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Molecular characterization of diarrheagenic *Escherichia coli* strains from stools samples and food products in Colombia

Laura Cristina Rúgeles¹, Jing Bai², Aída Juliana Martínez¹, María Consuelo Vanegas^{1,*}, and Oscar Gilberto Gómez-Duarte^{2,*}

¹ Laboratorio de Ecología Microbiana de Alimentos, Departamento de Ciencias Biológicas, Universidad de los Andes.

² Division of Infectious Diseases, Department of Pediatrics, University of Iowa Children's Hospital.

Abstract

The prevalence of diarrheagenic *E. coli* in childhood diarrhea and the role of contaminated food products in disease transmission in Colombia are largely unknown. The aim of this study is to identify *E. coli* pathotypes, including *E. coli* O157:H7, from 108 stool samples from children with acute diarrhea, 38 meat samples and 38 vegetable samples. Multiplex PCR and Bax Dupont systems were used for *E. coli* pathotype detection. Eighteen (9.8%) *E. coli* diarrheagenic pathotypes were detected among all clinical and food product samples tested. Four different pathotypes were identified from clinical samples, including enteroaggregative *E. coli*, enterotoxigenic *E. coli*, shiga-toxin producing *E. coli*, and enteropathogenic *E. coli*. Food product samples were positive for enteroaggregative and shiga-toxin producing *E. coli*, suggesting that meat and vegetables may be involved in transmission of these *E. coli* pathotypes in the community. Most *E. coli* strains identified belong to the phylogenetic groups A and B1, known to be associated with intestinal rather than extraintestinal *E. coli* clones. Our data is the first molecular *E. coli* report that confirms the presence of *E. coli* pathotypes circulating in Colombia among children with diarrhea and food products for human consumption. Implementation of multiplex PCR technology in Latin America and other countries with limited resources may provide an important epidemiological tool for the surveillance of *E. coli* pathotypes from clinical isolates as well as from water and food product samples.

Keywords

E. coli; multiplex PCR; pathotypes; Colombia; diarrhea

INTRODUCTION

Diarrheagenic *E. coli* are classified on the basis of its epidemiological, clinical and pathogenic characteristics in the following six different pathotypes: enteropathogenic *E. coli* (EPEC),

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*Corresponding authors: Mailing address for Oscar Gilberto Gómez-Duarte: Division of Pediatric Infectious Diseases, Department of Pediatrics, University of Iowa Children's Hospital, 200 Hawkins drive, Iowa City, IA 52242, USA. Telephone: 319 356 2270, Facsimile: 319 356 4855. oscargomez@uiowa.edu. Mailing address for Maria Consuelo Vanegas, Laboratorio de Ecología Microbiana y Alimentos-LEMA, Universidad de los Andes. Cra 1^a N° 18^a-70 J209, Bogotá, Colombia. Telephone: 571-33 94949 Extensión 2784 Facsimile: 571-3394949 Extensión 3339. mvanegas@uniandes.edu.co..

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shiga-toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffuse adherent *E. coli* (DAEC) (Levine, 1987; Nataro and Kaper, 1998). Each pathotype expresses a unique set of virulence and colonization factors encoded in the chromosome or in episomal structures. The genes encoding these virulence factors are conserved among strains isolated from different continents (Aranda et al., 2007, Brandal et al., 2007; Nguyen et al., 2005, Stacy-Phipps et al., 1995; Vidal et al., 2005). Specific DNA amplification of these genes (Table 1) has been used for detection of diarrheagenic *E. coli* from clinical (Gomez-Duarte et al., 2009) and environmental samples (CDC, 1996; Martinez et al., 2007; Meichtri et al., 2004).

In the last decade, molecular biology techniques are being reported in the literature for rapid identification of *E. coli* pathotypes including the use of the multiplex polymerase chain reaction (PCR) assays. Several Latin American countries have reported diarrheagenic *E. coli* by the use of these technology including Argentina (Rivas et al., 2006), Brazil (Aranda et al., 2007; Moreno et al., 2008), Chile (Vidal et al., 2005); and Mexico (Estrada-Garcia et al., 2009). While most reports rely on technology only available in biomedical research laboratories and not in clinical laboratories in developing countries, a recently reported three-sample PCR assay was designed to be implemented in areas of the world with limited resources. This method uses plasmid DNA as controls rather than bacterial prototype strains avoiding the need for expensive freezing conditions for reagents storage (Gomez-Duarte et al., 2009).

