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Identification of Human Enterovirulent *Escherichia coli* Strains by Multiplex PCR

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Some strains of *Escherichia coli* are involved in enteric infections in both adults and children. However the classical diagnostic methods can not differenciate pathogenic from nonpathogenic *E. coli*, because of the lack of phenotypic differences. In this study, we developed multiplex PCR in order to amplify fragments of specific virulence genes of the five main *E. coli* pathotypes. Fragments of the expected size were obtained using previously or newly designed primers and allowed identification of 10 virulence genes in only 5 reactions. This method was applied to the detection of pathogenic *E. coli* isolated from 90 patients' stools specimens during an 18-month survey. Patients were suffering from diarrhea or hemolytic uremic syndrome and in 13 cases (14.4%), an enterovirulent *E. coli* strain was detected. This diagnostic method could therefore represent an important technique in clinical laboratories which lack standard tests for these pathogens. J. Clin. Lab. Anal. 15:100–103, 2001.

Key words: diagnosis; polymerase chain reaction; *E. coli*; diarrhea

INTRODUCTION

Although *Escherichia coli* is the predominant facultative anaerobe of the human colonic flora, some strains are responsible for enteric disease. The clinical signs associated with these infections range from diarrhea to more complicated syndromes such as colitis or hemolytic uremic syndrome (HUS). However, the identification of these pathogens is rarely performed; in some cases, patients resolve their diarrhea long before they come to medical attention for stool culture. Moreover, identification of these pathogens requires that these organisms be differentiated from nonpathogenic members of the flora and so far, no simple and reliable diagnosis method has been described.

At least five main categories of enteric *E. coli* pathogens have been described: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasif *E. coli* (EIEC), and enteroggregative *E. coli* (EAEC) (1). Classification is based on the clinical signs observed during the infection, as well as the presence of different chromosomal or plasmid-encoded virulence genes. For this reason, specific detection of virulence genes represents the best reliable technique for differentiating diarrheagenic strains from nonpathogenic members of the stool flora and distinguish one category from another. Many probes have been designed and used in hybridization assays; they target either toxin- or adherence factors-encoding genes (for a review see (1)). However the standard hybridization procedure may take 2 to 3 days to complete. Shorter procedures such as PCR detection have been applied to identify gene sequences from pathogenic enteric *E. coli* strains (2–9). However the screening of bacterial isolates for several virulence genes requires a large number of individual PCRs if single primers sets are used in separate reactions. The present study examined the use of specific oligonucleotide primers in the amplification of 10 virulence genes of human enterovirulent *E. coli*. Multiplex reactions were developed and allowed detection of the different virulence genes in less than 24 hours. Using this diagnosis method, an 18-month survey was carried out including patients with clinical enteric manifestations, hospitalized in Clermont-Ferrand, France.

MATERIALS AND METHODS

Patients and Specimens

Stools specimens were collected from 90 patients hospitalized in the different wards of the Clermont-Ferrand hospi-

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tals between March 1999 and September 2000. The screening was systematically performed when clinical signs indicated an enterovirulent *E. coli* strain was potentially involved—patients enrolled in the study had diarrhea with or without blood or mucus, colitis, or any symptoms of the HUS—and when no other pathogen (bacteria, virus, or parasites) were isolated. The different PCRs were not systematically performed on each sample, but chosen according to clinical and general data. Briefly, PCR specific for EHEC was mainly performed when patients were suffering from either HUS or thrombocytopenia (26%), or from bloody diarrhea (28%). EPEC specific reactions were realized when aqueous diarrhea were observed (14%); enterotoxins-encoding genes (ETEC) were investigated only if the patients had been traveling in endemic countries (8%). EIEC and EHEC specific reactions were performed whenever inflammatory signs were observed (11%) and in the case of persistant or unexplained diarrhea the search for EAEC specific genes was included (13%).

