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Detection of *Escherichia coli* Enteropathogens by Multiplex Polymerase Chain Reaction from Children's Diarrheal Stools in Two Caribbean–Colombian Cities

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Abstract

Acute diarrheal disease is a leading cause of childhood morbidity and mortality in the developing world and *Escherichia coli* intestinal pathogens are important causative agents. Information on the epidemiology of *E. coli* intestinal pathogens and their association with diarrheal disease is limited because no diagnostic testing is available in countries with limited resources. To evaluate the prevalence of *E. coli* intestinal pathogens in a Caribbean–Colombian region, *E. coli* clinical isolates from children with diarrhea were analyzed by a recently reported two-reaction multiplex polymerase chain reaction (Gomez-Duarte *et al.*, Diagn Microbiol Infect Dis 2009;63:1–9). The phylogenetic group from all *E. coli* isolates was also typed by a single-reaction multiplex polymerase chain reaction. We found that among 139 *E. coli* strains analyzed, 20 (14.4%) corresponded to *E. coli* diarrheagenic pathotypes. Enterotoxigenic, shiga-toxin–producing, enteroaggregative, diffuse adherent, and enteropathogenic *E. coli* pathotypes were detected, and most of them belonged to the phylogenetic groups A and B1, known to be associated with intestinal pathogens. This is the first report on the molecular characterization of *E. coli* diarrheogenic isolates in Colombia and the first report on the potential role of *E. coli* in childhood diarrhea in this geographic area.

Introduction

Diarrheal disease is a leading cause of mortality and morbidity in children living in underserved countries. Close to 2 millions of children below 5 years of age are estimated to die every year because of diarrheal diseases (Bryce *et al.*, 2005; Boschi-Pinto *et al.*, 2008). In Colombia, South America, several studies report the importance of rotavirus as a cause of infectious diarrhea (Correa *et al.*, 1999; Urbina *et al.*, 2003, 2004; Caceres *et al.*, 2006; Gutierrez *et al.*, 2006), and to a lesser extent, there are studies describing the role of bacterial infections in diarrheal disease, such as *Shigella, Salmonella*, and *Vibrio cholerae* infections (Mattar, 1992; Hidalgo *et al.*, 2002; Manrique-Abril *et al.*, 2006; Muñoz *et al.*, 2006). Stool samples from children with diarrhea in the cities of Sincelejo and Cartagena, Colombia, tested positive for rota virus

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(36.6%), *Salmonella* spp. (9.0%), *Shigella* spp. (8.0%), and pathogenic *Escherichia coli* (2.8%) (Urbina *et al.*, 2003). In our study, the definition of pathogenic *E. coli* was based on detection of serotypes associated to enteropathogenic *E. coli* (EPEC) and the shiga-toxin–producing *E. coli* (STEC). No other *E. coli* pathotypes were evaluated.

Cartagena, Colombia, is a tourist destination and an important industrial city with a highly dynamic population. In contrast, Sincelejo is a city with an agricultural-based economy and minimal population movement. Of interest, rotavirus serotypes from children with diarrhea were significantly diverse in Cartagena, whereas only two serotypes were recognized in all samples tested from Sincelejo (Urbina *et al.*, 2004). No information is available on the role of *E. coli* pathotypes in childhood diarrhea in these locations or whether pathotypes vary from city to city. Information on the role of *E. coli* in childhood diarrhea in Colombia is limited to reports describing the identification of the *E. coli* O157:H7 (Mattar and Vasquez, 1998) and non-O157:H7 STEC (Martinez *et al.*, 2007) associated with cases of gastroenteritis. Information on enteropathogens associated with infectious diarrhea and the role of *E. coli* pathotypes is essential to understand the prevalence of these infections in the population at risk, to evaluate potential sources of transmission, and most importantly, to implement medical care and prevention strategies directed to decrease childhood morbidity and mortality due to diarrhea.

There are six *E. coli* intestinal pathotypes described in the literature, associated with acute diarrheal disease based on the array of virulence factors they express (Levine, 1987; Gomez-Duarte *et al.*, 2009). These include STEC, EPEC, enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffuse adherent *E. coli* (DAEC), and enteroinvasive *E. coli* (EIEC). Extraintestinal *E. coli* pathotypes are also known to express a distinct set of virulence genes; however, because they are not associated with acute diarrhea they will not be analyzed in our study.

