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Rapid Identification and Differentiation of Clinical Isolates of Enteropathogenic *Escherichia coli* (EPEC), Atypical EPEC, and Shiga Toxin-Producing *Escherichia coli* by a One-Step Multiplex PCR Method

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Enteropathogenic *Escherichia coli* (EPEC), atypical enteropathogenic *E. coli*, and Shiga toxin-producing *E. coli* differ in their virulence factor profiles, clinical manifestations, and prognosis, and they require different therapeutic measures. We developed and evaluated a robust multiplex PCR to identify these pathogroups based on sequences complementary to *escV*, *bfpB*, *stx*$_1$, and *stx*$_2$.

Pathogenic *Escherichia coli* strains are responsible for a broad spectrum of intestinal and extraintestinal diseases, including diarrhea, urinary tract infections, septicemia, and neonatal meningitis (11). Enteropathogenic *E. coli* (EPEC) and the majority of clinical isolates of Shiga toxin (Stx)-producing *E. coli* (STEC) harbor the "locus of enterocyte effacement" (LEE), a pathogenicity island that is responsible for the phenotype of attaching-and-effacing (A/E) lesions (6, 11).

EPEC are a major cause of human infantile diarrhea predominantly in less-developed countries but are also identified with increasing frequency in industrialized areas (1, 11, 17). These pathogens colonize the small intestine, induce the degeneration of epithelial microvilli, and intimately adhere to the host cell. Comparable to a "molecular syringe," the chromosomally encoded type III secretion system injects "effector" proteins into the host cell, inducing a characteristic rearrangement of the actin cytoskeleton resulting in the formation of "pedestals." These characteristic histopathological alterations are summarized as "A/E lesions."

The genes responsible for the A/E lesions are located on an ~35-kb pathogenicity island, known as the locus of enterocyte effacement (LEE). Typical EPEC harbor an additional 60-MDa plasmid, the EPEC adherence factor (EAF) plasmid (16), that is not present in atypical EPEC (here abbreviated as ATEC) strains (3, 28). The EAF plasmid harbors the bundle-forming pilus (*bfp*) operon, encoding the type IV pili responsible for localized adherence and the formation of microcolonies on host cells. ATEC strains harbor homologues of the LEE pathogenicity island but, due to the lack of the EAF plasmid (3, 8), they mostly adhere in a diffuse pattern to epithelial cells. Recent epidemiological evidence indicates an increasing prevalence of ATEC particularly in developed countries (see, for example, references 1, 17, 21, and 28) but also in developing countries (see, for example, references 9 and 27). This also indicates that in the field the EAF plasmid is not essential to cause disease.

Like ATEC strains, the closely related STEC responsible for sporadic infections as well as serious outbreaks worldwide, mostly harbor the LEE pathogenicity island and lack the BFP-encoding EAF plasmid. STEC strains differ geno- and phenotypically from ATEC by their production of Stx. These pathogens cause an acute inflammation of the colon, resulting in hemorrhagic colitis with rare but serious sequelae including neurological disorders and the hemolytic-uremic syndrome (HUS), the leading cause of acute renal failure in children (11, 12).

Identification of EPEC, ATEC, and STEC strains is currently usually based on serotyping with specific antisera in a time-consuming process demanding some technical expertise. To facilitate diagnostic and therapeutic measures, we developed a single multiplex PCR (MPCR) for the simultaneous and rapid identification and differentiation of diarrheagenic *E. coli* belonging to EPEC, ATEC, or STEC pathotypes.

TABLE 1. Primer pairs used for detection of the pathotype marker genes used in this study

Primer pairs[a]	Target gene	Primer sequence (5′ to 3′)	Product size (bp)
MP-escV-F	*escV*	GGCTCTCTTCTTCTTTATGGCTG	534
MP-escV-R		CCTTTTACAAACTTCATCGCC	
MP-bfpB-F	*bfpB*	GATAAAACTGATACTGGGCAGC	826
MP-bfpB-R		AGTGACTGTTCGGGAAGCAC	
MP2-stx1A-F	*stx*$_1$	GGCGTTCTTATGTAATGACTGC	250
MP2-stx1A-R		ATCCCACGGACTCTTCCATC	
MP2-stx2A-F	*stx*$_2$	CGTTTTGACCATCTTCGTCTG	325
MP2-stx2A-R		AGCGTAAGGCTTCTGCTGTG	

[a] -F, forward primer; -R, reverse primer.

