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Multicenter Evaluation of the BD Max Enteric Bacterial Panel PCR Assay for Rapid Detection of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. (*C. jejuni* and *C. coli*), and Shiga Toxin 1 and 2 Genes

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Diarrhea due to enteric bacterial pathogens causes significant morbidity and mortality in the United States and worldwide. However, bacterial pathogens may be infrequently identified. Currently, culture and enzyme immunoassays (EIAs) are the primary methods used by clinical laboratories to detect enteric bacterial pathogens. We conducted a multicenter evaluation of the BD Max enteric bacterial panel (EBP) PCR assay in comparison to culture for the detection of *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, and *Campylobacter coli* and an EIA for Shiga toxins 1 and 2. A total of 4,242 preserved or unpreserved stool specimens, including 3,457 specimens collected prospectively and 785 frozen, retrospective samples, were evaluated. Compared to culture or EIA, the positive percent agreement (PPA) and negative percent agreement (NPA) values for the BD Max EBP assay for all specimens combined were as follows: 97.1% and 99.2% for *Salmonella* spp., 99.1% and 99.7% for *Shigella* spp., 97.2% and 98.4% for *C. jejuni* and *C. coli*, and 97.4% and 99.3% for Shiga toxins, respectively. Discrepant results for prospective samples were resolved with alternate PCR assays and bidirectional sequencing of amplicons. Following discrepant analysis, PPA and NPA values were as follows: 97.3% and 99.8% for *Salmonella* spp., 99.2% and 100% for *Shigella* spp., 97.5% and 99.0% for *C. jejuni* and *C. coli*, and 100% and 99.7% for Shiga toxins, respectively. No differences in detection were observed for samples preserved in Cary-Blair medium and unpreserved samples. In this large, multicenter study, the BD Max EBP assay showed superior sensitivity compared to conventional methods and excellent specificity for the detection of enteric bacterial pathogens in stool specimens.

The World Health Organization reports that, worldwide, there are nearly 1.7 billion cases of diarrheal disease every year and that diarrheal disease is the second leading cause of death of children <5 years old. Each year, diarrhea kills ~760,000 children under the age of 5 years, and most importantly, it is preventable and treatable. Diarrhea is also a leading cause of malnutrition in this same age group. Most of this disease is related to unsafe drinking water, inadequate sanitation, and poor hygiene (1, 2).

Acute gastroenteritis is caused by a number of bacterial, viral, and parasitic agents (3). In the United States, noroviruses cause most cases of self-limited, acute gastroenteritis. However, *Salmonella*, *Campylobacter*, *Shigella*, and Shiga toxin-producing *Escherichia coli* (STEC) are the most common diarrheagenic bacteria, and they are usually associated with foodborne illness (4). Furthermore, *Shigella* is more frequently transmitted from person to person due to the low infectious dose. Importantly, agents of gastroenteritis may not be distinguished clinically. Identifying the cause of diarrhea is important for both the treatment of individual patients and public health intervention through outbreak management (5).

Conventional microbiological cultures remain the gold standard for identification, despite their limited sensitivity. Additionally, traditional methods are time-consuming and labor-intensive and require considerable technical skill. The application of nucleic acid amplification methods could have significant impact on diagnosis and treatment, as well as our understanding of the epidemiology of this disease (6, 7).

The BD Max enteric bacterial panel (EBP) is a multiplex nucleic acid amplification assay designed for the detection of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp. (*C. jejuni* and *C. coli*), and Shiga-like toxin genes (stx_1 and/or stx_2) in stool specimens with the BD Max system (BD Diagnostics, Baltimore, MD, USA). The BD Max system is a walkaway PCR instrument that can process the specimen and amplify and detect nucleic acids in a batch of up to 24 samples in ~3 h using only 1.25 min of hands-on time

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per sample (8). In contrast, routine diagnostic culture methods typically require 24 to 72 h of incubation, with additional technical time for the identification of pathogens by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) or biochemical methods.

A number of commercially available diagnostic systems for the detection of enteric pathogens have recently been developed. These systems use a variety of approaches and differ as to the number and type of targets incorporated in the assay and the overall platform design and throughput. The Prodesse ProGastro SSCS assay (Gen-Probe Prodesse, San Diego, CA) detects the same pathogens as those detected by the BD Max EBP assay, but unlike the BD Max assay, the ProGastro SSCS assay requires external extraction on a separate instrument, increasing the hands-on time and technical complexity (9). Other systems have been designed to detect viral and parasitic enteric pathogens as well as additional bacterial targets. The Luminex xTag gastrointestinal pathogen panel (GPP) assay (Luminex Corp., Austin, TX) can detect up to 15 targets (9 bacteria, 3 viruses, and 3 parasites) (6, 10), and the FilmArray gastrointestinal (GI) panel (Biofire Diagnostics, Inc., Salt Lake City, UT) contains 23 targets, including 14 bacterial, 5 viral, and 4 parasitic targets (9). The Luminex methodology is a high-throughput assay but also requires an external extraction step. The FilmArray assay requires very little technical time, as all analyses are performed with a single instrument, with results being available in an hour. Throughput is lacking, however, as only one sample at a time can be tested on each instrument. The Verigene Enteric Pathogens (EP) test (Nanosphere, Northbrook, IL), detects 7 bacterial and 2 viral targets. Like the FilmArray assay, throughput is limited. Single stool specimens are extracted and amplified on a processing instrument and moved to the Verigene reader for final analysis (11). From the description of these assays, it is clear that laboratories can choose from a variety of test platforms that may be implemented depending on whether a focused or broad approach to pathogen detection is desired. The need for throughput and proficiency of the technical staff with molecular assays may weigh heavily on the choice of assay. The BD Max EBP assay focuses on only the most prevalent bacterial enteric pathogens, might be characterized as having medium throughput, and requires almost no previous technical expertise in molecular diagnostics.

