

Multiplex PCR for Detection of Three Plasmid-Borne Genes of Enterotoxigenic *Escherichia coli* Strains

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We developed a novel multiplex PCR assay for enterotoxigenic *Escherichia coli* (EAEC) detection, by using three plasmid-borne genes (the aggregative adherence [AA] probe, *aap*, and *aggR*). One or more of the loci were detected in 24 (86%) of 28 patient isolates analyzed. The multiplex PCR assay is a fast, convenient, and sensitive molecular test to detect EAEC.

The importance of enterotoxigenic *Escherichia coli* (EAEC) strains in public health around the world is becoming increasingly clear. The pathogenicity of these strains has been proven in adult volunteers (12, 18) and in several studies from around the world (3, 4, 5, 9, 14). EAEC strains have been associated classically with persistent diarrhea (≥ 14 days), which represents a disproportionate share of diarrheal mortality. EAEC strains have been shown to elicit damage to the intestinal mucosa (C. Eslava, J. Villaseca, R. Morales, A. Navarro, and A. Cravioto, Abstr. 93rd Gen. Meet. Am. Soc. Microbiol. 1993, abstr. B-105, p. 44, 1993), and recent data link EAEC with growth retardation in infants (19, 24). Thus, detection of EAEC strains can make a significant contribution to public health in many areas.

EAEC diagnosis has long been problematic. The pathogen was initially defined by the presence of a characteristic stacked brick pattern, designated aggregative adherence (AA) in the HEP-2 cell adherence assay (14). Although this pattern provided the first EAEC epidemiologic associations with diarrhea, there has long been a desire to base detection on the presence of requisite virulence factors.

The pathogenesis of EAEC infection is only partly understood. EAEC adheres to the intestinal mucosa by means of aggregative adherence fimbriae (15) and then elicits damage to the mucosal epithelium, which is manifested as exfoliation of epithelial cells (19). A cytotoxin and several enterotoxins have been described (19), each of which may contribute to secretory diarrhea. EAEC diarrhea exhibits some features of an inflammatory enteritis, and the flagellin protein has been implicated in the release of interleukin-8 (IL-8) (24).

Many of the EAEC virulence factors have been localized to the 60- to 65-MDa AA plasmid, including the aggregative adherence fimbrial adhesin (16), the Pet (8) and EAST (19) enterotoxins, the transcriptional activator AggR (17), and a novel antiaggregation protein (dispersin) encoded by the *aap* gene (formerly known as *aspU*) (23). Furthermore, several other loci on the plasmid appear to be specific for EAEC,

although they have not yet been implicated in virulence. The importance of the AA plasmid is underscored by the fact that an empirically derived gene probe, designated the AA or CVD432 probe, has been reported to be sensitive and specific for EAEC detection (2). In the initial publication, the AA probe hybridized with 89% of EAEC strains, although this number can vary dramatically from study to study (4, 20, 21).

It has also become apparent that strains carrying the AA plasmid represent an important subgroup of EAEC strains and that many strains which do not hybridize with the AA probe nevertheless share plasmid-borne and chromosomal virulence determinants. Indeed, it has recently been shown that adult travelers carrying EAEC strains which harbored one of more of the AA plasmid loci were significantly more likely to shed fecal IL-8 and IL-1 than if their EAEC strains lacked all plasmid-borne factors (10). Thus, developing improved methods for detecting AA plasmid-positive EAEC strains is a priority. Here, we have developed a multiplex PCR assay to detect three AA plasmid-borne genes and show that these loci are commonly but not inevitably linked.

For the sake of comparison, all EAEC reference and patients strains were first characterized by the HEP-2 assay, which was performed as described previously (6). Strains were then further characterized by the standard colony blot method (1) for the identification of the plasmid-borne genes *aap* and *aggR* and for the AA probe (7). We next developed a multiplex PCR with previously described primers for the AA probe (22). For *aggR* detection, we used a previously described *aggR* forward primer (7) and a newly chosen reverse primer (GenBank accession no. 232523). *Aap* primers were chosen (GenBank accession no. 232523) and optimized specifically for the present study (Table 1). For standardization purposes, we used three previously described EAEC reference strains: JM221, 042, 17-2, and 12 additional diarrheagenic *E. coli* reference strains (11) (three enterotoxigenic, three enteroinvasive, three Shiga-like toxin producing, and three enteropathogenic), plus two *E. coli* negative controls strains: 3030 and K-12. Bacterial lysates were prepared by resuspending a single colony in 1 ml of deionized water (MilliQ) in a polypropylene tube, followed by boiling for 1 min. Each PCR tube contained 23 μ l of reaction mix comprised of (final concentrations): Tris-HCl (pH 8.3), 10 mM; KCl, 50 mM; MgCl₂, 2 mM; gelatin, 100 μ g/ml;

