Improvement in Detection of Enterotoxigenic *Escherichia coli* in Patients with Travelers’ Diarrhea by Increasing the Number of *E. coli* Colonies Tested

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**Abstract.** No cause of one-third of travelers’ diarrhea (TD) cases can be detected despite microbiologic assessment. We propose that these pathogen-negative TD cases include undetected enterotoxigenic *Escherichia coli* (ETEC). As a standard in diagnostic microbiology, samples of five *E. coli* colonies were tested to detect ETEC from stool cultures. We compared the sensitivities and the number of ETEC detected by 20 colonies with those of 5, 10, and 15 colonies. The percent detection of ETEC with five *E. coli* colonies was significantly different from that using 20 *E. coli* colonies. Of the 116 subjects studied, the number positive for ETEC for 5, 10, 15 and 20 colonies tested were 22 (19.0%), 37 (31.9%), 45 (38.8%) and 46 (39.7%), respectively. Based on our results, we recommend the testing of at least 10 *E. coli* colonies for optimum detection of ETEC in patients with TD.

**INTRODUCTION**

Enterotoxigenic *Escherichia coli* (ETEC) is the leading cause of travelers’ diarrhea (TD) and is identified in close to half the case-patients with diarrhea.1–4 Unlike most other bacterial pathogens causing diarrhea, which can be detected by culturing patient stool specimens on selective media, *E. coli* pathogens cannot be distinguished in this manner. Non-virulent *E. coli* is part of the biota living symbiotically and lining the gastrointestinal tract of healthy persons. Therefore, pathogenic *E. coli* strains causing diarrhea needs to be differentially identified with molecular techniques being widely used for this purpose.

ETEC causes diarrhea by colonizing the small intestine and produces one or two enterotoxins, a heat-labile enterotoxin (LT) and a heat-stable enterotoxin (ST).5 Previous methods for the detection of ETEC and other *E. coli* pathogens causing diarrhea included several types of DNA hybridization assays, also known as colony hybridizations.5–7 Currently, polymerase chain reaction (PCR) techniques are commonly used in enteric diagnostic laboratories for the detection of ETEC.8–11 Iijima and others recently suggested that quantitative real-time PCR (qPCR) will better identify diarrhea-producing *E. coli* (DEC).12

Conventionally, five *E. coli*-like colonies selected from stool cultures of patients with TD are tested for ETEC and other DEC.1,2 This procedure has been standardized to use five *E. coli* colonies for ETEC toxin assay without scientific validation.13,14 The underlining assumption has been that most *E. coli* present in stool specimens of patients with acute diarrhea are of the pathogenic form. This assumption may or may not be correct because the extent of DEC present may be related to duration of illness at time of study. A study showed that ratios of DEC to non-pathogenic *E. coli* in stools of patients with diarrhea were low, with the exception of ETEC and Shiga toxin–producing *E. coli* (STECl), which were present in proportions as low as 40% of total *E. coli*.12 Moreover, more than one-third of patients with TD studied remain negative for all known enteropathogens.5 We hypothesized that some of these pathogen-negative patients have ETEC that simply go undetected. We proposed that an increase in number of *E. coli* colonies selected and tested will more accurately detect cases of TD caused by ETEC. Our proposition was further supported by studies showing a positive antibacterial drug effective for in the treatment of pathogen-negative TD.15,16

**MATERIALS AND METHODS**

**Patients and *E. coli* colonies tested.** De-identified stool specimens collected from 116 patients with TD acquired in Guadalajara, Mexico, during the summer of 2006 were used for our study. A case-patient was defined as a foreign traveler who passed unformed stools three or more times during a 24-hour period with at least one abdominal symptom of enteric infection.

The collected stool samples were processed in our field laboratory for enteric bacterial pathogens (including *Salmonella* spp., *Shigella* spp., *Vibrio* spp., *Campylobacter jejuni*, *Yersinia enterocolitica*, *Aeromonas* spp., and *Plesiomonas shigelloides*) and parasites (*Giardia lamblia*, *Cryptosporidium* species, and *Entamoeba histolytica*) by previously described methods.17

After we cultured these stools separately on selective MacConkey agar, 20 colonies of *E. coli* for each patient were selected and saved in peptone stubs. The study sample consisted of 116 sets of 20 *E. coli* colonies. The sets of 20 *E. coli* colonies for each stool specimen were randomly numbered from 1 to 20 and divided into four sub-groups of five colonies: 1–5, 6–10, 11–15, and 16–20.

