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Single Multiplex PCR Assay To Identify Simultaneously the Six Categories of Diarrheagenic *Escherichia coli* Associated with Enteric Infections

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We designed a multiplex PCR for the detection of all categories of diarrheagenic *Escherichia coli*. This method proved to be specific and rapid in detecting virulence genes from Shiga toxin-producing (*stx*$_1$, *stx*$_2$, and *eae*), enteropathogenic (*eae* and *bfp*), enterotoxigenic (*stII* and *lt*), enteroinvasive (*virF* and *ipaH*), enteroaggregative (*aafII*), and diffuse adherent (*daaE*) *Escherichia coli* in stool samples.

Most *Escherichia coli* strains are commensal; however, there are several highly adapted clones that have the capacity to cause human illness. Strains that cause enteric infections are designated diarrheagenic *E. coli*, a group that includes emergent pathogens with public health relevance worldwide (13). Six categories of diarrheagenic *E. coli* that differ in their virulence factors have been described (13). The most commonly reported diarrheagenic *E. coli* strains in Chile are enterotoxigenic *E. coli* (ETEC), which produces one or more enterotoxins that are heat labile LT (LT-1 and LT-2) or heat stable ST (STa and STb) (11); enteropathogenic *E. coli* (EPEC), which harbors a pathogenicity island that encodes a series of proteins involved in the attaching and effacement lesions of the intestinal microvilli of the host cell (8); and the presence of the large EPEC adherence factor (EAF) plasmid, on which also the cluster of genes encoding bundle-forming pili (*bfp*) is present (9). Based on these, EPEC strains are classified as typical when they possess the EAF plasmid, whereas atypical EPEC strains do not possess the EAF plasmid (18); Shiga toxin-producing *E. coli* (STEC) is characterized by the production of two potent cytotoxins denominated Shiga-like toxins 1 and 2 (Stx1 and Stx2) (17) and in some strains the presence of the LEE locus related to the attaching and effacement lesion (7, 16). The three other categories seem to be less prevalent. Enteroinvasive *E. coli* (EIEC) has biochemical, physiological, and genetic properties similar to those of *Shigella*, invading the epithelial cells of the colon, where it proliferates and causes necrosis of the tissue. The genes related to invasion are located in a virulence plasmid (pInv) of 140 MDa that encodes a type III secretion system (1, 12). Enteroaggregative *E. coli* (EAggEC), first discovered by studies of adherence to HEp-2 cells, displays a pattern of adherence characterized by self-agglutination that is denominated aggregative adherence (AA). Fimbrial structures denominated AA fimbriae I and II (AAF-I and -II) have been associated with adhesion to HEp-2 cells and human erythrocytes (5). The AAF-II fimbriae (coded in the pAA plasmid) seem to be more prevalent and are related to the capacity for adherence of EAggEC to the intestinal surface (14). The most recently characterized category corresponds to diffuse adherent *E. coli* (DAEC), strains that are capable of adhering to HEp-2 cells in a nonlocalized pattern. A surface fimbria (denominated F1845) has been proposed as a putative virulence factor that could be mediating this adherence phenotype (2). The "gold standard" method for detection of DAEC strains is based on the diffuse adherence phenotype in tissue cultures or by detection of the gene *daa* that is necessary for the expression of the F1845 fimbriae (4, 20).

Identification of different diarrheagenic *E. coli* pathotypes is not routinely performed because it is cumbersome and techniques are not readily available. Diagnosis is currently recommended for cases of persistent diarrhea, especially among tourists, children with severe diarrhea unresponsive to treatment, and immunodeficient patients with moderate to severe diarrhea, and in epidemic outbreaks of gastroenteritis (13).

Considering the epidemiological impact of diarrheagenic *E. coli* worldwide, especially ETEC, STEC, and EPEC, we previously designed a multiplex PCR to detect these three enteropathogens that proved to be sensitive and specific (19). In the present study we incorporated into this one-step multiplex PCR the detection of the remaining categories of diarrheagenic *E. coli*, EIEC, EAggEC, and diffuse adherence *E. coli*.

