JOURNAL OF CLINICAL MICROBIOLOGY, June 1998, p. 1795–1797 Vol. 36, No. 6
0095-1137/98/$04.00+0
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Multiplex PCR for Enterotoxigenic, Attaching and Effacing, and Shiga Toxin-Producing *Escherichia coli* Strains from Calves

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Received 16 December 1997/Returned for modification 13 February 1998/Accepted 4 March 1998

A multiplex PCR was developed to identify enterotoxigenic, attaching and effacing, and Shiga toxin-producing *Escherichia coli* strains by amplifying genes encoding K99 and F41 fimbriae, heat-stable enterotoxin a, intimin, and Shiga toxins 1 and 2. This multiplex PCR was specific and sensitive. It will be useful for identification of *E. coli* strains which cause diarrhea in calves.

Diarrhea in calves is commonly caused by enterotoxigenic *Escherichia coli* (ETEC). More recently, attaching and effacing *E. coli* (AEEC) and Shiga toxin-producing *E. coli* (STEC) have also been identified as causes of diarrhea and dysentery in calves (2, 15).

ETEC has two groups of virulence factors: fimbriae (pili) and enterotoxins. K99 and/or F41 fimbriae mediate adherence to the ileum and are found on most calf ETEC (1, 9, 12). Calf ETEC produces heat-stable enterotoxin a (STa), which causes hypersecretion into the gut lumen (2).

Intestinal lesions caused by AEEC are termed attaching and effacing (AE) because of their intimate attachment to the enterocyte and effacement of the microvillus border (18). A chromosomal gene, *eaeA*, encodes the protein intimin, which is involved in AE activity (10). AEEC which causes disease and does not produce enterotoxins or Shiga toxins is referred to as enteropathogenic *E. coli* (EPEC).

STEC produces two types of *E. coli* Shiga toxins, those that are immunologically similar to the Shiga toxin produced by *Shigella dysenteriae* (Stx1) and those that are immunologically distinct from *Shigella dysenteriae* Shiga toxin (Stx2) (11). Bovine STEC produces either Stx1, Stx2, or both. These toxins act by inhibiting protein synthesis and are lethal for in vitro cultured Vero cells (8). Some *E. coli* strains, such as the zoonotic pathogen *E. coli* O157:H7, which produces Shiga toxins and has AE activity, are often termed enterohemorrhagic *E. coli* (EHEC) because of their association with hemorrhagic colitis in humans.

Diagnosis of *E. coli* infection currently relies on the phenotypic differentiation of pathogenic strains from nonpathogenic normal flora *E. coli* via bioassays or immunoassays for toxins and fimbriae. These tests can be time-consuming and complicated and are not routinely used in many clinical laboratories. Histological examination of the intestine may indicate involvement of ETEC or AEEC, but this is a postmortem diagnosis. Genotypic diagnosis may be accomplished by DNA colony blot hybridization to identify genes encoding virulence factors (17). However, the use of radioactive isotopes and the time required make this method unsuitable for many diagnostic laboratories. PCR is a useful diagnostic tool because it is quick, specific, sensitive, and relatively inexpensive. A multiplex PCR which detects genes encoding intimin and Stx1 and Stx2 has been developed (4). This paper reports the identification and differentiation of ETEC, AEEC, and STEC via multiplex PCR amplification of virulence-associated genes commonly found in these *E. coli* strains. Primers specific for genes encoding the fimbrial subunits of K99 and F41, STa enterotoxin, intimin, and the A subunits of Shiga toxins Stx1 and Stx2 were incor-

