JOURNAL OF CLINICAL MICROBIOLOGY, June 2003, p. 2669–2671 Vol. 41, No. 6
0095-1137/03/$08.00+0 DOI: 10.1128/JCM.41.6.2669–2671.2003

Multiplex PCR Assay for Identification of Human Diarrheagenic *Escherichia coli*

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Received 16 December 2002/Accepted 25 February 2003

A multiplex PCR assay for the identification of human diarrheagenic *Escherichia coli* was developed. The targets selected for each category were *eae* for enteropathogenic *E. coli*, *stx* for Shiga toxin-producing *E. coli*, *elt* and *est* for enterotoxigenic *E. coli*, *ipaH* for enteroinvasive *E. coli*, and *aggR* for enteroaggregative *E. coli*. This assay allowed the categorization of a diarrheagenic *E. coli* strain in a single reaction tube.

Escherichia coli is the most important etiologic agent of childhood diarrhea and represents a major public health problem in developing countries (8). Identification of diarrheagenic *E. coli* strains requires that these organisms be differentiated from nonpathogenic members of the normal flora. Serogrouping of O antigen is not sufficient to identify a strain as diarrheagenic, because it does not correlate, in most cases, with the presence of virulence factors (18). Thus, identification of diarrheagenic *E. coli* strains needs to detect factors that determine the virulence of these organisms. With the advent of PCR, it has become possible to detect pathogenic genes in bacterial isolates, allowing the rapid diagnosis of diarrheagenic *E. coli*. PCR methods using single primer sets have been reported elsewhere (5, 10, 17, 20), but screening of bacterial isolates requires a large number of individual PCRs if single primer sets are used in separate reactions. To reduce the number of tests needed for diagnosis of diarrheagenic *E. coli*, several multiplex PCR systems have been reported previously (7, 9, 12, 13, 15). However, usually more than one multiplex PCR is required for identification of a diarrheagenic *E. coli* strain. Recently, Pass et al. (11) reported a multiplex PCR to detect 11 virulence genes, but it has not been fully evaluated against a large panel of isolates. This study attempted to develop a multiplex PCR for identification of enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), and Shiga toxin-producing *E. coli* (STEC).

Thirteen *E. coli* control strains were used in this study (Table 1). EPEC, STEC, and EIEC strains were characterized in previous studies and confirmed to have the relevant gene by single PCRs and phenotypic assays (1, 6). For ETEC strains, the production of heat-labile enterotoxin was determined by a reversed passive latex agglutination test (Denka Seiken, Co., Ltd., Tokyo, Japan), and the production of heat-stable enterotoxin (ST) was determined by an enzyme immunoassay kit (Denka Seiken Co., Ltd.). For EAEC strains, the HEp-2 cell adherence assay was performed as described by Cravioto et al. (2).

The targets selected for each category were *eae* for EPEC, *stx* for STEC, *elt* and *est* for ETEC, and *ipaH* for EIEC. The primers to detect the *bfpA* (bundle-forming pilus) gene, which is present in typical EPEC, were not included in this multiplex PCR since the presence of *eae* is sufficient to define EPEC. On the other hand, atypical EPEC strains which do not possess *bfpA* and a high rate of spontaneous cure of the EAF plasmid have been reported previously (8). For each of the target genes, different pairs of primers were selected from the literature (Table 2) and tested in a single PCR. Universal primers were selected when different alleles could be present to reduce the number of primer sets. Therefore, primer set SK1-SK2 (10), which can detect all the intimin variants, was used for detection of *eae*; primer set VTcom-u–VTcom-d, which allows amplification of stx_1, stx_2, and its variants (21), was selected for *stx*; and the primer set AL65-AL125 (4), which reacts with the two ST-I toxin genes (ST-Ia and ST-Ib), was used for detection of the *est* gene. Primers sets LT_L-LT_R (20) and ipaIII-ipaIV (17) were selected to detect *elt* and *ipaH*, respectively, so that PCR products were sufficiently different in size to be distinguishable by agarose gel electrophoresis.

