16 strains (41%) as EPEC. An 88.18% (34/39) of STEC and EPEC strains were obtained from healthy alpacas and only 11.82% (5/39) from diarrheic alpacas considering this specie as potential zoonotic reservoir of STEC and EPEC.

Keywords

Shiga Toxin Producing *Escherichia coli*, Enteropathogenic *Escherichia coli*, Diarrhea, Young Alpacas

1. Introduction

Escherichia coli is a worldwide pathogen causing intestinal and extraintestinal disease in man, children, and

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animals, especially young animals. Nowadays, there are six pathotypes which cause diarrhea*:* Shiga toxin producing *E. coli* (STEC), enteropathogenic *E. coli* (typical or atypical EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffusely adherent (DAEC), enteroaggregative *E. coli* (EAEC), from which, enterohemorrhagic *E. coli* (EHEC), a subtype or subgroup of STEC is associated to two major diseases in humans, the urine-hemolytic syndrome (UHS) and the hemorrhagic colitis (HC), being the serotype O157:H7 its main representative [1]. In Peru, STEC and EPEC are two of the most important E. *coli* pathotypes that produce diarrheic syndrome in children [2]-[4]. Peru has the greatest alpaca population with approximately four million of alpacas [5], and constitutes an important economic resource to Andean communities with almost 200,000 habitants involved in this production system. However, insufficient information of zoonotic potential of *E. coli* strains from alpacas was generated. The objective of this study was to evaluate the presence of virulence factors, its association with diarrhea [6]-[9], and the evaluation of alpaca as potential reservoirs of *E. coli* zoonotic pathotypes.

2. Materials and Methods

2.1. Animals and Samples

The samples were collected from Andean communities in the department of Puno during January to March of 2007 (mating-breeding season). Three hundred twenty four samples of rectal and anal mucosal swab were collected from young alpacas up to 60 days old. The samples were divided in two paired groups (age, sex and origin): 1) clinical diarrhea (N = 162); and 2) without diarrhea (N = 162). Samples were placed in Cary-Blair transport medium and stored at 8˚C until their processing.

2.2. Biochemical and Virulence Factors Identification

Samples were cultured in McConkey agar and incubated at 37˚C for 18 to 24 hours. Evaluation of bacterial colonies and biochemistry were realized, strains that showed compatible results with *E. coli* were selected [8]. Molecular confirmation was performed using a simple PCR for universal stress protein (uspA) [10]. Genomic DNA was extracted using Promega Wizard Genomic ADN Isolation kit (PROMEGA) according to manufacturer. PCR for 7 virulence factor genes: Shiga toxin 1 (stx1A) [11], Shiga toxin 2 (stx2A) [11], Intimin (eaeA) [12], Bundle forming pilus (bfp) [13], Flagelar antigen H7 (fliCH7) [14], Somatic antigen O157 (rfbO157) [15], Enterohemolysin (hlyA) [16] were performed. In brief, the PCR reaction was realized using a final volume of 20 uL containing 10 ng genomic DNA, 1X PCR buffer (20 mM Tris-HCl, 20 mM KCl, 5 mM $(NH_4)_2SO_4$), 2 mM $MgCl_2$, between 0.75 to 4 pmol of each primer (**Table 1**), 0.2 mM of dNTPs, 0.5 U Maxima HotStart-Taq DNA

Table 1. Target gene, primer sequences, primer concentration and PCR product size.

Target gene	Primer	Sequence 5'-3'	Concentration (pmol)	PCR product size (bp)
Shiga toxin 1 (*stx*1*A*)	LP30	CAGTTAATGTCGTGGCGAAGG	1.4	348
	LP31	CACCAGACAATGTAACCGCTG		
Shiga toxin 2 (*stx*2*A*)	LP43	ATCCTATTCCCGGGAGTTTACG	1	584
	LP44	GCGTCATCGTATACACAGGAGC		
Intimin (*eaeA*)	Int-Fc	CCGGAATTCGGGATCGATTACCGTCAT	2	840
	Int-Rc	CCCAAGCTTTTATTTATCAGCCTTAATCTC		
Bundle forming pilus (*bfp*)	Bfp-F	GGAAGTCAAATTCATGGGGGTAT	1.5	254
	Bfp-R	GGAATCAGACGCAGACTGGTAGT		
Flagelar antigen H7(*fliCH7*)	FLICH7-F	GCGCTGTCGAGTTCTATCGAGC	2	625
	FLICH7-R	CAACGGTGACTTTATCGCCATTCC		
Somátic antigen O157 (*rfbO*157)	PF8	CGTGATGATGTTGAGTTG	0.75	420
	PR8	AGATTGGTTGGCATTACTG		
Enterohemolysin (*hlyA*)	hlyA-F	GCATCATCAAGCGTACGTTCC	4	534
	hlyA-R	AATGAGCCAAGCTGGTTAAGCT		

polymerase (Fermentas). Thermal cycles were the following: initial denaturation of 94˚C for 3 minutes, 30 cycles of 94˚C for 30 seconds, 53˚C for 60 seconds, 72˚C for 30 seconds and a final extension of 72˚C for 5 minutes. The PCR amplification was carried out using a Veriti Thermal cycler (Applied Biosystems). The PCR products were separated in 2% agarose gel TBE 1X electrophoresis and visualized by fluorescence using an ethidium bromide solution (0.1 ug/mL) and UV light using a BioDoc-It system.

