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PCR-Based Assay Using Occult Blood Detection Cards for Detection of Diarrheagenic *Escherichia coli* in Specimens from U.S. Travelers to Mexico with Acute Diarrhea▽

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Large field studies of travelers' diarrhea for multiple destinations are limited by the need to perform stool cultures on site in a timely manner. A method for the collection, transport, and storage of fecal specimens that does not require immediate processing and refrigeration and that is stable for months would be advantageous. This study was designed to determine if enterotoxigenic *Escherichia coli* (ETEC) and enteroaggregative *E. coli* (EAEC) DNA could be identified from cards that were processed for the evaluation of fecal occult blood. U.S. students traveling to Mexico during 2005 to 2007 were monitored for the occurrence of diarrheal illness. When ill, students provided a stool specimen for culture and occult blood by the standard methods. Cards then were stored at room temperature prior to DNA extraction. Fecal PCR was performed to identify ETEC and EAEC in DNA extracted from stools and from occult blood cards. Significantly more EAEC cases were identified by PCR that was performed on DNA that was extracted from cards (49%) or from frozen feces (40%) than from culture methods that used HEp-2 adherence assays (13%) ($P < 0.001$). Similarly, more ETEC cases were detected from card DNA (38%) than from fecal DNA (30%) or by culture that was followed by hybridization (10%) ($P < 0.001$). The sensitivity and specificity of the card test were 75 and 62%, respectively, compared to those for EAEC by culture and were 50 and 63%, respectively, compared to those for ETEC. DNA extracted from fecal cards that was used for the detection of occult blood is of use in identifying diarrheagenic *E. coli*.

Travelers' diarrhea (TD) is one of the most common illnesses affecting individuals from industrialized countries who visit developing countries. Bacterial pathogens account for about 60 to 85% of diarrheal cases. Worldwide, diarrheagenic *Escherichia coli*, especially enteroaggregative *E. coli* (EAEC) and enterotoxigenic *E. coli* (ETEC), account for the majority of the cases of TD (1, 5, 12). Individuals who live in developed countries such as the United States do not have acquired immunity to these organisms and are at high risk for developing symptomatic infection.

Large epidemiologic field studies of TD at multiple destinations are limited by the need to perform stool cultures on site in a timely manner. If stool cultures are not performed on site, the transportation of stool specimens back to a central laboratory in another country is both expensive and limited by the need for biosafety precautions. Additionally, transportation delays in the delivery of specimens can result in the loss of specimens and/or organism viability. These factors limit the ability to perform epidemiologic investigations in areas with limited resources. A method for the collection, transport, and storage of fecal specimens that does not require immediate processing and refrigeration and that is stable for months would be advantageous. Also, a method that could serve to create backup specimens in case specimens are lost or electricity fails would be helpful. We hypothesized that one potential method was to test cards that are routinely used to screen stools for occult blood for the presence of DNA from diarrheagenic bacteria.

Previous studies demonstrated that filter paper cards are a reliable means of collecting and storing DNA from infectious agents and that fecal DNA collected in such a way can be processed for PCR amplification (3, 10, 14). Bacterial and viral enteropathogene DNA can be detected from stools blotted on paper filter discs (3, 10, 14). Blood and saliva blotted on filter paper also can be used to detect viral DNA (2, 11, 13). However, there is no evidence, to our knowledge, in the literature that cards routinely processed to detect occult blood have been used to collect, transport, or store bacterial DNA for laboratory analysis.

The objective of this study was to determine if *E. coli* DNA could be identified from cards that were routinely used for the evaluation of fecal occult blood from subjects with TD in Mexico. We sought to determine if fecal bacterial DNA fixed on the cards was stable at room temperature and for long periods of time. Since the presence of occult blood in feces commonly is done by using cards impregnated with gum guaiac, a natural resin extracted from the wood of the plant

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TABLE 1. PCR primers used in this study

