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Molecular Detection and Identification of Intimin Alleles in Pathogenic *Escherichia coli* by Multiplex PCR

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A multiplex PCR was designed to detect the *eae* gene and simultaneously identify specific alleles in pathogenic *Escherichia coli*. The method was tested on 87 strains representing the diarrheagenic *E. coli* clones. The results show that the PCR assay accurately detects *eae* and resolves alleles encoding the α, β, and γ intimin variants.

Two groups of pathogenic *Escherichia coli* have evolved similar mechanisms of adhering to the intestinal epithelium that result in a characteristic attaching-and-effacing (A/E) histopathology (7). Both enteropathogenic *E. coli* (EPEC), a major cause of infantile diarrhea in the developing world, and enterohemorrhagic *E. coli* (EHEC), the agent responsible for foodborne epidemics of hemorrhagic colitis in North America, Europe, and Japan (3–5), can produce A/E lesions which contribute to the severity of diarrheal disease. Production of A/E lesions is associated with the expression of intimin, an outer membrane protein encoded by a gene (*eae*) that is part of the LEE (locus of enterocyte effacement) pathogenicity island (2, 7).

Evolutionary analysis has shown that *E. coli* strains with the virulence properties and serotypes of EPEC and EHEC are subdivided into four distinct groups of clones (EPEC 1, EPEC 2, EHEC 1, and EHEC 2) (8–10). The clonal lineages differ in the site where LEE is inserted in the genome (11), and they carry distinct intimin alleles (1, 6). Three variants of intimin—Int-α, Int-β, and Int-γ—are characteristic of EPEC 1, EPEC 2, and EHEC 1 respectively. A fourth intimin (Int-δ), found in EPEC strains of serotype O86:H34, has greater homology to the intimin homologue of *Citrobacter rodentium* than to Int-α of EPEC strain E2348/69 (1). Most members of EHEC 2 (e.g., O26:H11) express Int-β, with the exception of a closely related group of bacteria of serotypes O111:H8, O111:H11, and O111:H− whose intimin allele has yet to be determined.

The objective of the present study was to devise a multiplex PCR for rapid detection of *eae* and identification of the specific intimin alleles in *E. coli* strains. To accomplish this, we designed oligonucleotide primers for multiplex PCR based on the multiple sequence alignment of *eae* alleles by McGraw et al. (6). Primers *eae* P1 (5′-CTGAACGGCGATTACGCGAA-3′) and *eae* P2 (5′-CCAGACGATACGATCCAG-3′) were constructed in the N-terminal conserved region of the gene at positions 544 and 1461, respectively. PCR with *eae* P1 and *eae* P2 generated a 917-bp fragment, indicating the presence of the *eae* gene (Fig. 1). Primers designed to determine the specific *eae* allele were constructed in the same orientation as *eae* P2 on the noncoding strand in the part of the gene specifying the variable C-terminal region of the protein (Fig. 1). Eco*eae*α (5′-CTGGAGTTGTCGATGTT-3′) was located at position 2192, generating a 1,648-bp fragment indicative of the *eae* allele specifying Int-α. Eco*eae*β (5′-GTAATTGTGGCACTC C-3′), positioned at bp 2470, generated a 1,926-bp fragment indicative of the allele specifying Int-β. Eco*eae*γ (5′-GCCTC TGACATTGTTAC-3′), positioned at bp 2314, produced a 1,770-bp fragment indicative of the allele specifying Int-γ. All primers were synthesized by a Beckman 1000 oligonucleotide synthesizer (Beckman, Fullerton, Calif.).

To test the allele-specific PCR assay, we examined 87 strains of the diarrheagenic *E. coli* (DEC) collection, which have been characterized by electrophoretic type based on multilocus enzyme electrophoresis of 20 housekeeping genes (Table 1). The DEC strains represent 15 common clones associated with diarrheal disease and were examined previously for the presence of *eae* and several other virulence factors (10). The *eae* genes of DEC strains 3a, 3f, 5d, 11a, and 12a have also been sequenced (6).