Colombia, located at the North-western corner of South America, has 45,6 million inhabitants, 29% less than 15 years of age. Bogota is the Colombian capital and potable water is available to 96% of its population (WHO, 2006). The diarrheal disease morbidity rate in Colombia is however 110 cases per 100,000 inhabitants according to data collected between 1990 and 1998 (Manrique, et al., 2006). Information on the molecular characterization of diarrheagenic *E. coli* strains associated to pediatric gastrointestinal infections in Colombia is limited. Similarly, information of *E. coli* contaminants in food products is only limited to the identification of plain *E. coli* strains as a safe indicator. One study reported the presence of *E. coli* O157:H7 in pig, cattle and meat derived from these animals (Mattar et al., 2002) and a subsequent study reported predominantly non-O157:H7 STEC strains carrying shiga toxin 1 (*stx-1*) and/or shiga-toxin 2 (*stx-2*) genes (Martínez, et al, 2007).

Due to the importance of *E. coli* pathotypes as etiologic diarrheal agents affecting mainly children less than five years of age and the possible link between diarrhea and contaminated food, the aim of this study is to implement molecular and traditional techniques to identify *E. coli* pathotypes from food products and stools from children with acute diarrhea in Colombia. In addition to detecting pathotypes, *E. coli* isolates will be characterized at the phylogenetic level by a multiplex PCR (Clermont et al., 2000). Phylogenetic analysis of *E. coli* strains based on detection of chromosomal markers has shown that *E. coli* strains evolve from distinctive clonal groups (Ochman and Selander, 1984). *E. coli* strains that belong to phylogenetic groups A and B1 tend to associate with intestinal pathogens, while strains in groups B2 and D are more associated with extraintestinal pathogens (Gordon et al., 2008). Pathotyping and phylogenetic grouping of *E. coli* isolates will provide important information on the *E. coli* circulating in Colombia among children with diarrhea and contaminated food products for human consumption.

MATERIALS AND METHODS

Clinical and food product isolates

Between October 2006 and February 2007, 108 stool samples from children with diarrhea attending six hospitals in Colombia were obtained from clinical laboratories. Most samples were obtained from Bogota (86%) and the remaining from three non-coastal Colombian cities

(24%). Among the 76 food samples collected, 38 corresponded to meat samples from two supermarkets in Bogotá and 38 were vegetable samples (lettuce, spinach) from eight retail markets in Bogotá. The identification of *E. coli* strains from clinical and food samples were done by conventional microbiologic techniques. Stools and food samples collected in lauril sulphate broth (Oxoid, Hampshire, UK) were cultured on EMB (Eosin, Methylene blue agar) plates overnight at 37°C. Lactose fermenter Gram negative coccobacilli were then tested with conventional biochemical assays for identification of *E. coli*. Up to five colonies were evaluated per sample, with some samples having no colonies and some having five *E. coli* colonies. Presumptive *E. coli* strains capable to ferment lactose, beta-glucuronidase, positive indol, negative citrate, and positive motility were confirmed as *E. coli*. A total of 299 *E. coli* strains from all 184 samples were preserved at -70°C in 1% Glycerol and Brain Heart Infusion (BHI) Broth (Oxoid, Hampshire, UK).

DNA templates for PCR reactions

Crude genomic DNA samples were used as templates for PCR reactions. Genomic DNA preparations were obtained from 2 ml of Luria broth (Oxoid) inoculated with bacterial strains and incubated for 18h at 37°C. The bacterial suspension was centrifuged 10min at 13,000 rpm (Sorvall RT), resuspended in 1mL of TE buffer (10mM Tris -HCL pH 8.0, 5 mM EDTA), and followed by boiling for 10 min. After centrifugation the supernatant containing crude genomic DNA was stored at 4°C until used. Plasmids carrying gene targets originally amplified from each one of the six *E. coli* pathotypes (Table 1) were used as positive DNA template controls for multiplex PCR experiments as described before (Gomez-Duarte et al., 2009a). Plasmid DNA vector pSC-A (Invitrogen, Carlsbad, CA) and *E. coli* DH5alpha genomic DNA were used as negative controls. Plasmids were preserved in TE buffer at 4 °C.