Identification of *E. coli* pathotypes in association with diarrhea is limited in many developing countries because conventional microbiological testing is unable to distinguish between normal flora and pathogenic strains of *E. coli*. Although molecular diagnostic systems may detect intestinal *E. coli* pathogens, countries with limited resources may not afford their implementation. Despite multiple assays have been developed for typing *E. coli* intestinal pathotypes (Stacy-Phipps *et al.*, 1995; Nguyen *et al*, 2005; Aranda *et al*, 2007; Brandal *et al*, 2007), most assays rely on technologies not available in most clinical laboratories. A recently reported three-sample multiplex polymerase chain reaction (PCR) was developed for use in any clinical laboratory, including those with limited resources (Gomez-Duarte *et al.*, 2009). It is capable of identifying diarrheagenic *E. coli* strains as well as other non-*E. coli* pathogens. This method uses plasrnid DNA as control DNA templates instead of prototype wild-type strains. This factor obviates the need for −70°C freezers for strain storage. Further, the assay uses master mix Taq polymerase (this mix contains polymerase, polymerase buffer, nucleotides, and magnesium chloride) to eliminate the need to prepare multiple reagents.

Phylogenetic analysis of *E. coli* clinical isolates classifies *E. coli* strains into four phylogenetic or clonal groups, namely, A, B1, B2, and D (Herzer *et al.*, 1990). Each clonal group is believed to be associated with specific pathogenesis determinants. Intestinal *E. coli* pathotypes tend to associate with phylogenetic groups A and B1, but extraintestinal *E. coli* pathotypes generally associate with phylogenetic groups B2 and D (Boyd and Hartl, 1998; Johnson *et al.*, 2002). This type of analysis is believed to be superior to serotyping because lipopolysaccharide (O) and fiagellar (F) antigens undergo significant variation independently of virulence. Several *E. coli* phylogenetic typing methods exist, including multilocus enzyme electrophoresis (Ochman and Selander, 1984), multilocus sequencing typing (Maiden *et al.*, 1998), and PCR (Clermont, 2000). Although multilocus sequencing typing is more discriminatory and suitable for analysis

of strains from infection outbreaks, PCR phylogenetic testing provides information on the main phylogenetic groups in relation to intestinal versus extraintestinal *E. coli* strains, as previously described (Boyd and Hartl, 1998; Johnson *et al.*, 2002). PCR phylogenetic testing, however, is simple, reproducible, and accessible to most laboratories in countries with limited resources.

The goal of our study was to evaluate the prevalence of *E. coli* pathotypes among children with diarrhea in Colombia using a two-sample multiplex PCR. *E. coli* clinical isolates were obtained from children below 5 years of age and living in two Caribbean–Colombian cities. We evaluated the differences in *E. coli* pathotypes as well as in phylogenetic groups, cities of origin, and children's age. Phylogenetic grouping will be assessed by a second multiplex PCR, with the goal of determining the degree of *E. coli* diversity among all clinical isolates and specifically among *E. coli* intestinal pathotypes.

Materiais and Methods

Study design

This is a prevalence study for evaluating the number of *E. coli* pathotypes circulating in children with diarrhea in two Northern Colombian cities. Stool samples were collected from children less than 5 years of age with diarrhea and living in Sincelejo and Cartagena, Colombia, after informed consent was obtained from their parents. All children evaluated at health clinics fulfilled the World Health Organization criteria for acute diarrheal disease (WHO, 2005). Subjects were identified by age, date, and location. A total of 267 stool samples were collected and processed during a 1-year period from January to November 2007. Among them, 139 *E. coli* isolates were recovered, 28 from Cartagena and 111 from Sincelejo.

Sample processing

All stool samples used in our study had been previously collected by healthcare personnel at each medical center in Cartagena or Sincelejo for diagnosis purposes. A cotton swab was used to obtain a stool sample from the original specimen. The samples were placed in Stuart transport media (Oxoid, Basingstoke, UK) and taken to the Microbiology Laboratory, University of Cartagena, for further processing. The samples that could no be processed immediately were transported and stored at 4°C. *E. coli* isolates were obtained by culturing stool samples on McConkey or eosin methylene blue agar. Lactose-fermenting Gram-negative coccobacilli were tested with conventional biochemical assay for identification of *E. coli. E. coli* isolates were further confirmed by PCR amplification of the β-D-glucuronidase (*uigA*) gene using *E. coli* specific primers (Table 1) (McDaniels *et al*, 1996).