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TABLE 1. Primers, concentrations in the PCR mix, and amplicon sizes

Locus	Primers		Primer amt (pMol) in mix	Amplicon size (bp)
	O ^a	Sequence		
<i>aap</i>	F	5'-CTT GGG TAT CAG CCT GAA TG-3'	10	310
	R	5'-AAC CCA TTC GGT TAG AGC AC-3'		
<i>aggR</i>	F	5'-CTA ATT GTA CAA TCG ATG TA-3'	15	457
	R	5'-AGA GTC CAT CTC TTT GAT AAG-3'		
AA probe	F	5'-CTG GCG AAA GAC TGT ATC AT-3'	20	629
	R	5'-CAA TGT ATA GAA ATC CGC TGT T-3'		

^a O, orientation: F, forward; R, reverse.

glycerol, 5% (vol/vol); dATP, dCTP, dGTP, and dTTP (Roche), 200 μ M each; AmpliTaq polymerase (Gibco-BRL), 1 U/25 μ l. Also included were a mixture of the six primers (Table 1) and a 2- μ l portion of the bacterial lysates. The solutions were then subjected to the following cycling conditions: 50°C (2 min, 1 cycle); 95°C (5 min, 1 cycle); 95, 55, and 72°C (45 s at each temperature, 40 cycles); and a final extension step (10 min, 72°C) in a thermal cycler (Bio-Rad). Then, 4 μ l of the PCR mixture was visualized by ethidium bromide staining after electrophoresis in a 2.5% agarose gel in Tris-acetate-EDTA buffer (Gibco-BRL); the amplicon sizes are shown in Table 1 and Fig. 1. Only the presence of the correct sized gene product(s) was interpreted as a positive test.

All three reference strains—JM221, 042, and 17-2—were found to be positive for the AA probe and *aap* and *aggR* genes (Table 2). In contrast, none of the diarrheagenic *E. coli* or negative controls were positive for any of the analyzed genes. The minimal concentrations of CFU detected by this assay were 322 CFU (AA probe), 471 CFU (*aggR* gene), and 70 CFU (*aap* gene)/ml of saline buffer.

The multiplex assay was further evaluated against 28 EAEC strains isolated from patients and two other *E. coli*-negative control strains (from the collections of the Center of Vaccine Development, University of Maryland School of Medicine) (Table 2). All strains had been tested for HEP-2 adherence and

dot blot for the EAEC genes tested here. It was observed that 24 (86%) of 28 strains were positive for at least one gene, 23 (82%) of 28 strains were positive for the three genes, none of the strains were positive for just two genes, and 4 (14%) of 28 strains were negative for the three genes (Table 2). In the 28 strains analyzed, the PCR method identified 70 genes (23 AA probe, 24 *aap*, and 23 *aggR*), and the colony blot identified only 65 genes (22 AA probe, 23 *aap*, and 20 *aggR*) revealing that the PCR method is more sensitive. This PCR sensitivity was confirmed by the fact that 23 (82%) of 28 strains were positive for the three loci by PCR compared to 20 (71%) of 28 strains found to be positive by colony blot (Table 2).

There is a need for a convenient assay to detect EAEC strains based on molecular characteristics. The HEP-2 assay remains the "gold standard" for identification of EAEC when these strains exhibit the AA pattern. However, this technique requires special expertise and facilities and is time-consuming; thus, it is only performed in a few laboratories around the world, limiting both EAEC diagnosis and the epidemiological studies. Although a simplified version of the HEP-2 assay have been reported (13), the main problem remains that the strains exhibiting the AA pattern almost certainly encompass both pathogenic and nonpathogenic clones (20). Thus, we have developed a multiplex PCR that specifically and sensitively detects loci on the AA plasmid. The test appears to be more