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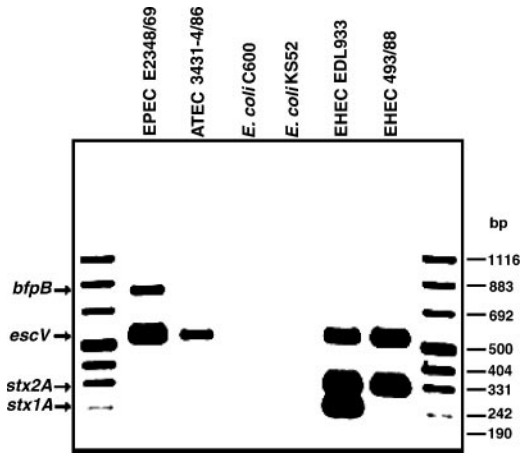

FIG. 1. Whole-cell multiplex-PCR pattern of reference strains EPEC E2348/69 (carrying LEE and the EAF plasmid but no *stx* genes), ATEC 3431-4/86 (carrying LEE, but lacking the EAF plasmid and *stx*), *E. coli* C600 (lacking LEE, the EAF plasmid and *stx*), uropathogenic *E. coli* KS52 (lacking LEE, the EAF plasmid and *stx*), STEC EDL933 (carrying LEE, stx_1 and stx_2, but lacking the EAF plasmid), and STEC 493/89 (carrying LEE and stx_2 but lacking the EAF plasmid). To identify the LEE pathogenicity island, we used primer pairs specifically for *escV*; for the EAF plasmid we designed primer pairs for the *bfpB* gene, and for the Shiga toxins (Stx) of STEC strains we used the primers MP2-stx1A-F/R and MP2-stx2A-F/R that correctly detected stx_1 and stx_2 and their variants stx_{1c}, stx_{1d}, stx_{2c}, stx_{2d}, and stx_{2e}.

MPCR development: selection of target genes and primer design. For the development of a single MPCR, we designed primer pairs (Table 1) that share similar temperature-related properties and that give rise to DNA fragments of sufficiently different sizes to be unequivocally resolved by agarose gel electrophoresis (Fig. 1). Therefore, for the specific identification of EPEC, ATEC, and STEC strains, we chose four marker genes (*escV*, *bfpB*, stx_1, and stx_2) exhibiting the highest degree of homology among the corresponding sequences found in the databases. The MPCR was performed in a 25-μl reaction mixture consisting of 1 U of *Taq* DNA polymerase with the corresponding *Taq* polymerase buffer (Segenetic; Borken), a 0.3 mM concentration of each deoxynucleoside triphosphate, and a 0.4 μM concentration of each PCR primer. Thermocycling conditions were as follows: 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min. The identity of the amplified fragments was determined by sequencing (SEQLAB; Göttingen).

Specificity and evaluation of the MPCR. The specificity of the MPCR was tested on LEE-positive and LEE-negative reference strains (EPEC E2348/69, ATEC 3431–4/86, STEC EDL933, STEC 493/89, uropathogenic *E. coli* KS52, and apathogenic *E. coli* C600). As shown in Fig. 1, the specific DNA fragments corresponding to the genes defining the appropriate LEE-harboring phenotypes (EPEC carrying LEE and *bfp* but not *stx*, ATEC carrying LEE but not *bfp* or *stx*, and STEC lacking *bfp* and/or LEE but carrying *stx*) were easily detected by MPCR in a single experiment using the listed primer pairs (Table 1). The *escV* gene was detected in reference strains encompassing all of the intimin subtypes (α, β, γ, ε, ζ, η, θ, ι, and κ) known thus far (30) and validated by sequencing.