TABLE 1. Primers used in multiplex PCR of ETEC, AEEC, and STEC

Virulence factor	Accession number	Primer sequence 5′-3′	Position in open reading frame	Size of product (bp)	Reference
Stx1	Z36899	TTCGCTCTGCAATAGGTA	125-142 of A subunit	555	21
		TTCCCCAGTTCAATGTAAGAT	659-679 of A subunit		
Intimin	Z11541	ATATCCGTTTTAATGGCTATCT	992-1013 of *eaeA*	425	10, 28
	S90827	AATCTTCTGCGTACTGTGTTCA	1395-1416 of *aeaA*		
F41	X14354	GCATCAGCGGCAGTATCT	34-51 of fimbrial subunit	380	7
		GTCCCTAGCTCAGTATTATCACCT	390-413 of fimbrial subunit		
K99	M35282	TATTATCTTAGGTGGTATGG	21-40 of fimbrial subunit	314	23
		GGTATCCTTTAGCAGCAGTATTTC	311-334 of fimbrial subunit		
Sta	M25607	GCTAATGTTGGCAATTTTTATTTCTGTA	9-36	190	25
		AGGATTACAACAAAGTTCACAGCAGTAA	171-198		
Stx2	L11078	GTGCCTGTTACTGGGTTTTTCTTC	30-53 of A subunit	118	22
		AGGGGTCGATATCTCTGTCC	128-147 of A subunit		

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TABLE 2. Reference *E. coli* strains tested by multiplex PCR

Literature no.	Phenotypic characterization	Genotypic characterization[a]	PCR result	Reference(s)
B41	Calf ETEC	K99, F41, STa	K99, F41, STa	19, 20
B44	Calf ETEC	K99, F41, STa	K99, F41, STa	19, 20
431	Pig ETEC	K99, F41, STa	K99, F41, STa	16
637	Pig ETEC	K99, STa	K99, STa	26
3081	Calf STEC O157: H7	Intimin, Stx1, Stx2	Intimin, Stx1, Stx2	5
933	Human STEC O157: H7	Intimin, Stx1, Stx2	Intimin, Stx1, Stx2	6, 27
CHMC#6	Human EPEC	Intimin	Intimin	24
E2348-69	Human EPEC	Intimin	Intimin	13
RDEC-1	Rabbit EPEC	Intimin	Intimin	3

[a] Genes encoding the indicated virulence factors were determined previously by DNA colony blot hybridization.

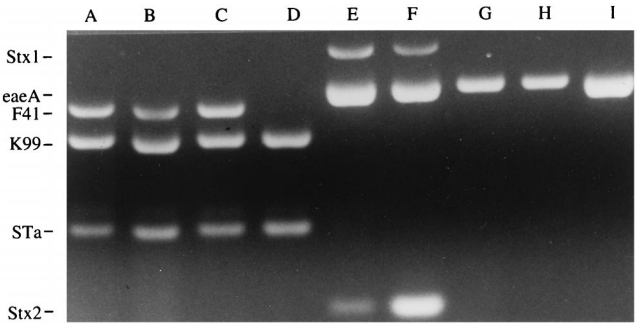

FIG. 1. Multiplex PCR of reference strains. DNA from reference strains was used as template DNA for PCR. Products were separated on a 3% agarose gel and compared to a positive control. Bands corresponding to Stx1, *eaeA*, F41, K99, STa, and Stx2 are indicated. Lane A, strain B41; lane B, strain B44; lane C, strain 431; lane D, strain 637; lane E, strain 3081; lane F, strain 933; lane G, strain CHMC6; lane H, strain E2348-69; lane I, RDEC-1.

porated into a multiplex PCR format. This multiplex PCR was then tested on 99 *E. coli* strains obtained from the *E. coli* collection at the National Animal Disease Center in Ames, Iowa, and from the Center for Vaccine Development, University of Maryland. Strains were previously probed by colony blot hybridization for genes encoding these virulence factors.

Primers were chosen from published sequences with the aid of the Primer Select software (DNASTAR Inc, Madison, Wis.). Table 1 includes the primer sequences, the position of the primer in the open reading frame of the gene, and the predicted sizes of amplified products. Primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, Iowa). Because the EHEC and EPEC genes which encode intimin have considerable heterogeneity on the 3′ end, the *eaeA* primers were made to amplify a constant 5′ region of the EHEC and EPEC genes (14). Other primers were chosen from the open reading frames of the published sequences. The primers were designed to have similar annealing temperatures and minimal interactions and resulted in different-sized products.