Organism and target gene	Amplicon size (bp)	Primer sequence
EAEC		
aggR[a]	324	5′-CTAATTGTACAATCGATGTA-3′
aggR[b]	433	5′-CTGAAGTAATTCTTGAAT-3′
		5′GCAGCGATACATTAAGACGCCT-3′
		5′-GCAACAGCAATGCTGCTTTGCT-3′
aatA[a]	630	5′-CTGGCGAAAGACTGTATCAT-3′
		5′-CAATGTATAGAAATCCGCTGTT-3′
ETEC		
est[a]	190	5′-ATTTTTCTTTCTGTATTGTCT-3′
		5′-CACCCGGTACAAGCAGGATT-3′
elt[a]	450	5′-GGCGACAGATTATACCGTGC-3′
		5′-CGGTCTCTATATTCCCTGTT-3′
Enterococcus faecalis		
ace[b]	327	5′-ACCAGACGCACTAGTTGGAATG-3′
		5′-TCTCCAGCCAAATCGCCTACTT-3′

[a] Used for card PCR and multiplex PCR.
[b] Used for card PCR only.

Guaiacum officianale (to which 80% ethanol and 4% H_2O_2 has been added), we also sought to determine if the occult blood assay itself interfered with PCR.

(This study was presented in part at the 44th Annual Meeting of Infectious Diseases Society of America, Toronto, Canada, 12 to 15 October 2006.)

MATERIALS AND METHODS

Study population. U.S. students, aged 18 years and older or between 16 and 18 with parental consent, traveling to Mexico from 2005 to 2007 were enrolled in a parent study to assess genetic polymorphisms that lead to susceptibility to TD in Mexico (8). The study was approved by the University of Texas Health Science Center Committee for the Protection of Human Subjects (the institutional review board). Subjects were monitored for the occurrence of diarrheal illness during their stay in Mexico. Diarrheal illness was defined as ≥3 unformed stools in a 24-h period accompanied by at least one gastrointestinal or systemic symptom (abdominal pain, excess gas or bloating, nausea, vomiting, fecal urgency, tenesmus, or fever). When ill, students provided a stool specimen, which was evaluated for occult blood by the standard method. Stool cultures were performed as previously described to detect the presence of ETEC and EAEC (4).

Pilot studies. Stool samples that were positive for EAEC were smeared onto Hemoccult cards (Beckman Coulter Inc., Fullerton, CA) and air dried. A developer then was placed in the usual fashion on half of the cards, which were developed according to the manufacturer's instructions. Cards were kept at room temperature for 3 to 400 days. Positive controls included stool specimens that were not placed on cards. Known positive stool samples for both EAEC and ETEC were smeared on cards, air dried at room temperature, and then used as the spiked control. As an internal control, the *ace* gene was amplified using specific primers to detect *Enterococcus faecalis* (Table 1) in spiked controls.

DNA extraction protocol. For DNA extraction, cards were placed on a clean surface, and the paper in the card was punched using a 2-mm Harris Uni-core punch (Whatman Inc., Florham Park, NJ). DNA then was extracted from three paper punches per card using a Qiagen QIA DNA stool mini kit (Qiagen Inc., Valencia, CA). Briefly, the paper punches were placed into 150 μl of stool lysis buffer, mixed, and incubated at 70°C for 10 min. The sample then was separated by centrifugation (14,000 × g for 1 min), and the supernatant was transferred into a new tube containing 50 μl of InhibitEX solution. After further mixing for 1 min and an incubation at room temperature for 5 min, the sample was centrifuged at 14,000 × *g* for 3 min. After the addition of 5 μl proteinase K and 25 μl of buffer to the supernatant, the sample was incubated at 70°C for 10 min. Fifty microliters of ethanol was added to the lysate, and the sample was loaded onto a QIAamp spin column. The column then was centrifuged at 14,000 × *g* for 1 min. The column was washed twice with 100 μl of wash buffer per the manufacturer's instructions. DNA was eluted from the column with 50 μl of elution buffer and stored at −80°C.

PCR. Primer sequences that were specific for the heat-labile toxin gene (*elt*) and the heat-stable toxin gene (*est*) of ETEC and the *aatA* and *aggR* genes of EAEC were used in this study (Table 1) (6, 7, 9). Appropriate negative controls, including elution buffer, water, DNA from nonpathogenic *E. coli*, and enterococcal DNA, were used. Positive controls included DNA extracted from EAEC and ETEC directly from laboratory cultures.

