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Atypical enteropathogenic *Escherichia coli* (aEPEC) in children under five years old with diarrhea in Quito (Ecuador)

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Summary. Enteropathogenic *Escherichia coli* (EPEC) remain one the most important pathogens infecting children and they are one of the main causes of persistent diarrhea worldwide. In this study, we have isolated EPEC from 94 stool samples of children under five years old with diarrheal illness in the area of Quito (Ecuador), and we have determined the occurrence of the two subtypes of EPEC, typical EPEC (tEPEC) and atypical (aEPEC), by PCR amplification of the genes *eae* (attaching and effacing) and *bfp* (bundle- forming pilus). Typical EPEC is positive for *eae* and *bfp* genes while aEPEC is positive only for *eae*. Our results suggest that aEPEC is the most prevalent subtype in Quito (89.36 %), while subtype tEPEC is less prevalent (10.64 %). [**Int Microbiol** 19(3):157-160 (2016)]

Keywords: *Escherichia coli* · atypical EPEC · genes *eae* and *bfp* · diarrhea in children · Quito (Ecuador)

Introduction

Diseases that affect the gastrointestinal system are one of the main causes of child morbidity and mortality in developing countries. The diarrhea produced by *Escherichia coli* agents have high incidence in those countries [12]. The diarrheagenic *E. coli* are subdivided into six pathotypes [8]. The most prevalent pathotype worldwide is EPEC (enteropathogenic *E. coli*), which cause 79,000 children deaths under 5-year-old around the world per year [10]. Since 1995, it is documented that EPEC presents two subtypes: the typical (tEPEC) and atypical (aEPEC). Typical EPEC is characterized by the plasmid gene *bfp* (bundle- forming pilus) and the chromosomal gene *eae* (attaching and effacing), and a single human reservoir. Only aEPEC has *eae* gene and infects humans and cattle. The typical subtype is positive for genes *eae* and *bfp* and atypical subtype only to *eae* gene [11]. To identify EPEC it is necessary first identify the presence of *eae* gene. The *eae* gene is present in all EPEC and absent in normal microbiota of *E. coli* [13]. To our knowledge, only two studies of EPEC prevalence in Ecuadorian population [14,15] have been published. How-

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ever, in those studies, only the presence of *bfp* gene (only present in subtype tEPEC) was determined and in consequence, aEPEC prevalence was underestimated. In this manuscript we attempted to quantify the prevalence of atypical and typical EPEC in children <5 by PCR amplification of genes *eae* and *bfp* in the Quito area.

Materials and methods

Bacterial strains. Fecal samples of children under five years old with diarrhea illness were collected during February and June 2013 by clinical laboratories (Netlab; Zurita & Zurita; Pazmiño & Narváez) in Quito. Samples were screened for *Escherichia coli* pathotypes determination by multiplex PCR [2]. The samples included in the study were those with EPEC gene amplification (*eae* gene).

DNA extraction. To purify and quantify the DNA of the EPEC strains, they were grown in LB medium (BBL, Sparks, MD, USA) to avoid the reagents present in the selective medium MacConkey, which interfere in the process of DNA extraction and quantification. DNA was extracted using the Wizard genomic DNA purification Kit (Promega, Madison, WI, USA). DNA concentration and purity was measured using a Nanodrop 2000/2000c® (Thermo Scientific,Wilmington, DE, USA) according to the manufacturer's protocol .

EPEC typification. The 94 EPEC strains identified by pathotype determination were analyzed for *eae* and *bfp* genes by duplex PCR (Table 1). The reaction contained 12,5 µl of GoTaq® Green Master Mix 2x (Promega, Madison, WI, USA), 0,3 µM of each forward and reverse primer, 2 ng Template DNA and PCR-grade water up to a total reaction volume of 25 µl. DNA amplification was carried out in a PCR Thermal Cycler Multigene Optimax (Labnet, Edison, NJ, USA). The Thermal Cycling protocol was as follow: initial denaturation for 5 min at 94°C followed by 30 cycles of 45 s at 94°C, 45 s at 57°C and 45 s at 72°C and a final extension of 10 min at 74°C [7,15]. Detection of PCR products was by electrophoresis in 1% ultrapure agarose gel (Invitrogen, Waltham, MA, USA) for 60 min at 90V and 120 mAmp. DNA was stained with Green® (Sigma Aldrich) and visualized under UV light and the lanes was compared with 100 bp DNA ladder (Promega, Madison, WI, USA). To quality control the EPEC strains positive for *eae* and *bfp* genes were provided by James Nataro (Virginia University) and Roberto Vidal (University of Chile) (Table 1).