A total of 509 stool samples were obtained from Chilean children younger than 9 years of age with acute diarrhea attending different outpatient clinics in Santiago between April 2004 and January 2005. Study protocols of acute diarrhea that considered evaluation of one stool sample per diarrhea episode were approved by the Institutional Review Board of the Faculty of Medicine, University of Chile, and the Ethics Committee of the Servicio de Salud Metropolitano Norte.

Diarrheagenic *E. coli* reference strains 933J (*stx*$_1$ *stx*$_2$ *eae*), C600J (*stx*$_1$), C600W (*stx*$_2$), 2348/69 (*eae*), H10407 (*st lt*), STEC O159 (*st*), STEC O8 (*lt*), STEC O6 (*lt*), EI-34 (*ipaH virF*), F-1845 (*daaE*), and O42 (*aafII*) were used as positive controls.

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TABLE 1. Bacterial strains tested by multiplex PCR, including reference diarrheagenic *E. coli*

Bacterial strain, source[a]	No. of strains showing positive PCR result/no. of strains tested									
	stx$_1$	*stx*$_2$	*eae*	*bfp*	*lt*	*st*	*vir*F	*ipaH*	*daaE*	*aafII*[b]
Enterohemorrhagic *E. coli*										
933J O157:H7, CVD	5/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
C600J O157, CVD	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
C600W O157, CVD	0/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Enteropathogenic *E. coli*										
2348/69, CVD	0/5	0/5	5/5	5/5	0/5	0/5	0/5	0/5	0/5	0/5
E. coli negative controls										
HS, CVD	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
60120, CVD	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Enterotoxigenic *E. coli*										
O159, MP	0/5	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5
O8, MP	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5
O6, MP	0/5	0/5	0/5	0/5	5/5	0/5	0/5	0/5	0/5	0/5
H10407, CVD	0/5	0/5	0/5	0/5	5/5	5/5	0/5	0/5	0/5	0/5
Enteroinvasive *E. coli*										
EI-34, CVD	0/5	0/5	0/5	0/5	0/5	0/5	5/5	5/5	0/5	0/5
Enteroaggregative *E. coli*										
O42, CVD	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5
Diffuse adherent *E. coli*										
F-1845, CVD	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	5/5	0/5
Enterobacter sp., MP	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Proteus mirabilis, MP	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Klebsiella oxytoca, MP	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Shigella sonnei, MP	0/5	0/5	0/5	0/5	0/5	0/5	5/5	5/5	0/5	0/5
Shigella flexneri, MP	0/5	0/5	0/5	0/5	0/5	0/5	5/5	5/5	0/5	0/5
Salmonella group B, MP	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Salmonella group D, MP	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
S. enterica serovar, MP	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

[a] CVD, Center for Vaccine Development, University of Maryland; MP, Microbiology Program, University of Chile.
[b] Region 2 of the gene.

To determine the specificity of the primers, other members of the *Enterobacteriaceae* family, e.g., *Shigella sonnei*, *Shigella flexneri*, *Enterobacter* sp., *Proteus mirabilis*, *Klebsiella oxytoca*, *Salmonella* group B, *Salmonella* group D, *Salmonella enterica* serovar Typhi, "normal" colonic flora *Escherichia coli* HS, and nonenteropathogenic *Escherichia coli* 60120, were included as negative controls (Table 1) (10).

PCR primers specific for *stx*$_1$ and *stx*$_2$ were previously described by Cebula et al. (3) and those for *eae*, *bfp*, *stII*, and *lt* were described by Vidal et al. (19). Primers for *virF*, *ipaH*, *daaE*, and *aafII* region 2 were designed from sequences available in the GenBank database using OMIGA 2.0 software for alignment and the Primer 3 program for primer design. Sequences, sizes of PCR products, and references are shown in Table 2.

