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Development of PCR for Screening of Enteroaggregative *Escherichia coli*

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In this study, we determined the sequence of the *Eco*RI-*Pst*I fragment of the plasmid pCVD432, also termed the enteroaggregative *Escherichia coli* (EAggEC) probe. A primer pair complementary to this probe was designed for PCR amplification of a 630-bp region. Comparison of the analysis of the EAggEC probe sequence with those in database libraries revealed no significant similarity to any known bacterial gene. Pure cultures of *E. coli* cells, as well as mixed cultures from stool specimens, were investigated with the PCR assay, the EAggEC probe test, and the adherence test. Of 50 *E. coli* strains which demonstrated aggregative adherence to HEp-2 cells, 43 (86%) were positive with the EAggEC PCR. All 43 of these strains reacted with the EAggEC probe. Six EAggEC strains gave negative results by both molecular techniques. In contrast, only 4 of 418 (0.96%) strains representing other categories of diarrheagenic *E. coli* demonstrated a positive PCR result. The PCR was also successful in screening for the presence of EAggEC in enriched cultures grown from stool specimens. Compared with cell culture assays and colony hybridization, our findings revealed that the PCR assay was more rapid, simple, and highly sensitive and can therefore be recommended as a screening method for EAggEC in the clinical laboratory.

Escherichia coli strains which cause diarrhea in humans constitute a heterogenous group of organisms that are distinct in their virulence properties, epidemiology, and disease association. Genetic techniques have been shown to be highly effective in detecting and identifying diarrheagenic *E. coli* (9). Recently, PCR has been developed to detect enterohemorrhagic *E. coli* (8), enterotoxigenic *E. coli* (13), class 1 enteropathogenic *E. coli* (6), and enteroinvasive *E. coli* (7). However, there is no PCR technique which can assist in the detection of the enteroaggregative *E. coli* (EAggEC). EAggEC has been epidemiologically implicated as one of the etiological agents of diarrhea in developing countries as well as in Europe (3, 5, 15, 16).

EAggEC strains are characterized by their unique patterns of adherence to HEp-2 cells (11). The bacteria appear as "stacked brick" clumps which adhere to both the HEp-2 cells and the glass coverslip (17). Their characteristic aggregative adherence pattern is associated with the presence of large plasmids (17). These plasmids are closely linked to their virulence mechanism; genes encoding bundle-forming fimbriae involved in adherence and a heat-stable enterotoxin have been identified on these plasmids (10, 12, 14). In addition to their putative role in the pathogenesis of diarrheal disease, fragments from the large plasmid have been used as DNA probes to diagnose EAggEC infections (2). The EAggEC probe currently used for diagnosis consists of a fragment from the plasmid of the EAggEC strain 17-2 (2). Because no sequence information is available for the EAggEC probe, we determined the sequence of this probe completely not only to investigate its function but also to develop a PCR with primers complementary to the EAggEC probe. Next, we investigated the correlation between the results from the EAggEC probe, EAggEC PCR, and adherence test. Finally, we developed a rapid and sensitive screening technique for EAggEC in mixed cultures grown from stool samples.

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MATERIALS AND METHODS

Bacterial strains. We analyzed 468 *E. coli* strains of different categories which are summarized in Table 1. These included 50 strains of EAggEC, of which 38 strains were from patients with diarrhea and obtained as pure cultures from the *E. coli* strain collections (Referenzzentrum für *Escherichia coli*, Robert Koch Institut, Berlin, Germany, and Nationales Referenzzentrum für Enteritiserreger, Hamburg, Germany). These 38 strains were detected by performing the HEp-2 cell assay as described in reference 16. The remaining 12 EAggEC strains were isolated during this study; their characteristics, disease associations, and methods of detection are listed in Table 2. The pCVD432-harboring strain has been described previously (2) and was kindly provided by J. Kaper, Center for Vaccine Development, Baltimore, Md.

Study population. The population used in this study to determine the frequency of EAggEC has been previously described (15). Briefly, the population consisted of 237 patients with diarrhea and the same number of controls (age range, 1 month to 86 years). The age, sex, and seasonal distributions of controls and subjects with diarrhea were similar.