Two-sample multiplex PCR assay

All DNA templates from 299 strains were subjected to two-sample multiplex PCR assays using two sets of specific primer pairs as described before (Gomez-Duarte et al., 2009b). Multiplex PCR reaction 1 contained primer mix 1 (M1) for the detection of the following virulence markers: *E. coli* attaching and effacing (*eae*) gene (for detection of STEC or EPEC), shiga-toxin (*stx*) genes (for detection of STEC), bundle-forming pilus (BFP) major subunit (*bfpA*) (for detection of typical EPEC strains), and EAEC global regulator *aggR* (for detection of EAEC strains). Multiplex PCR reaction 2 contained primer mix 2 (M2) specific for heat labile toxin (LT) and heat stable toxin (ST) (for detection of ETEC strains), invasion genes *virF* and *ipaH* (for detection of EIEC) and *daaE* (for detection of DAEC) (Table 1). Primer oligonucleotide DNA sequences and PCR products sizes are shown in Table 1. The multiplex PCR reaction 1 or 2 was carried out with a 25 µl reaction mixture containing 23 µl of Platinum Blue PCR SuperMix polymerase (Invitrogen, Carlsbad, CA), containing 1.0 µl of primer mix (M1 or M2), and 1 µl of template DNA. The amplification cycles consisted of an initial denaturation of 94°C for 2 min, followed by 40 cycles of 92°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 30 sec and a final extension at 72°C for 5 min in a Gene Cyclor™ Thermocycler (Bio-Rad Laboratories, Inc. USA). PCR products were separated on 2% (w/v) agarose gel in Tris Borate EDTA buffer (pH 8.2) stained with ethidium bromide (10µg/ml) and visualized with the ChemiDoc XRS UV transilluminator system (BioRad, Laboratories, Inc. USA).

Strains that tested positive with the generic *stx* primers were further tested with specific primer pairs for the presence of *stx-1* and/or *stx-2*. The *stx-1* (Vidal, 2004) and *stx-2* (Nguyen, 2004) forward and reverse primers were used for single PCR amplification as described (Vidal, 2004; Nguyen, 2004).

BAX system screening for *E. coli* 0157:H7

The BAX system (Hochberg et al., 2000) is a commercially available product used to specifically detect *E. coli* 0157:H7 strains. The lysate preparation contained 5 µl of an overnight culture grown in BHI and 200 µl of buffer with protease. The solution was treated at 37°C for 20 min and then chilled on ice for 5 min. Fifty microliters of lysate was added to PCR tablets in 0.2 ml tubes. Samples were cycled in a BAX system thermal cycler for 4 h, and results were analyzed with the BAX system software following manufacturer instructions.

Single multiplex PCR for identification of *E. coli* phylogenetic groups

Identification of the phylogenetic group for each *E. coli* strain was determined by the amplification of a set of DNA targets using a single multiplex PCR as described before (Clermont et al., 2000). In brief, genomic DNA from each strain was used for multiplex PCR DNA amplification of genes *chuA*, *yjaA* and DNA region TSPE4.C2. The reaction contained three primer pairs, including: ChuA.1 (5'-GACGAACCAACGGTCAGGAT-3') and ChuA.2 (5'-TGCCGCCAGTACCAAAGACA-3'), Yja.1 (5'-TGAAGTGTCAGGAGACGCTG-3') and Yja.2 (5'-ATGGAGAATGCGTTCCTCAAC-3'), and TspE4C2.1 (5'-GAGTAATGTCGGGGCATTCA-3) and TspE4C2.2 (5'-CGCGCCAACAAAGTATTACG-3'). Amplified DNA from each strain separated onto a 2.0% agarose and analyzed as described above. Based on the number and type of target amplified each isolate was assigned to a phylogenetic group. Phylogenetic group A was assigned to isolates *chuA* negative and TspE4.C2 negative; group B1 for isolates *chuA* negative and TspE4.C2 positive; group B2 for isolates *chuA* positive and *yjaA* positive; and group D for isolated *chuA* positive and *yjaA* negative.

