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Timely accurate diagnosis of Shiga toxin-producing Escherichia coli (STEC) infections is important. We evaluated a laboratory-developed real-time PCR (LD-PCR) assay targeting stx1, stx2, and rfbEO157 with 2,386 qualifying stool samples submitted to the microbiology laboratory of a tertiary care pediatric center between July 2011 and December 2013. Both cultures of PCR-positive samples were tested for Shiga toxins by enzyme immunoassay (EIA) (ImmunoCard STAT! enterohemorrhagic E. coli [EHEC]; Meridian Bioscience) and cultured in attempts to recover both O157 and non-O157 STEC. E. coli O157 and non-O157 STEC were detected in 35 and 18 cases, respectively. Hemolytic uremic syndrome (HUS) occurred in 12 patients (10 infected with STEC O157, one infected with STEC O125ac, and one with PCR evidence of STEC but no resulting isolate). Among the 59 PCR-positive STEC specimens from 53 patients, only 29 (54.7%) of the associated specimens were toxin positive by EIA. LD-PCR differentiated STEC O157 from non-O157 using rfbEO157 and LD-PCR results prompted successful recovery of E. coli O157 (n = 25) and non-O157 STEC (n = 8) isolates, although the primary cultures and toxin assays were frequently negative. A rapid “mega”-multiplex PCR (FilmArray gastrointestinal panel; BioFire Diagnostics) was used retrospectively, and results correlated with LD-PCR findings in 25 (89%) of the 28 sorbitol-MacConkey agar culture-negative STEC cases. These findings demonstrate that PCR is more sensitive than EIA and/or culture and distinguishes between O157 and non-O157 STEC in clinical samples and that E. coli O157:H7 remains the predominant cause of HUS in our institution. PCR is highly recommended for rapid diagnosis of pediatric STEC infections.

Shiga toxin-producing Escherichia coli (STEC) and especially E. coli O157:H7 are worldwide pathogens. Their importance is derived from the severity of the illnesses they cause, including diarrhea, bloody diarrhea, and hemolytic uremic syndrome (HUS), and their epidemic potential. Good clinical management depends on rapid accurate microbiological diagnosis (1, 2), because etiological information leads to avoidance of antibiotics (1, 3) and prompt hospital admissions and contact precautions to minimize secondary spread within the community (4) and nephroprotective intravenous volume expansion to avert hemocencentration, which has a poor prognosis if HUS develops (2, 5).

Diagnostic strategies to detect STEC vary according to serotype. STEC O157:H7 has the strongest and most enduring association with HUS (6, 7) and is best isolated on sorbitol-MacConkey (SMAC) agar, which exploits the unusual non-sorbitol-fermenting phenotype of this serotype. Most other STEC serotypes ferment sorbitol (as do most commensal E. coli strains) and have few unifying phenotypes other than toxin production; therefore, toxin assays of overnight broth cultures are used to detect these agents in stools. The Centers for Disease Control and Prevention (CDC) recommends routine laboratory testing for Shiga toxin-producing bacteria (8), but toxin assays are inexplicably suboptimal for the detection of toxin-producing E. coli O157:H7 (9).

Molecular testing for STEC is not routinely used in clinical settings, despite supportive data (10–12). PCR assays have routinely used stx1 and stx2 as targets but cannot, by themselves, distinguish O157 from non-O157 STEC, although stx1-positive signals are unlikely to originate from O157 STEC unless stx2 is also detected (13, 14).