**Testing of 20 colonies per subject for ETEC.** Each patient with TD was tested for ETEC in four steps, corresponding to the four sub-groups of five *E. coli* colonies. First, five colonies (colonies 1–5) of all 116 stool cultures were tested for ETEC, by identifying ST or LT toxin–encoding genes using PCR (see below for methods). Patients who were positive for ST, LT, or ST/LT for these five *E. coli* colonies were considered positive for ETEC. The remaining 15 *E. coli* colonies for each of these patients positive for ETEC were not tested further. Patients who were negative for ST and LT for the first five *E. coli* colonies were taken to the second step, where the next five colonies (colonies 6–10) of each patient were tested for ETEC using PCR. Similarly, those patients positive for ST, LT, or ST/LT in the second five *E. coli* colonies were considered positive
for ETEC and was not tested beyond the first 10 *E. coli* colonies. The patients that remained negative for ETEC were taken to the third step. The same process was repeated for the third set of five *E. coli* colonies (colonies 11–15) and then for the fourth set (colonies 16–20). After testing for ETEC in up to 20 *E. coli* colonies, patients negative for ST and LT toxin–encoding genes were considered to be truly negative for ETEC.

**DNA extraction and pooled DNA preparation.** In groups of five colonies per patient, *E. coli* from peptone stubs were grown separately on MacConkey agar plates at 37°C overnight and collected individually into 200 µL of deionized water in 1.5-mL microfuge tubes. Each sample in microfuge tubes were heated at 100°C for 10 minutes, subjected to vortex for 10 seconds, and centrifuged at 12,000 rpm for 2 minutes. Supernatants containing the DNA extracts were transferred into separate 0.5-mL microfuge tubes and labeled. Pooled DNA samples were prepared by mixing 10 µL of each of the five DNA extractions corresponding to a patient into another 0.5-mL microfuge tube. Six DNA extracts for each patient, five for the individual *E. coli* colonies, and one for the pooled DNA sample were stored in their 0.5-mL microfuge tube at −20°C.

**PCR amplification and ETEC identification.** Conventional PCR was used to amplify the ST and LT toxin–encoding genes in the pooled DNA extracts of *E. coli* colonies prepared for each patient. The PCR mixture per DNA extract contained 2.0 µL of 10× buffer, 4.0 µL of 5Q buffer, 0.8 µL of 2.5 mM MgCl₂, 1.5 µL of 2.5 µM dNTPs, 1.0 µL of forward primer, 1.0 µL of reverse primer, and 6.7 µL of double-distilled deionized water. A total of 0.5 µL of Taq DNA polymerase was used per 100 µL of PCR mixture. In each PCR tube, 3 µL of pooled DNA extract was combined with 17 µL of PCR reaction mixture. Amplification of ST and LT were done separately using 100 µL of PCR mixture. In each PCR tube, 3 µL of pooled DNA extract was combined with 17 µL of PCR reaction mixture. Amplification of ST and LT were done separately using 100 µL of PCR mixture. The PCR mixture per DNA extract contained 2.0 µL of 10× buffer, 4.0 µL of 5Q buffer, 0.8 µL of 2.5 mM MgCl₂, 1.5 µL of 2.5 µM dNTPs, 1.0 µL of forward primer, 1.0 µL of reverse primer, and 6.7 µL of double-distilled deionized water. A total of 0.5 µL of Taq DNA polymerase was used per 100 µL of PCR mixture. In each PCR tube, 3 µL of pooled DNA extract was combined with 17 µL of PCR reaction mixture.

