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Clinical Evaluation and Cost Analysis of Great Basin Shiga Toxin Direct Molecular Assay for Detection of Shiga Toxin-Producing *Escherichia coli* in Diarrheal Stool Specimens

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ABSTRACT The Shiga Toxin Direct molecular assay (ST Direct) relies on nucleic acid amplification and solid array-based amplicon detection to identify Shiga toxin-producing *Escherichia coli* (STEC) in preserved stool specimens. Genes encoding Shiga toxin (stx_1 and stx_2), as well as the *E. coli* serotype O:157-specific marker *rfbE*, are simultaneously detected within 2 h. ST Direct was evaluated using 1,084 prospectively collected preserved stool specimens across five clinical centers. An additional 55 retrospectively collected, frozen specimens were included to increase the number of positive specimens evaluated. Results were compared to results from routine culture and an enzyme immunoassay (EIA) specific for the recovery and identification of STEC. ST Direct was found to be 93.2% sensitive and 99.3% specific for detection of stx_1 and stx_2 and 95.7% sensitive and 99.3% specific for detection of *E. coli* serotype O:157. All specimens with false-positive results were found to contain stx_1 or stx_2 or were found to be positive for serotype O:157 when analyzed using alternative molecular methods. All 4 false-negative stx_1 or stx_2 results were reported for frozen, retrospectively tested specimens. In all cases, the specimens tested positive for *stx* by an alternative FDA-cleared nucleic acid amplification test (NAAT) but were negative for stx_1 and stx_2 following nucleic acid sequence analysis. Based on these data, culture and EIA-based methods for detection of STEC are only 33% sensitive compared to molecular tests. A retrospective cost analysis demonstrated 59% of the cost of routine stool culture to be attributable to the identification of STEC. Taken together, these data suggest that ST Direct may provide a cost-effective, rapid molecular alternative to routine culture for the identification of STEC in preserved stool specimens.

KEYWORDS molecular diagnostics, STEC, Shiga toxins, microarray

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Infection with Shiga toxin (Stx)-producing *Escherichia coli* (STEC) is an uncommon but potentially serious illness, particularly in pediatric and geriatric patient populations. The most serious condition associated with STEC infection is hemolytic-uremic syndrome (HUS), in which toxin-induced damage to the renal endothelium causes hemolytic anemia and acute kidney injury (1). HUS resulting from STEC infection occurs in approximately 6% of all cases but can reach 18% in children 1 to 5 years of age, in whom it carries a 4.6% mortality rate (2). While HUS is most commonly associated with STEC serotype O157:H7, it has also been reported following infection with many other STEC serotypes (3). Rapid identification of STEC is of clinical importance since antimo-

tility agents or antibiotics commonly prescribed to treat acute gastroenteritis (AGE) may actually increase the risk of HUS (1, 4, 5). Specifically, ciprofloxacin is associated with a 10- to 100-fold increase in transcription of stx_2 and up to a 1,000-fold-higher titer of toxin (4). Based on these data, optimal management of patients with STEC infection relies on intravenous rehydration and other supportive measures and should not include antibiotic treatment (1, 5).

The Centers for Disease Control and Prevention (CDC) recommend that all stool specimens submitted from patients with diarrheal illness be tested for Shiga toxins (Stx1 and Stx2) as well as *E. coli* serotype O:157 (6). Traditional methods for detection of STEC require multiple steps, including direct plating of stool specimens to MacConkey agar with sorbitol (SMAC), serotyping of presumptive serotype O:157 (sorbitol-negative) colonies, and detection of Stx1 and Stx2 using a Shiga toxin enzyme immunoassay (EIA) (6). These approaches are labor-intensive and costly and have poor sensitivity, ranging from 24 to 74% (7–11). Further, results are not available for 36 to 48 h following specimen culture, which can delay appropriate management of the patient.

Multiple studies have demonstrated sensitivity of >97% for nucleic acid amplification tests (NAATs) designed to detect STEC (stx_1 and stx_2) and *E. coli* serotype O:157 (*rfbE*) directly from preserved stool specimens in as little as 1 to 4 h (7, 12–15). Some of these NAATs are specific for STEC, while others are highly multiplexed to enable detection of numerous bacterial, viral, and toxin targets associated with AGE. While broad panels may be useful for specific populations at risk for severe disease (e.g., pediatric and immunocompromised patients), the high cost per test may not be warranted for routine use on all stool specimens submitted to the laboratory.