RESULTS AND DISCUSSION

In the present manuscript we describe the use of a two-sample PCR assay to identify the most common *E. coli* pathotypes from children with diarrhea and from food products in Colombia to examine the potential association between food product contaminated with *E. coli* and human infection. This is the first report, to our knowledge, to identify four *E. coli* diarrheagenic pathotypes from clinical and food product samples by molecular methods in Colombia.

To evaluate the number of *E. coli* pathotypes from clinical and food product samples, *E. coli* were isolated from stools from children with diarrhea and from food products available at retail stores. The analysis of diarrheagenic *E. coli* pathotypes is based on samples rather than isolates. Each sample tested was either negative for *E. coli* or positive for *E. coli* (regardless the number of *E. coli* colonies tested per sample). Similarly, each *E. coli* positive sample was either positive for a diarrheagenic *E. coli* pathotype or negative. No more than one *E. coli* pathotype was detected per sample in this study (Table 2).

The isolation and identification of *E. coli* strains by traditional microbiology showed a high recovery of this microorganism from clinical and food samples (meat, vegetables) (Table 2). In this study we analyzed a total of 95 samples positive for *E. coli* from a total of 184 samples. Sixty-seven samples positive for *E. coli* strains were isolated from stools of children with diarrhea, 16 from meat samples and 12 from vegetable samples (Table 2).

The two-sample Multiplex PCR assay was used for identification of *E. coli* pathotypes among all 184 clinical and food product samples. A total of 18 (9.8%) samples were positive for any of the *E. coli* pathotypes or positive for any of the virulence factors analyzed, 12 (11.1%) among all clinical isolates and 6 (7.9%) among food product samples (Tables 2 and 3). While EPEC was the most common pathotype among all *E. coli* positive clinical (9.0%) and total samples (6.3%), STEC was more common among *E. coli* positive food product samples (7.1%) (Table

3). All EPEC strains were positive for *eae* and negative for *bfpA*, indicating that they were atypical EPEC (Kaper, 1996; Trabulsi et al., 2002).

We also detected the presence of *bfpA* + *E. coli* strain in food product samples. The *bfpA* gene is the structural subunit of BFP and a *bfpA* mutant of an EPEC strains showed decreased virulence in clinical studies performed in volunteers indicating that BFP is essential in virulence (Bieber et al., 1998). Our *bfpA* + strains are however not EPEC, since they lack the *eae* gene and they were not identified in stools from children with diarrhea. The *bfpA* + *eae*- *E. coli* strains have been detected in stools from children with diarrhea in the Colombian Caribbean suggesting that *bfpA* + *E. coli* strains may be associated with diarrheal disease (Gomez-Duarte et al., 2009a). We speculate that these strains may carry virulence factors that may result in diarrheal disease by a perhaps unique mechanism of pathogenesis no yet identified.

STEC, ETEC, EAEC and EPEC pathotypes were detected from clinical samples. Only STEC, EAEC, and *bfpA* + *E. coli* were detected from food product samples (Table 3). These four *E. coli* pathotypes were detected in children with diarrhea in our recent study in the Colombian Caribbean region (Gomez-Duarte et al., 2009a). They were also reported in the US (Cohen et al., 2005) and other Latin American countries, including Brazil (Bueris et al., 2007), and Mexico (Estrada-Garcia et al., 2007).

No DAEC or EIEC pathotypes were detected in this study. This is in contrast with studies done in Mexico (Estrada-Garcia et al., 2007), Brazil (Moreno et al., 2008) and Southeast Asian countries (Hien et al., 2008) where these pathotypes are prevalent. DAEC strains are prevalent in Brazil and may be associated with diarrheal disease in an age-specific dependent manner (Spano et al., 2008). DAEC was also recognized in the Colombian Caribbean region (Gomez-Duarte et al., 2009a). More studies will be necessary to determine if other geographic regions in Colombia may be prevalent for DAEC and/or EIEC.