**Confirmation and detection of ETEC-positive colonies.** The PCR was conducted with pooled DNA extracts of groups of five *E. coli* colonies per patient. Once an ST- or LT-positive sample was detected in one of the pooled DNA extracts, this result was confirmed and determined as to which individual colonies were positive. This confirmation was conducted by repeating the PCR for either ST or LT on individual DNA extracts of the pooled DNA extracts that were positive for ST or LT. This method indicated how many ST- or LT-positive colonies were present within a sample of five *E. coli* colonies tested. In cases with an ST- and an LT-positive pooled DNA extract, the PCR for both ST and LT was repeated separately on individual DNA extracts. The same colony or DNA extract had to be positive for ST and LT to be identified as positive for ST/LT.

**RESULTS**

**Detection of ETEC and assay sensitivities.** The 5-colony assay detected 22 (19%) of 116 patients positive for ETEC: 15 positive for ST, 6 positive for LT, and 1 positive for ST and LT (Table 1). The 10-colony assay detected 37 (31.9%) patients positive for ETEC: 26 positive for ST, 10 positive for LT, and 1 positive for ST and LT. The 15-colony assay detected 45 (38.8%) patients positive for ETEC: 26 positive for ST, 17 positive for LT and 2 positive for ST and LT. The 20-colony assay detected 46 (39.7%) patients positive for ETEC: 26 positive for ST, 17 positive for LT, and 3 positive for ST and LT. The 5-colony assay had the lowest sensitivity, and sensitivities increased with use of more *E. coli* colonies. The 10-, 15-, and 20-colony assays were 1.68, 2.05, and 2.09 times, respectively, increased with use of more *E. coli* colonies. The 5-colony assay had the lowest sensitivity, and sensitivities increased with use of more *E. coli* colonies. The 10-, 15-, and 20-colony assays were 1.68, 2.05, and 2.09 times, respectively.

**Table 1**

<table>
<thead>
<tr>
<th>Toxin detected</th>
<th>No. (%) of stools with positive results (n = 116)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 colonies</td>
</tr>
<tr>
<td>Heat stable</td>
<td>15 (12.9%)</td>
</tr>
<tr>
<td>Heat labile</td>
<td>6 (5.1%)</td>
</tr>
<tr>
<td>Both toxins</td>
<td>1 (0.9%)</td>
</tr>
<tr>
<td>Total ETEC</td>
<td>22 (19.0%)</td>
</tr>
</tbody>
</table>

* ETEC = enterotoxigenic *Escherichia coli*; PCR = polymerase chain reaction.
more sensitive than the 5-colony assay. If the 20-colony assay was assumed to have a sensitivity of 100%, the sensitivities of the 5-, 10-, and 15-colony assays would be 47.8%, 80.4% and 97.8%, respectively.

Comparison of the 5-, 10-, and 15-colony assays with the 20-colony assay. Results of the 5-colony assay were significantly different from those obtained with the 20-colony assay in the number of ST (15 versus 26; \( P = 0.0072 \)), LT (6 versus 17; \( P = 0.0019 \)), and ETEC (22 versus 46; \( P < 0.0001 \)) forms detected among the 116 case-patients. However, there was no significant difference between the results of the 5-colony assay and the 20-colony assay in detecting ST/LT (1 versus 3; \( P = 0.1210 \)). Results of the 10-colony assay were not significantly different from those obtained with the 20-colony assay in the number of ST (26 versus 26; \( P = 0.5000 \)), LT (10 versus 17; \( P = 0.0295 \)), ST/LT (1 versus 3; \( P = 0.1210 \)), and ETEC (37 versus 46; \( P = 0.0438 \)) forms detected. Similar to the 10-colony assay, the results of the 15-colony assay were not significantly different from those obtained with the 20-colony assay in the number of ST (26 versus 26; \( P = 0.5000 \)), LT (17 versus 17; \( P = 0.5000 \)), ST/LT (2 versus 3; \( P = 0.2793 \)), and ETEC (45 versus 46; \( P = 0.4247 \)) forms detected.