Card PCR. Primer sequences specific for the heat-labile toxin gene (*elt*) and the heat-stable toxin gene (*est*) of ETEC and the *aatA* and *aggR* genes of EAEC were used in this study (Table 1) (6, 7, 9). PCR was carried out using a HotStarTaq Master mix kit (Qiagen Inc., Valencia, CA) with 2 μl of fecal card DNA in a volume of 10 μl that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, and 2.5 U of *Taq* DNA polymerase. Thermocycling conditions were as follows: 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, and then incubation at 42°C for *aggR*, 50°C for *elt* and *est*, and 55°C for *aatA* for 1 min, with a final elongation at 72°C for 1 min. Positive controls were EAEC DNA extracted in the pilot project (from stool) and EAEC and ETEC DNA from culture. Appropriate negative controls, including elution buffer, water, DNA from nonpathogenic *E. coli*, and enterococcal DNA, were used.

Fecal multiplex PCR. The primers used for the multiplex fecal PCR are presented in Table 1. The multiplex fecal PCR assay was performed with specific primers in 10 μl of reaction mixture that contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate, 2.5 U of *Taq* DNA polymerase, 1 μl of the stool DNA template, and premixed PCR primers (7). The PCR mixtures then were subjected to the following cycling conditions. Samples that had been heated to 95°C for 5 min were amplified for 40 cycles using a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems Inc., Foster City, CA). Each cycle consisted of denaturation at 95°C for 45 s, annealing at 50°C for 45 s, and elongation at 72°C for 45 s, with a final elongation at 72°C for 10 min. PCR products were visualized after electrophoresis in 2% agarose gels in Tris-borate-EDTA buffer containing ethidium bromide stain. The amplicons were identified based on the size of the amplified product with DNA markers. PCR done on DNA from Hemoccult cards was performed with specific primers for EAEC and ETEC in separate reactions.

Statistical analysis. The proportions of positive test results from each one of the extraction methods were compared by chi square tests to determine significant differences between methods. Statistical analyses between groups were performed with STATA 9.0. The sensitivity, specificity, and kappa statistics of the PCR that was performed on DNA cards were estimated by comparing the results to the gold standard, which in this case was routine stool culture, and to the results of multiplex fecal PCR that was performed directly on stool samples.

RESULTS

There were 236 study participants from whom stored occult blood cards, which had been obtained from 2005 to 2007, were available for PCR. A total of 54 study participants had to be excluded; 39 of them did not experience diarrhea and 15 participants provided duplicate samples, leaving a total of 182 subjects for study. Fecal culture data was available from 39 individuals for ETEC and from 70 subjects for EAEC.

Travelers with diarrhea predominantly were female (71%), white (91%), and between the ages of 20 and 29 (maximum age, 57; mean age, 31). Overall, the microbiology findings were consistent with the previously reported frequency of diarrheagenic *E. coli* in U.S. adults traveling to Mexico (5, 12).

EAEC was identified more frequently by card PCR (49%) than by PCR done on fecal DNA (40%) or fecal cultures (13%) ($P < 0.001$ for the result for card PCR compared to that for culture) (Tables 2 and 3). Card PCR also was superior to fecal PCR and culture in detecting ETEC (38, 35, and 10%, respectively; $P < 0.001$ for the result for card PCR compared to that for culture) (Tables 2 and 3).

The sensitivity and specificity of the card DNA method, compared to those for the culture method for the detection of EAEC, were 75 and 62%, respectively. In the case of ETEC,

TABLE 2. Detection rates of EAEC and ETEC by fecal culture or by PCR performed on DNA extracted from Hemoccult cards

Organism	No. detected/total no. of samples (proportion detected) tested by:		*P* value
	Culture	Card PCR	
EAEC[a]	13/98 (0.13)	90/182 (0.49)	<0.001
ETEC[b]	4/39 (0.10)	70/182 (0.38)	0.001
ETEC LT[c]	1/39 (0.03)	26/182 (0.14)	0.043
ETEC ST[d]	2/39 (0.05)	26/182 (0.14)	0.119
ETEC LT/ST	1/39 (0.03)	18/182 (0.10)	0.139

[a] The sensitivity and specificity for EAEC were 0.75 and 0.62, respectively.
[b] The sensitivity and specificity for ETEC were 0.50 and 0.63, respectively.
[c] LT, heat-labile toxin producing.
[d] ST, heat-stable toxin producing.

