Diarrheagenic *Escherichia coli* in Children from Costa Rica

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Abstract. More than 5,000 diarrheal cases per year receive medical care at the National Children's Hospital of Costa Rica, and nearly 5% of them require hospitalization. A total of 173 *Escherichia coli* strains isolated from children with diarrhea were characterized at the molecular, serologic, and phenotypic level. Multiplex and duplex polymerase chain reactions were used to detect the six categories of diarrheagenic *E. coli*. Thirty percent ($n = 52$) of the strains were positive, indicating a high prevalence among the pediatric population. Enteropathogenic *E. coli* and enteroinvasive *E. coli* pathotypes were the most prevalent (21% and 19%, respectively). Pathogenic strains were distributed among the four *E. coli* phylogenetic groups A, B1, B2, and D, with groups A and B1 the most commonly found. This study used molecular typing to evaluate the prevalence of diarrheagenic *E. coli* reported in Costa Rica and demonstrated the importance of these pathotypes in the pediatric population.

INTRODUCTION

After rotavirus, diarrheagenic *Escherichia coli* are the second most common cause of diarrhea in children less than five years of age. There are six categories of intestinal *E. coli* pathotypes implicated in diarrheal disease. These are enterohemorrhagic (shiga toxin-producing *E. coli* [STEC]) *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC). The categories differ mainly in their virulence genetic makeup.

EPEC is associated with protracted diarrhea in infants in developing countries.¹ Typical EPEC strains express *eae*, which encodes intimin, and the bundle-forming pilus (BFP) responsible for the localized adherence phenotype and enterocyte attaching and effacing lesions.^{2,3} ETEC strains are defined by the presence of one or two plasmid-encoded enterotoxins, the thermostable toxin (*st*) and the thermolabile toxin (*lt*). This pathogen is the most common cause of childhood diarrhea among all *E. coli* pathotypes and the most frequent cause of diarrhea in travelers to developing countries.^{4,5} In addition to toxins, they also express fimbrial colonization factors.^{6,7} EHEC is associated with bloody diarrhea and with hemolytic uremic syndrome.^{8,9} EHEC contains the locus of the enterocyte effacement pathogenicity island also present in EPEC and expresses one or two shiga-like toxin encoding genes (*stx*₁ and *stx*₂).¹⁰ The most common serotype associated with outbreaks in the United States and Europe is the O157:H7. EIEC shows pathogenic, phenotypic, and genetic similarities with *Shigella*. Different probes and primers have been designed for a specific approach, including those encoding for invasins such as the *ipaH* and *ial* genes.^{11,12} EAEC is commonly associated with traveler's diarrhea in developing countries. The enteroaggregative adherence is characterized by a stacked-brick formation of bacterial cells on cell culture assays.¹³ The pathogenesis of EAEC infection includes adherence of bacteria to the terminal ileum and colon and a variety of virulence factors regulated by the aggregative factor (AggR).¹⁴ The last cate-

gory of diarrheagenic *E. coli*, DAEC, is defined by the diffuse pattern of adherence on Hep2 cell culture assays. A fimbrial adhesin known as F1845 mediates the adherence of bacteria to epithelial cells. The gene cluster encoding F1845 (*daaE*) can be found on either the bacterial chromosome or on a plasmid.^{15,16}

The polymerase chain reaction (PCR) for detecting virulence genes is commonly used, and PCR modifications such as multiplex PCR make them suitable as a routine test in developing countries, where expensive analyses are difficult to perform.^{17,18} Phylogenetic studies show that *E. coli* strains associated with disease in humans has diversified and distributed into four distinct clonal groups. The commensal *E. coli* strains tend to associate within phylogenetic groups A and B1 and the extra-intestinal pathotypes within phylogenetic groups B2 and D.^{19–21}

We used a multiplex PCR based on the one described by López-Saucedo et al. for detecting EPEC, ETEC, EHEC, and EIEC. Furthermore, we developed a duplex PCR for detection of *aggR* and *daaE* from EAEC and DAEC, respectively, based on a multiplex PCR assay recently described.¹⁷ The purpose of this study was to investigate the presence and properties of the six main categories of diarrheagenic *E. coli* among pediatric population who attended to the Hospital Nacional de Niños in San José, Costa Rica.