Moreover, the MPCR primer pairs MP2-stx1A-F/R and MP2-stx2A-F/R detected STEC belonging to each of the four different seropathotypes that have been identified among human STEC isolates by Karmali et al. (12). Most of the clinical STEC isolates possess genes encoding Stx1, Stx2 or their variants (4, 7, 14, 23, 24). These include stx_{1c}, stx_{1d}, stx_{2c}, stx_{2d}, and stx_{2e} that were all correctly detected using the primers MP2-stx1A-F/R and MP2-stx2A-F/R included in the MPCR. As expected, stx_{2f}, which is the prevalent *stx* gene in STEC strains isolated from pigeons (22, 25), could not be detected by the novel MPCR because its sequence identity to stx_2 of *E. coli* O157:H7 strain EDL933 is only 63.4 and 57.4% for the A- and B-subunit genes, respectively (22). Although a single case of diarrhea caused by an stx_{2f}-harboring *E. coli* strain has been recently reported (24), this *stx* allele appears to be extremely rare among human isolates (7).

Validation of MPCR with clinical isolates comprising different pathogroups. The comparison of the analysis of 184 of 281 well-defined clinical isolates comprising EPEC, ATEC, and STEC by MPCR and single PCRs targeting *eae*, stx_1, and

TABLE 2. Detection of virulence genes in clinical isolates using MPCR

Pathogroup	Serotype (no. of strains)	No. of strains							
		Results of single PCRs targeting:				Results of MPCR targeting:			
		eae	*bfp*	stx_1	stx_2	*escV*	*bfp*	stx_1	stx_2
Classical EPEC	O55:H6 (10)	10	10	–	–	10	10	–	–
	O86:H− (3)	3	3	–	–	3	3	–	–
	O111:H− (3)	3	3	–	–	3	3	–	–
	O111:H2 (8)	8	8	–	–	8	8	–	–
	O114:H2 (1)	1	1	–	–	1	1	–	–
	O127:H− (1)	1	1	–	–	1	1	–	–
	O128:H2 (3)	3	3	–	–	3	3	–	–
	O142:H6 (2)	2	2	–	–	2	2	–	–
	O144:H2 (1)	1	1	–	–	1	1	–	–
ATEC	O8:H− (1)	1	–	–	–	1	–	–	–
	O26:H− (1)	1	–	–	–	1	–	–	–
	O26:H11 (23)	23	–	–	–	23	–	–	–
	O55:H− (1)	1	–	–	–	1	–	–	–
	O55:H6 (1)	1	–	–	–	1	–	–	–
	O55:H7 (7)	7	–	–	–	7	–	–	–
	O86:H8 (1)	1	–	–	–	1	–	–	–
	O119:H9 (1)	1	–	–	–	1	–	–	–
	O127:H40 (2)	2	–	–	–	2	–	–	–
	O128:H− (1)	1	–	–	–	1	–	–	–
	O128:H2 (3)	3	–	–	–	3	–	–	–
	O128:H8 (1)	1	–	–	–	1	–	–	–
STEC	O157:H7 (3)	3	–	3	–	3	–	3	–
	O157:H7 (25)	25	–	–	25	25	–	–	25
	O157:H7 (5)	5	–	5	5	5	–	5	5
	SF O157:NM (25)	25	–	–	25	25	–	–	25
	O26:H11 (10)	10	–	10	–	10	–	10	–
	O26:H11 (15)	15	–	–	15	15	–	–	15
	O26:H11 (10)	10	–	10	10	10	–	10	10
	O111:H− (1)	1	–	1	–	1	–	1	–
	O111:H8 (1)	1	–	1	1	1	–	1	1
	O111:H8 (2)	2	–	2	–	2	–	2	–
	O145:H25 (1)	1	–	1	1	1	–	1	1
	O145:NM (10)	10	–	–	10	10	–	–	10
	O60:H− (1)	–	–	–	1	–	–	–	1
	O91:H21 (3)	–	–	–	3	–	–	–	3
	O101:H9 (1)	–	–	–	1	–	–	–	1
	O126:H29 (1)	–	–	1	–	–	–	1	–
	O128:H2 (1)	1	–	–	–[a]	1	–	–	–

[a] The stx_{2f} gene was present in this strain, which was recently isolated from a stool of a child with diarrhea (25). –, Strains harboring the respective virulence gene were not detected.