As EAEC strains are heterogenous (3, 16, 19) and no DNA sequence was demonstrated to be present in all strains, the

TABLE 1. Control strains used in this study

Strain	Description	Serogroup	Positive gene(s)	Reference
E22-5	EPEC	O152	*eae*	6
E23-8	EPEC	O164	*eae*	6
O111-19	STEC	O111	*eae*, stx_1	6
O157-29	STEC	O157	*eae*, stx_2	6
O157-6	STEC	O157	*eae*, stx_1, stx_2	6
EBa 35	ETEC	O167	*elt*	This study
O126-53	ETEC	O126	*est*	This study
KNH172	ETEC	O148	*elt*, *est*	This study
O126-32	EAEC	O126	*aggR*, *aspU*, CVD432	This study
O111-15	EAEC	O111	*aggR*, *aspU*, CVD432	This study
C-481	EIEC	O143	*ipaH*	1
D36f	EIEC	O124	*ipaH*	1
O115-3	None	O115		This study

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TABLE 2. PCR primers used in this study

Designation	Sequence (5′ to 3′)	Target gene	Amplicon size (bp)	Reference
SK1	CCCGAATTCGGCACAAGCATAAGC	*eae*	881	10
SK2	CCCGGATCCGTCTCGCCAGTATTCG			
VTcom-u	GAGCGAAATAATTTATATGTG	*stx*	518	21
VTcom-d	TGATGATGGCAATTCAGTAT			
AL65	TTAATAGCACCCGGTACAAGCAGG	*est*	147	4
AL125	CCTGACTCTTCAAAAGAGAAAATTAC			
LT_L	TCTCTATGTGCATACGGAGC	*elt*	322	20
LT_R	CCATACTGATTGCCGCAAT			
ipaIII	GTTCCTTGACCGCCTTTCCGATACCGTC	*ipaH*	619	17
ipaIV	GCCGGTCAGCCACCCTCTGAGAGTAC			
aggRks1	GTATACACAAAAGAAGGAAGC	*aggR*	254	14
aggRkas2	ACAGAATCGTCAGCATCAGC			
Eaggfp	AGACTCTGGCGAAAGACTGTATC	CVD432	194	11
Eaggbp	ATGGCTGTCTGTAATAGATGAGAAC			
aspU-3	GCCTTTGCGGGTGGTAGCGG	*aspU*	282	This study
aspU-2	AACCCATTCGGTTAGAGCAC			

HEp-2 cell adherence assay is still the best method of defining this category. To determine the appropriate target gene to define EAEC, we investigated the presence of *aggR* (transcriptional activator of AAF/I and AAF/II), CVD432 probe (cryptic open reading frame), and *aspU* (EAEC-secreted protein U) genes described as prevalent in this *E. coli* category by Czeczulin et al. (3). Twenty EAEC strains, defined by HEp-2 cell adherence pattern, were studied by single PCR with use of the primer pairs aggRks1-aggRkas2 for *aggR* (14), Eaggfp-Eaggbp for the CVD432 probe (11), and aspU-3–aspU-2 for *aspU* (Table 2). Ten strains (50%) were positive for *aggR* and the CVD432 probe, while 11 strains (55%) were positive for *aspU*. Nine strains (45%) were negative for the three sets of primers. According to the results obtained, each primer set was tested in combination with the primer sets to define EPEC, ETEC, EIEC, and STEC.

For the PCR, DNAs were extracted from control strains by the method described by Yokoyama (22). Briefly, control strains were cultured in 2 ml of Luria-Bertani broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37°C with shaking. Thirty-six microliters of broth culture was added to 4 μl of 10× Tris-EDTA buffer (100 mM Tris-HCl, 10 mM EDTA, pH 8.3), and 60 μl of 2× proteinase K buffer (100 mM KCl, 20 mM Tris-HCl, 5 mM $MgCl_2$, 1% Tween 20, 800 μg of proteinase K/ml, pH 8.3) was added. After incubation for 90 min at 56°C and 10 min at 95°C, the sample was centrifuged at 10,000 × *g* for 1 min, and the supernatant was used as DNA template. Having confirmed the specificity of each primer set by single PCR, we combined six primer sets in different ratios and tested the control strains in several PCR cycling protocols. The optimized protocol was carried out with a 50-μl mixture containing 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 0.1% Triton X-100; 1.5 mM $MgCl_2$; 2.5 U of *Taq* DNA polymerase (Toyobo, Osaka, Japan); 0.2 mM deoxynucleoside triphosphate; a 0.125 μM concentration (each) of primers SK1, SK2, ipaIII, and ipaIV; a 0.25 μM concentration (each) of primers VTcom-u, VTcom-d, LT_L, LT_R, aggRks1, and aggRkas2; a 0.5 μM concentration (each) of primers AL65 and AL125; and 5 μl of the DNA template. The PCR program was 95°C for 1 min, 52°C for 1 min, and 72°C for 1 min, for 30 cycles, and 72°C for 10 min. PCR products were then electrophoresed on a 2.5% agarose gel (AmpliSize; Bio-Rad Laboratories), stained