MATERIALS AND METHODS

Study design. This prevalence study evaluated the number of *E. coli* pathotypes among children with diarrhea who received care from the hospital outpatient clinic or from the inpatient services, from August 2005 through August 2007 at the National Children's Hospital Dr. Carlos Sáenz Herrera, in San José, Costa Rica. Children less than five years of age with loose or watery stools, at least three times in a 24-hour period (diarrhea definition, World Health Organization, 2005), and who received no antibiotics after onset of diarrhea, were enrolled in the study. According to the Statistics Department and the Laboratory Information System (Nexus, Brighton, United Kingdom), the hospital received 14,055 patients with diarrhea, and 12,065 stool samples were processed at the laboratory. Stool samples were analyzed for general diagnostic purposes, searching for parasites, rotavirus, *Salmonella enteritidis*, *Shigella*, *Campylobacter*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, and *Vibrio cholera*. A protocol for additional culture in Tergitol 7 and MacConkey-sorbitol agar

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were performed to isolate *E. coli* colonies in stool cultures. Because the study was limited to investigate *E. coli* intestinal pathogens, all samples that were positive for parasites, rotavirus, and the other bacteria described above were excluded.

Bacterial strains and growth conditions. A total of 173 of 1,042 strains of diarrhea-associated *E. coli* were randomly selected during the study period on the basis of the inclusion criteria. Suspected *E. coli* were plated on blood agar and single-colony suspensions were prepared for bacterial identification by automated identification (Vitek®; bioMérieux, Marcy l'Etoile, France), according to manufacturer's specifications. Identified *E. coli* isolates were frozen for further analysis. *Escherichia coli* isolates were obtained from 55 (31.8%) hospitalized patients, and 118 (68.2%) from outpatients living in San José, Costa Rica during the two-year period of the study.

Prototype EPEC, ETEC, EHEC, and EIEC strains for the experiments controls were kindly provided by the Instituto Costarricense de Investigación y Enseñanza en Nutrición y Salud (San José, Costa Rica). Prototypes for EAEC and DAEC were obtained from the University of Iowa (Iowa City, IA). Each control strain carries the specific set of genes to be amplified by PCR, namely, *eaeA*, *stx*₁/*stx*₂ (EHEC), *st* and *lt* (ETEC), *bfpA* and *eaeA* (EPEC), *ial* (EIEC), *daaE* (DAEC), and *aggR* (EAEC). *Escherichia coli* ATCC 25922 and *E. coli* K12 were used as negative controls.

Strain serotyping. O group serologic typing of strains was performed by agglutination testing by using a commercial kit (Bio-Rad, Hercules, CA) that contained 12 frequent EPEC-related serotypes (O26, O55, O86, O111, O114, O119, O124, O125, O126, O127, O128, and O142). Testing for STEC (EHEC) O157:H7 (Denka, Tokyo, Japan) were also included.

Antimicrobial drug susceptibility tests. Resistance to antimicrobial drugs was examined by using automated testing methods (Vitek®; BioMérieux). Strains activity was tested against amoxicillin/clavulanic acid, ampicillin, carbenicillin, cefazolin, ceftriaxone, cefuroxime, cephalothin, ciprofloxacin,

gentamicin, levofloxacin, minocycline, nalidixic acid, nitrofurantoin, norfloxacin, ticarcillin/clavulanic acid, tobramycin, and trimethoprim/sulfamethoxazole.