We conducted a large, multicenter evaluation of the BD Max EBP assay in comparison to routine diagnostic culture for *Salmonella* spp., *Shigella* spp., *C. coli*, and *C. jejuni* and an enzyme immunoassay (EIA) for Shiga toxins. Stool samples were collected prospectively and included specimens preserved in Cary-Blair transport medium and unpreserved specimens.

(The results of this study were presented in part at the 114th General Meeting of the American Society for Microbiology, Boston, MA, 17 to 20 May 2014, and at the 24th European Congress of Clinical Microbiology and Infectious Diseases, Barcelona, Spain, 10 to 13 May 2014.)

MATERIALS AND METHODS

Specimens. Soft or diarrheal stool specimens ($n = 4,242$) from pediatric or adult patients submitted for routine analysis of bacterial stool pathogens were included in the study. Formed stools and rectal swabs were excluded. Samples were submitted either in a clean, dry container or preserved in Cary-Blair transport medium (e.g., Para-Pak [Meridian Bioscience, Cincinnati, OH]). Prospective and retrospective specimens were collected and tested at six clinical centers in the United States and one in Canada. Additionally, specimens were provided by collection sites (2 in the United States, 1 in Canada, and 1 in Mexico). For some clinical centers, reference cultures were performed by Microbiology Specialists, Inc. (Houston, TX). Specimens were collected in compliance with site-specific Institutional Review Board (IRB) protocols. Between December 2012 and September 2013, 3,457 samples were collected prospectively: 1,345 (38.9%) were unpreserved and 2,112 (61.1%) were preserved. To increase the number of positive results, samples yielding pathogens by culture or Shiga toxin EIA collected between 2007 and 2013 and frozen at a temperature of −20°C or lower were included. The majority of previously characterized and archived specimens (84%) were collected and stored between March 2012 and August 2013. Retrospective specimens were thawed prior to testing and did not undergo other freeze-thaw cycles. When possible, positive retrospective samples were paired with one or more culture/EIA-negative specimens from the same time period. A total of 785 retrospective specimens were initially included: 321 (40.9%) were unpreserved, and 464 (59.1%) were preserved. Since targets in retrospective samples may have degraded during storage, prior to testing with the BD Max EBP assay, results for retrospective samples were confirmed by alternate PCR, according to previously reported methods (12, 13). Specimens with historical results that were not confirmed were excluded from further analysis.

Reference culture and EIA methods. Preserved specimens were stored at 2°C to 25°C and planted for culture or tested for Shiga toxins by an EIA within 96 h of collection. Reference methods for unpreserved specimens were initiated upon receipt in the clinical laboratory, according to established laboratory protocols. Clinical Laboratory Improvement Amendment (CLIA) (31)-compliant culture methods for standard patient care were used at each site, and appropriate quality control was documented according to Clinical and Laboratory Standards Institute (CLSI) M22-A3 guidelines (14). Stool specimens were planted on primary culture medium for the detection of *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., and *E. coli* O157. However, specific media varied somewhat. Media included MacConkey agar, selective campylobacter agar, Trypticase soy blood agar, xylose-lysine-deoxycholate agar, Hektoen enteric agar, salmonella-shigella agar, eosin-methylene blue agar, sorbitol MacConkey agar, CHROMagar Salmonella, and O157 CHROMagar and Gram-negative (GN) or selenite broth. Cultures were incubated for 48 h at 35°C for the detection of *Salmonella* spp., *Shigella* spp., and *E. coli* O157 and at 42°C under microaerophilic conditions for 72 h for the detection of *Campylobacter* spp. Identification of colonies suspicious for stool pathogens was performed with tube biochemicals, including API 20 (bioMérieux, Inc., Durham, NC), Vitek (bioMérieux, Marcy l'Etoile, France), Phoenix (BD, Sparks, MD), or MicroScan (Beckman Coulter, Brea, CA). *Campylobacter* isolates with negative hippurate and positive indoxyl acetate results were confirmed to be *C. jejuni* or *C. coli* by 16S rRNA sequence analysis. Non-sorbitol-fermenting colonies identified as *E. coli* were tested with O157-specific antiserum. Assessment for Shiga toxins 1 and/or 2 was performed following enrichment in GN or MacConkey broth for 18 to 24 h with either the Immunocard Stat! enterohemorrhagic *E. coli* (EHEC) or the Premier EHEC test according to the manufacturer's instructions (Meridian Bioscience, Cincinnati, OH).