Sequence analysis of the pCVD432 probe. Sequence analysis of the pCVD432 probe was performed with M13 sequencing and reverse sequencing oligonucleotides (Boehringer GmbH, Mannheim, Germany). A total of 5 μg of double-stranded plasmid DNA was subjected to *Taq* cycle sequencing reactions with the Prism Ready Reaction Dye Deoxy Terminator Cycle sequencing kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. Electrophoresis of the sequencing products was performed on 7% polyacrylamide gels with an automatic sequencer (model 373 A; Applied Biosystems). Nucleotide sequencing was carried out in triplicate and analyzed by the DNASIS program, version 2.0 (Hitachi Software, San Bruno, Calif.).

EAggEC PCR. PCR was performed with the primers pCVD432/start (5′-CTG GCG AAA GAC TGT ATC AT-3′) and pCVD432/stop (5′-CAA TGT ATA GAA ATC CGC TGT T-3′) for 30 cycles. Samples were incubated at 94°C for 40 s to denature DNA, at 53°C for 1 min to anneal the oligonucleotides, and then at 72°C for 1 min for DNA extension.

Restriction enzyme analysis. After PCR with primers pCVD432/start and pCVD432/stop, restriction analysis of the PCR products was carried out with *Dra*I and *Alu*I. On the basis of the nucleotide sequence of pCVD432 analyzed (Fig. 1), restriction with *Dra*I should yield two DNA fragments of 273 and 357 bp, whereas *Alu*I cuts the PCR product into DNA fragments of 391 and 239 bp.

Analysis of stool samples by PCR assay, DNA probe test, and HEp-2 cell adherence test. For isolation of *E. coli*, approximately 1 g of stool was suspended in 1 ml of 0.9% NaCl and 100 μl was streaked onto a MacConkey agar plate. The

TABLE 1. Rate of EAggEC PCR positivity of various categories of *E. coli* that were either EAggEC DNA probe positive or negative

Class of *E. coli*	No of strains tested	No. of strains reactive with:	
		EAggEC probe	EAggEC PCR
E. coli with aggregative adherence	50	44	43[a]
Enteropathogenic *E. coli* with diffuse adherence	33	3	3
E. coli with localized adherence	125	0	0
Enterohemorrhagic *E. coli*	82	0	0
Enteroinvasive *E. coli*	7	0	0
Enterotoxigenic *E. coli*	46	0	0
Nonadherent intestinal *E. coli* flora from patients	125	1	1

[a] These strains were also positive with the EAggEC probe.

plate was incubated overnight and then flooded with 1 ml of 0.9% NaCl to retrieve the colonies. This suspension was then diluted 1:20 with 0.9% NaCl. Fifteen microliters was subjected to the EAggEC PCR as described above, and the remaining suspension was serially diluted to give 120 to 150 CFU on Luria broth agar for the purpose of colony hybridization. The hybridization assay was performed as described by Baudry et al. (2). In addition, five colonies from each MacConkey agar plate were tested for their adherence pattern as described by Scotland et al. (16). The samples were independently and blind tested by all three methods.

Clump formation test. The clump formation test was performed as described by Albert et al. (1). Scum formation was recognized after 20 h of growth of EAggEC colonies in Mueller-Hinton broth and Luria broth.

Nucleotide sequence accession number. The entire nucleotide sequence of the pCVD432 probe was submitted to the EMBL database library and assigned the accession number X81423.

RESULTS

Nucleotide sequence analysis of the EAggEC probe. The nucleotide sequence of the *Eco*RI-*Pst*I DNA fragment of pCVD432 was determined and is shown in Fig. 1. Comparison of the complete sequence with those of genes published in EMBL and GenBank data libraries revealed no significant similarity (less than 50%) to any known bacterial gene.

Oligonucleotides for amplification of EAggEC-specific DNA fragments. The positions of the selected oligonucleotide primers pCVD432/start and pCVD432/stop within the pCVD432 probe are depicted in Fig. 2. With this primer pair, a single 630-bp DNA fragment was amplified (Fig. 3, lanes 1 to 3).