Adherence assays. Adherence patterns on HEp-2 cells were examined by using a modification of the protocol described by Nataro and others.²² Briefly, HEp-2 cells were grown on glass coverslips until 75% confluence was reached, and 15 µL of bacterial culture containing 1 mL of Dulbecco's minimal essential medium plus 1% D-mannose was then added to each well. Infected cells were incubated for 3 hours at 37°C in an atmosphere of 5% CO₂. After incubation, cells were washed, fixed, and stained with Giemsa. Coverslips were examined under by light microscopy.

Fluorescent actin polymerization assay. An attaching and effacement effect caused by actin polymerization was examined by using a fluorescent action polymerization assay as described.²³ HEp-2 cells were grown, infected, and fixed. Fluorescein isothiocyanate-phalloidin and 4'-6-diamidino-2-phenylindole staining was performed for visualization of cytoskeleton and DNA, respectively. Staining with 4'-diamidino-2-phenylindole was used for improving the visualization by its counterstaining effect. Coverslips were examined by epifluorescent microscopy.

Gentamicin protection assay. Invasiveness of strains positive for the *ial* gene (EIEC) was examined by protection assay with gentamicin. Experiments were performed in quadruplicate. HEp-2 cells were grown as described for adherence assays. Bacterial culture grown were inoculated on each well to reach a final concentration of 10⁸ colony-forming units per well. Infection was conducted for 3 hours, and cells were then washed with phosphate-buffered saline. Extracellular bacteria were killed by incubating cells for one hour with Dulbecco's minimal essential medium plus 1% D-mannose and 60 µg/mL of gentamicin. The cells were then lysed and serial dilutions were spread on MacConkey agar and incubated 16 hours for colony counting.

DNA amplification by PCR. Primers used for PCR amplification of each target gene are described in Table 1. The

TABLE 1

Specific genes, primers, and expected products for PCR assays for analysis of diarrheagenic *Escherichia coli* from children in Costa Rica*

Pathotype or assay	Specific gene	Primers (5'→3')†	Product size, basepairs	Primer (pmol) in mixture	Reference
EPEC	<i>bfpA</i>	F: AAT GGT GCT TGC GCT TGC TGC R: GCC GCT TTA TCC AAC CTG GTA	324	6.0	24
EPEC	<i>eaeA</i>	F: GAC CCG GCA CAA GCA TAA GC R: CCA CCT GCA GCA ACA AGA GG	384	6.3	18, 25
ETEC	<i>st</i>	F: ATT TTT CTT TCT GTA TTG TCT T R: CAC CCG GTA CAA GCA GGA TT	190	6.3	26
ETEC	<i>lt</i>	F: GGC GAC AGA TTA TAC CGT GC R: CGG TCT CTA TAT TCC CTG TT	450	6.3	26
EHEC	<i>stx</i> ₁	F: CTG GAT TTA ATG TCG CAT AGT G R: AGA ACG CCC ACT GAG ATC ATC	150	6.0	25
EHEC	<i>stx</i> ₂	F: GGC ACT GTC TGA AAC TGC TCC R: TCG CCA GTT ATC TGA CAT TCT G	255	6.0	25
EIEC	<i>ial</i>	F: GGT ATG ATG ATG ATG AGT CCA R: GGA GGC CAA CAA TTA TTT CC	650	6.3	18
EAEC	<i>aggR</i>	F: GTA TAC ACA AAA GAA GGA AGC R: ACA GAA TCG TCA GCA TCA GC	254	5.0	27
DAEC	<i>daaE</i>	F: GAA CGT TGG TTA ATG TGG GGT AA R: TAT TCA CCG GTC GGT TAT CAG	542	5.0	28
Phylogenetic assay	ChuA.1	F: GAC GAA CCA ACG GTC AGG AT R: TGC CGC CAG TAC CAA AGA CA	279	10.0	29
Phylogenetic assay	YjaA.1	F: TGA AGT GTC AGG AGA CGC TG R: ATG GAG AAT GCG TTC CTC AAC	211	10.0	29
Phylogenetic assay	TspE4C2.1	F: GAG TAA TGT CGG GGC ATT CA R: CGC GCC AAC AAA GTA TTA CG	152	10.0	29

* EPEC = enteropathogenic *E. coli*; ETEC = enterotoxigenic *E. coli*; EHEC = enterohemorrhagic *E. coli*; EIEC = enteroinvasive *E. coli*; EAEC = enteroaggregative *E. coli*; DAEC = diffusely adherent *E. coli*.