DNA techniques

Unless otherwise specified, standard methods were used for plasmid isolation, genomic DNA isolation, and agarose electrophoresis DNA separation (Sambrook and Russell, 2001).

E. coli pathotype identification by multiplex PCR

E. coli clinical isolates were processed for isolation of genomic DNA as previously described (Gomez-Duarte *et al*., 2009). In brief, overnight liquid cultures were centrifuged, and the pellet was resuspended in water, boiled for 10 min, and centrifuged again. The supernatant containing a crude DNA extract was used as a DNA template on a multiplex PCR for identification of *E. coli* pathotypes, namely, EPEC, STEC, EAEC, ETEC, DAEC, and EIEC. The *E. coli* pathotype two-sample multiplex PCR was carried out using plasmid DNA with cloned targets as positive controls and plasmid DNA vectors as negative clones, as previously described (Gomez-Duarte *et al*., 2009). In brief, there was one plasmid clone for each gene target, while plasmid vectors pCR2.1 and pSC-A and *E. coli* flora genomic DNA were used as negative controls. The PCR

1 contained Ml primers for amplification of *eae*, *bfpA*, VT, and *aggR* genes for identification of STEC, EPEC, and EAEC pathotypes (Table 1). The PCR 2 contained M2 primers for amplification of LT, ST, *daaE*, *ipaH*, and *virF* gene targets for identification of ETEC, DAEC, and EIEC pathotypes. One microliter of genomic DNA was mixed with 24 μL of a pre-made mix containing primers at a 0.2 μM final concentration and Platinum Blue PCR SuperMix polymerase (Invitrogen, Carlsbad, CA). The PCR program used for amplification consisted of 2 min at 94°C denaturing temperature, followed by 40 cycles of 30 sec at 92°C denaturing temperature, 30 sec at 59°C annealing temperature, and 30 sec at 72°C extension temperature. At the end of 40 cycles and a 5-min extension at 72°C, samples were separated onto a 2% agarose ethidium bromide-stained gel, and DNA bands were visualized and recorded under ultraviolet light for further analysis.

Those *E. coli* isolates identified as STEC were further analyzed for determination of the type of verotoxin they carry. This was done by a standard single PCR using specific shiga-like toxin 1 (VT1) (Vidal *et al*., 2005) and shiga-like toxin 2 (VT2) (Nguyen *el al*., 2005) oligonucleotide primers (Table 1).

E. coli phyiogenetic grouping

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 previously described (Clermont *et al*., 2000). In brief, genomic DNA from each strain was used for multiplex PCR DNA amplification of genes *chuA*, *yjaA* and of DNA region TSPE4.C2. The reaction contained three primer pairs, including ChuA.F and ChuAR, YjaAF and YjaAR, and TspE4C2F and TspE4C2R (Table 1). Amplified DNA from each strain was separated onto a 2.0% agarose and analyzed as described earlier. Based on the number and type of target amplified the isolate was assigned a phylogenetic group. Phylogenetic group A was assigned to isolates *chuA* negative and TspE4.C2 negative; group B1 was assigned to isolates *chuA* negative and TspE4.C2 positive; group B2 was assigned to isolates *chuA* positive and *yjaA* positive; and group D was assigned to isolates *chuA* positive and *yjaA* negative. All PCRs were performed more than once to confirm the results. Previously reported *E. coli* strains (Boyd and Hartl, 1998) were used as phylogenetic group controls: *E. coli* ECOR1 for group A, ECOR29 for group B1, ECOR53 for group B2, and ECOR47 for group D. Strains were kindly provided by Dr. Weismann at the University of Washington.

Serologic testing for *E. coli* O157:H7

E. coli strains positive for VT by PCR were tested for the presence of O157 antigen and H7 flagellar antigen. Anti-O157 antiserum (Difco, Sparks, MD) and anti-H7 antiserum (Difco) were added to bacterial suspension to evaluate for agglutination. A clinically isolated O157:H7 STEC strain was used as positive control and the O8:H9 E9034A ETEC strain (Gomez-Duarte *et al*., 2007) was used as negative control. The agglutination protocol was carried out as recommended by the antiserum suppliers.