In preparation for PCR, each *E. coli* strain was grown overnight at 37°C in 10 ml of nutrient broth (Difco, Detroit, Mich.) in a shaking water bath. Chromosomal DNA was isolated according to the instructions in the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, Minn.). Aliquots (1 μl) of DNA samples were each amplified in a 50-μl reaction mixture

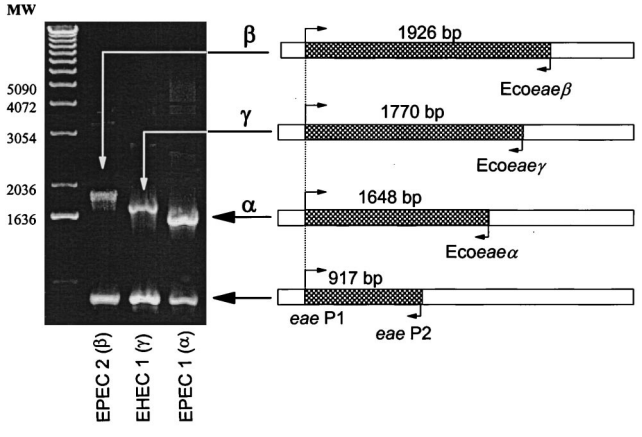

FIG. 1. Primer locations and fragment sizes for multiplex PCR with five primers: *eae* P1, *eae* P2, Eco*eae*α, Eco*eae*β, and Eco*eae*γ. PCR results are given for three standards (DEC 12a [EPEC 2], DEC 4f [EHEC 1], and E2348/69 [EPEC 1]) representing the three intimin alleles (Int-β, Int-γ, and Int-α, respectively).

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TABLE 1. Characteristics of 86 DEC strains examined by multiplex PCR to detect Int-α, Int-β, and Int-γ alleles