3. Results and Discussion

A total of 39 isolates (12.88%) showed amplification for one or more virulence factor genes: Shiga toxin 1 (stx1A), Shiga toxin 2 (stx2A), Intimin (eaeA), Flagelar antigen H7 (fliCH7) and Enterohemolysin (hlyA) (**Figure 1**), constituted 11 *E. coli* toxinotypes, 1 in EPEC and 10 at STEC. A total of 23 strains of *E. coli* (59%) were identified as STEC and 16 (41%) as atypical EPEC (EPECa) due to absence of the *bfpA gene* (**Table 2**). A 2.1% *of E. coli* strains (3/149) isolated from alpacas with diarrhea was identified as STEC, and 1.3% (2/149) as EPECa in contrast with 12.9% (20/154) for STEC and 9.1% (14/154) for EPECa from alpacas without diarrhea (**Table 2**).

Previous studies in alpacas from Peru [6] [8] [9] reported a STEC as the predominant pathotype in samples of young alpacas with diarrhea. Also, a study in guanaco with diarrhea from Argentina reported the presence of STEC [17]. However, the presence of STEC and EPECa strains in higher proportion in alpacas without diarrhea in comparison to alpacas with diarrhea contrasts with previous reports [7]. Of all strains of STEC detected in alpacas without diarrhea, sxt2A gene as a single gene or in combination with stx1A, eaeA and hlyA genes was most frequent gene (11/20), similar to reported by other authors [8] [18] in contrast to reported in cattle from Lima (year 2010) where the stx1 gene was most frequent [19].

The EPECa is among the most important pathogens causing acute and persistent diarrhea in children in Peru [2] [3]. Studies in cattle, sheep and pigs also showed that these animal species may be a source of strains EPECa and STEC [19] [20]. Therefore, the detection of 14 strains of EPECa in alpacas without diarrhea in this study showed preliminary evidence that alpacas can be EPECa reservoir. Additionally, the presence of STEC strain with the stx2 gene, alone or in combination with *eae*, could be associated with the development of UHS and HC

Table 2. Detected genes by multiplex PCR in *E. coli* strains isolated from feces of 303 young alpacas.

E. coli genotypes (Detected genes)	*E. coli* strains isolated		
	With diarrhea N (%)	Without diarrhea N (%)	Total N (%)
EPEC			
eae	2 (1.3)	14 (9.1)	16 (5.3)
STEC			
sxt1	1 (0.7)	2 (1.3)	3 (1.0)
sxt2	1 (0.7)	7 (4.6)	8 (2.6)
sxt1+sxt2	0	1 (0.6)	1 (0.3)
sxt1+eae	0	2 (1.3)	2 (0.7)
sxt2+eae	0	1 (0.6)	1 (0.3)
sxt2+hlyA	1 (0.7)	1 (0.6)	2 (0.7)
sxt1+hlyA	0	2 (1.3)	2 (0.7)
sxt1+eae+hlyA	0	2 (1.3)	2 (0.7)
sxt2+eae+hlyA	0	1 (0.6)	1 (0.3)
sxt1+hlyA+fliCH7	0	1 (0.6)	1 (0.3)
None	144 (96.6)	120 (78)	264 (87)
Total	149 (100)	154 (100)	303 (100)

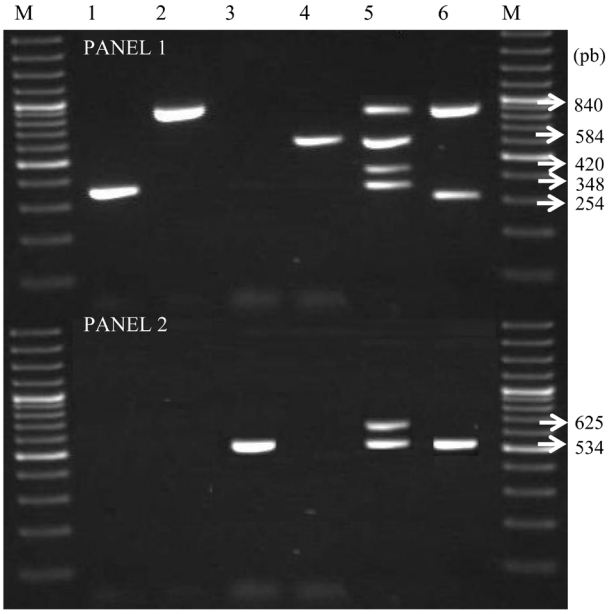

Figure 1. Panel 1: Line 1, *stx*1*A* positive sample, line 2, *eaeA* positive sample, line 3, ETEC H10407, line 4, sxt2 positive sample; line 5, EHEC O157: H7 (*eaeA*, *stx*2*A*, *rfbO*157 and stx1A positive), line 6, EPEC 2348/69 (*eaeA* and *bfpA* positive) and line M, molecular marker of 100 pb. Panel 2: Line 1 and 2 negative samples, line 3, *hlyA* positive sample, line 4, ETEC H10407; line 5, EHEC O157: H7 (*fliCH*7 and *hlyA* positive), line 6, STEC 3153-86 (*hlyA* positive).

in humans [1]. The presence of theses virulence factors in healthy alpacas and the breeding management in Peru allows close contact between animals and humans, increasing the risk of transmission, primarily through contamination of food or water with alpaca feces.

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