We conducted a multicenter clinical evaluation of the FDA-cleared Shiga Toxin Direct (ST Direct) test (Great Basin, Salt Lake City, UT). This is a sample-to-answer NAAT capable of detecting stx_1, stx_2, and *rfbE* (serotype O:157) in preserved stool specimens within 2 h. Results were compared to those from routine culture and EIA methods, and all positive specimens were also tested using an alternative FDA-cleared NAAT (FilmArray GI; BioFire, Salt Lake City, UT) as a molecular comparator.

(A portion of these data was presented at the ASM Microbe meeting in Boston, MA, June 2016.)

RESULTS

Study enrollment. A total of 1,084 prospectively collected stool specimens were tested using ST Direct and the reference culture method. Two specimens (0.2%) were repeatedly reported as invalid by ST Direct, indicating the presence of inhibitory substances. Of the remaining 1,082 specimens, 4 (0.4%) were positive for Stx by the reference EIA method. No *E. coli* serotype O:157 organisms were recovered using culture. Because of the low positivity rate for ST Direct targets, a set of retrospectively collected specimens positive for stx_1, stx_2, and *E. coli* O:157 were included in this evaluation. This retrospective specimen cohort comprised a total of 88 specimens: 31 positive for STEC non-O:157, 24 positive for STEC O:157, and 33 negative for STEC but positive for other enteric pathogens, including norovirus, *Clostridium difficile*, *Campylobacter* spp., and enteropathogenic *E. coli* (EPEC).

Comparison of the Shiga Toxin Direct test compared to routine culture and EIA in prospectively collected specimens. ST Direct reported "Shiga toxin positive" for all 4 prospectively collected specimens that were positive for Stx by EIA (Table 1). Serotype O:157 was reported as negative by ST Direct for all 4 specimens, which was in agreement with reference culture and typing results. An additional 8 specimens that were not identified by EIA were reported as Shiga toxin positive, 2 of which were also reported as serotype O:157 positive by ST Direct. These data demonstrate 100% (4/4) sensitivity and 99.3% (1,070/1,078) specificity for STEC among prospectively collected clinical specimens giving a valid result. The ST Direct test repeatedly reported two specimens (0.2%) as invalid. If these were included as false negative, the overall sensitivity would fall to 66.7% (4/6). With an overall prevalence of only 0.4%, considering even a single invalid result as false negative has a significant impact on test

TABLE 1 Performance of the Shiga Toxin Direct test compared to routine culture and EIA[a]

Organism and sample type	No. of TP	No. of FP	No. of TN	No. of FN	Sensitivity,[b] % (CI)	Specificity,[c] % (CI)	PPV, % (CI)	NPV, % (CI)
STEC								
Fresh, prospective	4	8[e]	1,070	0	100 (40–100)	99.3 (98–100)	33.3 (11–65)	100 (99–100)
Frozen, retrospective	51	0	33	4[f]	92.7 (82–98)	100 (87–100)	100 (91–100)	89.2 (74–96)
Total	55	8	1,103	4	93.2 (83–98)	99.3 (99–100)	87.3 (76–94)	99.6 (99–100)
O:157[d]								
Fresh, prospective	0	2[g]	4	0	ND	66.7 (24–94)	0 (0–80)	100 (99–100)
Frozen, retrospective	22	0	24	1[h]	95.7 (76–100)	100 (83–100)	100 (82–100)	96.0 (78–100)
Total	22	2	28	1	95.7 (76–100)	93.3 (76–99)	91.7 (72–99)	69.6 (80–100)