A pool of five *E. coli* colonies from cultures of reference strains and stool samples were analyzed by multiplex PCR for detection of virulence genes (*stx*$_1$, *stx*$_2$, *eae*, *bfp*, *stII*, *lt*, *virF*, *ipaH*, *daaE*, and *aafII*). When multiplex PCR was positive for the pool, each separate isolate was tested by multiplex PCR and then biochemically identified.

The multiplex PCR assay was performed as follows. Each 50 μl of reaction mixture contained 1 mM deoxynucleoside triphosphates, 2 pmol of each primer, 1.5 mM $MgCl_2$, 1× reaction buffer (10 mM Tris-HCl, 50 mM KCl), 0.2 μl of *Taq* DNA polymerase, and 3 μl of template DNA. The crude cell lysate used as template DNA was prepared by boiling five colonies of *E. coli* in 0.5% Triton X-100 for 20 min. The hot start technique was used to prevent nonspecific amplification: 40 μl of the reaction mixture was preheated to 94°C for 5 min before *Taq* DNA polymerase (2 U in a 10-μl reaction mixture) was added. Samples were amplified for 35 cycles, with each cycle consisting of 1.5 min at 94°C for denaturation, 1.5 min at 60°C for primer annealing, and 1.5 min at 72°C for strand elongation. PCR products were visualized following electrophoresis through 1.5% agarose gels stained with ethidium bromide, and the amplicons were identified based only on the size of the amplified product.

Specificity of the multiplex PCR was tested with reference strains (Table 1). The different sizes of the amplification products for the *stx*$_1$, *stx*$_2$, *eae*, *bfp*, *stII*, *lt*, *virF*, *ipaH*, *daaE*, and *aafII* genes are shown in Table 2. EPEC and STEC strains detected by multiplex PCR were serotyped by an agglutination test using a commercial antiserum (PROBAC, Sao Paulo, Brazil).

The multiplex PCR assay designed in this study incorporated 20 primers for the amplification of 10 virulence genes (Table 2). The assay proved to be specific for the different categories of diarrheagenic *E. coli* when applied to prototype reference strains. Also, the laboratory protocol design allowed us to detect some of the most frequent categories and serogroups of diarrheagenic *E. coli* isolated from stool samples in Chilean children with acute diarrhea (Tables 1 and 3; Fig. 1) (15). All stool samples were cultured on MacConkey, SS, and XLD agar (Oxoid) for isolation of *Escherichia coli*, *Salmonella* spp., and

TABLE 2. Primers used in the multiplex PCR for amplification of diarrheagenic *E. coli* genes[a]

Gene	Primer sequence (5′–3′)	Size of product (bp)	Reference
stx_1	CAG TTA ATG TGG TGG CGA AGG CAC CAG ACA ATG TAA CCG CTG	348	3
stx_2	ATC CTA TTC CCG GGA GTT TAC G GCG TCA TCG TAT ACA CAG GAG C	584	3
eae	TCA ATG CAG TTC CGT TAT CAG TT GTA AAG TCC GTT ACC CCA ACC TG	482	16
bfp	GGA AGT CAA ATT CAT GGG GGT AT GGA ATC AGA CGC AGA CTG GTA GT	300	16
lt	GCA CAC GGA GCT CCT CAG TC TCC TTC ATC CTT TCA ATG GCT TT	218	16
stII	AAA GGA GAG CTT CGT CAC ATT TT AAT GTC CGT CTT GCG TTA GGA C	129	16
virF	AGC TCA GGC AAT GAA ACT TTG AC TGG GCT TGA TAT TCC GAT AAG TC	618	Current study
ipaH	CTC GGC ACG TTT TAA TAG TCT GG GTG GAG AGC TGA AGT TTC TCT GC	933	Current study
daaE	GAA CGT TGG TTA ATG TGG GGT AA TAT TCA CCG GTC GGT TAT CAG T	542	Current study
aafII	CAC AGG CAA CTG AAA TAA GTC TGG ATT CCC ATG ATG TCA AGC ACT TC	378	Current study

[a] Specific primers used for amplification of diarrheagenic *E. coli* virulence genes were designed from sequences obtained from the following GenBank accession numbers: *virF*, AY206433, AF386526, AF348706, AL391753, X16661, X58464, and M29172; *ipaH*, AF386526, AL391753, AF348706, M76445, and M32063; *daaE*, M27725; *aafII*, AF114828.