TABLE 3. Comparison of marker gene detection by single PCR and MPCR in stool enrichment cultures of patients with diarrhea and HUS

Isolated strain serotype (no.)	Pathogroup designation	No. of samples	No. of strains positive for gene[e]						
			Single PCR			MPCR			
			eae	*stx*$_1$	*stx*$_2$	*escV*	*bfp*	*stx*$_1$	*stx*$_2$
ND[a]	NA[b]	35	–	–	–	–	–	–	–
O157:H7 (25)	STEC	25	25	5	24	25	–	5	24
O157:NM[c] (15)	STEC	15	15	–	15	15	–	–	15
O26:H11 (7)	STEC	7	7	–	7	7	–	–	7
O145:NM (6)	STEC	6	6	3	6	6	–	3	6
O111:NM (4)	STEC	4	4	4	4	4	–	4	4
O26:NM (3)	ATEC	3	3	–	–	3	–	–	–
Other non-O157 serotypes (5)[d]	STEC	5	–	1	4	–	–	1	4

[a] ND, serotype not determined.
[b] NA, not applicable.
[c] NM, nonmotile; SF, sorbitol-fermenting.
[d] O91:H21NM (2), O113:H21, O128:H2, and O146:H21.
[e] –, A strain harboring the respective virulence gene was not detected.

stx$_2$ as single genes demonstrated that both approaches yielded the same virulence gene patterns (Table 2). Moreover, 97 of 281 *E. coli* strains belonging to enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, and enteroaggregative *E. coli* tested negative in the MPCR.

Validation of MPCR with stool samples from healthy volunteers and patients with diarrhea and HUS. Stool samples from healthy volunteers ($n = 50$) were all negative, emphasizing the specificity of the MPCR. We then tested 100 stool samples from patients with HUS and diarrhea in parallel by single PCRs targeting *eae*, *stx*$_1$, and *stx*$_2$ and by MPCR. As shown in Table 3, there was 100% agreement between both methods for the identification of ATEC and STEC. Classical EPEC strains were not found in any of these samples.

Numerous MPCR methods have been developed for the identification of *E. coli* pathotypes (2, 15, 18–20, 26, 29), including approaches by real-time PCR (see, for example, references 5 and 18). However, most of the MPCR methods in the literature harbor limitations in terms of the number of targeted genes, specificity, the resolution of amplified fragments in agarose electrophoresis, nonspecific amplification, and the inability to differentiate between EPEC and ATEC strains. Recently, an MPCR has been introduced by Kimata et al. (13) that targets 12 genes to differentiate between diarrheagenic *E. coli* pathotypes. However, it appears that a differentiation between EPEC and ATEC might not be straightforward, and thus the additional investigation of HEp-2 adherence patterns has been suggested. Real-time PCR and DNA array (10) approaches have the advantage of higher sensitivity but are usually quite expensive.

Therefore, in the present study, a novel single MPCR has been developed that allows for the specific differential detection of LEE-harboring EPEC, ATEC, and STEC isolates and also identifies LEE-negative STEC strains in a straightforward and robust reaction. By several rounds of redesign and optimization, four highly specific primer pairs were developed which, combined in one reaction, gave rise to amplicons that are well resolved by agarose gel electrophoresis. The specificity of the MPCR was demonstrated with reference strains, as well as with numerous well-characterized clinical isolates. The eight primers developed in the present study proved to be specific for the corresponding four genes, since we could not observe any cross-priming or the amplification of nonspecific DNA fragments. Further evaluation of the tetrameric MPCR with 281 clinical *E. coli* isolates and 150 stool samples demonstrated that the MPCR is highly specific and reliable. We conclude that the newly designed MPCR is a specific method for the identification and differentiation of EPEC, ATEC, STEC, and LEE-negative STEC strains. As a fast, straightforward, and robust technique, it might be introduced into routine diagnostic in clinical microbiological laboratories.

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