[a]A total of 1,170 specimens were tested. Abbreviations: TP, true positives; FP, false positives; TN, true negatives; FN, false negatives; PPV, positive predictive value; NPV, negative predictive value; CI, 95% confidence interval.
[b]Positive percent agreement for frozen, retrospective specimens.
[c]Negative percent agreement for frozen, retrospective specimens.
[d]O:157 result is reported only if specimens were positive for STEC.
[e]Preserved stool specimens and enrichment broths were subjected to bidirectional sequence analysis (LOD ~ 10^4 CFU/ml). Sequence for stx_1, stx_2, or both was confirmed in all 8 samples.
[f]All 4 specimens were positive by alternative molecular IVD test but negative by bidirectional sequence analysis.
[g]Both specimens were reported as positive for *E. coli* O:157 by an alternative molecular IVD test.
[h]Specimen was reported as positive for serotype *E. coli* O:157 by an alternative molecular IVD test.

sensitivity. However, because of the combined low rate of invalid results (0.2%) and positive specimens (0.4%), the likelihood of an invalid result occurring in a positive specimen by random chance is only 0.0008% (1/125,000).

Shiga toxin-specific PCR and bidirectional sequencing were performed on all 8 discordant specimens that were positive by ST Direct only. Results confirmed the presence of stx_1 ($n = 1$), stx_2 ($n = 1$), or both ($n = 6$) in all 8 specimens, indicating increased sensitivity of ST Direct compared to that of reference EIA. Both specimens that were positive for *E. coli* O:157 by ST Direct were analyzed by an alternative molecular *in vitro* diagnostic (IVD) test, which also reported the presence of *E. coli* O:157. Therefore, after analysis of discordant results, ST Direct was 100% sensitive and specific for STEC and *E. coli* O:157 among prospectively collected specimens.

Performance of ST Direct in a retrospectively collected specimens. Within the set of 88 characterized and retrospectively tested specimens, ST Direct demonstrated a 92.7% (51/55) positive agreement and 100% (33/33) negative agreement for detection of STEC. All 4 specimens that were classified as false negative for ST Direct tested positive by an alternative molecular IVD test but negative for Shiga toxin by PCR and bidirectional sequence analysis. The established limit of detection (LOD) for the PCR used prior to sequence analysis was ~5×10^3 CFU/ml, whereas the alternative molecular IVD test has a slightly lower stated LOD, ~1×10^3 (FilmArray GI product insert), which may account for the discordant results between sequence analysis and the alternative molecular IVD test. Among 51 specimens reported as Shiga toxin positive, ST Direct demonstrated 95.7% (22/23) positive agreement for detection of serotype O:157. The single false-negative O:157 result was reported as positive by the alternative molecular IVD test.

Cost associated with routine workup of stool culture. A comparison of materials and labor costs required for routine bacterial culture and STEC-specific culture/EIA is presented in Table 2. Data were collected over a 4-month period at one laboratory in which 926 stool specimens were submitted to the microbiology laboratory for routine stool culture.

Six standard plating media and an AnaeroPack for isolation of *Campylobacter* were required for all specimens and totaled $1.63. Initial accessioning, labeling of media, and inoculation required ~ 6 min of technologist time per specimen, totaling $3.19. Review of culture plates and entry of updated data into the laboratory information system (LIS) required ~3 min and was conducted following 24, 48, and 72 h of incubation for a total of 9 min ($4.80) per culture. In total, 77.3% (716/926) of specimens were negative by

TABLE 2 Cost associated with routine workup of stool specimens

Step or item	Routine bacterial culture (per specimen)			STEC-specific culture/EIA (per specimen)		
	Time (min:s)	Labor[a] cost ($)	Reagent cost ($)	Time (min:s)	Labor cost ($)	Reagent cost ($)
Preanalytical steps (ordering test and labeling and inoculation of media)	6:00	3.19		2:00	1.07	
Media						
5% sheep blood			0.21			
MacConkey			0.21			
XLD			0.22			
Hektoen enteric			0.24			
CIN			0.36			
Campy agar			0.29			
Sorbitol MacConkey						0.32
MacConkey broth						0.74
Reagent						
AnaeroPack[b]			0.10			
API20E[c]			0.64			
Stx EIA						11.12
EIA controls[d]						1.85
Culture workup						
Plate reading[e]	9:00	4.80		1:00	0.53	
Additional workup[f]	3:27	1.76				
EIA specimen processing[g]				1:30	0.79	
EIA plate processing[h]				2:00	1.07	
Entry of results into LIS	0:45	0.40		0:45	0.40	
Subtotal	19:12	10.15	2.27	7:15	3.86	14.03
Total cost/specimen			12.42			17.89