DEC strain no.	Original strain no.	Serotype	Isolation data[a]			PCR fragment size (bp)				*eae* allele
			Yr	Locale	Host	917	1,648	1,770	1,926	
1a	572-56	O55:H6	1956	USA	Human	+	+			α
1b	C54-58	O55:H6	1958	Suriname	Human	+	+			α
1c	F196-51	O55:H6	1951	Germany	Human	+	+			α
1d	F563-55	O55:H6	1955	Egypt	Human	+	+			α
1e	AC-C21	O55:H6	1986	Mexico	Human	+	+			α
2a	3787-62	O55:H6	1962	Congo	Human	+	+			α
2b	5513-56	O55:H−	1956	USA	Human	+	+			α
2c	607-54	O55:H6	1954	USA	Human	+	+			α
2d	F60-51	O55:H6	1951	France	Human	+	+			α
2e	2087-77	O55:H6	1977	USA	Human	+	+			α
3a	3299-85	O157:H7	1985	USA	Human	+		+		γ
3b	46240	O157:H7	1990	USA	Human	+		+		γ
3c	3104-88	O157:H7	1988	USA	Human	+		+		γ
3d	3009-88	O157:H7	1988	USA	Human	+		+		γ
3e	3077-88	O157:H7	1988	Canada	Human	+		+		γ
3f	493/89	O157:H−	1989	Germany	Human	+		+		γ
4a	C1520-77	O157:H7	1977	Argentina	Calf	+		+		γ
4b	C999-87	O157:H7	1987	Denmark	Human	+		+		γ
4c	C374-83	O157:H7	1983	Egypt	Buffalo	+		+		γ
4d	C681-87	O157:H7	1987	Japan	Calf	+		+		γ
4e	C7-88	O157:H7	1988	Denmark	Human	+		+		γ
4f	EDL-933	O157:H7	1982	USA	Meat	+		+		γ
5a	5624-50	O55:H7	1950	USA	Human	+		+		γ
5b	660-79	O55:H7	1979	USA	Human	+		+		γ
5c	5380-66	O55:H7	1966	USA	Human	+		+		γ
5d	C586-65	O55:H7	1965	Sri Lanka	Human	+		+		γ
5e	C997-63	O55:H7	1963	Iran	Human	+		+		γ
6a	5338-66	O111:H21	1966	USA	Human					
6b	C142-54	O111:H12	1954	Germany	Human					
6c	2277-67	O111:H12	1967	Guatemala	Human					
6d	F436-51	O111:H12	1951	Italy	Human					
6e	184-83	O111:H12	1983	Brazil	Human					
7a	750001	O157:H43	1975	USA	Pig					
7b	902034	O149:H−	1990	USA	Pig					
7c	820691	O157:H43	1982	USA	Pig					
7d	831015	O157:H43	1983	USA	Pig					
7e	861575	O157:H−	1986	USA	Pig					
8a	2198077	O111:H−	1977	USA	Human	+				?
8b	3030A-86	O111:H8	1986	USA	Human	+				?
8c	8610049	O111:H−	1986	USA	Calf	+				?
8d	C130-53	O111:H11	1953	Cuba	Human	+				?
8e	C194-65	O111:H8	1965	Denmark	Human	+				?
9a	3323-61	O26:H11	1961	USA	Human	+			+	β
9b	2262-79	O26:H−	1979	USA	Human	+			+	β
9c	C240-52	O26:H−	1952	Switzerland	Human	+			+	β
9d	C814-67	O26:H11	1967	Denmark	Human	+			+	β
9e	45	O26:H11	1986	Mexico	Human	+			+	β
10a	H30	O26:H11	ND	Canada	Human	+			+	β
10b	3047-86	O26:H11	1986	Australia	Human	+			+	β
10c	1557-77	O26:H11	1977	USA	Human	+			+	β
10d	C12-52	O26:H11	1952	France	Human	+			+	β
10e	900105	O26:H11	1990	USA	Calf	+			+	β
10f	RDEC-1	O15:H−	1970s	USA	Rabbit	+			+	β
10g	C309-64	O128:H8	1964	ND	Human	+			+	β
10h	C186-61	O119:H11	1961	ND	Human	+			+	β
10i	87-1713	O145:H6	1987	Canada	Human	+			+	β
10j	88817	O70:H11	1988	Canada	Human	+			+	β
11a	2254-75	O128:H2	1975	USA	Human	+			+	β
11b	3733-71	O128:H2	1971	USA	Human	+			+	β
11c	A9619-c2	O45:H2	1983	USA	Human	+			+	β
11d	E335021	O128:H2	1989	UK	Human	+			+	β
11e	WM-63	O128:H2	ND	Brazil	Human	+			+	β
12a	F1-50	O111:H2	1950	UK	Human	+			+	β
12b	2966-56	O111:H2	1956	USA	Human	+			+	β
12c	3942-67	O111:H−	1967	Panama	Human	+			+	β
12d	9101-83	O111:H2	1983	Peru	Human	+			+	β
12e	3291-86	O111:H−	1986	Kenya	Human	+			+	β

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TABLE 1—*Continiued*

DEC strain no.	Original strain no.	Serotype	Isolation data[a]			PCR fragment size (bp)				*eae* allele
			Yr	Locale	Host	917	1,648	1,770	1,926	
13a	3350-73	O128:H7	1973	USA	Human					
13b	5024-71	O128:H7	1971	USA	Human					
13c	C500-74	O128:H7	1974	Tanzania	Human					
13d	C1083-79B	O128:H7	1979	Rwanda	Human					
13e	2384-81	O128:H47	1981	USA	Human					
14a	C916-70	O128:H21	1970	Peru	Human					
14b	C691-71	O128:H21	1971	India	Human					
14c	9088-83	O128:H21	1983	Peru	Human					
14d	1791-79	O128:H−	1979	USA	Human					
14e	C639-77	O128:H21	1977	Bangladesh	Human					
15a	5430-66	O111:H21	1966	USA	Human					
15b	448-71	O111:H21	1971	USA	Human					
15c	2660-77	O111:H21	1977	USA	Human					
15d	2708-78	O111:H21	1978	USA	Human					
15e	2394-80	O111:H21	1980	USA	Human					

[a] USA, United States; ND, not determined; UK, United Kingdom.

that contained 5.0 μl of PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 15 mM $MgCl_2$, 1% Triton, 0.05% gelatin), 2.5 μl of primer *eae* P1 at 200 ng/μl, 1.0 μl each of primers *eae* P2, Eco*eae*α, Eco*eae*β, and Eco*eae*γ at 200 ng/μl, 1.25 mM deoxynucleoside triphosphate mixture, 5 units of displayTAQ (Display Systems Biotech), and distilled H_2O to volume. Amplification in a Perkin-Elmer 480 DNA thermal cycler utilized an initial denaturing step at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 53°C for 2 min, and 72°C for 3 min. Positive and negative controls were included with each set of strains tested. PCR products were visualized on ethidium bromide-stained gels by transillumination with UV light.

