JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 2004, p. 1787–1789 Vol. 42, No. 4
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.4.1787–1789.2004

Multiplex PCR for Diagnosis of Enteric Infections Associated with Diarrheagenic *Escherichia coli*

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Received 30 July 2003/Returned for modification 22 September 2003/Accepted 6 January 2004

A multiplex PCR for detection of three categories of diarrheagenic *Escherichia coli* was developed. With this method, enterohemorrhagic *E. coli*, enteropathogenic *E. coli*, and enterotoxigenic *E. coli* were identified in fecal samples from patients with hemorrhagic colitis, watery diarrhea, or hemolytic-uremic syndrome and from food-borne outbreaks.

Enteric infections remain an important cause of morbidity in Chile. Incidence rates of diarrhea in Chilean children range from 1.3 to 4.5 per 100,000 inhabitants (8), and in Santiago, the incidence rates of food-borne outbreaks were reported as 3, 4.1, and 5.1 from 1999 to 2001, respectively (7). In an etiologic study in Chilean children with diarrhea, Levine et al. (5) showed that different categories of diarrheagenic *Escherichia coli* organisms have an important role as the causes of enteric infections. However, these pathogens are probably underestimated due to inappropriate diagnostic methods in clinical practice (4, 5).

Six categories of diarrheagenic *E. coli* organisms that differ in their virulence factors have been described (6). The most commonly reported diarrheagenic *E. coli* organisms in Chile are enteropathogenic *E. coli* strains that have a pathogenicity island (locus of enterocyte effacement), encoding proteins involved in the formation of attaching and effacing lesions on host intestinal cells (3); enterotoxigenic *E. coli* strains, which produce heat-labile and/or heat-stable enterotoxins (4); and enterohemorrhagic *E. coli* strains, important pathogens around the world (9), characterized by the presence of the locus of enterocyte effacement (2) and the production of Shiga toxins (Stx1 and Stx2) (10). Our study was undertaken to develop a multiplex PCR to simultaneously detect enterohemorrhagic *E. coli*, enterotoxigenic *E. coli*, and enteropathogenic *E. coli* in different fecal samples.

Diarrheagenic *E. coli* reference strains 933J (*stx*$_1$ *stx*$_2$ *eae*), C600J (*stx*$_1$), C600W (*stx*$_2$), 2348/69 (*eae*), and H10407 (*st* and *lt*) were used as positive controls. We also used *Shigella* and *Salmonella* strains to define the accuracy of the method (Table 1). Clinical samples included samples from 20 children with hemolytic uremic syndrome, 27 patients involved in six different food-borne outbreaks in Santiago during 2000, and 1,468 stool samples from sporadic diarrheal episodes in children less than 5 years old. We also incorporated one stool sample from an adult patient diagnosed with hemorrhagic colitis by colonoscopy and histopathological procedures. We used PCR primers specific for *stx*$_1$ and *stx*$_2$, as previously described (1), and the primers for *eae*, *bfp*, *stII*, and *lt* were obtained from sequences available in the GenBank database with the OMIGA software for alignment and Primer3 program for primer design (Table 2).

The DNA sequences of the primers and the sizes of PCR products are shown in Table 2. Fecal *E. coli* isolates were analyzed by multiplex PCR to detect virulence genes (*stx*$_1$, *stx*$_2$, *eae*, *bfp*, *stII*, and *lt*). Each multiplex PCR assay was performed in 50 μl of reaction mixture containing 1 mM deoxynucleoside triphosphate mix, 10 pmol of each primer, 1.5 mM $MgCl_2$, 1× reaction buffer (10 mM Tris-HCl, 50 mM KCl), 0.25 U of *Taq* DNA polymerase, and 3 μl of DNA as the template. The crude cell lysate used to obtain the DNA was obtained by boiling five colonies of the *E. coli* isolate for 20 min in 0.5% Triton X-100. The hot-start technique was used to prevent nonspecific amplification (40 μl of the reaction mixture was heated to 94°C for 5 min before *Taq* DNA polymerase was added [2 U in 10 μl of reaction mixture]). The samples were amplified for 35 cycles, and each cycle consisted of 1.5 min at 94°C, 1.5 min at 64°C, and 1.5 min at 72°C. The PCR products were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide.

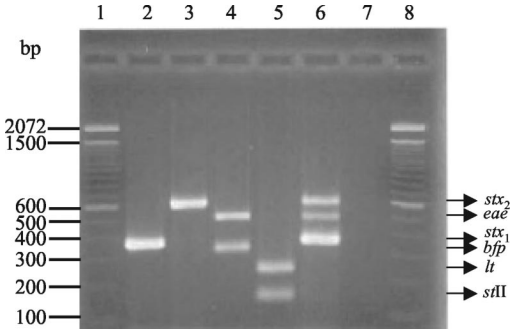

FIG. 1. Multiplex PCR of selected reference strains. Bands corresponding to *eae*, *bfp*, *stx*$_2$, *stx*$_1$, *lt*, and *stII* are indicated. Lane 1, 100-bp size ladder; lane 2, *E. coli* C600J (*stx*$_1$); lane 3, *E. coli* C600W (*stx*$_2$); lane 4, enteropathogenic *E. coli* 2348/69 (*eae bfp*); lane 5, enterotoxigenic *E. coli* H10407 (*lt st*); lane 6, enterohemorrhagic *E. coli* 933J (*stx*$_1$ *stx*$_2$ *eae*); lane 7, reagent control; lane 8, 100-bp size ladder.