BD Max EBP automated PCR. Prospective samples were tested with the BD Max EBP assay within 48 h if stored at 8°C to 25°C and within 120 h if kept at 2°C to 8°C, according to the manufacturer's instructions. When thawed, retrospective samples were tested within the same time frames as those required for prospective samples. The BD Max EBP assay was performed according to the IUO (investigational use only) package insert for the BD Max instrument (15). BD Max and BD Max EBP are licensed trademarks of Becton, Dickinson and Company. By using a calibrated bacteriological loop, 10 μl of a homogenized stool specimen was placed into a sample buffer tube (SBT) containing diluent formulated to minimize inhibition associated with stool matrices. Individualized unitized reagent strips (URSs) containing reagents and consumables for DNA

extraction were placed onto the BD Max instrument. A sample processing control (SPC) containing a plasmid with a proprietary synthetic target DNA sequence was incorporated into the extraction tube to monitor the effectiveness of the DNA extraction, amplification, and detection steps. Also loaded into the URSs were small tubes containing EBP-specific primers, TaqMan probes, and PCR master mix. The system performs lysis and DNA extraction and automatically pipettes the extracted sample into the PCR master mix. Tests for samples with a failed SPC result were repeated with the same SBT. If the repeat SPC test failed, a second SBT was inoculated from the original sample. Up to three repeats were allowed, provided that samples from the SBT were tested within 120 h and stored at 2°C to 8°C. Samples with repeatedly negative SPC results were reported as unresolved.

The BD Max EBP multiplex real-time PCR assay uses four primer sets: *spaO* for *Salmonella* spp., *ipaH* for *Shigella* spp. and enteroinvasive *E. coli*, stx_1 and stx_2 for Shiga toxin-producing organisms, and *tuf* for *Campylobacter coli* and *C. jejuni*. The two *Campylobacter* species and stx_1 and stx_2 are not differentiated from each other with these primer sets. Primers and probe sequences are proprietary and not available for publication. The BD Max instrument has five different optical channels for the detection of fluorescent signals. Discrimination of pathogen-specific amplicons and the SPC was accomplished with TaqMan probes with different fluorometric properties for each target. Amplification and detection occur in a disposable microfluidic cartridge. A software algorithm within the BD Max instrument interprets the amplification data and provides a positive or negative result. Each cartridge may be used for up to 12 samples. The total run time, including sample processing, PCR, and result reporting, was <3 h.

A positive and a negative external control were included at minimum in the first run of each day. The positive control was one of the four targets, alternated on a daily basis. Each species was tested at least once with each BD Max EBP kit lot. *S. enterica* subsp. *enterica* serovar Typhimurium (ATCC 14028), *Shigella sonnei* (ATCC 9290), and Shiga toxin-producing *E. coli* (ATCC 43890) were subcultured on 5% Trypticase soy sheep blood and incubated for 18 to 24 h. *C. jejuni* (ATCC 33291) was subcultured on brucella agar with 5% sheep blood, hemin, and vitamin K and incubated for 2 to 3 days in a microaerophilic environment. A 1:100 dilution of a suspension equivalent to a 0.5 McFarland turbidity standard was prepared from a fresh culture. By using a bacteriological loop, 10 μl was inoculated into an SBT prior to testing with the BD Max system. The negative control was saline.

Result interpretation and discrepant analysis. Results for each analyte could not be reported for every sample for several possible reasons, including discrepancies in compliance with the study protocol, an unresolved SPC result for at least one target, or instrument failure. Samples with results that could not be reported after repeat testing due to system failure or instrument failure were termed indeterminate or incomplete. Results for noncompliant, unresolved, indeterminate, and incomplete samples were removed from the performance analysis.

The reference method for the detection of *Shigella* spp., *Salmonella* spp., and *C. coli* and *C. jejuni* was culture. EIA was the reference method for Shiga toxins 1 and 2. If an *E. coli* O157 isolate was identified by culture and the EIA result was negative, the EIA was repeated. If the result of the repeated EIA was negative, the final result was considered negative because the BD Max EBP assay detects toxin genes, irrespective of species. Culture results for *E. coli* O157 were used as supplemental information and did not contribute to performance statistics.

Prospective samples with discrepant results between culture or EIA and the BD Max EBP assay were tested by an alternate PCR method (10, 11) (performed at BD Diagnostics, Sparks, MD) and bidirectional sequencing of amplicons (ILS Genomics, LLC, Research Triangle Park, NC). The alternate PCR assessed different targets for *Salmonella* spp. (*invA*) and *Campylobacter coli* and *C. jejuni* (*cadF*) and utilized alternate primers for *Shigella* spp. (*ipaH*) and Shiga toxins (stx_1 and stx_2). For samples that were culture negative and BD Max EBP positive and for those with any results discrepant between the Shiga toxin EIA and BD Max EBP assay, the results of the alternate PCR were used as the final result. Growth of *Salmonella* spp., *Shigella* spp., *Campylobacter coli*, or *C. jejuni* in culture was considered a positive result, regardless of results with the alternate PCR. As retrospective samples were qualified prior to enrollment, they were not subject to additional discrepant analysis.

Statistical analysis. The prevalence for each target was defined as the number of prospective specimens positive by standard culture or EIA reference methods divided by the total number of compliant prospective specimens. As no true reference method was available, including clinical criteria to determine whether subjects had disease or did not have disease, percent agreement calculations rather than clinical sensitivity and specificity estimates were performed (16). Positive percent agreement (PPA) is the number of samples positive by both the reference and test methods divided by the number of samples positive by both methods plus the number positive by the reference method only. Negative percent agreement (NPA) is the number of samples negative by both the reference and the test methods divided by the number of samples negative by both methods plus the number negative by the reference method only. PPA and NPA were calculated with 95% confidence intervals for all samples with compliant results for both the reference and BD Max EBP methods. The chi-square test was used for comparisons of PPA and NPA for preserved and unpreserved specimen types and prospective and retrospective data sets when the number of values in denominator for comparison was ≥5. Otherwise, Fisher's exact test was performed.