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1 ATCGAGTGAGACACATATTATACTCATTTCTTGCAATAAATGCTTATCTG  50
  TTTTCGACACAGACTCTGGCGAAAGACTGTATCATTGATAATTTCTTTCA 100
  GAAAAGCATCCAGTTTAATTCTTATTCTCTTGATATCGAAGAGTTAGATA 150
  TTAATAAACATAACAATATAAAAACGATGTTACCAGATATAAATATAGGG 200
  TTAGGGCAGTATATAAACAACAATCAATGGTTCTCATCTATTACAGACAG 250
  CCATTTTTATTTATCATTATCCTATAATCTTCTATCGGCTTATGAAGCAA 300
  AAATGCAGAATAATAAATTGGATATTGCTAATTATTTAAAATATATTGAA 350
  ATGCTTAGTGAGAGGAACAACTACATAATTAATTTGCTCTCGGAAATTAT 400
  TAACTATAAGATAAAAAAATCTCACCTGATGTTGATGCTCGAGAGATATA 450
  GGAAGCTCAATAAAGAATACGAAATTGCAAAGCATAAAATGTCAATTGGA 500
  TTAATATCTGCTCTTGATGTAGAGATGATATATAATATTTTACAAAAAAT 550
  CAGGTTTGATATTGATGTCCTTGAGGAGGAGGAAAGTTTACTGTCAGATA 600
  AAATCTCGAGAGAATATCATGTTCCTGAGAGTGCAATCCCAGACATTACA 650
  TATCATAAGTTAAAAGAGTGTAAAACAGCGGATTTCTATACATTATTAGC 700
  TGAAAACAAAAAACTCAAGATTAAGGCTGCTGATATAGATAATGATATAA 750
  GGAAACTATCTGAGA                                     765
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FIG. 1. Nucleotide sequence of the 765-bp EAggEC probe derived from pCVD432.

These PCR products were derived from *E. coli* strains belonging to three different serogroups (O44, O86, and O78). The identity of the PCR products was confirmed by endonuclease digestion with *Alu*I, yielding two fragments of 391 and 239 bp (Fig. 3, lanes 4 to 6), and with *Dra*I, generating fragments of 273 and 357 bp (Fig. 3, lanes 7 to 9).

When serially diluted broth cultures of three EAggEC strains were subjected to amplification, a visible band was seen in samples with 10 bacteria with all three strains.

Comparison of the HEp-2 cell adherence test, EAggEC probe test, and the PCR assay for screening of EAggEC in stool samples. A total of 470 stool samples from patients (n = 235) and controls (n = 235) were subjected independently to the adherence test, the EAggEC probe test, and PCR assay, and these results are shown in Table 3.

Characteristics of the strains from patients and controls. Isolated colonies from 13 of the 14 positive samples were characterized further as shown in Table 2. One sample (from the patient group) could not be further characterized; although the PCR was positive, strains which reacted either with the probe or revealed adherence in the cell culture assay could not

TABLE 2. Characteristics of strains identified by adherence test or molecular methods

Subject no. (age [yr, mo])	Diarrhea	Serotype	Analysis of strains by:			
			EAggEC PCR	EAggEC probe	Adherence test[a]	Clump formation
1 (0,8)	Watery	O44:H18	Positive	Positive	AA	Positive
2 (0,4)	Watery	O44:H18	Positive	Positive	AA	Positive
3 (4,0)	Watery	O44:H18	Positive	Positive	AA	Positive
4 (42,2)	Watery	O44:H18	Positive	Positive	AA	Positive
5 (0,3)	Bloody	O119:H$^-$	Positive	Positive	AA	Negative
6 (0,1)	Watery	O126:H27	Positive	Positive	AA	Negative
7 (0,1)	Watery	O111:H2	Negative	Positive	AA	Negative
8 (63,1)	Watery	O128:H2	Positive	Positive	DA	Negative
9 (0,8)	No	O78:H11	Positive	Positive	AA	Positive
10 (32,0)	No	O86:H2	Positive	Positive	AA	Positive
11 (4,0)	No	O86:H2	Positive	Positive	AA	Positive
12 (0,6)	Watery	ONT	Negative	Negative	AA	Negative
13 (0,9)	No	O119:H$^-$	Negative	Negative	AA	Negative

[a] Abbreviations: AA, aggregative adherence; DA, diffuse adherence.