The STEC strains detected by the two-sample multiplex PCR were *stx* positive and *eae* negative. All STECs detected were *stx-2* positive and *stx-1* negative. All STEC strain were negative for O157:H7 according with the BAX- system indicating that STECs from non-O157:H7 serotypes were present in Colombia during the period tested. This is in contrast with the Colombian report published in 1998 indicating that the prevalence of STEC O157:H7 was up to 7.7% in children with diarrhea and that prevalence of cattle positive for STEC O157:H7 was 6.5% (Mattar and Vasquez, 1998). We may speculate that during the 10 years the prevalence of STEC serotypes may have changed and that non-O157:H7 STEC strains may predominate in Colombia. Our study, however, correlates well with a most recent study indicating that STEC strains are prevalent in Colombia even though O157:H7 strains were not detected among clinical isolates (Martinez et al., 2007). Similar reports indicate the non-O157:H7 infections may be more common (Johnson et al., 2006). In Latin America, Argentina is one of the countries that reports high incidence of hemolytic uremic syndrome associated with STEC O157:H7 strains (Miceli et al., 1999; Rivas et al., 2006). While infection may occur by consumption of contaminated food, person to person transmission may also contribute to infection as fecal shedding of STEC was reported in children attending day-care centers (Miliwebsky et al., 2007).

The US and European countries also report frequent outbreaks due to O157:H7 strains (CDC, 1996; Gould et al., 2009; Cohen et al., 2005; Sharp et al., 1994).

While STEC and EAEC strains were isolated from clinical and food product isolates, ETEC and EPEC were only detected in clinical isolates. This information suggests that meat and vegetables in retail stores in Colombia may be the source of STEC and EAEC infections in the community. In contrast, ETEC and EPEC infection transmission may result as a consequence of contaminated sources such as water or other food products not tested in this

study, or as a result of person to person transmission. Studies in Argentina report fecal shedding of STEC in children attending day-care centers (Miliwebsky et al., 2007).

All *E. coli* isolates were tested with a multiplex PCR to identify *E. coli* phylogenetic groups. Clinical isolates had representatives from all four phylogenetic groups. Groups A, B2 and D with 27.3%, and group B1 with 18.2%. In contrast, food product isolates were only from phylogenetic groups A (75%) and B1 (25%). Multiplicity of phylogenetic groups provides evidence that the samples were random and that several clonal groups are circulating in the community and among food products. From all *E. coli* pathotypes identified 50% belong to the phylogenetic groups A and B1, known to be associated with intestinal pathogens, and 50% to the phylogenetic groups B2 and D. EPEC and ETEC strains were identified as groups B2 and D, EAEC and *bfp+* strains as group A and B1, and STEC has representatives in group A and D (Data not shown). Reports in the literature indicate that ETEC strains as well as other pathotypes associated with childhood diarrhea have representatives along all phylogenetic groups (Escobar-Paramo et al., 2004) suggesting that horizontal transfer of plasmid-encoded virulence genes including enterotoxins and colonization factors may transfer among strains from different phylogenetic groups. *E. coli* strains acquiring virulence factors horizontally may require an appropriate genomic background to become intestinal pathogens.

More information is necessary to understand the role of *E. coli* pathotypes in children diarrhea in Colombia and the role of food products and water in disease transmission. There is a need to evaluate other sources of contamination such as fruits, milk, dairy products as well as water. Climate changes, and time during the year where outbreaks of *E. coli* diarrhea are more frequent has high epidemiological significance and may be facilitated by the two-sample multiplex PCR implemented in this study. Furthermore, stool testing from asymptomatic adults will provide evidence of carrier state for some of these *E. coli* pathotypes.

In summary, the implementation of a two-sample multiplex PCR in this study successfully identified four *E. coli* pathotypes (ETEC, EAEC, STEC and EPEC) from clinical and food samples. It also recognized that food products from retail stores in Colombia carrying STEC and EAEC strains maybe, in part, the source of infections in the community. We believe that this study provides the basis for the systematic evaluation of *E. coli* diarrheal pathogens in Colombia, not only from stools from children with diarrhea but also from food sources, and water samples for human consumption. Epidemiological surveillance data on *E. coli* pathotypes may provide means to guide prevention and control measures against *E. coli* diarrheal disease in Colombia.

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Table 1

Oligonucleotide primers for the two-sample multiplex PCR amplification.