RESULTS

A total of 4,242 stool specimens were collected. The age distribution of patients was as follows: 3.7% were <1 year of age, 10.3% were 1 to 4 years of age, 11.5% were 5 to 12 years of age, 10.5% were 13 to 18 years of age, 48.6% were 19 to 65 years of age, 15.3% were >65 years of age, and 0.1% were of an unknown age. Approximately 25% of specimens were from children <12 years of age. Eighty-one percent of specimens were collected prospectively ($n = 3,457$), and the remainder ($n = 785$) were retrospective samples. As not all specimens met study criteria for compliant results with all targets, the number of results for each target varies.

Results for prospective specimens. The results of the BD Max EBP assay compared to those of culture and Shiga toxin EIA for prospective specimens are shown in Table 1. Table 1 presents all data combined and stratified according to whether the specimen was preserved or not, prior to the resolution of discrepant results. PPA values were as follows: 97.8% for *Campylobacter* spp., 87.2% for *Salmonella* spp., 100% for *Shigella* spp., and 80.0% for Shiga toxins. PPA values tended to be higher for unpreserved specimens than for preserved specimens. However, differences were not statistically significant for any of the four targets. NPA values for prospective specimens were as follows: 98.2% for *Campylobacter* spp., 99.1% for *Salmonella* spp., 99.7% for *Shigella* spp., and 99.3% for Shiga toxins (Table 1). Although the overall NPA was 98.2% for *Campylobacter* spp., more false-positive results were noted for unpreserved specimens than for preserved specimens.

Results for retrospective specimens. Combined results and data for retrospective samples stratified according to whether the sample had been placed into preservative or not are shown in Table 2. PPA values were as follows: 97.0% for *Campylobacter* spp., 99.4% for *Salmonella* spp., 98.9% for *Shigella* spp., and 100% for Shiga toxins. Six samples were positive by culture and alternate PCR but were negative by BD Max EBP. These included four samples positive for *Campylobacter*, one positive for *Salmonella*, and one positive for *Shigella*. Four of the six samples were preserved specimens. There were no false-negative results for Shiga toxins

TABLE 1 Results for prospective specimens prior to resolution of discrepancies

Target and specimen type	No. of samples with BD Max EBP result with reference to culture/EIA				Total no. of samples	PPA (95% confidence interval)	*P* value for PPA[a]	NPA (95% confidence interval)	*P* value for NPA[a]
	True positive	False negative	False positive	True negative					
Campylobacter									
Total	44	1	51	2,797	2,893	97.8 (88.4–99.6)		98.2 (97.7–98.6)	
Preserved	24	1	21	1,681	1,727	96.0 (80.5–99.3)	1.000	98.8 (98.1–99.2)	0.010
Unpreserved	20	0	30	1,116	1,166	100 (83.9–100)		97.4 (96.3–98.2)	
Salmonella									
Total	34	5	26	2,857	2,922	87.2 (73.3–94.4)		99.1 (98.7–99.4)	
Preserved	16	3	15	1,721	1,755	84.2 (62.4–94.5)	0.951	99.1 (98.6–99.5)	0.950
Unpreserved	18	2	11	1,136	1,167	90.0 (69.9–97.2)		99.0 (98.3–99.5)	
Shigella									
Total	24	0	10	2,889	2,923	100 (86.2–100)		99.7 (99.4–99.8)	
Preserved	7	0	5	1,743	1,755	100 (64.6–100)	1.000	99.7 (99.3–99.9)	0.732
Unpreserved	17	0	5	1,146	1,168	100 (81.6–100)		99.6 (99.0–99.8)	
Shiga toxins									
Total	8	2	17	2,347	2,374	80.0 (49.0–94.3)		99.3 (98.9–99.6)	
Preserved	6	2	12	1,701	1,721	75.0 (40.9–92.9)	1.000	99.3 (98.8–99.6)	1.000
Unpreserved	2	0	5	646	653	100 (34.2–100)		99.2(98.2–99.7)	

[a] *P* value for testing of the significance of the difference between preserved and unpreserved specimens.

among retrospective samples. The NPA was ≥99.5% for all targets. Three unpreserved samples were positive by BD Max EBP but negative by culture and alternate PCR. These samples included two samples in which *Campylobacter* was detected and one that was positive for *Salmonella* spp. Among retrospective specimens, no false-positive results were determined for *Shigella* spp. or Shiga toxins.

Combined results after discrepant analysis. Prior to the resolution of discrepant results, PPA and NPA values for all sample types and prospective and retrospective samples combined were as follows: 97.2% and 98.4% for *C. jejuni* and *C. coli*, 97.1% and 99.2% for *Salmonella* spp., 99.1% and 99.7% for *Shigella* spp., and 97.4% and 99.3% for Shiga toxins, respectively. (Percentages were calculated from combined total data in Tables 1 and 2.) After