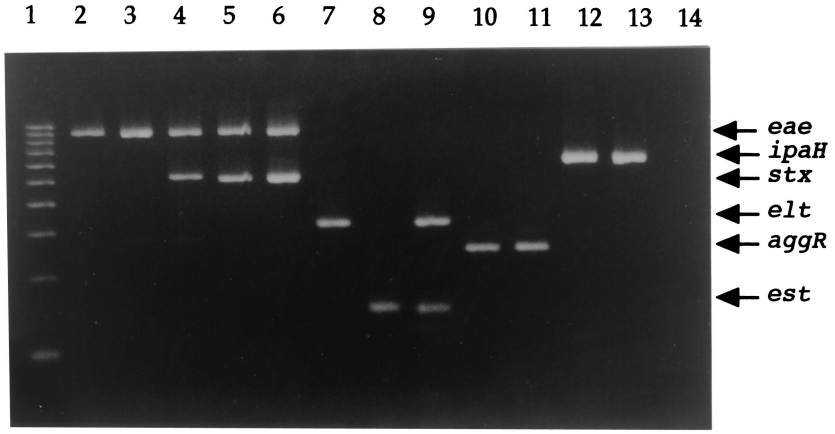

FIG. 1. Agarose gel electrophoresis of products from multiplex PCR with control strains. Lanes: 1, 100-bp DNA ladder; 2, E22-5 (EPEC); 3, E23-8 (EPEC); 4, O111-19 (STEC); 5, O157-29 (STEC); 6, O157-6 (STEC); 7, EBa 35 (ETEC); 8, O126-53 (ETEC); 9, KNH172 (ETEC); 10, O126-32 (EAEC); 11, O111-15 (EAEC); 12, C-481 (EIEC); 13, D36f (EIEC); 14, O115-3 (negative control).

TABLE 3. Results of single and multiplex PCRs

Category	No. of strains examined	Gene	No. of positives by PCR	
			Single	Multiplex
EPEC	27	*eae*	27	27
STEC	40	*eae*	37	37
		stx	40	40
EIEC	13	*ipaH*	13	13
ETEC	28	*elt*	7	7
		est	22	22
EAEC	30	*aggR*	30	30
None	18		0	0

with ethidium bromide, and visualized by UV transillumination. The buffer in the electrophoresis chamber and in the agarose gel was 0.5× Tris-borate-EDTA (11). The strains shown in Fig. 1 gave PCR products of the expected sizes for *eae* (881 bp, lanes 2 to 6), *stx* (518 bp, lanes 4 to 6), *elt* (322 bp, lanes 7 and 9), *est* (147 bp, lanes 8 and 9), *aggR* (254 bp, lanes 10 and 11), and *ipaH* (619 bp, lanes 12 and 13). PCR products were obtained for all six genes, and no PCR product was detected in the negative control (lane 14). To assess the sensitivity of the multiplex PCR, overnight cultures of control strains were serially 10-fold diluted and DNA was extracted as described above. Extracts of these samples were then subjected to multiplex PCR. The sensitivity of detection was 10^3 CFU per assay for *ipaH* and 10^4 CFU per assay for *eae*, *elt*, *est*, *aggR*, and *stx* (data not shown). Therefore, the presence of 10^4 CFU per assay must be ensured for detection of all the categories.

To demonstrate the utility of the multiplex PCR assay, 156 strains isolated from diarrheic patients were subjected to the optimized protocol, and the results were compared with those obtained by single PCR (Table 3). The EPEC strains tested included representatives of serogroups O55 (3 strains), O111 (11 strains), O119 (11 strains), O114 (1 strain), and O26 (1 strain). The STEC strains tested belonged to serogroups O26 (8 strains); O111 (10 strains); O157 (16 strains); O145 (2 strains); and O15, O121, O171, and OX3 (1 strain each). The EIEC strains tested belonged to serogroups O28ac (eight strains), O112ac (one strain), O124 (three strains), and O143 (one strain). The ETEC strains were of serogroups O26 (1 strain), O27 (2 strains), O126 (13 strains), and O128 (6 strains), and there were six strains that were O nontypeable or of unknown type. The EAEC strains belonged to serogroups O44 (six strains); O111 (eight strains); O126 (seven strains); O128 (two strains); O144 (two strains); and O127a, O157, O159, O164, and O166 (one strain each). There was agreement between the single and multiplex PCRs for all the strains.

Our multiplex PCR could identify EPEC, STEC, EIEC, and ETEC strains because the virulence markers for these four categories are well defined. To include the identification of EAEC strains in the multiplex PCR, we selected the primer set aggRks1-aggRkas2, which gave the best result when combined with the other five sets of primers. Some *aggR*-negative strains with aggregative adherence will not be detected by this assay. However, considering the difficulty of performing phenotypic assays in some laboratories, the multiplex PCR presented here is a practical and rapid diagnostic tool for identification of diarrheagenic *E. coli* in a single reaction tube.

The work was partly supported by the Uruma Trust Fund for Research into Science and Humanity of Japan.

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