Fecal samples from patients were collected and were cultured in Luria Bertani (LB) (Difco Laboratories, Detroit, MI) broth and streaked out on Drigalski plates (Biomérieux, La Balme les Grottes, France), and they were then incubated at 37 °C for 18 h. Bacteria from 1 ml of the LB culture or from at least 10 colonies grown on Drigalski agar and previously suspended in 1 ml of sterile water were harvested, resuspended in 200 µl of sterile water, and incubated at 100 °C for 10 min. Following centrifugation, 5 µl of the supernatant was used in PCR as described in the following section. Virulence genes-positive isolates were then identified biochemically by using API 20E test (Biomérieux). Improvement of PCR reactions was initially performed with reference enterovirulent *E. coli* strains (Table 1). They were grown in LB broth overnight at 37 °C. Total DNA was extracted from 1.5 ml of overnight broth culture by resuspending the bacteria in a small volume (200 µl) of sterile dezionized water and boiling the suspension for 10 min. The samples were centrifuged at 12,000*g* for 10 min, and 100 µl of the supernatant was collected for use as template in PCR.

PCR Procedure

PCR was performed with 0.2 ml Eppendorf tubes on a Perkin Elmer 2400 thermal cycler with a reaction volume of 50 µl. The DNA template (5 µl) was added to a mixture containing 0.1mM each dATP, dCTP, dGTP, and dUTP (Roche Diagnostics, Meylan, France); a buffer solution (Quantum-Appligène, Strasbourg, France) consisting of 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH 8.8 at 25 °C), and 0.01% Tween 20; 1.5 mM $MgCl_2$; and the PCR primers. One unit of Taq polymerase (Quantum-Appligène, Strasbourg, France) were added. Each PCR program was preceded by a denaturation step of 5 min at 94 °C, followed by 25 or 30 cycles of amplification (see Table 1) and a final 72 °C for 5 min.

Reaction products were separated by agarose gel electrophoresis by adding 5 µl of loading dye containing 0.25% bromophenol blue in 40% sucrose solution and loading onto a 2% agarose gel (Eurogentec, Herstal, Belgium) containing ethidium bromide (1 µg/ml). The buffer in the electrophoresis chamber was 0.5X Tris-Borate-EDTA and 100 mA was applied across the gel. DNA in the gel was visualized by exposing the gel to UV light and was photographied with the computer program VisioLab 200.

RESULTS AND DISCUSSION

The first step of this study was dedicated to the improvement of PCR using genomic DNA from reference enterovirulent *E. coli* strains. Four multiplex reactions were developed allowing the one-step detection of virulence factors from EPEC, EHEC, ETEC, and EAEC strains (Table 2),

TABLE 1. Bacterial strains and parameters of the simplex and multiplex PCRs

E. coli strains	Target genes	Primers Denaturation, hybridization and polymerisation temperatures Number of cycles
ECEH EDL933	*stx1-stx2-eae*	slt6-slt7, slt12-slt13, eae1B-eae5B 94 °C for 50 sec / 55 °C for 50 sec / 72 °C for 50 sec × 25
EPEC 2348/69	*eae-bfpA*	eae-I-eae-II, EP1-EP2 94 °C for 50 sec / 53 °C for 50 sec / 72 °C for 50 sec × 25
EIEC 0124	*ipaH*	ipaHIII-ipaHIV 94 °C for 50 sec / 50 °C for 50 sec / 68 °C for 50 sec × 25
ETEC H10407	TL-TS	J11-TW20, JW7bis-JW14 94 °C for 50 sec / 50 °C for 50 sec / 72 °C for 1 min 30 sec × 30
EAEC 17.2	*aggC-EAST-1*	aggC693-aggC694, east11a-east11b 94 °C for 50 sec / 45 °C for 50 sec / 72 °C for 50 sec × 25