† F = forward; R = reverse.

PCR was performed by using variable concentrations of each primer until optimal results were obtained. Primers (Fermentas, Glen Burnie, MD) were used at final concentrations of 100 μ M. Extraction of crude genomic DNA was performed by heating samples for 20 minutes at 95°C and high-speed centrifugation for 10 minutes at 5°C. Supernatants containing DNA were frozen until needed. The PCR was performed in a final reaction volume of 50 μ L. Multiplex PCR were performed to detect virulence factors belonging to EPEC, ETEC, EHEC, and EIEC. Each reaction tube was prepared by adding 20 μ L of primer mixture, 5 μ L of DNA template, and 25 μ L of Master Mix 2X (Fermentas). Cycling conditions were an initial denaturation at 94°C for 3 minutes; 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute; and a final extension at 72°C for 5 minutes using a Mastercycler® (Eppendorf, Hauppauge, NY). For EAEC and DAEC, a duplex PCR was performed as follows: 1 μ L of DNA template was added to 19 μ L of a mixture containing 12.5 μ L of Master Mix (Accuzyme®; Bio-Rad) and 1 μ L of each primer in a final volume of 20 μ L. Amplification by touchdown PCR was performed with an initial denaturation at 94°C for 2 minutes; 8 cycles at 94°C for 15 seconds, 66°C–59°C for 30 seconds, and 72°C for 30 seconds; 32 cycles at 94°C for 15 seconds, 59°C for 30 seconds, and 72°C for 30 seconds; and a final extension at 72°C for 5 minutes. Electrophoresis on 1.5% agarose gels and staining with ethidium bromide was used for visualization of amplified targets.

Phylogenetic grouping by PCR. A triplex PCR was used for phylogenetic grouping of clinical isolates by using a modification of a protocol previously described.²⁹ Briefly, the amplification mixture was adjusted to a final volume of 25 μ L and contained 5 μ L of DNA template, 12.5 μ L of Master Mix (Accuzyme®), and 10 μ M of each diluted primer. Cycling conditions were an initial denaturation at 94°C for 5 minutes; 30 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds; and a final extension step at 72°C for 7 minutes. Electrophoresis on 1.5% agarose gels and staining with ethidium bromide was used for visualization of amplified targets.

Statistical analyses. Statistical analyses were performed by using SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL) and Minitab version 15.1 (Minitab Inc., State College, PA). The Fisher exact test was used to compare resistance to antibiotics

on the basis of the presence or absence of virulence factors. A *P* value ≤ 0.05 was considered statistically significant.

RESULTS

Detection of pathotypes of intestinal *E. coli*. Multiplex and duplex PCR were performed to detect the main six categories of *E. coli*. A total of 52 isolates were positive for virulence factors related to diarrheagenic *E. coli*. The frequencies of distribution among the categories of diarrheagenic *E. coli* are shown in Table 2. A total of 40 (77%) strains identified as pathogenic *E. coli* were isolated from diarrheic illness from ambulatory healthcare services and distributed as follows: 22% EIEC (9 of 40), 20% (8 of 40) mixed biotypes, 15% (6 of 40) EAEC and EPEC, 12% (5 of 40) EHEC, 10% (4 of 40) DAEC, and 5% (2 of 40) ETEC. Moreover, 12 strains (13%) were identified from hospitalized patients: 42% (5 of 12) EPEC, 17% (2 of 12) ETEC and EAEC, and 8% EHEC, EIEC, and mixed biotypes (1 of 12). No DAEC pathotypes were detected for this subset of strains. The most frequent pathotypes were EPEC and EIEC among all clinical isolates. Because most of the strains that were positive for the *eaeA* gene were negative for *bfpA*, a confirmatory PCR using purified plasmid DNA was performed. Results confirmed only two strains harboring the *bfpA* gene, which indicated that atypical EPEC strains positive for *eaeA* and negative for *bfpA* are prevalent in San José, Costa Rica.