TABLE 2 Results for retrospective specimens

Target and specimen type	No. of specimens with BD Max EBP result with reference to culture/EIA confirmed by alternate PCR				Total no. of specimens[a]	PPA (95% confidence interval)	*P* value for PPA[b]	NPA (95% confidence interval)	*P* value for NPA[b]
	True positive	False negative	False positive	True negative					
Campylobacter									
Total	129	4	2	372	507	97.0 (92.5–98.8)		99.5 (98.1–99.9)	
Preserved	64	2	0	151	217	97.0 (89.6–99.2)	1.000	100 (97.5–100)	0.657
Unpreserved	65	2	2	221	290	97.0 (89.8–99.2)		99.1 (96.8–99.8)	
Salmonella									
Total	166	1	1	450	618	99.4 (96.7–99.9)		99.8 (98.8–100)	
Preserved	105	1	0	213	319	99.1 (94.8–99.8)	1.000	100 (98.2–100)	1.000
Unpreserved	61	0	1	237	299	100 (94.1–100)		99.6 (97.7–99.9)	
Shigella									
Total	91	1	0	451	543	98.9 (94.1–99.8)		100 (99.2–100)	
Preserved	50	1	0	187	238	98.0 (89.7–99.7)	1.000	100 (98.0–100)	1.000
Unpreserved	41	0	0	264	305	100 (91.4–100)		100 (98.6–100)	
Shiga toxins									
Total	66	0	0	90	156	100 (94.5–100)		100 (95.9–100)	
Preserved	41	0	0	79	120	100 (91.4–100)	1.000	100 (95.4–100)	1.000
Unpreserved	25	0	0	11	36	100 (86.7–100)		100 (74.1–100)	

[a] The number of specimens varies by target due to differences in compliance with the protocol and unresolved results.
[b] *P* value for testing of the significance of the difference between preserved and unpreserved specimens.

TABLE 3 Combined results after resolution of discrepancies

Target and specimen type	No. of specimens with BD Max EBP result with reference to culture/EIA or alternate PCR[a]				Total no. of specimens[c]	PPA (95% confidence interval)	*P* value for PPA[d]	NPA (95% confidence interval)	*P* value for NPA[d]
	True positive	False negative	False positive	True negative					
Campylobacter									
Total	195	5	31	3,170	3,401	97.5 (94.3–99.2)		99.0 (98.6–99.3)	
Prospective	66	1	29	2,797	2,893	98.5 (91.3–99.7)	0.867	99.0 (98.5–99.3)	0.525
Retrospective[b]	129	4	2	373	508	97.0 (92.5–98.8)		99.5 (98.1–99.9)	
Salmonella									
Total	219	6	8	3,307	3,540	97.3 (94.3–99.0)		99.8 (94.5–99.9)	
Prospective	53	5	7	2,857	2,922	91.4 (81.0–97.1)	0.005	99.8 (99.5–99.9)	1.000
Retrospective	166	1	1	450	618	99.4 (96.7–99.9)		99.8 (98.8–100)	
Shigella									
Total	124	1	1	3,340	3,466	99.2 (95.6–99.9)		100 (99.8–100)	
Prospective	33	0	1	2,889	2,923	100 (89.3–100)	1.000	100 (99.8–100)	1.000
Retrospective	91	1	0	451	543	98.9 (94.1–99.8)		100 (99.2–100)	
Shiga toxins									
Total	85	0	8	2,437	2,530	100 (95.7–100)		99.7 (99.4–99.9)	
Prospective	19	0	8	2,347	2,374	100 (82.2–100)	1.000	99.7(99.3–99.9)	1.000
Retrospective	66	0	0	90	156	100 (94.5–100)		100 (95.9–100)	

[a] For *Campylobacter* spp., *Salmonella* spp., and *Shigella* spp., a positive result by either culture or alternate PCR was considered a true-positive result. For the resolution of discrepant results for Shiga toxins, results of alternate PCR were considered true-positive results.
[b] Results for all retrospective samples were confirmed by alternate PCR and bidirectional sequencing prior to testing with the BD Max EBP assay. Discrepancies were not further resolved.
[c] The number of prospective specimens varies by target due to differences in compliance with the study protocol and unresolved results.
[d] *P* value for testing of the significance of the difference between prospective and retrospective data sets.

discrepant analysis, PPA and NPA values (with 95% confidence intervals) were as follows: 97.5% (94.3 to 99.2%) and 99.0% (98.6 to 99.3%) for *Campylobacter* spp., 97.3% (94.3 to 99.0%) and 99.8% (94.5 to 99.9) for *Salmonella* spp., 99.2% (95.6 to 99.9%) and 100% (99.9 to 100%) for *Shigella* spp., and 100% (95.7 to 100%) and 99.7% (99.4 to 99.9%) for Shiga toxins, respectively (Table 3). When prospective and retrospective samples were compared for each target, PPA and NPA were similar for all targets, with the exception of PPA for *Salmonella* spp.

Discrepant results for prospective specimens are detailed in Tables 4 and 5. Six samples were positive by culture but negative by BD Max EBP. One culture grew *Campylobacter* and five grew *Salmonella*, which were not detected by either BD Max EBP or alternate PCR. Four of these six samples were preserved samples. Two samples were positive by Shiga toxin EIA and negative by both BD Max EBP and alternate PCR. There were no samples that were positive by culture and negative by the BD Max EBP assay for *Shigella*.

Prior to the resolution of discrepancies, numbers of false-positive results for prospective samples with the BD Max EBP assay were 51 for *Campylobacter* spp., 26 for *Salmonella* spp., 10 for *Shigella* spp., and 17 for Shiga toxins. Discrepant analysis demonstrated that many samples were likely falsely negative by culture or Shiga toxin EIA. The numbers of discrepant samples positive by alternate PCR were 22/51 (43.1%) for *Campylobacter* spp., 19/26 (73.1%) for *Salmonella* spp., 9/10 (90%) for *Shigella* spp., and 9/17 (52.9%) for Shiga toxins (Table 5).

