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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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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. 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. The objective of this study was 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.
TABLE 1. PCR primers used in this study

<table>
<thead>
<tr>
<th>Organism and target gene</th>
<th>Amplicon size (bp)</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>EAEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aggR</td>
<td>324</td>
<td>5'-CTAAATGTGACATCGATGTA-3'</td>
</tr>
<tr>
<td>aggB</td>
<td>433</td>
<td>5'-CTGGAATTTACGTCTGTAAT-3'</td>
</tr>
<tr>
<td>eaatA</td>
<td>630</td>
<td>5'-CTGGGGGAAAAGCTGTAATC-3'</td>
</tr>
<tr>
<td>ETEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>estB</td>
<td>190</td>
<td>5'-ATTTTCTCCTTGGATTTGCT-3'</td>
</tr>
<tr>
<td>eltB</td>
<td>450</td>
<td>5'-GGGCCAGACATTACAACGGTG-3'</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aceA</td>
<td>327</td>
<td>5'-ACCAGACGACATCTGTTAGATG-3'</td>
</tr>
</tbody>
</table>

* Used for card PCR and multiplex PCR.
" Used for card PCR only.

Guaiacum officianale (to which 80% ethanol and 4% H₂O₂ has been added), we also sought to determine if the occult blood assay itself interfered with PCR.

(The 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.

PRC. 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 MgCl₂, 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 50°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 MgCl₂, 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 mixture then was 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,
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 affected the concordance of the two tests. (iv) Different 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 Hemocult card can be maintained and still reliably be used for DNA studies.

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