Adherence properties of *E. coli* pathotypes. Strains were classified according to the adherence pattern described.²² Adherence patterns and fluorescent actin polymerization assay confirmed that strains exhibited phenotypic properties according to their pathotypes. Similarly, the gentamicin protection assays demonstrated that all EIEC strains detected by multiplex PCR were able to invade HEp-2 cells.

Phylogenetic grouping. The triplex PCR described by Clermont and others²⁹ was used for phylogenetic grouping analysis. The method enabled detection of the four main phylogenetic groups of *E. coli* (A, B1, B2, and D). Among studied strains, 30.1% (52 of 173) were classified as group A, 21.4% (37 of 173) as group B1, 19.7% (34 of 173) as group B2, and 28.9% (50 of 173) as group D. Among pathogenic strains, 29% (15 of 52) were classified as group A, 35% (18 of 52) as

TABLE 2
Distribution of pathotypes and phylogenetic groups among *Escherichia coli* clinical isolates from children in Costa Rica*

Characteristic	Group A		Group B1		Group B2		Group D		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Pathogenic	15/52	28.8	18/52	34.6	12/52	23.1	7/52	13.5	52	100
Origin										
Outpatient	12/40	30.0	15/40	37.5	9/40	22.5	4/40	10.0	40/52	77.0
Hospitalized patient	3/12	25.0	3/12	25.0	3/12	25.0	3/12	25.0	12/52	23.0
Pathotype										
EPEC	2/11		2/11		7/11		0/11		11	
EHEC	1/6		3/6		0/6		2/6		6	
ETEC	1/4		2/4		0/4		1/4		4	
EIEC	2/10		7/10		1/10		0/10		10	
EAEC	4/8		2/8		2/8		0/8		8	
DAEC	2/4		0/4		1/4		1/4		4	
Mixture	3/9		2/9		1/9		3/9		9	
Non-pathogenic	37/121	30.6	19/121	15.7	22/121	18.2	43/121	35.5	121	100
Total	52/173	30.0	37/173	21.4	34/173	19.6	50/173	28.9	173	100

* EPEC = enteropathogenic *E. coli*; EHEC = enterohemorrhagic *E. coli*; ETEC = enterotoxigenic *E. coli*; EIEC = enteroinvasive *E. coli*; EAEC = enteroaggregative *E. coli*; DAEC = diffusely adherent *E. coli*.

group B1, 23% (18 of 52) as group B2, and 13% (13 of 52) as group D.

Serologic analyses. Serologic tests classified 28% (49 of 173) of the strains as diarrhea-associated serotypes. The most common were O55, O86, O111, O124 with a prevalence of 3.5% (6 of 173), followed by O26 and O127 with a prevalence of 2.9% (5 of 173). When we analyzed the presence of virulence factors, the most common serotypes were O124 (9.6%, 5 of 52) and O26 (7.7%, 4 of 52). Almost all (4 of 5) of the strains belonging to the O26 serotype were positive for a virulence factor by PCR. The O26 serotype has been related with intestinal illness in several countries.³⁰ In addition, 5 of 6 O124 strains were positive for *ial* encoded in EIEC. A total of 72% (n = 124) of the total strains studied and 58% (n = 30) of the PCR-detected pathogenic strains did not agglutinate with available antisera. Only 33% (2 of 6) of shiga toxin-positive strains were O157:H7. There was a significant relationship ($P < 0.05$, by Fisher exact test) between a positive serotyping result and the presence of virulence factors for diarrheagenic *E. coli*. The predictive values for serotyping were sensitivity = 42%, specificity = 78%, positive predictive value = 45%, negative predictive value = 76%, and efficiency = 67%.