Here, we compared a laboratory-developed real-time PCR (LD-PCR) assay targeting stx1, stx2, and rfbEO157, a commercial lateral flow enzyme immunoassay (EIA) (Meridian Bioscience, Inc., Cincinnati, OH), and sorbitol-MacConkey agar culture. The rfbEO157 gene encodes perosamine synthase, the first O157 STEC rfb locus to be cloned and sequenced (15), and has been identified in food and human samples (16, 17). To our knowledge, this is the first study to apply PCR for rfbEO157 to distinguish O157 from non-O157 STEC in a clinical microbiology laboratory. As part of this investigation, the specimens and their associated MacConkey (MAC) broth cultures that were positive by LD-PCR were archived for an additional molecular assay, the rapid “mega”-multiplex PCR (RM-PCR) with the FilmArray gastrointestinal panel (BioFire Diagnostics, Salt Lake City, UT).
MATERIALS AND METHODS

Setting. All qualifying stool samples from patients less than 21 years of age that were submitted to the microbiology laboratory at Seattle Children’s Hospital (Seattle, WA) between July 2011 and December 2013 for detection of bacterial pathogens were evaluated. From a total of 2,849 specimens, 2,386 were included in this analysis after the elimination of repeat specimens (i.e., specimens obtained from the same patient within a 2-week period and testing negative by PCR) (n = 156) and specimens not qualified for Shiga toxin testing (n = 307), such as specimens submitted on swabs (n = 4) and cultures with no Gram-negative bacterial growth (n = 68) or no bacterial growth at all (n = 235). This retrospective study was approved by the institutional review board of Seattle Children’s Hospital. Clinical records for all 54 patients with laboratory detection of stx1, stx2, and/or rfbEO157 were reviewed. HUS was defined as hemolytic anemia (hematocrit level of <30% with smear evidence of intravascular erythrocyte destruction), thrombocytopenia (platelet count of <150,000 platelets/mm3), and azotemia (serum creatinine level above the upper limit of the normal range for age) (18).

Conventional culture. All stool samples were plated on sheep blood, Hektoen enteric, MacConkey (MAC), Salmonella-Shigella, sorbitol-MacConkey (SMAC), and Campylobacter- and Yersinia-selective agar plates, according to our laboratory protocols (19, 20). The specimens were also inoculated into Selenite F broth for enrichment of Salmonella spp. and MAC broth for enrichment of all Enterobacteriaceae, including E. coli. Cultures were evaluated for Salmonella spp., Shigella spp., Campylobacter spp., E. coli O157, Yersinia spp., Aeromonas spp., Plesiomonas spp., and Vibrio spp. (19, 20). For growth-based detection of O157 STEC, SMAC agar plates were incubated for 18 to 22 h at 35°C and then examined for non-sorbitol-fermenting colonies. Colorless colonies were tested for the presence of the O157 lipopolysaccharide antigen by latex agglutination (Oxoid Ltd., Basingstoke, United Kingdom) (19, 20). Colonies positive for the O157 antigen were subjected to biochemical testing using the Vitek 2 Compact system (bioMérieux, Marcy l’Etoile, France) and LD-PCR.

Enzyme immunoassays. Following MAC broth enrichment and realtime PCR, 50 µl of any PCR-positive MAC broth was tested using the lateral flow EIA ImmunoCard STAT! enterohemorrhagic E. coli (EHEC) assay (Meridian Bioscience, Inc.), according to the manufacturer’s instructions. The EIA-positive samples were defined as samples with visible bands for stx1 and/or stx2.

LD-PCR for STEC. After incubation for 18 to 22 h at 35°C, bacterial growth in MAC broth was determined visually by turbidity and then the samples were submitted for PCR. For each sample, bacterial DNA was obtained using EasyMAG automated extraction (bioMérieux, Marcy l’Etoile, France). For specimens positive for any PCR locus, repeat testing using both the bacterial growth material from the same MAC broth and the heavy growth area from the MAC plates was performed to confirm the PCR results. According to the EasyMAG manufacturer’s instructions, 200 µl of incubated MAC broth was extracted, and DNA was eluted into 100 µl of elution buffer. O157 positive-control broth (positive for all targets) and/or RM-PCR for gastrointestinal pathogens. For MAC broths positive for STEC, 200 µl thawed broth archives were used for RM-PCR without the addition of Cary-Blair transport medium. Also, after thawing, freeze-dried direct stool specimens were diluted approximately 1 part specimen to 3 parts Protocol Cary-Blair transport medium (Thermo Scientific, Middletown, VA), according to the manufacturer’s recommendations (BioFire). The RM-PCR is characterized by a two-step process consisting of a primary single multiplex PCR followed by an array of organism-specific monoplex second-stage PCRs; it has high analytical sensitivity and specificity, as well as rapid turnaround time (22), and it is currently approved to detect 11 bacterial, 5 viral, and 4 protozoal confirmed or candidate pathogens, including Campylobacter, Clostridium difficile, Salmonella, Vibrio spp., Vibrio cholerae, Yersinia enterocolitica, enteraggregative E. coli (EAEC), enteropathogenic E. coli (EPEC), enterotoxigenic E. coli (ETEC), STEC, Shigella/enteroinvasive E. coli (EIEC), adenosivirus, F40/41, astrovirus, norovirus GI/GII, rotavirus A, sapovirus, Cryptosporidium, Cyclospora cayetanensis, Entamoeba histolytica, and Giardia lamblia.