Primer Mix	Primer Sequence	Orientation	Gene target	PCR Size	Strain	Plasmid control ^a
M1	5'-GAGCGAAATAATTTATATGTG-3'	Forward	<i>stx_b</i>	518	STEC	pOG401
	5'-TGATGATGGCAATTCAGTAT-3'	Reverse				
	5'-CTGAACGCGGATTACGCGAA-3'	Forward	<i>eae</i>	917	STEC, EPEC	pOG390
	5'-CGAGACGATACGATCCAG-3'	Reverse				
	5'-AATGGTGCTTGGCGTTGCTGG-3'	Forward	<i>bfpA</i>	326	EPEC	pOG394
	5'-GCCGCTTTATCCAACTGTGA-3'	Reverse				
	5'-GTATACACAAAAGAAAGGAAAGC-3'	Forward	<i>aggR</i>	254	EAEC	pOG395
	5'-ACAGAAATCGTCAGCATCAGC-3'	Reverse				
M2	5'-GCACACGGAGCTCCTCAGTC-3'	Forward	LT	218	ETEC	pWD299
	5'-TCCTTCATCCTTTCAATGGCTTT-3'	Reverse				
	5'-GCTAAACCAAGTAGAG(C)TCTTCAAAA-3'	Forward	ST	147	ETEC	pSLM004
	5'-CCCGGTACAG(A)GCAGGATTACAACA-3'	Reverse				
	5'-GAACGTTGGTTAATGTGGGGTAA-3'	Forward	<i>daaE</i>	542	DAEC	pOG391
	5'-TATTACACCGGTCGGTTATCAGT-3'	Reverse				
	5'-AGCTCAGGCAATGAAACTTTTGAC-3'	Forward	<i>virF</i>	618	EIEC	pOG392
	5'-TGGGCTTGATATCCGATAAGTC-3'	Reverse				
	5'-CTCGGCACGTTTTTAATAAGTCTGG-3'	Forward	<i>ipaH</i>	933	EIEC	pOG393
	5'-GTGGAGAGCTGAAGTTTCTCTGC-3'	Reverse				

^a Plasmid carrying specific target genes used as template DNA control for the two-sample multiplex PCR assay (Gómez-Duarte et al., 2009).

^b *stx* primer pair amplifies either *stx-1* or *stx-2* genes. To discriminate between the two *stx* genes a subsequent PCR was performed with specific *stx-1* and *stx-2* primer pairs, as described in Materials and Methods.

Table 2
Pathotypic and non-pathotypic *E. coli* strains among clinical and food products samples

Samples	Negative for <i>E. coli</i>		Positive for <i>E. coli</i>				Total Samples	
	Negative for <i>E. coli</i>		Positive Pathotype		Negative Pathotype		Total <i>E. coli</i>	
	No.	%	No.	%	No.	%	No.	%
Children's stool	41	38.0	12	11.1	55	50.9	67	62.0
Food product								
Vegetable	26	68.4	3	7.9	9	23.7	12	31.6
Meat	22	57.9	3	42.1	13	7.9	16	57.1
Total	48	63.2	6	7.9	22	28.9	28	36.8
Total	89	48.4	18	9.8	77	41.8	95	51.6
							184	100

Table 3
Number of *E. coli* pathotypes among all *E. coli* strains isolated from clinical and food products samples.

Pathotype	Meat		Vegetables		Total food Products		Clinical samples		Total	
	No.	%	No.	%	No.	%	No.	%	No.*	%
STEC	1	6.3	1	8.3	2	7.1	1	1.5	3	3.2
ETEC	0	0	0	0	0	0	2	3.0	2	2.1
EAEc	0	0	1	8.3	1	3.6	3	4.5	4	4.2
EPEC	0	0	0	0	0	0	6	9.0	6	6.3
<i>bfp+</i>	2	12.5	1	8.3	3	10.7	0	0	3	3.2
Negative	13	81.3	9	75.0	22	78.6	55	82.1	77	81.1
Total	16	100.0	12	100.0	28	100.0	67	100.0	95	100.0

* Each strain reported represents a single clinical or food product sample. No more than one *E. coli* pathotype was detected per sample.