Proportion of ETEC to \( E. coli \) stool cultures. The proportions (95% confidence intervals [CIs]) of ST, LT, and ST/LT toxin-encoding genes detected in patients positive for ETEC to total \( E. coli \) colonies present in these patients were 0.39 (0.29–0.49), 0.22 (0.18–0.32), and 0.11 (0.00–0.31), respectively. The proportion (95% CI) of ETEC to total \( E. coli \) was 0.31 (0.24–0.39) for the sample of patients in this study. It should be noted that in calculating the proportion estimates, the probability samples were not the same size, having 5, 10, 15, and at times, 20 colonies, depending upon the step at which the first positive sample was identified.

Mixed infection. Eight patients (7%) with ETEC infections were co-infected with another pathogen in this study. Co-infection with EAEC and ETEC accounted for six of the eight mixed infections. One patient with ETEC infection was co-infected with \( Cryptosporidium parvum \) and another was co-infected with \( Salmonella \) spp.

DISCUSSION

Our study showed a significant increase in the number of ETEC strains detected in patients with TD when 20 \( E. coli \) colonies were tested for ST and LT toxin-encoding genes than when five \( E. coli \) colonies were tested. The sensitivity of the PCR assay using 20 colonies was 2.09 times higher than that of the assay using five colonies. The 19.0% of ETEC-positive patients detected by the 5-colony assay was significantly different from the 39.7% of ETEC-positive patients detected by the 20-colony assay (\( P < 0.0001 \)). More than half the possible ETEC-positive patients (52.2%) went undetected with the 5-colony assay. Use of a probability sample of five \( E. coli \) colonies showed low sensitivity (< 50%) for detecting ETEC in patients with TD.

The sensitivity of the 20-colony assay was 1.24 times higher than that of the 10-colony assay. However, the 31.9% of ETEC-positive patients detected by the 10-colony assay was not significantly different from the 39.7% of ETEC-positive patients detected by the 20-colony assay (\( P = 0.0438 \)). There was also no statistically significant difference between the 10- and 20-colony assays for detection of ST, LT, and ST/LT individually. Increasing the number of colonies from a 15 to 20 detected only one more ST/LT-positive patient, but this difference was not statistically significant.

The low proportion of DEC found by Iijima and others when five \( E. coli \) colonies were tested is supportive of our findings. Their study also showed that ETEC and STEC were present in a proportion of approximately 40% among non-pathogenic \( E. coli \), which was much higher than the observed proportions for non-ETEC DEC. Their study and our results provide evidence that ETEC could be detected with less than 20 colonies. Their suggestion that real-time PCR is needed for effective detection of DEC may not be true for ETEC because we could identify most ETEC-positive sample with less than 20 \( E. coli \) colonies.

Use of the conventional assay with five \( E. coli \) colonies would have categorized the ETEC diarrhea that we detected in colonies 6–20 as pathogen-negative after screening for other known enteropathogens. Our findings may at least partially explain a portion of the pathogen-negative TD cases found in studies of etiology of TD.1 Our results also help explain in part the effectiveness of antibacterial drug therapy used to treat pathogen-negative TD.15

Although increasing the number of colonies tested increased the sensitivity and the percentage of ETEC detected, the specificity of the assays may decrease. The more colonies used, ETEC will be detected even when present in low quantities. Thus, this assay may detect ETEC that are not etiologically important in the acute diarrhea episode, which represent false-positives samples. Therefore choosing an optimum number of colonies for detection of ETEC is important. However, in our study, most ETEC-positive samples were detected with the first 15 colonies.

We recently reported that fecal DNA remained stable on hemoccult cards stored at room temperature for up to 14 months and could be used for PCR amplification for detection of diarrheagenic \( E. coli \).19 Significantly more ETEC cases were detected from hemoccult card DNA (38%) than from fecal DNA (30%) or by culture that was followed by hybridization (10%) (\( P < 0.001 \)). These results support those of the current study, which indicate that ETEC may go undetected when standard assay methods are used.

We recommend the use of at least 10 \( E. coli \) colonies for detection of ETEC in enteric diagnostic laboratories instead of the five \( E. coli \) colonies often used. This modification will increase the sensitivity of the current PCR assay and the number of ETEC detected in patients with diarrhea patients and lead to a lower percentage of unexplained pathogen-negative TD.

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REFERENCES