Limit detection. We have determined the detection limit of PCR amplification used. DNA concentration was determined with bacterial culture adjusted to 0.5 McFarland with a nephelometer (Hanna Industries, WoonSocket, RI, USA) approximately $1.5x10^8$ CFU/ml and diluted 1/10. Dilutions were analyzed by Nanodrop 2000/2000c to determinate the DNA concentration and used to amplify *eae* gene. GenPro Software (Media Cybernetics Inc., Rockville, MD) was used for calculation of the Relative Optical Density of PCR amplification products on agarose gels.

Statistical analysis. We Graph Pad Software (GraphPad Software Inc., San Diego, CA, USA) was used to analyze the results. The ANOVA one way and Student-Newman-Keuls post-test was used to determine the statistical significance.

Results and Discussion

During the period from February to June 2013, a total of 94 fecal samples were determined for EPEC from children aged <5 years old with diarrhea in Quito. They were positive for *eae* gene and negative for the genes: *vt1*; *vt2*; *aggR*; *eltB*; *estA*; *daaC* and *ipaH*. It was determined that 2 ng is the minimum amount of DNA to detect those genes. This amount of DNA corresponds to 10^3 CFU/ml (Fig. 1). The two subtypes of EPEC, tEPEC and aEPEC, were determined by duplex PCR amplification of the genes *eae* and *bfp*. The DNA concentration of the samples studied was comprised in a range between 45 and 80 ng/µl. The 94 samples were positive for the *eae* gene, and 10 samples were also positive for the *bfp* gene. Therefore, the relative frequency of subtype aEPEC in

Table 1. Primers used to amplify EPEC genes by real-time PCR

Category	Target	Localiz.	Orient.	Primer seq. (5'-3')	Amplicon size	Position*
EPEC	*eae*	Chromosome	F	5'-GTAAGTCTCAAACGCAAGCAACCAC-3'	167	122313-122479
			R	5'-AACCTTGTTGTCAATTTTCAGTTCATCA-3'		
	bfp	Plasmid	F	5'-AATGGTGCTTGCGCTTGCTGC-3'	268	2725-3008
			R	5'-GCCGCTTTATCCAACCTGGTA-3'		

*Reference sequence *Escherichia coli* chromosome (CP017444.1) and plasmid (NC_010862.1).

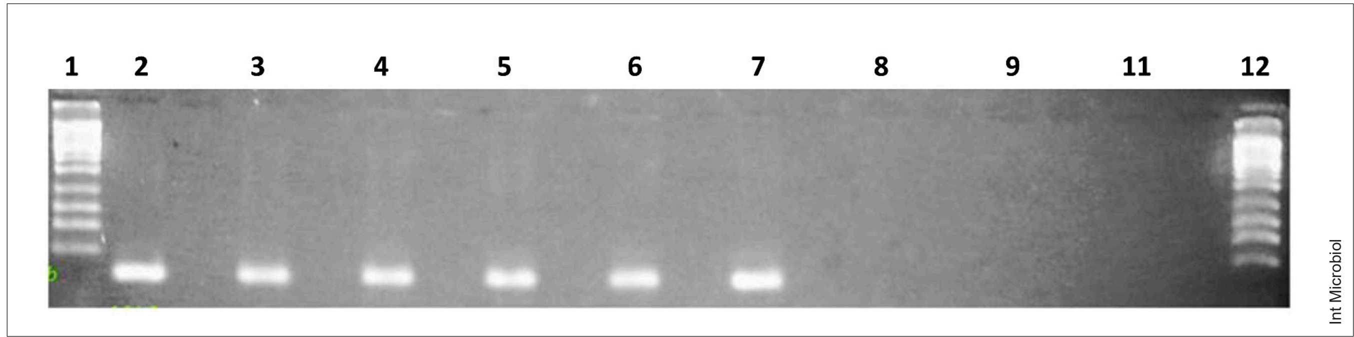
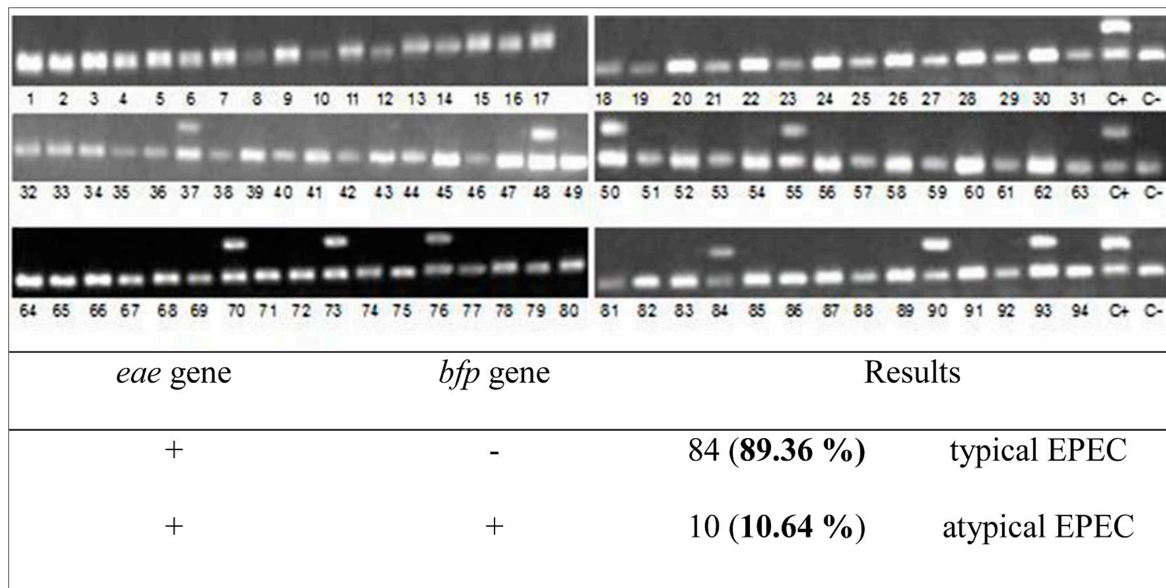