Resistance to antimicrobial drugs. For pathogenic strains, resistance to penicillins ranged from 8% (4 of 52) for ticarcillin/carbenicillin to 40% (21 of 52) for ampicillin. Resistance to first-generation cephalosporins was 11% (6 of 52), resistance to second-generation cephalosporins was 6% (3 of 52), and resistance to third-generation (ceftriaxone) cephalosporins was < 1%. Resistance to minocycline was 11% (6 of 52) and resistance to sulfamethoxazole was 13% (7 of 52). Resistance was not demonstrated for ciprofloxacin, levofloxacin, nalidixic acid, nitrofurantoin, and norfloxacin among pathogenic *E. coli* strains. The EPEC strains showed a significant susceptibility to ampicillin, carbenicillin, and STX. The EHEC strains were significantly resistant to cefazoline, and ETEC strains showed increased resistance to ampicillin, cefazoline, and ceftriaxone. The EIEC strains exhibited resistance against cefuroxime sodium and acetil. Nonetheless, borderline statistical significance was observed in ETEC for resistance to tobramycin, in EAEC for resistance to cefazoline, and in EIEC for resistance to cephalotin ($P = 0.068$, $P = 0.057$, and $P = 0.061$, respectively). Strains lacking diarrhea-associated virulence factors were significantly resistant to trimethoprim/sulfamethoxazole compared with strains having virulence factors.

DISCUSSION

During the past decade, molecular diagnosis has become a common technology used by clinical laboratories. The PCR enables detection of many pathogens through amplification of specific genes encoding virulence factors. If one considers the difficulty in diagnosis of diarrheagenic *E. coli* by conventional laboratory methods, the PCR becomes useful in clinical laboratories because of its sensitivity and specificity.

The EPEC was found to be the most frequent category among pathogenic *E. coli* in this study, followed by EIEC. If one considers the origin of the strains, 77% (40 of 52) of these pathogenic strains were isolated from patients who attended ambulatory health care services, and only 23% (12/52) were from hospitalized children. Of interest, only 2 from 11 EPEC harbored the *bfpA* gene, and consequently are the only typical EPEC. The remaining strains should be considered as atypical

EPEC. The *bfpA* gene encodes plasmid-borne type IV fimbriae and confers the phenotypic feature known as localized adherence.³¹ This subset of EPEC has become a frequently detected pathogen, and recent investigations focused on its role in persistent diarrhea, probably caused by the lack of the *bfp* gene and subsequent delay in apoptosis of enterocytes.³²⁻³⁵ Further research is necessary to demonstrate the relevance of these infections in the community, the burden of disease in children less than five years of age, and the potential role of antibiotics in medical management.

Antimicrobial drug therapy is recommended when diarrheal disease is severe to reduce duration of the symptoms, and concerns are increasing in studies reporting high levels of resistance to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole among diarrhea-associated bacteria.³⁶ Our data showed an EPEC subset highly susceptible to all antibiotics tested. In contrast, other pathotypes demonstrated multidrug resistance. This phenomenon included EHEC strains resistant to cefazoline and ETEC strains resistant to ampicillin, cefazoline, and ceftriaxone. Although some resistance to antimicrobial drugs could be demonstrated in this study, the overall data differ markedly from studies in southeast Asia, where resistance is prominent.³⁶ Furthermore, pathogenic strains as a whole did not show any differences regarding antimicrobial drug resistance compared with non-pathogenic strains ($P > 0.05$).