Shigella spp., and *Campylobacter* spp. and *Yersinia enterocolitica* were cultured on campylobacter blood-free selective and yersinia-selective agar (Oxoid), respectively. In the series of children with acute diarrhea, we observed mixed infections by different categories of diarrheagenic *E. coli* in only one patient (Fig. 1), and no other bacterial enteropathogens were isolated as mixed infection.

TABLE 3. Detection by multiplex PCR of different categories of diarrheagenic *Escherichia coli* in 2,545 colonies obtained from 509 stool samples from Chilean children with acute diarrhea

Diarrheagenic *E. coli* (no. of positive samples/ no. studied)	Genotype detected by multiplex PCR	*E. coli* serogroup
Total EPEC (54/509)		
8/509	*eae*	O119
2/509	*eae*	O158
2/509	*eae*	O55
2/509	*eae*	O125
1/509	*eae*	O26
1/509	*eae bfp*	O111
1/509	*eae*	O86
1/114	*eae*	O114
1/509	*eae*	O128
22/509	*eae*	ND[a]
13/509	*eae bfp*	ND
Total STEC (8/509)		
2/509	stx_2 *eae*	O157:H7
2/509	stx_1 *eae*	O26
1/509	stx_1 *eae*	O128
1/509	stx_1 *eae*	O126
1/509	stx_1 *eae*	ND
1/509	stx_1	O127
ETEC (10/509)	*lt*	ND
DAEC (3/509)	*daaE*	ND
EAggEC (1/509)	*aafII*	ND

[a] ND, not determined with available antisera.

With the exception of enteroinvasive *E. coli*, which was not detected in the 509 stool samples studied, we were able to differentiate five categories of diarrheagenic *E. coli*, including the less common DAEC and EAggEC and a variety of different serogroups of STEC and EPEC; results were comparable to those reported by Cebula et al. (3) and Vidal et al. (19) (Table 3). Eight STEC strains from patients with sporadic diarrhea were detected. Two of them had the stx_2 gene, the predominant toxin phenotype pattern described in countries of the Northern hemisphere (6). However, six strains were non-O157:H7 and harbored stx_1; these results are comparable with the toxigenic pattern of STEC strains observed in other studies in Chile (15).

The most frequent category of diarrheagenic *E. coli* detected was EPEC (54/509) (Table 3). To discriminate between typical and atypical EPEC, primers previously described for *bfp* gene detection were included in multiplex PCR (19). In this sample of children with acute diarrhea, 14 out of 54 EPEC strains were typical (Table 3).

The main challenge of designing a multiple PCR assay is the possibility for primer dimers and nonspecific products. So, it is necessary to design primers with close annealing temperatures, to begin the program with a hot start, and to use reference strains to determine reaction specificity (Table 1). The multiplex PCR is a rapid method for detecting multiple targets in a single reaction and in a short time.

Our results confirm that it is possible and feasible to perform a simultaneous amplification of the virulence genes from all categories of diarrheagenic *E. coli* (STEC, ETEC, typical or atypical EPEC, EIEC, DAEC, and EAggEC) and that this technique can be applied for the etiologic diagnosis of patients