PPA for culture compared to consensus results. Specimens positive by BD Max EBP and by alternate PCR and sequencing but negative by culture or EIA (Table 5) are likely to be true-positive results that were not detected by culture due to a loss of organism viability or a low organism burden. The PPA for culture was calculated for all specimen types combined. With the definition of a true-positive result being a specimen positive by either culture/EIA or BD Max EBP (confirmed by alternate PCR), the PPAs for

TABLE 4 Discrepant results for prospective samples negative by BD Max EBP[a]

Target	No. of BD Max EPB-negative specimens with result		
	Culture positive	EIA positive	Alternate PCR negative
Campylobacter	1	NA	1
Salmonella	5	NA	5
Shiga toxin	NA	2	2

[a] NA, not applicable.

TABLE 5 Discrepant results for prospective samples positive by BD Max EBP[a]

Target	No. of BD Max EPB-positive specimens with result		
	Culture negative	EIA negative	Alternate PCR positive
Campylobacter	51	NA	22
Salmonella	26	NA	19
Shigella	10	NA	9
Shiga toxin	NA	17	9

[a] NA, not applicable.

TABLE 6 Unresolved results

Testing site	% of unresolved test results			
	Initial	Initial SBT (1st repeat)	2nd SBT (2nd repeat)	2nd SBT (3rd repeat)
A	3.5	0.7	0.7	0.4
B	7.7	1.6	1.2	0.8
C	10.0	4.5	3.2	2.6
D	4.7	0.6	0.4	0.4
E	2.6	0.1	0.0	0.0
F	4.1	1.1	1.1	0.7
G	4.7	1.7	1.0	0.5
H	5.0	1.0	0.2	0.2
Overall	5.0	1.3	0.9	0.6

culture/EIA for each target were determined to be 90.1% for *Campylobacter* spp., 92.2% for *Salmonella* spp., 93.2% for *Shigella* spp., and 90.4% for Shiga toxins.

Prevalence and coinfections. Based on culture and EIA reference methods, the prevalences of each target in prospective samples were as follows: 45/2,893 samples (1.5%) for *Campylobacter* spp., 39/2,922 (1.2%) for *Salmonella* spp., 24/2,923 (0.8%) for *Shigella* spp., and 10/2,374 (0.4%) for Shiga toxin. With the addition of samples positive by BD Max EBP and alternate PCR, the prevalences of each target in prospective specimens were as follows: 67/2,893 (2.3%) for *Campylobacter* spp., 58/2,922 (2.0%) for *Salmonella* spp., 33/2,923 (1.1%) for *Shigella* spp., and 19/2,374 (0.8%) for Shiga toxin.

Ten coinfections were detected with the BD Max EBP assay. None were detected by culture. Discrepant analysis confirmed four coinfections. No significant patterns of coinfection were observed. *Campylobacter* spp. were present in six coinfections, *Salmonella* spp. were present in five, *Shigella* spp. were present in three, and Shiga toxins were present in six.

Unresolved results. Samples were considered unresolved if the SPC was not amplified and detected. Initially, the overall rate of unresolved results was 5.0% and was highest for testing sites B (7.7%) and C (10.0%) (Table 6). After a single repeat test from the same SBT, the overall rate of unresolved results was much lower (1.3%). Repeat testing from a redilution of original specimen in a new SBT lowered rates slightly more. A few more specimens could be resolved by a third repeat test, performed with the new SBT. The rate of unresolved results for site C remained higher (2.6%) than that for all other sites (≤0.8%). Rates of unresolved results for unpreserved and preserved samples were 7.1% and 3.7%, respectively. The final rate of unresolved results after repeat testing for unpreserved specimens (1.2%) was higher than that for preserved specimens (0.3%) ($P = 0.0006$).

DISCUSSION

The BD Max EBP assay is a multiplex real-time PCR assay for the detection of *Campylobacter jejuni*, *C. coli*, *Salmonella* spp., *Shigella* spp., and Shiga toxin 1 and 2 genes. The target used for the detection of *Shigella* is also found in enteroinvasive *E. coli* (EIEC), potentially allowing the detection of this pathogen as well. However, detection of EIEC was not specifically assessed. The BD Max EBP assay is performed with the BD Max instrument, a walkaway instrument that conducts all steps of the molecular assay from extraction to interpretation of results. Compared to routine stool culture, laboratories have demonstrated significant reductions in turnaround time with the use of molecular assays (17, 18). Additionally, the CDC recommends culture-based screening for *E. coli* O157 and EIA or molecular methods for Shiga toxins (19). Some laboratories also use EIAs to increase the sensitivity of detection of *Campylobacter* species (20). While the addition of EIA methods improves the detection of pathogens, EIA methods complicate the workflow of routine stool assessment and increase hands-on time. Thus, the BD Max EBP assay can save technical effort and improve the time to reporting of results.