We tested the multiplex PCR assay with three positive controls for which the complete *eae* sequence is known (E2348/69, DEC 4f, and DEC 12a) and observed PCR fragments of the predicted sizes (Fig. 1). We then tested the 87 DEC strains by multiplex PCR and found that 57 strains produced the 917-bp fragment indicative of the presence of the *eae* sequence (Table 1). The allele-specific fragments showed that Int-α occurs in DEC 1 and 2 strains (EPEC 1 group), Int-β occurs both in DEC 11 and 12 (EPEC 2) and in DEC 9 and 10 (EHEC 2) strains, and Int-γ occurs both in DEC 3 and 4 (EHEC 1) and in DEC 5 (atypical EPEC of serotype O55:H7) strains. PCR results for representative DEC strains are shown in Fig. 2. Interestingly, DEC 8 strains have an *eae* gene, but it is sufficiently different in sequence that it is not amplified by the allele-specific primers (Fig. 2). The genetic basis of this difference remains to be determined.

The results demonstrate that the multiplex PCR can accurately detect the presence of the *eae* gene and simultaneously identify specific *eae* alleles. Because the *eae* alleles encoding Int-α, Int-β, and Int-γ are lineage specific, this multiplex PCR method provides a rapid way to classify suspected pathogens into the major clonal groups of EPEC and EHEC.

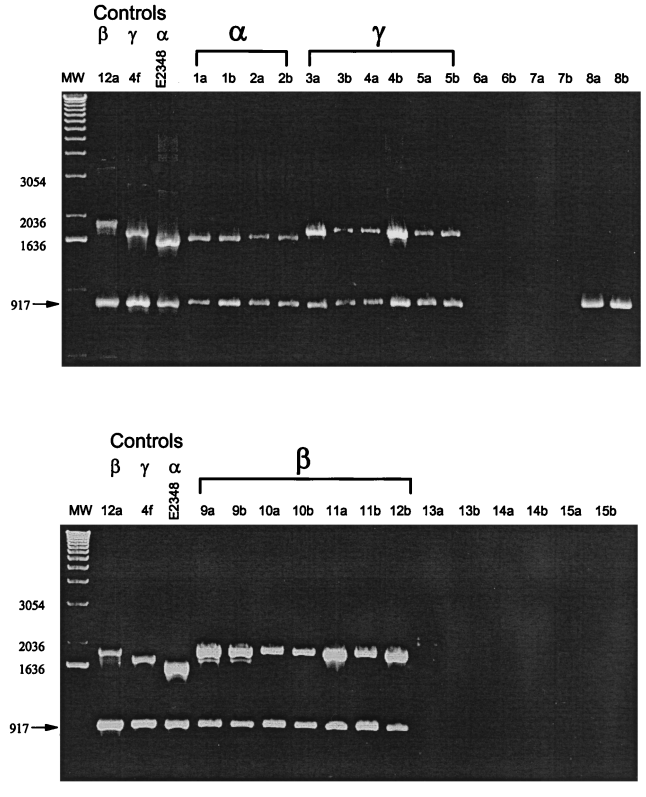

FIG. 2. Detection of the *eae* gene and specific intimin alleles in strains of the DEC collection. The first two strains of each of the 15 electrophoretic types of the DEC collection (Table 1) are shown. The presence of *eae* is indicated by a 917-bp fragment. Identification of specific intimin alleles is indicated by fragments of characteristic sizes (α, 1,648 bp; β, 1,926 bp; γ, 1,770 bp). Three controls (Fig. 1) are presented on the left side of each gel for comparison.

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