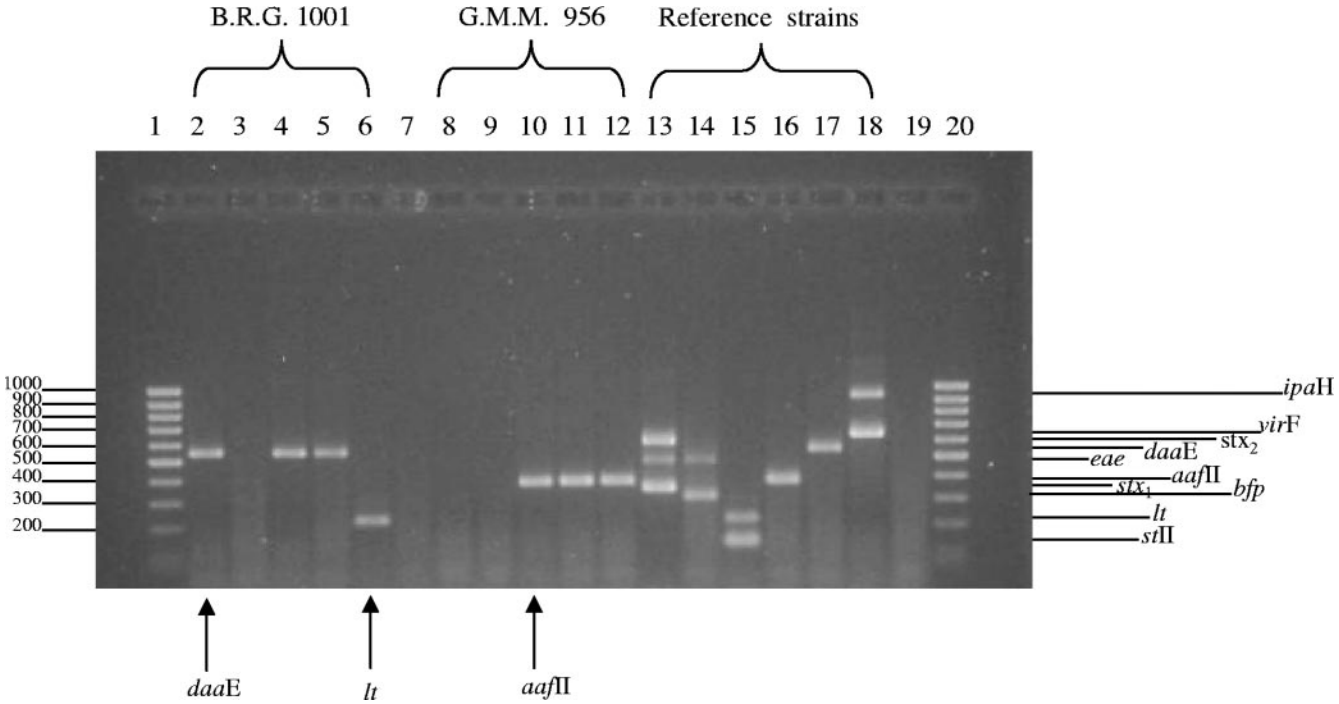

FIG. 1. Multiplex PCR analysis of stool samples from Chilean children with acute diarrhea and selected reference strains. Bands corresponding to stx_2, stx_1, *eae*, *bfp*, *st*, *lt*, *aafII*, *daaE*, *virF*, and *ipaH* are indicated. Lanes 1 and 20, 100-bp size ladder; lanes 2, 4, and 5, colonies in patient B.R.G. 1001 with mixed infection (DAEC *daaE* and ETEC *lt* genes, respectively); lanes 7 and 19, reagent control; lanes 10 to 12, patient G.M.M. 956 with EAggEC (three colonies); lanes 3, 8, and 9, commensal *E. coli* colonies without virulence factor in both studied patients; lane 13, enterohemorrhagic *E. coli* 933J (stx_1 stx_2 *eae*); lane 14, enteropathogenic *E. coli* 2348/69 (*eae bfp*); lane 15, enterotoxigenic *E. coli* H10407 (*lt st*); lane 16, enteroaggregative *E. coli* O42 (*aafII*); lane 17, diffuse adherent *E. coli* F-1845 (*daaE*); lane 18, enteroinvasive *E. coli* EI-34 (*virF ipaH*).

with sporadic diarrhea.This multiplex PCR showed high specificity for diarrheagenic *E. coli*, becoming a novel diagnostic tool for future epidemiological studies.

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