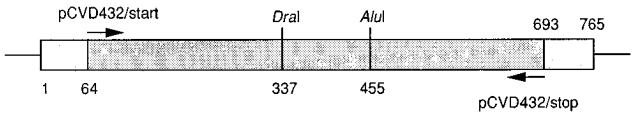

FIG. 2. EAggEC probe and position of the oligonucleotides pCVD432/start and pCVD432/stop used for amplification of the 630-bp PCR product. The restriction sites of the endonucleases *Dra*I and *Alu*I are indicated.

be isolated. In total, four of the strains were from controls and nine were from patients. The subjects with EAggEC infections were predominantly infants, although one subject was elderly. All of the patients reported vomiting as a symptom. Interestingly, one patient, 42 years old, who was diagnosed with AIDS, had watery diarrhea associated with EAggEC. No pathogens other than EAggEC were isolated in any of the patients. Upon comparison of the strain characteristics, two unusual findings appeared. Notably, one of the EAggEC probe- and PCR-positive strains displayed diffuse adherence; its pattern of adherence is shown in Fig. 4A. Moreover, two strains showed aggregative adherence but were negative with both the EAggEC probe and the PCR. The pattern of one of these strains is shown in Fig. 4B. In addition, we noted that one strain was EAggEC probe positive but gave a negative result in the PCR.

PCR with different categories of *E. coli*. In addition to the strains reported above, we also analyzed pure cultures; these results are summarized in Table 1.

DISCUSSION

E. coli strains with an aggregative adherence to HEp-2 cells have been associated with diarrhea, particularly in developing countries (3, 17). Currently, in many laboratories, EAggEC is detected from mixed cultures by analyzing individual colonies with the adherence test. However, this technique is cumbersome and inefficient if large numbers of colonies from stool samples must be analyzed. The DNA hybridization test using the EAggEC probe is an alternative method for identification and has been found to be both sensitive and specific (2). In this study, we performed a complete sequence analysis of this probe. Although no function could be ascribed to the sequence, we were able to construct primers for PCR amplification of a 630-bp fragment, which in this study was shown to be present in a majority of EAggEC strains. Moreover, we demonstrated that the PCR provides a rapid and sensitive screening technique for EAggEC in mixed cultures.

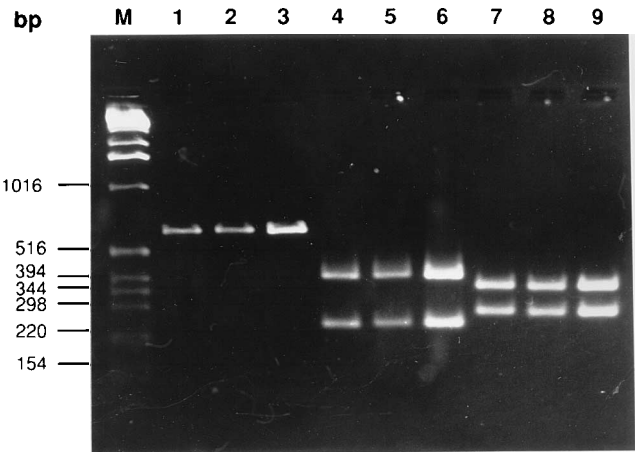

FIG. 3. Demonstration of EAggEC PCR and subsequent restriction analysis in three *E. coli* strains of distinct serogroups. Lanes 1 to 3, fragments obtained after PCR with strains of serogroups O44, O78, and O86, respectively. Digestion of the PCR products was performed with *Alu*I (lanes 4 to 6), generating fragments of 391 and 239 bp, and *Dra*I (lanes 7 to 9), yielding two fragments of 273 and 357 bp. M, molecular weight marker (1-kb DNA ladder [GIBCO BRL]).

TABLE 3. Comparison of results[a] with the EAggEC PCR, EAggEC probe, and cell culture assay used for screening of EAggEC in 470 stool samples from patients and controls

No. of samples	Agreement or disagreement by:		
	Cell culture assay	EAggEC probe	EAggEC PCR
456	−	−	−
5	+	+	+
1	−	−	+
6	−	+	+
2	+	−	−

[a] −, negative result; +, positive result.