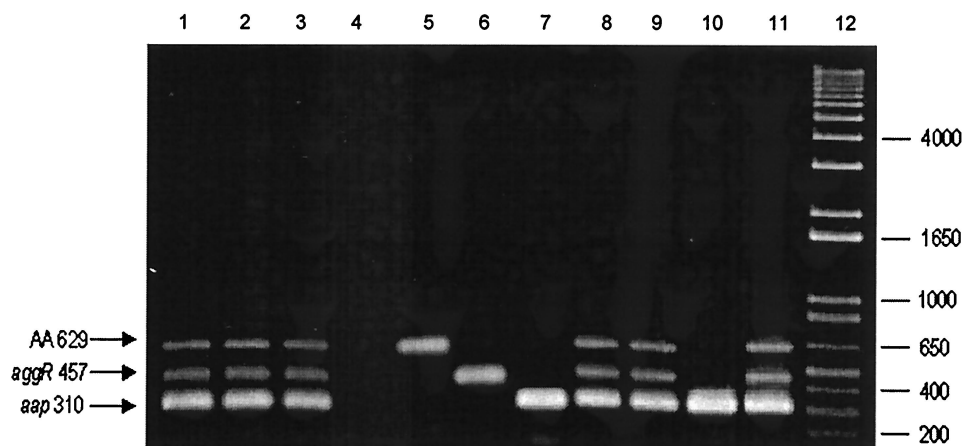


FIG. 1. PCR products. Lanes 1 to 4, PCR products (in base pairs) obtained with DNA of reference strains mix 17-2, 042, JM221, and 3030, respectively, along with the primer mix; lanes 5 to 7, PCR products obtained with the 17-2 strain and primers for the AA probe, *aggR*, and *aap* genes, respectively; lanes 8 to 11, PCR products obtained with DNA of patient isolates; lane 12, 1-kb molecular weight marker in base pairs.

TABLE 2. Results of control, reference, and patient EAEC strains as analyzed by HEP-2 assay, colony blot, and multiplex PCR techniques

Strain	HEP-2 assay result	Colony blot result			PCR multiplex result		
		AA probe	<i>aap</i>	<i>aggR</i>	AA probe	<i>aap</i>	<i>aggR</i>
EAEC strains							
101-1	AA	(-)	+	(-)	(-)	+	(-)
Mex 60A	AA	+	+	+	+	+	+
43977	AA	(-)	(-)	(-)	(-)	(-)	(-)
Peru H32-1	AA	+	+	+	+	+	+
Peru H38-1	AA	+	+	+	+	+	+
Peru H46-2	AA	+	+	+	+	+	+
Peru H72-2	AA	(-)	(-)	(-)	(-)	(-)	(-)
Peru H92-1	AA	+	+	+	+	+	+
Peru H133	AA	+	(-)	(-)	+	+	+
Peru H145-1	AA	+	+	+	+	+	+
Peru H191-1	AA	(-)	(-)	(-)	(-)	(-)	(-)
Peru H194-2	AA	+	+	+	+	+	+
Peru H223-1	AA	+	+	+	+	+	+
Peru H232-1	AA	+	+	+	+	+	+
Thai 6-1-1	AA	+	+	+	+	+	+
Thai 44-1-1	AA	+	+	+	+	+	+
Thai 103-1-1	AA	(-)	+	(-)	+	+	+
Thai 144-1-1	AA	+	+	+	+	+	+
Thai 199-1-4	AA	+	+	+	+	+	+
Thai 216-1-1	AA	+	+	+	+	+	+
Thai 232-1-1	AA	+	+	+	+	+	+
Thai 239-1-1	AA	+	+	(-)	+	+	+
Thai 253-1-1	AA	+	+	+	+	+	+
Thai 278-1-1	AA	+	+	+	+	+	+
Thai 309-1-1	AA	+	+	+	+	+	+
Thai 435-1-1	AA	+	+	+	+	+	+
Thai 495-1-1	AA	+	+	+	+	+	+
Thai 501-1-1	AA	(-)	(-)	(-)	(-)	(-)	(-)
Reference strains							
042	AA	+	+	+	+	+	+
17-2	AA	+	+	+	+	+	+
JM221	AA	+	+	+	+	+	+
Negative-control							
<i>E. coli</i> strains							
m61655 (K-12)	Nonadherent	(-)	(-)	(-)	(-)	(-)	(-)
HS	Nonadherent	(-)	(-)	(-)	(-)	(-)	(-)

sensitive than the colony blot technique (for the same three genes) and is also less time-consuming than the colony blot and HEP-2 assay.

Our multiplex PCR has demonstrated that most of the EAEC strains isolated from patients with diarrhea 23 (82%) of 28 were positive for the three loci, i.e., *aggR*, *aap*, and the AA probe, revealing that these plasmid-borne genes may be more frequently linked than was previously demonstrated by colony blot. These three loci have been reported in a majority of strains (7), and it has been suggested that they may be phylogenetically or pathogenetically linked (7, 23). Of course, it is possible that all three genes are markers for truly virulent EAEC clones, and this hypothesis remains to be tested. Importantly, however, the use of the multiplex assay increases both the sensitivity and the specificity of EAEC detection, which may permit earlier diagnosis of this infection and improve epidemiologic understanding of this emerging pathogen.

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