[a]Labor calculated at an average of $32.00/h, inclusive of salary and benefits.
[b]For isolation of *Campylobacter*, cost/specimen assumes 1 pack/12 plates.
[c]Cost/specimen is based on $2.76/test and 23% of specimens requiring workup of ≥1 potential pathogen.
[d]Based on inclusion of a positive and negative control with each batch, cost/specimen assumes 12 specimens/batch.
[e]Based on retrieval and examination of plates at 24, 48, and 72 h.
[f]Time/specimen based on 15 min for completion of Gram stain and setup of API20E and 23% of specimens requiring workup of ≥1 potential pathogen.
[g]Includes all steps required for each specimen (e.g., labeling, addition of sample diluent, addition of sample, and vortexing).
[h]Includes all steps performed as batch (e.g., 5 wash/reagent addition steps and reading). Time/specimen assumes 12 specimens per batch.

culture screen and required no additional workup beyond daily examination. Therefore, the total cost for a negative bacterial culture (excluding STEC) was $9.62.

At least one potential pathogen was present on the screening media in 23% (210/926) of cultures and required additional workup for full identification. This included Gram stain, basic biochemical tests, and an API20E test ($2.76) which required ~15 min ($8.00) of technologist time per isolate. Therefore, workup of a positive stool required an additional $10.76 in materials and labor. If the cost of additional workup for the 23% of screen-positive cultures is distributed across all specimens, the total average cost of routine bacterial culture is $12.42 per specimen.

In addition to routine bacterial culture, all specimens were inoculated to SMAC agar and MacConkey enrichment broth for identification of STEC. Combined, materials and labor for inoculation of each stool to these media cost $2.13 per specimen. Greater than 95% (883/926) of MacConkey broth cultures were positive and were subsequently tested using a Shiga toxin EIA. The materials for EIA are $11.12 per test, and positive and negative controls are included with each batch. Our laboratory runs an average of 12 samples per batch, so the total cost in test materials inclusive of controls is $12.97 per specimen. Labor for individual specimen processing steps plus batch steps distributed across an average batch size of 12 specimens totals 5 min 15 s (5:15 min) ($2.79) per specimen. This brings the total cost for identification of STEC to $17.89 per specimen. Therefore, STEC-specific testing accounts for 59% ($17.89/$30.31) of the total cost of routine stool culture.

DISCUSSION

Rapid and accurate identification of Shiga toxin-producing *E. coli* (STEC), including serotype O:157, is essential for individual patient care and also aids in identification of potential foodborne outbreaks. Historically, *E. coli* serotype O:157 was associated with the most severe cases of STEC gastroenteritis, including those resulting in HUS. This may be a combination of the increased risk of HUS associated with Stx2 and the high prevalence of stx_2 in STEC serotype O:157 isolates (16). Over the past 2 decades, reports of more than 100 additional non-O:157 STEC types associated with mild to severe gastroenteritis and HUS have raised awareness of the clinical significance of other STEC serotypes as well. Globally, 19 to 100% of gastroenteritis resulting from STEC are attributable to non-O157 serotypes, and these infections affect both adult and pediatric populations (3, 16–18). Therefore, direct detection of Shiga toxin in stool specimens is recommended by the CDC (6).

Current enzyme immunoassays for Stx1 and Stx2 have a limit of detection of 10^6 to 10^7 CFU/ml; therefore, broth enrichment of the stool prior to EIA is essential for optimal performance (8, 9). Importantly, even with broth enrichment the sensitivity of EIA is low, ranging from 29 to 74% (7–9, 19). Moreover, the necessity of broth enrichment prior to EIA delays results for 24 to 36 h, which, in turn, delays appropriate patient management. Because of the increased association of *E. coli* serotype O:157 with HUS, selective culture using MacConkey agar with sorbitol is also recommended, with confirmation of the O:157 serotype based on latex agglutination. Combined, these efforts to detect STEC account for up to 58% of the total cost associated with the routine workup of stool specimens (10, 11). A rapid and inexpensive NAAT can address many of the shortcomings of current methods for detection of STEC by eliminating the need for broth enrichment, selective culture, and serotyping of sorbitol-negative colonies.