Statistical analysis

Fisher's exact test or one-way analysis of variance was conducted to determine the differences and associations between *E. coli* isolates and pathotypes, phylogenetic groups, city of origin, and patient age. All statistical calculations used the GraphPad InStat version 3.06 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). For all calculations the *p*-value for statistical significance was set at <0.05.

Results

E. coli pathotypes identified among clinical isolates

Twenty (14.4%) *E. coli* strains out of 139 clinical isolates were positive for any of the six known *E. coli* pathotypes associated with diarrhea. This indicates that 14.4% of all *E. coli* isolates were *E. coli* intestinal pathotypes, and that 7.5% of all stool samples collected contained an *E. coli* intestinal pathotype. The most frequent pathotype was ETEC, (Table 2), which corresponded to 5% of the total number of *E. coli* isolates studied. Other *E. coli* pathotypes identified included STEC, EPEC, EAEC, and DAEC. There was a group made up of *E. coli* strains that were either positive for virulence factors from more that one pathotype, or were positive for a single virulence factor not sufficient to identify that strain as a typical pathotype. This group corresponded to 4% of the total *E. coli* isolates analyzed, and it was made of *E. coli* strains carrying any combination of VT, LT, ST, *eae*, or *bfpA* genes.

Low rates of STEC, EPEC, EAEC, and DAEC were identified, and these accounted for less than 7% of the isolates. Further, EPEC and STEC isolates were all atypical as EPEC isolates were only positive for *eae* and negative for *bfpA*, and all STEC isolates were positive for VT and negative for *eae*. STEC strains were positive for either VT1 or VT2, or both. None of the STEC strains identified were positive for serotype O157:H7 according to agglutination testing with anti-O157 or anti-H7 antisera.

These data indicate that *E. coli* pathotypes are circulating in the pediatric population of two Northern Colombian cities and that these isolates may be associated to children with diarrhea. While ETEC was the predominant pathotype, this difference was not statistically significant with respect to children's age, city of origin, or *E. coli* pathotypes.

Proportion of *E. coli* pathotypes based on children's age

All *E. coli* isolates from children with diarrhea were analyzed according to children age and *E. coli* pathotype. The proportion of *E. coli* positive for any intestinal pathotype was 18.6% in children less than 2 years of age and only 7.5% in children above 2 years this difference; however, it is not statistically significant. EAEC, EPEC, and DAEC isolates were identified in children less than 2 years of age only, whereas ETEC and STEC pathotypes were identified in both age groups, above 2 years and below 2 years. The number of enteric *E. coli* pathotypes was similar with respect to city of origin, and the difference in *E. coli* pathotypes between Cartagena and Sincelejo was not statistically significant (Table 2).

Phylogenetic groups A and B1 were the most common among *E. coli* pathotypes

One hundred eight *E. coli* isolates (77.1%) of all *E. coli* isolates belong to the phylogenetic groups A and B1, which are known to be associated with the gastrointestinal tract. The remaining isolates were distributed between phylogenetic groups B2 and D. B2 was the phylogenetic group least represented, with only 10 strains (Table 3). Among all enteric *E. coli* pathotypes, 85.0% belong to the phylogenetic groups A and B1 and the remaining to phylogenetic group D. There were no representatives from phylogenetic group B2 among the *E. coli* intestinal pathotypes. Among the 20 *E. coli* pathotypes identified, ETEC, EPEC, DAEC, and mixed pathotypes were members of the phylogenetic groups A and B1. Only STEC and EAEC had representatives from phylogenetic group D in addition to phylogenetic groups A and/or B (Fig. 1). No significant difference in phylogenetic groups was observed when *E. coli* isolated from children less than 2 years of age was compared with children above 2 years of age (Table 3). When phylogenetic group means were compared from the two age group populations, the difference between phylogenetic group A and the remaining groups was statistically significant ($p < 0.05$).