In comparing the results obtained with the EAggEC probe, the EAggEC PCR, and the cell culture assay, we noted some disagreement. First, one of the *E. coli* strains which hybridized with the EAggEC probe gave a negative PCR result. It is possible that this strain exhibits sequence variation in the primer binding sites. Second, one of the 47 EAggEC PCR-positive strains which was also EAggEC probe positive did not adhere to HEp-2 cells. A mutation in the genes necessary for adherence is one plausible explanation. Third, three of the EAggEC PCR (and EAggEC probe)-positive strains did not show the expected aggregative pattern but exhibited diffuse adherence. This finding is in contrast to the results of a previous study in which all of 43 strains with diffuse adherence were EAggEC probe negative (2). Fourth, six *E. coli* strains attached to HEp-2 cells in the characteristic aggregative pattern but were EAggEC probe and PCR negative. Previous studies have also shown that not all aggregative *E. coli* strains hybridize with the EAggEC probe (2, 16). Baudry et al. found that only 56 of 63 strains with the aggregative adherence pattern hybridized with the EAggEC probe (2). The reason why not all EAggEC strains hybridize with the probe is unknown. However, it has been hypothesized that different categories of EAggEC strains exist (2, 16). In addition, it would be interesting to assess whether heterogeneity of the strains with respect to probe hybridization is correlated with differences in their virulence.

The detection rates of the EAggEC PCR, the EAggEC probe, and adhesion tests on mixed cultures from stool specimens revealed that PCR was similar in sensitivity to that of the EAggEC probe. Only one sample was positive by PCR screening and negative in the colony hybridization assay. Two plausible explanations for this finding are that either the number of EAggEC cells was too low to be detected by the colony blot test or the DNA was from bacteria which were not viable. The PCR and EAggEC probe hybridization were superior to the adhesion assay as a screening test for EAggEC. However, it should be considered that only five single colonies from each stool sample were analyzed. When performing colony hybridization, we observed that the proportion of EAggEC probe-positive colonies to the *E. coli* colonies tested was low (5 to 10%) in some of adhesion test-negative stool samples. If more colonies were tested in the cell culture assay, there would be a greater probability of selecting *E. coli* strains with aggregative patterns. Although other investigators have analyzed only two colonies from a stool sample (3), it might be necessary to test large numbers of colonies to increase the sensitivity of the