STEC broth subculture. All MAC broth cultures positive for STEC by real-time PCR were subcultured onto SMAC, MAC, and Spectra urinary tract infection (UTI) (Remel) agar plates. Suspected non-sorbitol-fermenting colonies were worked up as described above. In cases of specimens associated with MAC broth cultures that were reproducibly positive only for either or both stx1 and stx2, starting from 5 or 6 colonies (and up to 30 suspected E. coli colonies, if necessary), colonies from primary and subculture plates of SMAC, MAC, and Spectra UTI agar plates were tested for the same set of targets by LD-PCR, to identify and to isolate non-O157 STEC. Each colony was added to water (0.5 McFarland units) and heated at 95°C for 10 min. Four microliters of the bacterial lysate was added to each PCR. All PCR-conformed STEC isolates were sent to the Washington State Public Health Laboratory, which reported their serotypes.

Statistical analyses. The chi-square test, Fisher’s exact test, and the McNemar test were used to evaluate categorical variables, as appropriate. Continuous variables were analyzed nonparametrically using the Mann-Whitney U test. Two-tailed P values of ≤0.05 were considered statistically significant. Statistical analyses were performed using Stata version 12.0 (StataCorp, College Station, TX), while MedCalc version 12.0 (MedCalc Software, Mariakerke, Belgium) was used for the McNemar test.

RESULTS

STEC diagnosis and patients. Of the 2,386 qualifying samples, 53 (2.2%) contained STEC pathogens by LD-PCR. The 53 patient specimens positive for STEC were further separated into 35 cases positive for O157 and 18 cases positive for non-O157 STEC, as differentiated by the specific rfbEO157 locus (Fig. 1). For four of the 35 patients with initial samples positive for STEC O157 by PCR, repeat samples obtained 2 to 7 days later resulted in various levels of loss of detection by either or both EIA and culture but no change in PCR detection (data not shown). For the two patients without diarrhea, non-O157 STEC was identified in the context of cultures requested to investigate failure to thrive and chronic abdominal pain (cases 6 and 7, respectively) (Table 1). HUS occurred in 12 STEC-infected patients (22.6%; 7 male and 5 female patients), 10 of whom were definitely infected with O157 (7 of
One culture-positive STEC O157 infection in a 2-year-old patient was accompanied by radiological evidence of intussusception (case 23, Table 1), which was positive for both stx1 and stx2. Patients infected with O157 (22/35 patients [62.9%]) were more frequently hospitalized than those infected with non-O157 STEC (5/18 patients [27.8%]) (odds ratio [OR], 4.4 [95% confidence interval [CI], 1.3 to 15.2]; P = 0.021), with greater frequency of blood present in their stools (P = 0.045) (Table 2). One culture-positive STEC O157 infection in a 2-year-old patient was accompanied by radiological evidence of intussusception.