The proportion of *E. coli* from two Colombian cities differs with respect to pathotypes and phylogenetic groups

To determine the distribution of *E. coli* pathotypes between the two cities, we compared the number of intestinal *E. coli* pathotypes and negative *E. coli* pathotypes between the two cities. In both cities the proportion of positive pathotypes was close to 14%. Similarly, the proportion of *E. coli* isolates from phylogenetic groups A, B1, and B2 was similar in Sincelejo and Cartagena, with the exception of group D isolates, which were twofold more common in Cartagena than in Sincelejo, a result that is statistically significant (Table 3). Further, the proportion of ETEC and STEC pathotypes was similar in both cities; however, the proportion of EAEC and DAEC was different because these pathotypes were not detected in Cartagena. This is likely related to the smaller number of *E. coli* clinical isolates analyzed in Cartagena; the difference was not statistically significant.

Discussion

Acute dehydrating diarrhea remains a leading cause of mortality in the developing world, and oral hydration is not sufficient to alter the disproportionate number of deaths due to the condition, especially in low-income nations (Forsberg *et al*., 2007). Surveillance for diarrheal etiologic agents in developing nations is necessary to understand the local epidemiology of infectious diarrhea and to measure the burden of disease in children (Gomez-Duarte, 2009; Gomez-Duarte *et al*., 2009). Based on this information it may be possible to implement public health measures directed to control and prevent specific causes of infectious diarrhea. In our study we have analyzed the presence of intestinal enteric *E. coli* pathotypes among children with infectious diarrhea in Colombia. To our knowledge, this is the first report describing the molecular characterization of *E. coli* enteropathogens in this country and the potential role of ETEC in childhood diarrhea in this part of the country.

Representatives of five *E. coli* pathotypes were isolated from stool samples from children with diarrhea in two coastal cities in Colombia, and ETEC was the most common pathotype. The calculated prevalence of 7.5% of enteric *E. coli* pathotypes among all stools tested corroborates previous results reporting 6% pathogenic *E. coli* in Colombia (Urbina *et al*., 2003). The predominance of the ETEC pathotype in our study is comparable with data reported in Tunisia (Al-Gallas *et al*., 2007), Bangladesh (Qadri *et al*., 2007), and Egypt (Shaheen *et al*., 2009). This is in contrast to the EAEC pathotype which was found to be the predominant pathotype in Brazil (Bueris *et al*., 2007), Tanzania (Moyo *et al*., 2007), and the United States (Cohen *et al*., 2005).

Our study also reveals that children less than 5 years of age are at risk of diarrheagenic *E. coll*. We speculate that children at this age are immunologically naive and may not possess specific immune response to new pathogens. Our study cannot answer the specific role of diarrheagenic *E. coli* in diarrhea, as no other pathogens were investigated, including non-*E. coli* bacterial pathogens or viral agents. One limitation of our study was the small number of strains isolated, mainly from Cartagena, to be analyzed for city of origin, children's age, pathotypes, and phylogenetic groups. A larger study comparing children with diarrhea with healthy controls, from both Sincelejo and Cartagena, will be necessary to determine the proportion of children of different ages who, after initial infection with diarrheagenic *E. coli*, remain colonized with these strains and become normal carriers. These studies may also calculate the proportion of children who, after recovery from infectious diarrhea, eradicate the infection and become immune to new infections.

Our study used a two-sample PCR assay to detect a specific set of virulence genes and clearly identified most *E. coli* pathotypes. The presence of LT or ST identified ETEC, VT identified STEC, *eae* (alone or in combination with *bfpA*) identified EPEC, and *virF* identified EIEC.

Scientific debate continues regarding the virulence gene-based definition of EAEC and DAEC pathotypes, because not all virulence genes associated with these strains are present in 100% of them or are associated with human disease. Until these debates are settled, we believe that the EAEC global virulence regulatory *aggR* gene and the DAEC fimbrial *daaE* gene are appropriate for detection.

Regarding phylogeny, most *E. coli* isolates including intestinal *E. coli* pathotypes belong to the phylogenetic groups A and B1. These data indicate that the majority of *E. coli* isolates analyzed are representatives of intestinal flora as well as intestinal pathogens. Most *E. coli* pathotypes in our study belong to the phylogenetic group A and B1, and some STEC and EAEC isolates were representatives of the phylogenetic group D. Because the virulence factors that define *E. coli* intestinal pathotypes are present in phages (VT toxin genes), pathoge-nicity islands (*eae*), or virulence plasmids (*aggR*), it is likely that horizontal transfer of these genes has occurred from group A or B1 *E. coli* ancestors to new group D and even group B2 *E. coli* permissive recipients, as recently reported (Escobar-Paramo *et al*., 2004). More studies are necessary to clarify the role of *E. coli* pathotypes from phylogenetic groups B2 and D to determine what proportion of theses strains are true intestinal pathogens.