TABLE 2. Target genes, sequences of PCR primers,[a] and product sizes

Pathotype	Genes		Primers	Product size (nucleotides)	Ref
EHEC	*stx1*	slt6	5′-ACCCTGTAACGAAGTTTGCG-3′	140	5
		slt7	5′-ATCTCATGCGACTACTTGAC-3′		
	stx2	slt12	5′-ATCCTATTCCCGGGAGTTTACG-3′	584	6
		slt13	5′-GCGTCATCGTATACACAGGAGC-3′		
	eae	eae1B	5′-GAACGGCAGAGGTTAATCTGC-3′	200	this study
		eae5B	5′-TCAATGAAGACGTTATAGCCC-3′		
EPEC	*eae*	eae-I	5′-GGTACTGAACGCAGTACGC-3′	831	10
		eae-II	5′-CGACATCGCTAACACGGG-3′		
	bfpA	EP1	5′-AATGGTGCTTGCGCTTGCTGC-3′	326	2
		EP2	5′-GCCGCTTTATCCAACCTGGTA-3′		
EIEC	*ipaH*	ipaHIII	5′-GTTCCTTGACCGCCTTTCCGATACCGTC-3′	600	9
		ipaHIV	5′-GCCGGTCAGCCACCCTCTGAGAGTAC-3′		
ETEC	LT	J11	5′-CGGTCTCTATATTCCCTGTT-3′	450	8
		TW20	5′-GGCGACAGATTATACCGTGC-3′		
	ST	JW7bis	5′-ACAGGCAGGATTACAACA-3′	185	8
		JW14	5′-ATTTTTMTTTCTGTATTRTCTT-3′		
EAEC	*aggC*	aggC693	5′-GCCAAGATCCGAGATTGA-3′	528	this study
		aggC694	5′-TATTAAACCATGGTAGCG-3′		
	east-1	east11a	5′-CCATCAACACAGTATATCCGA-3′	111	7
		east11b	5′-GGTCGCGAGTGACGGCTTTGT-3′		

[a]M = A and C; R = A and G.

respectively. The set of primers previously described for the detection of the *eae* gene in EPEC strains (10) could not be used to amplify the *eae* gene from EHEC isolates because of of the sequence's heterogeneity (11). Therefore, a new set of primers specific for the *eae* gene was designed and allowed detection of this target gene from EHEC strains (Table 1). These combinations of primers gave adequate amplification of their respective target genes—*bfp* and *eae* for EPEC; *stx1*, *stx2*, *eae* for EHEC; the heat-stable toxin sequence (TS) and the heat-labile toxin sequence (LT) for ETEC; *aggC* and *east* for EAEC—and the products were sufficiently different in size to be distinguishable by agarose gel electrophoresis (Fig. 1). An extra single reaction was necessary to detect the EIEC specific gene *ipaH*. The total length of each reaction was re-