Fig 1. Detection limit of EPEC (*eae* gene). Lanes 2 to 11, dilutions from 10^8 to 10^1 CFU/ml, respectively; lane 11, negative control. Lanes 1 and 12 DNA ladder (100 bp). The 198 bp products correlate with the *eae* gene.

Quito is 89.36 % and tEPEC subtype is 10.64% (Fig. 2). The *eae* and *bfp* genes were amplified at least three times. The relative optical density image analysis of the bands showed no significant differences greater than 25% between them ($P < 0.01$).

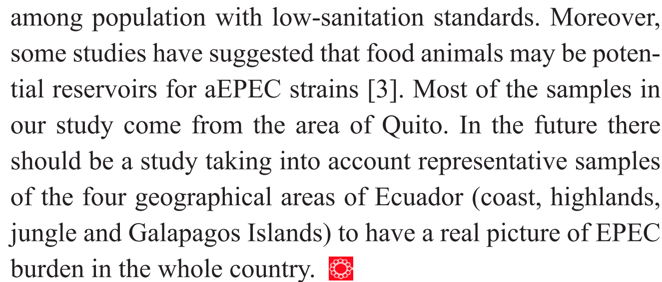
Our study shown that the prevalence of 90% aEPEC and 10% tEPEC in Quito properly correlated with the prevalence of aEPEC in the region: Peru with 76.8 % [3], Argentina with 93.1 % [5] and Venezuela with 88.9 % [6]. Previous studies conducted in Ecuador [14,15] underestimated the prevalence of *Escherichia coli* EPEC pathotypes. In those studies, only the amplified *bfp* gene was present in the tEPEC subtype. As it has been observed in the present work, tEPEC only accounts for the 10% of all EPECs isolated from clinical samples in Quito. For that reason, the percentages of total EPEC presented in previous works were underestimated.

It has been suggested that pathogens evolve through gene loss [1]. The basic genetic differences between the two subgroups of EPEC are the absences of plasmid pEAF and the plasmidic *bfp* gene [9] in the aEPEC subtype. In addition, the aEPEC subtype has several reservoirs described [7], this fact suggest that this subtype is already more adapted to circulate



eae gene	*bfp* gene	Results	
+	-	84 (**89.36 %**)	typical EPEC
+	+	10 (**10.64 %**)	atypical EPEC

Fig 2. Duplex PCR amplification of the *eae* (198 bp) and *bfp* (255 bp) genes. *Escherichia coli* EPEC samples are specified from 1 to 94. The controls are verified *E. coli* strains. Lane C+ tEPEC *E. coli* strain (James Nataro, U. of Virginia) and lane C- aEPEC *E. coli* strain (Roberto Vidal, U. of Chile). The 94 samples were positive for the *eae* gene and samples 37, 48, 50, 55, 70, 73, 76, 84, 90 and 93 were also positive for the *bfp* gene.



among population with low-sanitation standards. Moreover, some studies have suggested that food animals may be potential reservoirs for aEPEC strains [3]. Most of the samples in our study come from the area of Quito. In the future there should be a study taking into account representative samples of the four geographical areas of Ecuador (coast, highlands, jungle and Galapagos Islands) to have a real picture of EPEC burden in the whole country.

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Competing interests. None declared.

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