Our study also recognized the diversity of *E. coli* pathotypes in these Colombian cities by detecting clinical isolates carrying virulence genes from different pathotypes. The identification of this group of isolates indicates that *E. coli* pathotypes in this geographic region is highly diverse and it suggests that horizontal transfer of virulence genes is occurring among *E. coli* intestinal pathotypes. The successful colonization of these strains may suggest that these strains are well adapted to the human intestinal environment and may potentially be more virulent. Reports indicate that this phenomenon is not uncommon as EAEC was reported to acquire virulence markers from DAEC (Czeczulin *et al.*, 1999). A small number of EPEC and STEC strains were also identified; all of them were atypical as EPEC were only positive for *eae* and negative for *bfpA* and STEC were positive for VT and negative for *eae*.

All STEC strains identified were negative for the O157:H7 serotype. These data correlate with recent findings indicating that the prevalence of O157:H7 STEC in Colombia is low (Martinez *et al*, 2007), Non-O157:H7 STEC strains have been described in other Latin American countries such as Brazil (Bueris *et al*, 2007) and Mexico (Estrada-Garcia *et al.*, 2009). While O157:H7 is the predominant STEC associated with hemolytic–uremic syndrome outbreaks in the United States, Europe, Argentina, and Australia, non-0157:H7 STEC-associated outbreaks are increasingly recognized worldwide (Johnson *et al.*, 2006). In Iran, non-O157:H7 STEC strains are the predominant agents associated with severe diarrheal infections; in this country the most common STEC serotypes were O25:H3, O85:H32, and O162:H21 (Aslani and Bouzari, 2009). Similarly, all STEC isolates evaluated in Poland were non-O157:H7 (Sobieszczanska *et al.*, 2004), indicating that the prevalence of STEC O157:H7 and non-O157:H7 serotypes may widely vary according to geographic location. More studies in Colombia will be necessary to assess whether non-O157:H7 STEC strains predominate in all Colombian regions and neighbor countries, and also to evaluate the association of these strains with cases of bloody diarrhea and hemolytic–uremic syndrome in children and adults.

Although Cartagena is a tourist city, with highly dynamic population and significantly better infrastructure than Sincelejo, no statistically significant differences were noticed in the number and type of *E. coli* identified, except for the increased number of *E. coli* isolates from phylogenetic group D in Cartagena. We speculate that the increased number of group D *E. coli* strains may be the result of transmission from colonized travelers to the native population. More studies are needed to identify the most common diarrheagenic *E. coli* reservoirs and the transmission patterns within the children population. Studies in Colombia are currently testing meats and vegetables from retails stores for bacterial contamination. Preliminary results

indicate the presence of diarrheogenic *E. coli* contaminants in these food products (our unpublished results).

Living conditions in the Colombian Caribbean region may differ from other cities in Colombia. It is likely that the proportion of *E. coli* pathotypes vary according to the geographic region. Epidemiological studies using similar technology may provide clues to whether *E. coli* is widely spread or limited to temperate climates. PCR technology can facilitate the identification of possible sources of diarrheagenic *E. coli* contamination. These sources may include water for human consumption, food products, caregivers (healthcare and non-healthcare workers), and even city air. Epidemiological information obtained by these means may contribute to the control of infectious diarrhea at different levels. First, it will increase the understanding of the impact of *E. coli* on childhood diarrhea in Colombia. It may help implement mechanisms of disease control and prevention, including vaccines. Also, it may facilitate the implementation of strategies to manage diarrhea secondary to each one of the *E. coli* pathotypes.