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TABLE 1. Reference *E. coli* strains tested by multiplex PCR

Bacterial strain[a]	No. of strains showing positive PCR result/no. of strains tested					
	stx$_1$	*stx*$_2$	*eae*	*bfp*	*lt*	*st*
Enterohemorrhagic *E. coli*						
933J O157:H7 CVD	10/10	10/10	10/10	0/10	0/10	0/10
O157, ISP	0/5	5/5	5/5	0/5	0/5	0/5
C600J O157, CVD	10/10	0/10	0/10	0/10	0/10	0/10
C600W O157 CVD	0/10	10/10	0/10	0/10	0/10	0/10
Enteropathogenic *E. coli*						
2348/69, CVD	0/10	0/10	10/10	10/10	0/10	0/10
HS negative control, CVD	0/10	0/10	0/10	0/10	0/10	0/10
O142, SP	0/5	0/5	5/5	5/5	0/5	0/5
O25, ISP	0/5	0/5	5/5	5/5	0/5	0/5
Enterotoxigenic *E. coli*						
O159, MP	0/5	0/5	0/5	0/5	0/5	5/5
O8, MP	0/5	0/5	0/5	0/5	5/5	0/5
O6, MP	0/5	0/5	0/5	0/5	5/5	0/5
Nt, MP	0/10	0/10	0/10	0/10	3/10	10/10
H10407, CVD	0/10	0/10	0/10	0/10	10/10	10/10
Salmonella enteriditis, MP	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
Salmonella group B, MP	0/5	0/5	0/5	0/5	0/5	0/5
Salmonella spp., MP	0/5	0/5	0/5	0/5	0/5	0/5
Shigella flexneri, MP	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
Klebsiella oxytoca, MP	0/5	0/5	0/5	0/5	0/5	0/5
Proteus spp., MP	0/4	0/4	0/4	0/4	0/4	0/4

[a] CVD, Center for Vaccine Development, University of Maryland; ISP, Chilean Institute of Public Health; MP, Microbiology Program, University of Chile; Nt, not typeable.

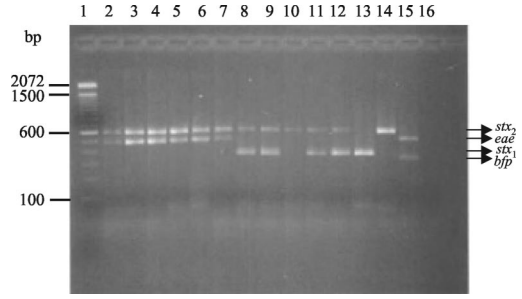

FIG. 2. Multiplex PCR analysis of clinical samples. Lane 1, 100-bp size ladder; lanes 2 to 6, samples from hemolytic uremic syndrome patients; lanes 7 to 12, samples from patients involved in food-borne outbreaks; lane 13, *E. coli* C600J; lane 14, *E. coli* C600W; lane 15, enteropathogenic *E. coli* 2348/69; lane 16, reagent control.

To develop the multiplex PCR, we tested the progressive incorporation of primers corresponding to the different genes and several combinations of melting temperatures and primer concentrations. The sensitivity and specificity of the reaction were assayed with the reference strains (Fig. 1). The PCR products for the *stx*$_1$, *stx*$_2$, *eae*, *bfp*, *stII*, and *lt* genes were 348, 584, 482, 254, 129, and 218 bp, respectively. Multiplex PCR-positive colonies were serotyped by agglutination with commercial antisera (Probac R, Sao Paulo, Brazil), and the results were confirmed at the Laboratory for Foodborne Zoonoses, Canada, or at the Chilean Institute of Public Health.

E. coli isolates from all hemolytic-uremic syndrome patients and from one adult patient with hemorrhagic colitis were positive for the *stx*$_2$ and *eae* genes (Fig. 2) and corresponded to enterohemorrhagic *E. coli* serotype O157:H7 (Table 3). Among the stool samples from patients involved in food-borne outbreaks, 122 *E. coli* colonies were isolated from 27 patients. For samples from two of the six outbreaks, we obtained positive multiplex PCR results. For four of the patients from the first outbreak, an enteropathogenic *E. coli* strain of serogroup O26 was detected, which was positive for the *eae* and *bfp* genes (Table 3). In the other outbreak, enterohemorrhagic *E. coli*-positive colonies were detected in samples from three of four patients affected. One patient was positive for enterohemorrhagic *E. coli* O157:H7 (with the genotype *stx*$_2$ *eae*), and in