Detection of STEC by LD-PCR and retrospective correlation with RM-PCR. Of the 53 specimens positive for STEC by LD-PCR, 28 (10 and 18 specimens positive for O157 and non-O157 STEC, respectively) were retrospectively evaluated by RM-PCR because of their lack of STEC isolation in routine SMAC agar cultures (Table 1 and Fig. 1). With the exception of three archived MAC broth-enriched specimens, RM-PCR produced the same stx genotype as generated by LD-PCR for 25 specimens, including 9 of the 10 culture-negative specimens (cases 20 to 28) (Table 1) positive for STEC O157 (Fig. 1). Case 23 (Table 1), which was unconfirmed by RM-PCR for STEC O157, involved a patient who was admitted with abdominal pain and bloody diarrhea. If positive samples detected by LD-PCR were considered true-positive samples, then RM-PCR achieved 89% sensitivity (positive for 25/28 samples) using the archived samples retrospectively. The difference between the 2 methods was 10.7% (95% CI, −4.5% to 10.7%) and was not statistically significant (McNemar test, P = 0.25).

There were seven direct fecal (frozen) samples available for RM-PCR (cases 3, 8, 13, 22, 23, 26, and 28) (Table 1). The RM-PCR results for these direct samples were identical to those for the corresponding frozen MAC broth samples, with RM-PCR again missing the STEC findings in the same two samples (cases 3 and 23 by LD-PCR) (Table 1). One additional sample (case 29, which was excluded from the 53 total STEC findings) was positive for rfbEO157 only by LD-PCR, but the isolate was a sorbitol-fermenting E. coli O157 strain that lacked stx1 and stx2, and the patient did not have diarrhea.

Among the 35 specimens characterized for the presence of E. coli O157 based on detection of rfbEO157, stx1, and stx2 were detected in 16 specimens (stx1+/stx2−) (Table 1), and stx1+ alone (i.e., in the absence of stx1) was detected in 19. These stx genotypes were confirmed in all 25 corresponding STEC O157 isolates (data not shown). In specimens from cases 27 and 28, the detection of all 3 targets, including rfbEO157, was diagnostic for STEC O157 by LD-PCR and was confirmed by RM-PCR, although findings were confirmed by the presence of an O186 STEC strain (case 27, which contained stx1, but not stx2) or an O111 STEC strain (case 28, which was positive for both stx1 and stx2) (Table 1). These two specimens were from 16- and 17-year-old patients, respectively, who had just returned from Mexico with vomiting and diarrhea, and the patient infected with STEC O111 had bloody diarrhea.

Among the 18 specimens positive for either or both stx determinants but negative for rfbEO157 by LD-PCR, 16 (88.9%) were determined by RM-PCR to contain non-O157 STEC (Table 1 and Fig. 1). The two RM-PCR nonconfirmatory STEC cases included case 3, which was positive for both stx1 and stx2 targets, and case 10, which was positive for stx2 only; the former patient had bloody diarrhea and the latter was coinfected with Campylobacter jejuni. Of the 18 patients infected with non-O157 STEC, positive LD-PCR results for a profile of stx1+/stx2− were found for 7 specimens, stx1+/stx2− for 9 specimens, and stx1−/stx2− for 2 specimens (Table 1).

Enzyme immunoassay. The lateral flow EIA (using overnight MAC broth-enriched specimens) provided evidence of STEC in specimens from 29 patients (54.7%) (Fig. 1) but failed to detect 11 (31.4%) of the 35 STEC O157 cases and 13 (72.2%) of the 18 non-O157 STEC cases. Specifically, EIA results were positive for only 2 (20%) of the 10 culture-negative specimens that were positive for O157 STEC by LD-PCR and 5 (28%) of the 18 specimens that were positive for non-O157 STEC (Table 1).

Culture. Corresponding STEC organisms were isolated from 33 (62.3%) of the 53 patients (excluding cases 27 and 28), and many would not have been further tested without PCR screening information, i.e., if the EIA for toxin had been used for follow-up culture evaluations. All corresponding STEC isolates recovered by culture were also confirmed by LD-PCR and produced consistent stx and rfbE results. The search for E. coli O157, in particular, was successful for only 25 (71.4%) of the 35 specimens that were PCR positive for rfbEO157, although five were toxin EIA and SMAC agar negative. The LD-PCR results prompted us to subculture the MAC broths on Spectra UTI agar (data not shown). Of the 18 patients whose stools contained non-O157 STEC, isolation would not have been routinely attempted.