The 20-μl PCR mixture contained 0.5 μM concentrations of each primer, 0.2 mM concentrations of each dNTP (di-deoxynucleotides), 1× AmpliTaq Gold buffer, 2.5 U of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Branchburg, N.J.), and bacterial DNA. All reagents except AmpliTaq Gold and template DNA were added to PCR tubes, lyophilized under centrifugation in a DNA Speed Vac (Savant Instruments, Farmingdale, N.Y.), and stored at −20°C. The use of tubes containing lyophilized reagents reduced the time for setup of the assay and decreased the possibility of false positives due to contamination. Bacterial DNA was obtained by suspending a colony of bacteria grown overnight on trypticase soy agar in 50 μl of H_2O and boiling at 100°C for 10 min. After addition of 19.5 μl of bacterial DNA and 0.5 μl of AmpliTaq Gold to tubes containing the lyophilized reaction mixture, samples were amplified in a GeneAmp PCR 2400 thermal cycler (Perkin-Elmer) under the following conditions: 25 cycles beginning with a 30-s denaturation at 94°C, primer annealing at 50°C for 45 s, followed by extension for 1 min 30 s at 70°C. The extension time was ramped for an additional 3 s per cycle, and a final extension for 10 min at 70°C was performed. Products were electrophoresed in a 3% NuSieve 3:1 agarose gel (FMC Bioproducts, Rockland, Maine) for 1 h at 100 V, stained with ethidium bromide, and photographed under UV light. Each experiment contained negative controls with all reagents except template DNA. Also, a positive control was included with bacterial DNA from two strains containing all six genes of interest.

The multiplex PCR was tested on several reference strains

TABLE 3. Results of multiplex PCR test on groups of *E. coli* representing different phenotypes

Group	Phenotypic characterization	Serogroup	Host species	Genotypic characterization[a]	PCR results (no. in agreement/no. tested)
a	ETEC	O101, O9	Calf and pig	K99, F41, STa	9/9
		O8	Calf	K99, STa	2/2
		O9	Pig	F41, STa	1/1
		O9, O20, O141, O148, O149, O78	Pig and human	STa	8/8
b	EPEC	O55, O111, O119, O125, O126, O127, O128, O142	Human	Intimin	16/16
c	STEC	O157:H7, O157 NM, O15, O17	Calf and human	Intimin, Stx1, Stx2	19/19
		O5, O84, O103, O133	Calf	Intimin, Stx1	10/10
d	Various	O101	Calf	F41	1/1
		O9	Calf	F41, K99	1/1
		O133, OX3, O22	Calf	Stx1, Stx2	3/3
		O98	Pig	Stx1	1/1
		O5, O8, O171	Calf	Stx2	3/3
		O139	Pig	Stx2e	1/1
		O8, O138	Pig	Stx2e, STa	3/3
		O119	Calf and pig	Stx2, STa	1/1
e	Nonpathogens		Calf and pig	No genes of interest	11/11

[a] Genes encoding the indicated virulence factors were determined previously by DNA colony blot hybridization.

with different phenotypes as illustrated in Fig. 1 and Table 2. These strains had been characterized phenotypically and by colony blot hybridization previously by others. The multiplex PCR assay correctly determined the presence or absence of the genes of interest in all of the reference strains.

Further isolates from the National Animal Disease Center and the Center for Vaccine Development were chosen to verify that the multiplex PCR could detect genes with a high level of agreement with colony blot hybridization (Table 3). The isolates tested included the following groups: a, 20 ETEC isolates from calves, pigs, or humans; b, 16 EPEC isolates from humans; c, 29 STEC isolates from calves or humans; d, 14 isolates representing various combinations of the six genes of interest; and e, 11 nonpathogenic *E. coli* isolates containing none of the six genes of interest. The multiplex PCR correctly detected the presence or absence of all genes of interest in all 90 isolates from groups a to e.

This multiplex PCR was highly specific, because it did not detect any genes which were determined to be absent by probing. The multiplex method was also highly sensitive, as it correctly detected all genes of interest in 100% (88 of 88) of the strains and isolates containing them. This assay will be useful in diagnostic situations for identification and characterization of *E. coli* isolated from calves with diarrhea. This multiplex PCR may also prove useful in detecting any of the genes of interest in *E. coli* from other host species.

This research was funded in part by Merck and Company, Inc., through the Merck Scholars program at Iowa State University and by the Frank K. Ramsey endowment.

We would like to thank Rob Schneider and James Kaper for providing the genotypically characterized strains.

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