Conventional serotyping methods for *E. coli* somatic and flagellar antigens are still important techniques in many laboratories for diagnosis and surveillance. We demonstrated the prevalence of diarrhea-associated serotypes among the children of Costa Rica, including the serotypes O26 and O124, which have been described in several countries.³⁰ Conversely, only a one-third of the positive EHEC strains were positive for O157:H7 antigens, which indicated that further research is needed to evaluate the prevalence of non-O157 EHEC (STEC) clones and their relationship with complications such as hemolytic-uremic syndrome. Our study demonstrated a congruent serotyping result with the presence of virulence factors among the studied diarrhea-associated *E. coli* strains. Even when some predictive values are low, the serotypes included in the study are suitable for an initial approach to identification of a diarrheagenic *E. coli* of any category, not only EPEC. Furthermore, serologic antigens are not directly involved in virulence, but can provide important information about the circulating serotypes in the communities and in outbreaks.¹ Other clinical, epidemiologic, and laboratory evidence that associate *E. coli* strains with a particular diarrheic syndrome should also be included in serologic analysis.

Four phylogenetic groups are found in *E. coli* (A, B1, B2, and D). One study showed that strains containing extraintestinal virulence factors belong mostly to groups B2 and D, whereas most commensal *E. coli* belong to group A.²⁹ However, diarrhea-associated *E. coli* strains are distributed among all phylogenetic groups.²¹ Pathogenicity of *E. coli* has been associated with some phylogenetic groups based on the presence of virulence factors carried by plasmids, phages, or pathogenicity islands within the bacterial genome. Although these virulence determinants can be acquired by horizontal gene transfer, a specific genetic background may be necessary for their correct expression. Approximately 30% of the pathogenic strains included in this study belong to phylogenetic groups A and B1, which is consistent with available data.^{20,29}

The remaining strains belonged to groups B2 and D (23% and 13%, respectively).

Phylogenetic analysis has become another important key in understanding bacterial virulence. Our findings demonstrate a high percentage of extraintestinal-related strains harboring intestinal-related virulence factors. This genetic heterogeneity in strains from Costa Rica suggests a high potential for horizontal gene transfer, even among traditional extraintestinal *E. coli* isolates. Phylogenetic analyses have demonstrated that *E. coli* pathotypes isolated in San Jose, Costa Rica belong to the phylogenetic groups associated with intestinal disease, including phylogenetic groups A, B1, and B2 (Table 2). It is unclear if the *E. coli* isolates positive for virulence genes that belong to phylogenetic group D may be considered pathogens. Further research may be necessary to elucidate the virulence potential of these strains in the development of intestinal disease.

Diagnosis of diarrheagenic *E. coli* by PCR has been demonstrated.¹ Use of this technology in developing countries has been delayed mainly because of economic limitations of local health authorities and local health services. The social security system was improved in Costa Rica beginning in 1940 and provides a successful integrated health attention model, a network that refers patients from basic to more specialized levels according their need. This health system is used by the entire population of Costa Rica. The Hospital Nacional de Niños Dr. Carlos Sáenz Herrera in Costa Rica is a reference hospital and a primary center for acute illnesses such as diarrhea. Nearly 5,000 patients with cases of diarrhea are admitted per year, and approximately 5% of children with diarrhea need additional hospitalization because of the severity of the disease.

The PCR technology has recently being introduced into Costa Rica and many other countries in Latin America, Africa, and southeast Asia. Standardization of this multiplex and duplex PCR technology provides laboratories with a highly specific, sensitive, and useful tool for identification of diarrheagenic *E. coli*, including EHEC, ETEC, EIEC, EPEC, EAEC and DAEC, which was only possible in the past in research laboratories in the United States and Europe. Our study shows that identification of *E. coli* is possible and necessary because the prevalence of these pathogens is high. We were able to validate the method with cell culture adherence assays, fluorescent actin staining, and invasion assays, which corroborated the PCR findings.

This study provided relevant information on the prevalence, phylogenetic distribution, and antimicrobial drug resistance of *E. coli* pathotypes in San Jose, Costa Rica. The multiplex and duplex PCR assays will be instrumental for epidemiologic surveillance of children with diarrhea. They may also be used in epidemiologic surveillance of water and food samples for *E. coli* contamination and to determine the risk of infection. Information acquired by this these molecular detection assays may also facilitate rapid diagnosis, guide medical management, and direct public health measures for the prevention of infectious diarrheal disease in children.

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