Culture had a sensitivity for detection of O157 STEC of 0.71...
(95% CI, 0.5 to 0.9) (Fig. 1). Isolation of non-O157 STEC was successful for only 8 of the 18 specimens, with a sensitivity of 0.44 (95% CI, 0.2 to 0.7), yielding STEC O26 (n = 3), O121 (n = 2), and O6:H34, O125ac, and O145 (n = 1 each). All O157 STEC strains were PCR rfbE<sub>O157</sub> positive, and all non-O157 STEC strains were PCR rfbE<sub>O157</sub> negative.

**Coisolation of other enteric bacterial pathogens.** Bacterial enteric pathogens, including *Salmonella* spp. (n = 57), STEC (n = 53), *Campylobacter* spp. (n = 43), *Aeromonas* spp. (n = 37), *Yersinia enterocolitica* (n = 10), *Shigella* spp. (n = 9), and *Plesiomonas shigelloides* (n = 1), were documented in 210 (8.8%) of the 2,386 patient specimens. Stool specimens from nine of the 53 STEC-positive patients yielded additional bacterial pathogens, including mixed STEC serotypes (n = 2 cases), *Aeromonas* spp. (n = 2 cases), *C. jejuni* (n = 3 case), and *Salmonella* (n = 2 cases).

**DISCUSSION**

Multitarget PCR sensitively detects pediatric STEC infections with a resolution that discriminates O157 from non-O157 STEC. Except for missing 3 cases (cases 3, 10, and 23) (Table 1) by RM-PCR, the detection of stx<sub>1</sub> and/or stx<sub>2</sub>, as well as rfbE<sub>O157</sub>, was in agreement for LD-PCR and RM-PCR, including detection of O157 STEC (n = 9) and non-O157 STEC (n = 16). Detection of STEC would not have been made for 24 patients (45%), including 3 with severe infections and HUS, had lateral flow EIA been used alone (Fig. 1). LD-PCR was superior to EIA and culture for samples with low densities of STEC, in accordance with previous reports (23). This increased sensitivity is a clear advantage, as bacterial loads decrease rapidly during infection, particularly if cultures are not performed early in the illness (7, 14). Low EIA sensitivity was also
TABLE 2 Characteristics of 53 patients (excluding a case with a nontoxinogenic isolate) with O157:H7 and non-O157:H7 Shiga toxin-producing E. coli

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total STEC (n = 53)</th>
<th>O157:H7 (n = 35)</th>
<th>Non-O157:H7 (n = 18)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (median [total no. [%])]</td>
<td>28/53 (52.8)</td>
<td>17/35 (48.6)</td>
<td>11/18 (58.8)</td>
<td>0.386</td>
</tr>
<tr>
<td>Age (median [range] (IQR) (yr)</td>
<td>4 (0.75–19) (1.9–8)</td>
<td>4.0 (0.92–19) (2–8)</td>
<td>3.0 (0.75–17) (1.8–8)</td>
<td>0.376</td>
</tr>
<tr>
<td>Blood in stool (no./total no. [%])</td>
<td>36/53 (67.9)</td>
<td>27/35 (77.1)</td>
<td>9/18 (50.0)</td>
<td>0.045</td>
</tr>
<tr>
<td>Admission to hospital (no./total no. [%])</td>
<td>27/53 (50.9)</td>
<td>22/35 (62.9)</td>
<td>5/18 (27.8)</td>
<td>0.021</td>
</tr>
<tr>
<td>Hemolytic uremic syndrome (no./total no. [%])</td>
<td>11/53 (20.8)</td>
<td>9/35 (25.7)</td>
<td>2/18 (11.1)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

a IQR, interquartile range.
b Two-tailed Mann-Whitney test.
c Two-tailed Fisher’s exact test.

reported in other studies when PCR was used as the gold standard (10), as well as in comparison to culture (9).