After the resolution of discrepant samples, the overall PPA for the BD Max EBP assay compared to culture and alternate PCR was ≥97.3% for all targets. The BD Max EBP assay was more sensitive than culture, detecting an additional 22 specimens with *Campylobacter* spp., 19 specimens with *Salmonella* spp., 9 specimens with *Shigella* spp., and 9 specimens with Shiga toxins from among specimens collected prospectively. A review of the literature indicates that the performance of the BD Max EBP assay is similar to those of other multiplex assays developed recently. The sensitivity and specificity of the Prodesse ProGastro SSCS assay compared to culture and EIA determined by one multicenter study of 1,244 specimens were 100% and ≥99.4%, respectively, for all four bacterial targets (9). Early studies of the Luminex xTAG GPP assay demonstrated sensitivities ranging from 83 to 93% for *Salmonella* spp., 90 to 97% for *Campylobacter* spp., 93 to 100% for *Shigella* spp., and 94% to 100% for STEC (6, 21). The reported sensitivities based on a recent large multicenter study were higher for some targets with prospective samples (22). The FilmArray GI panel was evaluated in a multicenter study of 1,556 samples. The reported sensitivities were as follows: 100% for *Salmonella* spp., 95.9% for *Shigella* spp., 97.1% for *Campylobacter* spp., and 100% for Shiga toxins (23). An assessment of 611 prospective and 839 contrived specimens was conducted with the Verigene Enteric Pathogens test. The reported sensitivities were 96.5% for *Salmonella* spp., 93.0% for *Campylobacter* spp., 100% for *Shigella* spp., 100% for stx_1, and 97.4% for stx_2 (11). These studies demonstrated that multiplex PCR assays always outperformed culture and have very good sensitivity. User preferences regarding target selection, throughput, and the technical expertise needed to perform the test will contribute largely to assay selection.

Of the 12 positive samples (5 positive for *Campylobacter*, 6 positive for *Salmonella*, and 1 positive for *Shigella*) not detected with the BD Max EBP assay, 1 *Campylobacter* and 5 *Salmonella* specimens were also not detected by the alternate PCR. Although a previous study of the analytical sensitivity of the BD Max EBP assay in comparison with culture for Cary-Blair medium-preserved stool specimens spiked with pathogens demonstrated a superior sensitivity of the BD Max EBP assay for all pathogens at every organism concentration (24), we hypothesize that the organism load in these study samples may have been close to the limiting dilution for detection by PCR. The limit of detection (LOD) for the BD Max EBP assay has been reported to be between 10 and 910 CFU/ml, depending on the target (15). The alternate PCR assay was based on a design reported previously by Cunningham et al. (12). The LOD for the alternate PCR was similar to that for the BD Max EBP assay and was reported to be between 16 and 990 CFU/ml for all targets with the exception of *C. coli* diluted in fresh stool. Although PCR assays are very sensitive and increase the overall number of positive samples detected, if low numbers of pathogens are present, the volume of the specimen tested can im-

pact detection due to sampling bias. The BD Max EBP assay uses only 10 μl of sample. When cultures are set up, a drop (~25 to 50 μl) is generally planted on a minimum of two agar media. However, neither the BD Max EBP assay nor the alternate PCR was performed on larger volumes of specimen to assess this hypothesis. A less likely explanation for false-negative results is the possibility that some isolates were misidentified or lacked the molecular target. The pathogens isolated in this study were not confirmed in a reference laboratory or assessed for the presence of the target by PCR. However, all laboratories were CLIA certified and used standard procedures for the reference methods.

With the exception of results for *Salmonella* spp. in the prospective arm, there was little difference in performance between prospective and retrospective data sets (Table 3). Reduced sensitivity for *Salmonella* in comparison to culture has been reported (12, 17, 21, 25). Cunningham et al. attributed reduced sensitivity to the enhancement of the culture method with a selenite enrichment broth (12). Selenite broth was used at three of the centers in our study, and three of the six false-negative results were from laboratories that routinely used selenite broth for culture of *Salmonella*. The use of an enrichment broth incubated overnight is recommended for improving the detection of group B *Streptococcus* in vaginal-rectal specimens whether PCR- or culture-based methods are used (26). Overnight enrichment may similarly enhance the detection of *Salmonella* from stool specimens by PCR. Evaluation of the benefit of a broth enrichment step prior to multiplex PCR for enteric pathogens may be warranted.

Two prospective specimens that were positive by the Shiga toxin EIA and negative by BD Max EBP were also negative by alternate PCR (Table 4). The analytical sensitivity of the ImmunoCard Stat! EHEC assay was reported to be 10^7 CFU/ml, and that of the Premier EHEC EIA was 10^6 CFU/ml (27). Given the limited analytical sensitivity of the EIA methods, it is unlikely that PCR was insensitive compared to the EIA. The specificity of the EIA has been reported to be 99.7% (27, 28), which is consistent with our data. These apparently false-negative BD Max EBP results may represent either false-positive EIA results or the presence of toxin in specimens in which stx_1 or stx_2 nucleic acids were degraded.

In the prospective arm, the PPA tended to be higher for unpreserved specimens, although differences between specimen types were not statistically significant. It is reasonable to expect specimen preservative to maintain viability and thereby enhance recovery. However, when the organism is in low numbers, preservative may dilute the target below the limit of detection. Since PCR assays do not require viable organisms, further study of the role of preservative for maintaining stool specimens submitted for PCR assays may be of value.