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
eae	TCA ATG CAG TTC CGT TAT CAG TT GTA AAG TCC GTT ACC CCA ACC TG	482	This study
bfp	GGA AGT CAA ATT CAT GGG GGT AT GGA ATC AGA CGC AGA CTG GTA GT	254	This study
stx$_1$	CAG TTA ATG TGG TGG CGA AGG CAC CAG ACA ATG TAA CCG CTG	348	1
stx$_2$	ATC CTA TTC CCG GGA GTT TAC G GCG TCA TCG TAT ACA CAG GAG C	584	1
lt	GCA CAC GGA GCT CCT CAG TC TCC TTC ATC CTT TCA ATG GCT TT	218	This study
stII	AAA GGA GAG CTT CGT CAC ATT TT AAT GTC CGT CTT GCG TTA GGA C	129	This study

[a] Specific primers used for amplification of diarrheagenic *E. coli* virulence genes were designed from sequences obtained from the indicated GenBank accession numbers: *eae*, AB040740, AF025311, AF043226, AF065628, AF116899, AF200363, AF449418, AF530555, AF530556, AF530557, Z11541, AJ271407, AJ298279, AJ308550, AJ308551, AJ308552, M58154, U38618, and U66102; *bfp*, AF119170 and U27184; *lt*, gi1648865; and *stII*, AY028790 and M35586.

TABLE 3. Results of the multiplex PCR assay with *E. coli* strains obtained from clinical samples

Disease	No. of patients	No. of colonies tested	Multiplex PCR results (no. of positive patients/ no. studied)	Genotype detected by multiplex PCR	*E. coli* serotype[a]
Hemolytic-uremic syndrome	20	85	5/20	*stx*$_2$ *eae*	O157:H7
Hemorrhagic colitis	1	2	1/1	*stx*$_2$ *eae*	O157:H7
Sporadic diarrhea episodes	1,468	2,936	4/1,468	*stx*$_1$	ND
			1/1,468	*stx*$_1$	O26
			1/1,468	*stx*$_1$	O111
			2/1,468	*stx*$_1$ *eae*	ND
			1/1,468	*stx*$_2$	O125
			78/1,468	*eae bfp*	ND
			12/1,468	*stII*	ND
			25/1,468	*lt*	ND
			8/1,468	*stII lt*	ND
Food-borne outbreaks					
1	4	17	4/4	*eae-bfp*	O26
2	4	19	3/4	*stx*$_1$-*eae*	O157:H7
				stx$_1$-*stx*$_2$	O174:H28
				stx$_2$	O9:H21
6[b]	6	27	0/6		
9	3	12	0/3		
10	3	11	0/3		
12	7	35	0/7		

[a] ND, not done.
[b] *Shigella* spp. found in six of six patients.

samples from another patient, we detected colonies of three different genotypes and serotypes (*stx*$_2$ *eae*/O157:H7, *stx*$_2$/O91:H21, and *stx*$_1$ *stx*$_2$/O174:H28). A third patient was positive for *stx*$_1$ *stx*$_2$/O174:H28, suggesting that this particular food-borne outbreak was a mixed infection (Fig. 2, Table 3). A third outbreak was caused by *Shigella* spp., and for the three remaining outbreaks, we did not isolate any enteric pathogen. Analysis of 1,468 *E. coli* isolates from children with sporadic diarrheal episodes in the metropolitan region of Santiago from November 2002 to April 2003 revealed enterohemorrhagic *E. coli* strains in nine patients (0.6%), enteropathogenic *E. coli* strains in 78 children (5.3%), and enterotoxigenic *E. coli* strains in 45 patients (3.1%) (Table 3).

In the present study, we designed specific primers for the *eae*, *bfp*, *stII*, and *lt* genes. For the *eae* gene, 14 different subtypes have been identified so far, and their differences were incorporated into the primer design so that they can recognize most of the variants that have been described. For the *bfp*, *stII*, and *lt* genes, there are few sequences in the databases, but they were considered in the primer design.

The multiplex PCR showed sensitivity and specificity for the most common categories of diarrheagenic *E. coli* strains of different clinical origins, genotypes, and serotypes. The results of this study indicate that it is possible to perform simultaneous amplification of virulence genes from differentiate enterohemorrhagic, enterotoxigenic, and enteropathogenic *E. coli* strains and apply this technique to the diagnosis of patients with hemolytic-uremic syndrome, those involved in food-borne outbreaks, and patients with sporadic enterocolitis.

We thank Mohamed Karmali, Laboratory for Foodborne Zoonoses, Guelph, Ontario, Canada, for assistance with serotyping and James P. Nataro, Center for Vaccine Development, University of Maryland at Baltimore, and Alfredo Torres, Medical Branch Department of Microbiology and Immunology, University of Texas, for careful review of the manuscript and helpful discussions.

This work was supported by FONDECYT grant 1000636.

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