The isolation of either STEC O186 or STEC O111 (both isolates negative for rfbE) from two specimens that were reproducibly positive for all three targets, including rfbE (Table 1), indicates possible mixed STEC infections. Indeed, an inexplicably large proportion of patients infected with non-O157 STEC are also infected with O157 STEC (24, 25).

Based on this study, the rate of clinical HUS following a positive test for STEC of 22.6% (12/53 cases) is similar to reported rates at academic pediatric institutions (26, 27). However, the proportion of STEC O157 specimens with the stx1/stx2 genotype, i.e., 54% during this study period, differs from findings reported for our area (28). STEC O125ac, which was identified in case 13, has been implicated as a human pathogen (29) in the Netherlands. In some situations (cases 2 and 13), we cannot completely exclude the possibility of an E. coli O157 infection although rfbE results were negative. Specifically, it is possible that rfbE was present below the level of detection, especially in light of the presence of either a single locus per cell, compared to stx1 and stx2, which are encoded on inducible bacteriophages. Similar to previous findings, patients infected with O157 STEC were more likely to have blood in their stools (P = 0.045) (Table 2) and to require hospitalization than were those infected with non-O157 STEC (P = 0.021) (Table 2) (26, 30).

The scope of the clinical impact of our PCR assay is yet to be appreciated. All but two of the STEC patients were acutely symptomatic (Table 1), and we question the value of seeking these pathogens (or any other enteric bacterial pathogens) in individuals without acute diarrhea. However, it is interesting to note that Denno et al. failed to find STEC in any of 452 community control children without diarrhea (19), but Bokete et al. did find a non-O157 STEC strain in the stool of a child with chronic diarrhea (31). With the advent of molecular technology, STEC will be detected in situations other than acute gastroenteritis (11, 27). As we enter an era of expanded use of culture-independent enteric diagnosis, clinical context will be critical to patient management, and it is important that test-ordering algorithms be designed to minimize the risk of finding results that are not relevant or misleading (32).

Our study was unable to compare the performance characteristics between LD-PCR and RM-PCR and between culture and EIA because of the selective use of EIA for PCR-positive specimens; only a subset of specimens were tested by all four methods. Another limitation of our LD-PCR assay is the requirement for overnight culture enrichment, which does not permit same-day notification of the critical findings. However, RM-PCR not only provided a rapid platform but also produced comparable sensitivity for a limited number of direct samples (Table 1). Although both PCR assays effectively distinguished O157 from non-O157 STEC causes, recovery of the pathogen is still vital for STEC serotyping and genotyping and for disease control interventions.

In summary, our LD-PCR assay using enriched stool specimens demonstrated greatly improved sensitivity for detection of STEC, especially in pediatric settings where rapid sensitive diagnosis of STEC infections can prompt specific clinical actions and avoidance of antibiotics (1, 2). Rapid diagnoses of gastrointestinal infections caused by bacteria, viruses, and protozoa may have become available (e.g., RM-PCR assays with direct stool specimens). Multiple etiologies were found at higher frequencies (17%) among our 53 STEC-positive cases than were reported (3.6%) through FoodNet sites (33). Diagnostic ability to differentiate O157 from non-O157 STEC has significant clinical implications, as infections caused by STEC O157 are more likely to be admitted to hospitals for supportive care and have a very much higher risk of progressing to HUS. For the present, we would consider any child with diarrhea who is determined to be infected with STEC to be at risk for HUS, with treatment as if infected with E. coli O157:H7 (34).

ACKNOWLEDGMENTS

We thank Michael Astion for academic sponsorship supporting the focused research activities of E.G. We thank Amanda Adler for assistance with statistical analysis and the microbiology laboratory staff at Seattle Children’s Hospital for technical assistance.

P.I.T. was supported by the National Institutes of Health (grant 5P30 DK052574).

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