Commercial and laboratory-developed NAATs demonstrate an LOD as much as 4 $\log_{10}$ lower ($\sim 10^2$ to 10^4 CFU/ml) than that of broth-enriched Stx EIA (9, 20). This increased sensitivity enables the identification of STEC in 2 to 3 times more specimens than routine culture and EIA methods (7, 12, 15). Increased sensitivity and more rapid turnaround time (TAT) not only benefit individual patient management but also can aid in infection control efforts (e.g., identification of patients with low bacterial load who are still infectious) and identification of potential outbreaks 24 to 72 h sooner than current culture methods. An unintended consequence of implementing culture-independent diagnostic tests (CIDTs) for STEC is the impact on public health and epidemiologic investigations. Without a culture isolate, strain typing using traditional methods is not possible. The added cost and expertise required for molecular typing or whole-genome sequence analysis directly from clinical specimens are currently not practical for most public health laboratories. However, the isolation and identification of non-O:157 STEC are hampered by the limitation of current methods to differentiate STEC from other resident *E. coli* organisms in specimens that test positive for Stx by EIA but do not contain sorbitol-negative colonies.

The performance of ST Direct in this study is comparable to that in previous evaluations of other molecular tests, including the BD MAX EBP, ProGastro SSCS, and FilmArray GI (7, 12, 15). Specifically, the sensitivity for detection of STEC was 100% compared to reference culture and EIA. ST Direct also correctly identified an additional 8/12 (67%) specimens positive for stx_1 or stx_2 and two specimens positive for *E. coli* serotype O:157 that were missed by routine methods. The clinical significance of these additional positive results could not be fully assessed due to the use of residual deidentified specimens for this study; however, all results were confirmed upon further analysis using alternative molecular methods. Therefore, compared to ST Direct, the reference EIA was only 33% sensitive for STEC and culture was 0% sensitive for O:157 among 1,082 prospectively collected specimens.

Stool culture remains one of the most cost and labor-intensive methods in the laboratory. In our analysis, each specimen required an average of more than 26 min (22:15 min for negative cultures and 37:15 min for positive cultures) of technologist

time and had a total average cost of $30.31 ($28.15 for negative cultures and $38.91 for positive cultures), inclusive of materials and labor. Within the stool culture, direct and enriched culture and EIA specific for identification of STEC account almost one-third of the hands-on time and 59% of the total cost. These data are in agreement with those of others who have reported over 50% of stool culture costs being attributable to STEC-specific testing (10, 11). Despite these efforts, the detection of STEC remains low, typically around 1:1,000 specimens in nonoutbreak settings. The combination of high cost and low prevalence can equate to an astounding $18,300 per positive result (10). In our prospective analysis, only 4/1,082 (0.4%) were positive for STEC by the culture method, equating to $4,800 per positive result. In either case, the combination of high labor and materials cost, low prevalence, and poor culture and EIA sensitivity result in significant inefficiency. The ST Direct test is currently marketed for $24.00 per test, which is similar to that of culture and EIA, but it increases positive identifications of STEC 3- to 4-fold over routine methods. In our example, adoption of the ST Direct test would add $5,557.86 over current STEC culture and EIA for 926 stools, but detection of 8 additional positive specimens actually decreases the cost per positive result by 62% ($4,800.00 versus $1,852.00).

In conclusion, the ST Direct test offers increased sensitivity and decreased TAT for the identification of STEC and *E. coli* serotype O:157 in preserved stool specimens compared to those of culture and EIA methods. ST Direct provides a cost-effective molecular alternative to fulfill the CDC recommendation for STEC screening and enables a definitive diagnosis and appropriate treatment more quickly for patients with STEC-related gastroenteritis.