TABLE 3. Detection rates of EAEC and ETEC by PCR performed using DNA extracted from Hemoccult cards or directly from fecal DNA

Organism and/or target gene	No. detected/total no. of samples (proportion detected) tested by:		*P* value
	Fecal PCR	Card PCR	
EAEC[a]	64/159 (0.40)	90/182 (0.49)	0.089
EAEC *aggR*	0/159 (0)	20/182 (0.11)	<0.001
EAEC *aatA*	53/159 (0.33)	29/182 (0.16)	<0.001
EAEC *aggR* and *aatA*	11/159 (0.07)	41/182 (0.23)	<0.001
ETEC[b]	55/159 (0.35)	70/182 (0.38)	0.459
ETEC *elt*	29/159 (0.18)	26/182 (0.14)	0.322
ETEC *est*	6/159 (0.04)	26/182 (0.14)	0.001
ETEC *elt* and *est*	20/159 (0.13)	18/182 (0.10)	0.431

[a] The sensitivity and specificity for EAEC were 0.53 and 0.51, respectively.
[b] The sensitivity and specificity for ETEC were 0.56 and 0.70, respectively.

the card DNA demonstrated a sensitivity and specificity of 50 and 63%, respectively (Table 2). Compared to those for fecal PCR, the card DNA had a sensitivity and specificity of 53 and 51%, respectively, for EAEC and 56 and 70%, respectively, for ETEC. Kappa statistics indicated slight to fair concordance (Table 3) between EAEC card DNA test results and those from culture (57%) or fecal PCR and ETEC card DNA test results (57%). The highest kappa value was observed between the ETEC card PCR and fecal PCR tests (Table 3).

DISCUSSION

The guaiac-based card method is an established and effective means of identifying small amounts of blood in fecal specimens. We sought to determine whether, once cards were fixed and developed, DNA from enteric pathogens could still be identified by PCR. This would provide an easy and economical means of collecting and transporting bacterial fecal DNA for the PCR identification of diarrheagenic *E. coli*. In our pilot study, we demonstrated that DNA from *Enterococcus faecalis*, an organism that is representative of fecal flora, could be identified by PCR after card fixation. Similarly, we demonstrated that DNA for the detection of EAEC and ETEC (using spiked samples) could be extracted from the cards after many days of storage. The use of the developer/fixative might have helped to stabilize the DNA and inactivate DNAse due to the high content of alcohol. The card and fixatives also may have inactivated other enzymes that could degrade DNA.

In this study, we demonstrate that fecal DNA that has been fixed on cards and stored at room temperature for up to 14 months was stable enough for PCR amplification. The potential advantages of this method include its use as a backup for the identification of enteropathogens, the elimination for the need for cold chain for transportation, the elimination of on-site culture processing, and the stabilization of DNA for long periods of time prior to processing. A possible commercial and clinical application includes the ability to detect pathogens from a rectal exam after examination for occult blood without the need for the collection of a stool specimen. A disadvantage of this method is that it does not yield viable organisms for phenotypic analysis, such as antimicrobial susceptibility testing, adherence patterns, or studies that deal with the molecular epidemiology of the pathogen(s).

Although the PCR performed on card DNA was the method that detected the highest number of *E. coli* pathogens, the correlation between results of card PCR and culture or fecal multiplex PCR was modest. This may be explained by several reasons. (i) Stool cultures were done from samples that had been transported in Cary-Blair vials for several days prior to being processed, and this may have decreased the viability of some of the bacteria. It is plausible that if cultures had been performed on site, the yield would have been higher. (ii) Fecal PCR was done on specimens that were frozen and then thawed. The presence of DNases in intestinal secretions may have decreased the amount of DNA present. (iii) The conditions used for the card PCR and the multiplex fecal PCR could have affected the concordance of the two tests. (iv) Different parts and amounts of the fecal specimen were sampled for each one of the tests. Additional studies that take these aspects into consideration are warranted.

We conclude that after prolonged storage, fixated cards that are used for the detection of occult blood are a source of DNA for the detection of diarrheagenic *E. coli*. Our study emphasizes the need for alternative means of the collection, transport, and storage of enteric pathogens for epidemiologic purposes that can replace or complement current methods. We plan studies to examine the detection of other non-*E. coli* enteropathogens by using this technique. We do not know how much longer than 14 months the Hemoccult card can be maintained and still reliably be used for DNA studies.

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