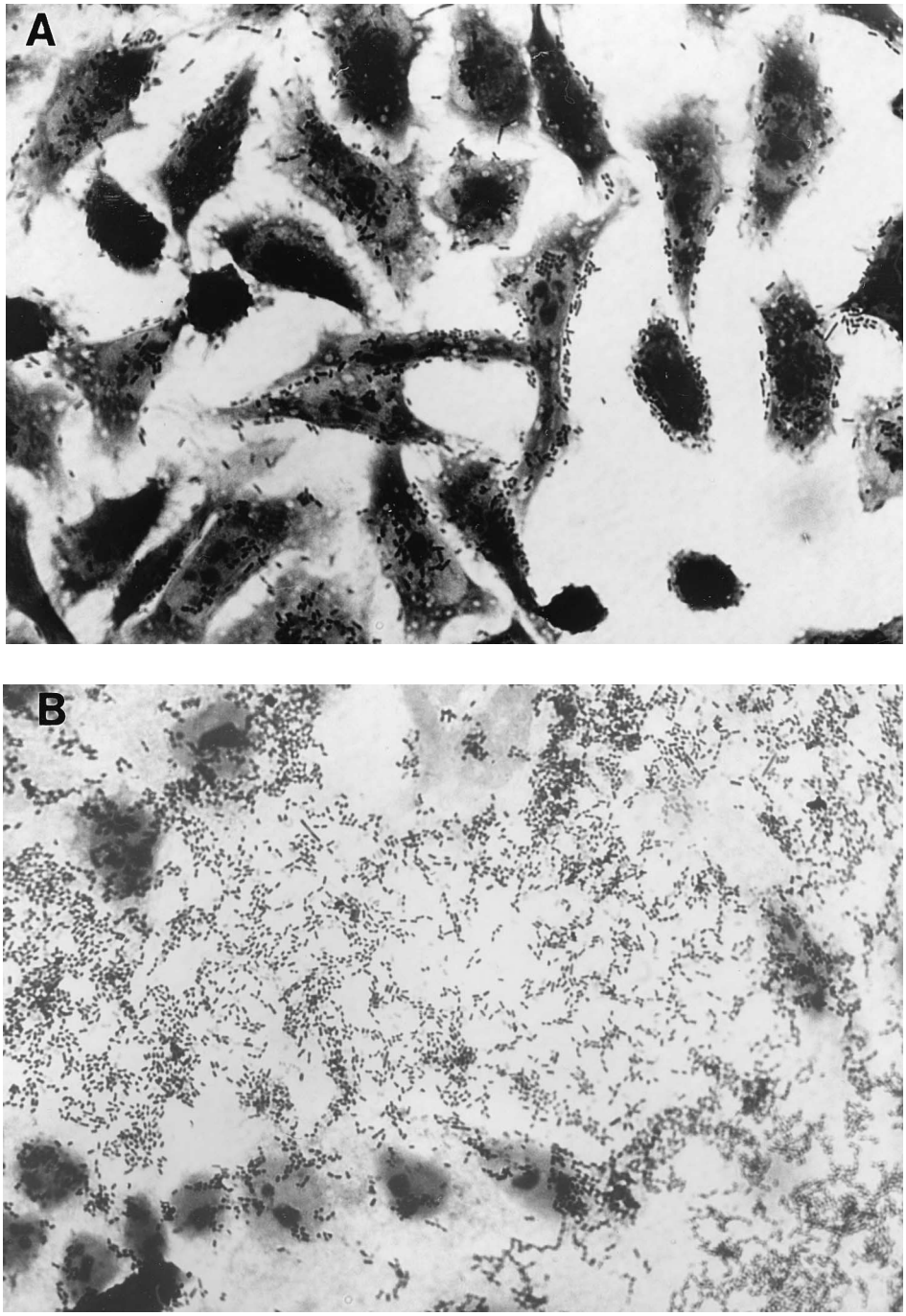

FIG. 4. Micrographs of HEp-2 cells infected with an EAggEC probe-positive strain showing diffuse adherence (A) and an aggregative-adherent but EAggEC probe-negative strain (B).

adhesion assay. However, this is impractical and time-consuming.

All 41 EAggEC strains from a previous study were reported to produce a bacterial clump formation at the surface of liquid cultures (1). From the 50 strains tested in this study, only 40 produced clump formation. The 10 strains without clump formation included all 6 aggregative *E. coli* strains which were EAggEC probe negative. The high level of correlation described by Albert et al. (1) seems to be due to a preselection of their strains; i.e., only strains which were EAggEC probe positive were included. In our study, this clump formation was either not observed in mixed cultures grown from stool specimens or was not prominent enough to be considered an accurate screening method.

Little information on the role of EAggEC as an enteric pathogen is available. This includes the serotypes associated with disease, its mode of transmission, and its spectrum of clinical illness. The difficulty in identifying these strains has been a primary reason for this lack of knowledge. The PCR technique used here enables a more rapid diagnosis of

EAggEC than the other techniques currently available. However, the EAggEC probe or the adherence test must still supplement the PCR to identify the disease-causing strains. A three-step schedule for EAggEC identification may be practical for the clinical laboratory. In the first step, overnight cultures from stools are screened by the PCR method as described above. The PCR may also be directly performed with stool samples, but such a procedure will need to be standardized. In the second step, only samples which are PCR-positive should be analyzed for strain identification. This must be performed for single colonies with either the cell culture tests or the EAggEC probe. Because one EAggEC probe-positive strain was found to be negative in the cell culture assay, the typical aggregative pattern can only be determined by cell culture. Therefore, the adhesion test appears to have the highest level of specificity and is recommended to be used as a definitive confirmation test. The third step should include species identification by biochemical testing of positive colonies; this can be followed by O and H antigen determination. For epidemiological purposes, molecular typing by DNA fingerprinting may be helpful to determine the clonal relatedness of strains. We are aware that about 10% of strains with an aggregative pattern in the HEp-2 cell assays which do not harbor the EAggEC probe sequences will not be detected. Molecular studies are under way to identify sequences specific for these strains in order to fill this gap.

It is critical to rely on PCR as the sole method for diagnosing the etiologic agent for diarrheal diseases—as recently stated for enterohemorrhagic *E. coli* (4). Isolated strains will be needed in order to assess the chain of infection and to implement effective control measures.

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