MATERIALS AND METHODS

Study enrollment. Specimen enrollment and testing were conducted at five clinical centers representing geographically distinct regions within the United States. Each site enrolled and tested between 35 and 307 remnant prospective stool specimens in accordance with individual institutional review board (IRB)-approved protocols. To be included in the study, prospective specimens had to be transferred to nonnutritive stool preservation medium (e.g., Cary-Blair or Para-Pak C&S) within 2 h of collection. A set of retrospectively collected stools that were reported as positive for Stx and/or *E. coli* serotype O:157 were included to augment prospective testing and increase the number of total positive specimens. These specimens were received as frozen stool specimens in preservation medium and were originally characterized by each laboratory's standard-of-care method. To confirm the original result, each retrospective stool was subjected to direct PCR and bidirectional sequence analysis for stx_1 and stx_2 (Clarient Diagnostic Services, Houston, TX), as well as analysis using the FDA-cleared FilmArray GI test. Any specimen for which the initial clinical result could not be confirmed by either method was omitted from the study. A panel of 55 specimens positive for STEC and 33 negative stools were randomized, deidentified, and distributed to selected study sites for testing.

ST Direct test. The Shiga Toxin Direct test (ST Direct) consists of a single-use test cassette and a benchtop analyzer. The test cassette contains all reagents necessary for nucleic acid extraction, target amplification, and amplicon detection. Preserved stool specimens were tested within 4 h if kept at room temperature or within 5 days if refrigerated. A 50-μl aliquot of preserved stool specimen was loaded into the sample port of the test cassette using a calibrated pipette, and the cassette was loaded into the analyzer for automated processing. Target amplification is achieved using standard PCR and biotin-labeled target-specific primers (stx_1, stx_2, and *rfbE*). Array-based capture of the resulting biotinylated amplicon(s) is achieved using immobilized complementary capture probes. Detection is achieved by incubation of the immobilized amplicon-probe duplex with horseradish peroxidase (HRP)-conjugated anti-biotin antibody and tetramethylbenzidine (TMB), a substrate that forms HRP-mediated precipitate. The chip surface is then imaged and detection of precipitate is used to indicate a positive result. An internal process control within the test cassette is used to monitor for specimen inhibition. Results are reported as Shiga toxin negative, Shiga toxin positive, serotype O:157 positive, or invalid. A serotype O:157 result (positive or negative) is reported only for specimens with a positive Shiga toxin result. The time to result is approximately 2 h. Any specimen reported as invalid was retested a single time, and the repeat result was reported.

Reference method. Bacterial culture including Shiga toxin EIA was conducted at each study site in parallel with ST Direct testing. Each preserved specimen was directly inoculated to sorbitol MacConkey agar using a 10-μl loop. Cultures were examined for the presence of sorbitol-negative (colorless) colonies following 16 to 24 h of incubation at 35°C. Colorless colonies were typed using a serotype O:157-specific latex agglutination test. MacConkey broth medium (5 ml) was inoculated with 175 μl of preserved stool and incubated 16 to 24 h at 35 to 37°C. Positive broths (turbid and yellow) were tested for the presence of Shiga toxin using the ImmunoCard STAT! enterohemorrhagic *E. coli* (EHEC) EIA (Meridian Bioscience, Cincinnati, OH) in accordance with the manufacturer's product insert.

Resolution of discordant results. An aliquot of each preserved stool and enriched MacConkey broth was stored at −70°C for resolution of discordant results between ST Direct and the reference culture and EIA method. For any discordant STEC results, the preserved stool and enriched MacConkey broth were subjected to Shiga toxin-specific PCR and bidirectional sequence analysis as previously described (7). Briefly, nucleic acid was extracted from the preserved stool or enriched broth and Shiga toxin-specific primers were used to amplify the stx_1 and/or stx_2 template present in the sample. The limit of detection for PCR and sequence analysis was similar to that of the ST Direct test (approximately 5.0×10^3 CFU/ml). The resulting amplicon was subjected to bidirectional sequencing using the Sanger chain termination method. Resulting sequence data were subjected to BLAST alignment using established criteria for positive identification (sequence of >200 nucleotides in length with a minimum 90% of sequence having a PHRED score of ≥20, i.e., a <1% error rate, and ≥95% identity with the reference consensus sequence). Discordant results for *E. coli* serotype O:157 were resolved using an FDA-cleared molecular IVD test (FilmArray GI) as an arbiter in accordance with the manufacturer's instructions.

Statistical analysis. Clinical performance characteristics, including sensitivity, specificity, and positive and negative predictive values, were calculated using standard methods. Ninety-five percent confidence intervals were calculated using binomial expansion.

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