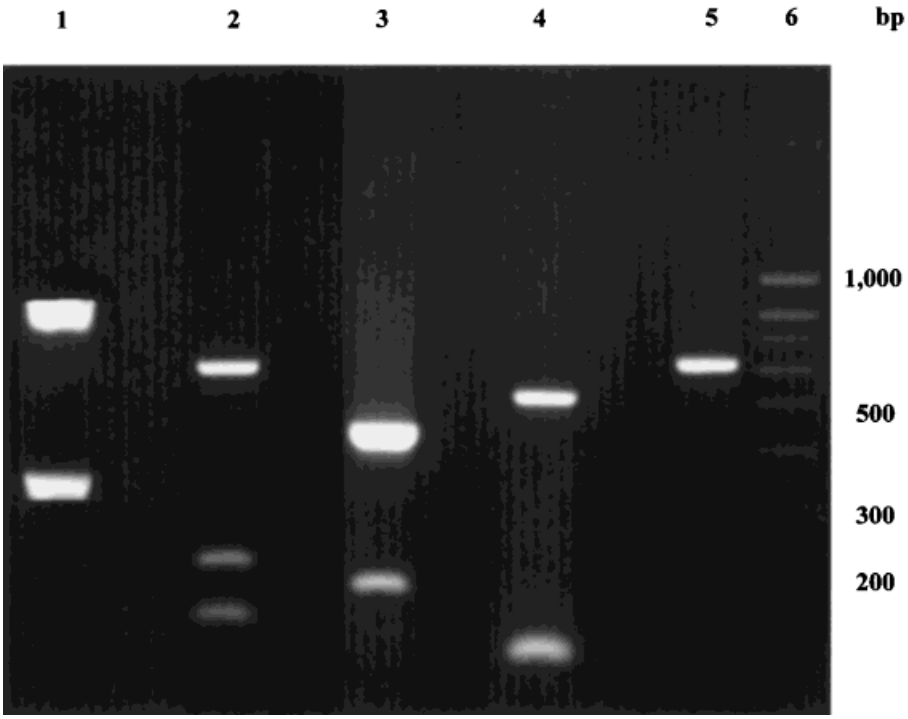

Fig. 1. Agarose gel analysis of PCR-amplified DNA from enteric *E. coli* strains. Lane 1, EPEC template contained genes for *eae* (831 bp) and *bfpA* (326 bp); lane 2, EHEC template contained genes for *stx2* (584 bp), *stx1* (140 bp) and *eae* (200 bp); lane 3, ETEC template contained genes for LT (450 bp) and ST (185 bp); lane 4, EAEC template contained genes for *aggC* (528 bp) and *east-1* (111 bp); lane 5, EIEC template contained genes for *ipaH* (600 bp); lane 6, molecular mass marker.

TABLE 3. General and clinical data of patients and characteristics of the *E. coli* strain isolated from stool specimens

Patients			*E. coli* virulence genes		
Pt	Sex, Age (yr)	Basic clinical data	Non-detected	Detected	Pathotype
1.	F, 1	Hemolytic uremic syndrome	*stx2*	*stx1* and *eae*	EHEC
2.	M, 20	Proctorrhagia, fever	*ipaH, stx2*	*stx1* and *eae*	EHEC
3.	F, 0.6	Hemolytic uremic syndrome	*stx1*	*stx2* and *eae*	EHEC
4.	M, 71	Diarrhea	*stx1*	*stx2* and *eae*	EHEC
5.	F, 21	Hemolytic uremic syndrome	*stx1, eae*	*stx2*	EHEC
6.	M, 1	Bloody diarrhea	*stx2*	*stx1* and *eae*	EHEC
7.	M, 78	Bloody diarrhea	*stx1, stx2, eae*	*east*	EAEC
8.	M, 2	Diarrhea	ST, LT	*east*	EAEC
9.	F, 5	Diarrhea	ST, LT	*east*	EAEC
10.	M, 32	Dysenteria	ST	LT	ETEC
11.	F, 24	Diarrhea		LT and ST	ETEC
12.	F, 13	Diarrhea	*stx1, stx2*	*eae* and *bfp*	EPEC
13.	F, 37	Bloody diarrhea		*ipaH*	EIEC

duced by decreasing either the amplification step length or the number of cycles, and easily detectable signals were obtained by performing PCRs in less than two hours (Table 1). The specificity of the amplified fragments was checked by dot blot hybridization using internal DNA probes (data not shown). Therefore only 5 PCR were necessary to screen for the main enterovirulent *E. coli* strains.

This diagnosis method was then used during an 18-month study period, during which we assessed 90 patients from different wards of the Clermont-Ferrand hospital. The results are presented in Table 3. Thirteen patients (14.4%) with enterovirulent *E. coli* in their feces were identified. They suffered from diarrhea or dysenteria (n = 10), or developed HUS (n = 3). Six EHEC stains were isolated, harboring either both *stx1* and *eae* (3), or *stx2* and *eae*, or *stx2* alone. Two ETEC were isolated from patients traveling back from endemic countries (patients #10 and #11). EAgEC isolates were detected in stools from one adult and two children suffering from diarrhea. One EPEC (*bfpA* and *eae*-positive) was isolated from a 13-year-old girl with aqueous diarrhea; this strain did not agglutinate with any of the so-called classical EPEC serotypes (data not shown) showing the usefulness of the serotyping method as a diagnosis tool. Finally an EIEC strain was detected in the stool of a 37-year-old female suffering from bloody diarrhea. The presence of these enterovirulent strains in the patients' stools was detected in less than 24 hours.

In summary, we have developed and improved PCRs to detect human enterovirulent *E. coli* strains. These combinations were used with DNA extract from pure reference strains cultures, but we also successfully amplified virulence genes in cultures from patients' stools samples. Therefore, this method offers a pratical possibility for rapid identification of pathogenic mechanisms in enteric *E. coli* infections.

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