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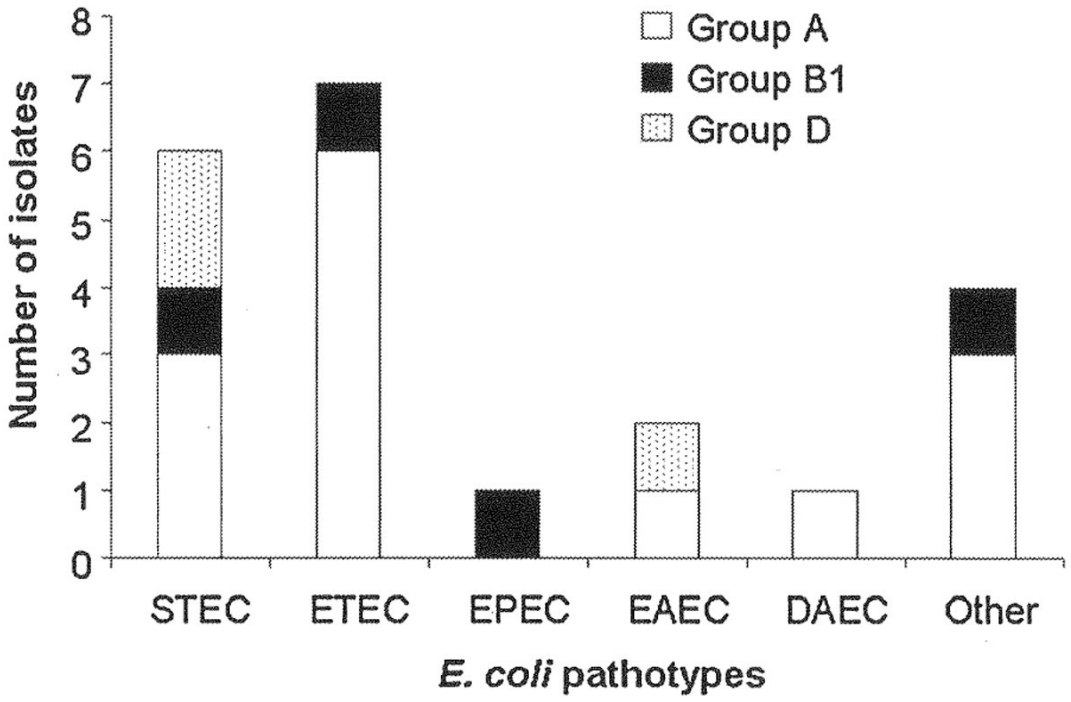

FIG. 1.

Distribution of phylogenetic groups according to *Escherichia coli* pathotypes. *E. coli* pathotypes were individually analyzed with respect to their phylogenetic group. Only three phylogenetic groups (A, B1, and D) were represented because no *E. coli* pathotype strain from phylogenetic group B2 was identified.

Table 1

Oligonucleotide Primers for Multiplex and Single Polymerase Chain Reaction Assays

Primer mix	Primer sequence	Primer name	Gene target	PCR Size (bp)	Pathotype	Plasmid control[a]
M1[b]	5′-GAGCGAAATAATTTATATGTG-3′	VT.F	VT	518	STEC	pOG401
	5′-TGATGATGGCAATTCAGTAT-3′	VT.R				
	5′-CTGAACGGCGATTACGCGAA-3′	eae.F	*eae*	917	STEC, EPEC	pOG390
	5′-CGAGACGATACGATCCAG-3′	eae.R				
	5′-AATGGTGCTTGCGCTTGCTGC-3′	bfpA.F	*bfpA*	326	EPEC	pOG394
	5′-GCCGCTTTATCCAACCTGGTA-3′	bfpA.R				
	5′-GTATACACAAAAGAAGGAAGC-3′	aggR.F	*aggR*	254	EAEC	pOG395
	5′-ACAGAATCGTCAGCATCAGC-3′	aggR.F				
M2[b]	5′-GCACACGGAGCTCCTCAGTC-3′	LT.F	LT	218	ETEC	pWD299
	5′-TCCTTCATCCTTTCAATGGCTTT-3′	LT.R				
	5′-GCTAAACCAGTAGAG(C)TCTTCAAAA-3′	ST.F	ST	147	ETEC	pSLm004
	5′-CCCGGTACAG(A)GCAGGATTACAACA-3′	ST.R				
	5′-GAACGTTGGTTAATGTGGGGTAA-3′	daaE.F	*daaE*	542	DAEC	pOG391
	5′-TATTCACCGGTCGGTTATCAGT-3′	daaE.R				
	5′-AGCTCAGGCAATGAAACTTTGAC-3′	virF.F	*virF*	618	EIEC	pOG392
	5′-TGGGCTTGATATTCCGATAAGTC-3′	virF.R				
	5′-CTCGGCACGTTTTAATAGTCTGG-3′	ipaH.F	*ipaH*	933	EIEC	pOG393
	5′-GTGGAGAGCTGAAGTTTCTCTGC-3′	IpaH.R				
p[c]	5′-GACGAACCAACGGTCAGGAT-3′	chuA.F	*chuA*	279	NA	-
	5′-TGCCGCCAGTACCAAAGACA-3′	chuA.R				
	5′-TGAAGTGTCAGGAGACGCTG-3′	yiaA.F	*yiaA*	211	NA	-
	5′-ATGGAGAATGCGTTCCTCAAC-3′	yiaA.R				
	5′-GAGTAATGTCGGGGCATTCA-3′	TspE4C2.F	TspE4C2	152	NA	-
	5′-CGCGCCAACAAAGTATTACG-3′	TspE4C2.R				
VT1	5′-CAGTTAATGTGGTGGCGAAGG-3	VT1.F	*VT1*	348	STEC	-
	5′CACCAGACAATGTAACCGCTG-3′	VT1.R				
VT2	5′-ACCGTTTTTCAGATTTT(G/A)CACATA-3′	VT2.F	*VT2*	298	STEC	-
	5′-TACACAGGAGCAGTTTCAGACAGT-3′	VT2.R				