The NPA was very high and increased to ≥99% following assessment of discrepant prospective specimens. For all targets combined, 57% (59/104) of the apparent false-positive BD Max EBP results were confirmed by alternate PCR. Particularly striking were results for *Salmonella* and *Shigella*, where 73.1% and 90% of the apparent false-positive BD Max EBP results, respectively, were confirmed by discriminant analysis. Some BD Max EBP PCR-positive results were not supported by alternate PCR results. These included 31 specimens positive for *Campylobacter* spp., 8 specimens positive for *Salmonella* spp., 1 specimen positive for *Shigella* spp., and 8 specimens positive for Shiga toxins (results from Tables 2 and 5 combined). Apparent false-positive results with multiplex PCR assays may truly reflect enhanced sensitivity in the presence of targets in low copy numbers or the presence of nonviable organisms. False-positive results for *Campylobacter* are particularly notable and call the confirmatory method into question. The reported analytical sensitivities of the alternate PCR were as low as 16 CFU/ml for *C. jejuni* and between 400 and 4,000 CFU/ml for *C. coli*, depending on the matrix (12). Although a slightly modified version of the published assay was used in our study, the previously published results suggest that unless *C. coli* was responsible for the apparent false-positive BD Max results, the alternate PCR was adequately sensitive. Buchan et al. also reported that 7/13 (53.4%) specimens positive for *Campylobacter* spp. by the ProGastro SSCS PCR and negative by culture were not confirmed by an alternate PCR method (9). Buss et al. noted 5/24 (21%) samples false positive for *Campylobacter* by FilmArray that were not confirmed by alternate PCR, and Coste reported confirming 9/15 (60%) false-positive results by using an alternate PCR and EIA methods (23, 29). False-positive results could also be due to cross-reactivity of primers and probes with other targets. Stool is a vastly complex matrix full of nucleic acids from organisms that may be uncharacterized and that could potentially share sequences with enteric pathogens. Nine species of *Campylobacter* were evaluated in premarket specificity studies, and none were cross-reactive with the BD Max EBP (15). Other, less well-characterized or unculturable species that may or may not have a role in diarrheal disease could have cross-reactive potential. Finally, false-positive results may reflect laboratory contamination. However, prior to the start of the clinical study and each week thereafter, each testing laboratory performed a wipe test of 10 work area locations to assess and control for contamination of all targets. Of 5,520 total wipe tests, only 10 (<0.2%) were positive. All contamination events were resolved after cleaning. Thus, we do not believe that contamination contributed to our findings.

Initially, the overall rate of unresolved results for all targets and all sites combined was 5.0%. A repeat of the initial SBT lowered the rate of unresolved results to 1.3%. Although the results for some samples could be resolved by diluting the original sample in a new SBT and repeating the assay a second or third time, this process provided less benefit than did the initial repeat (Table 6). Study sites B and C had the highest rates of unresolved results. These two sites included only unpreserved specimens. Sites A and F included both preserved and unpreserved specimens. All other sites included only specimens preserved in Cary-Blair medium. The rate of unresolved results for unpreserved specimens was higher than that for specimens in preservative, providing a plausible explanation for the higher rates at sites B and C. It might be expected that dilution of the stool matrix in Cary-Blair medium would reduce the effects of inhibitory substances. Thus, after a single repeat test, an unresolved rate of slightly more than 1% may be expected if samples are not preserved, and a lower rate is expected if preserved samples are tested.

The combined prevalence of all 4 targets in prospective specimens increased from 3.9% based on culture for *Salmonella* spp., *Campylobacter* spp., and *Shigella* spp. and the Shiga toxin EIA to 6.2% with the additional positive results detected by the BD Max EBP assay. Large, multicenter studies conducted in North American laboratories reported prevalence rates similar to those detected by the Prodesse ProGastro SSCS assay (8.3%) (6) and the Luminex xTAG GPP assay (7.1%) (19). While prevalence rates remain relatively low, those studies all demonstrate the increased sensitivity of molecular over traditional methods.

Real-time PCR methods can bring great advances in turnaround time and analysis for multiple pathogens simultaneously. Not only are these assays more sensitive than routine culture and EIA methods, they are also more likely to detect coinfections, as evidenced in this evaluation and others (6, 10, 21). However, multiplex assays that report bacterial, viral, and parasitic targets simultaneously may generate results that were not requested by clinicians and create complexities in reporting as well as in interpretation (17, 29, 30). For example, detection of *Clostridium difficile* in colonized individuals who actually harbor an agent of acute bacterial enteritis may lead to overtreatment for this pathogen. Multiplex assays with many targets might be best suited to limited patient populations, such as international travelers, immunocompromised individuals, or those with highly suspect disease who have negative results from assays with more limited menus.

The strengths of this study are the large number of specimens tested and the use of multiple testing sites. Specimens were collected across the United States, in Canada, and in Mexico, providing an adequate number of positive samples and a broad population base. Although serotyping and strain typing were not performed, it is likely that large numbers of clones and serotypes are represented among the pathogens detected. Another strength was the inclusion of both preserved and unpreserved samples. The BD Max EBP assay has been cleared by the FDA for testing of both specimen types. The ability to test either specimen type may allow better flexibility in specimen collection practices than those required for some other methods. One potential weakness is the comparison to culture and EIA methods, which are known to lack sensitivity for enteric pathogens (6, 9, 12, 17). However, the use of alternate PCR and bidirectional sequencing allows a more direct comparison of similar methods for specimens that gave discrepant results. In summary, this large, multicenter clinical study demonstrated very good performance of the BD Max EBP multiplex PCR assay. The throughput and ease of use may provide advantages to many laboratories, improving the detection of bacterial stool pathogens and time to reporting of results.

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