Primer mix	Primer sequence	Primer name	Gene target	PCR Size (bp)	Pathotype	Plasmid control[a]
uigA	5′-GCGTCTGTTGACTGGCAGGTGGTGG-3′	uidA.F	*uidA*	503	NA	-
	5′-GTTGCCCGCTTCGAAACCAATGCCT-3′	uidA.R				

PCR, polymerase chain reaction; EPEC, enteropathogenic *Escherichia coli*; STEC, shiga-toxin producing *E. coli*; EAEC, enteroaggregative *E. coli*; ETEC, enterotoxigenic *E. coli*; DAEC, diffuse adherent *E. coli*; EIEC, enteroinvasive *E. coli*; NA, not applicable.

[a] M1 and M2 are primer mixes to be used for the two-sample multiplex PCR assay for detection of *Escherichia coli* pathotypes.

[b] Plasmids carrying each one of the *E. coli* pathotype gene targets. Plasmids are used as template DNA control for the two-sample multiplex PCR assay as previously described (Gomez-Duarte *et al.*, 2009).

[c] P is the primer mix to be used for a single-multiplex PCR for identification of phylogenetic group (Clermont *et al.*, 2000).

Table 2

Distribution of *Escherichia coli* Clinical Isolates According to Pathotypes, Host's Agb, and City of Origin

	Children's age					City of origin				
	≤2 years		>2 years			Cartagena		Sincelejo		
Pathotypes	**No.**	**%**	**No.**	**%**	***p*-Value**	**No.**	**%**	**No.**	**%**	***p*-Value**
STEC	3	3.5	2	3.8	>0.99	1	3.6	4	3.6	>0.99
EPEC	1	1.2	0	0	>0.99	1	3.6	0	0	0.201
EAEC	2	2.3	0	0	0.524	0	0	2	1.8	>0.99
ETEC	6	6.9	1	1.9	0.251	2	7.1	5	4.5	0.628
DAEC	1	1.2	0	0	>0.99	0	0	1	0.9	>0.99
Mix	3	3.5	1	1.9	>0.99	0	0	4	3.6	0.582
Negative	70	81.4	49	92.5	0.084	24	85.7	95	85.6	>0.99
Total	86	100	53	100		28	100	111	100	

Table 3

Distribution of *Escherichia coli* Clinical Isolates According to Phylogenetic Groups, Host's age, and City of Origin

	Children's age					City of origin				
	≤2 years		>2 years			Cartagena		Sincelejo		
Phylogenetic group	**No.**	**%**	**No.**	**%**	***p*-Value**	**No.**	**%**	**No.**	**%**	***p*-Value**
Group A	49	56.32	36	67.92	0.212	15	53.57	71	63.96	0.384
Group B1	15	17.24	8	15.09	0.817	3	10.71	19	17.12	0.056
Group B2	7	8.05	3	5.66	0742	2	7.14	8	7.21	>0.99
Group D	16	18.39	6	11.32	0.341	8	28.57	13	11.71	0.37[a]
Total	87	100.00	53	100.00		28	100.00	111	100.00	

[a]Statistically significant *p*-value of <0.005.