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A multicenter evaluation of a sample to answer real-time PCR assay for toxigenic *C. difficile* in symptomatic subjects

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ABSTRACT

We evaluated the performance of the Luminex ARIES® *C. difficile* Assay on 984 stool specimens prospectively collected from patients being tested for CDI at 4 clinical laboratories in the United States. Results were compared to direct and enriched toxigenic culture. Positive percent agreement (PPA) of the ARIES® *C. difficile* Assay was 98.1% versus direct toxigenic culture, and sensitivity versus direct plus enriched toxigenic culture was 90.5%. Negative percent agreement (NPA) of the ARIES® *C. difficile* Assay against direct culture was 92.6%, and specificity versus direct plus enriched toxigenic culture was 95.8%. The ARIES® *C. difficile* Assay was also compared to the results of routine (molecular, antigen, and/or toxin) methods for *C. difficile* testing used at each institution. The PPA of the ARIES® *C. difficile* Assay ranged from 82.9% to 100%. NPA values against these commercial assays ranged from 94.5% to 100%.

1. Introduction

Clostridioides difficile (C. difficile) is an intestinal bacterium that causes symptoms ranging from mild diarrhea to life-threatening inflammation of the colon (Lawson et al., 2016). *C. difficile* infection (CDI) often occurs as a complication of antibiotic treatment and is the most common infectious cause of hospital-acquired diarrhea. Recently published data from an active population- and laboratory-based surveillance study funded by the U.S. Centers for Disease Control and Prevention estimated that *C. difficile* was responsible for 453,000 incident infections and was associated with approximately 29,000 deaths in the United States in 2011(Lessa, Winston, and McDonald, 2015). The health economic burden of CDI is also significant. In 2008, CDI may have resulted in as much as $4.8 billion in excess health care costs in acute-care facilities alone (Dubberke and Olsen, 2012). A systematic review of U.S.-based studies estimated the economic healthcare costs associated with primary CDI to be between $2871 and $4846 per case, while incremental cost estimates ranged from $13,655 to $18,067 per case for recurrent CDI (Ghantoji et al., 2010).

Various methods are available for testing for CDI. Laboratories have employed the 2-step algorithm that uses glutamate dehydrogenase (GDH) assays to screen for *C. difficile* in stool specimens followed by enzyme immunoassay (EIA), cell culture neutralization assay (CCNA) testing, toxigenic culture, or nucleic acid amplification test (NAAT) to identify toxin-producing *C. difficile*. Others have adopted a more sensitive NAAT approach as a stand-alone assay or in conjunction with EIA, CCNA testing, and toxigenic culture. Currently, there are a total of 19 NAATs that have been FDA cleared for the direct detection of *C. difficile* toxin genes, and as compared to other non–culture-based methods, NAATs are the most sensitive tests available. The reported sensitivities/positive percent agreements (PPAs) of these assays (against direct and combined direct and enriched toxigenic culture) range from 86.1% to 99.0% and 80.5% to 95.2%, respectively, and the specificity/negative percent agreement (NPA) values range from 87.6% to 97.0% and 92.5% to 98.9% (Nucleic Acid Based Tests, 2018). Thus, the performance among the platforms is generally comparable, although a single trial comparing them all has yet to be performed.

In this study, we assessed the diagnostic accuracy of the ARIES® *C. difficile* Assay in prospectively collected, deidentified, remnant stool

Abbreviations: PPA, positive predictive agreement; NPA, negative predictive agreement; CDI, *C. difficile* infection; CCNA, cell culture neutralization assay; EIA, enzyme immunoassay; NAAT, nucleic acid amplification test.

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specimens from patients suspected of having and being tested for CDI. The ARIES® *C. difficile* Assay uses Luminex's MultiCode® real-time PCR chemistry in combination with the Luminex ARIES® Systems. ARIES® Systems are capable of automated nucleic acid extraction and purification, real-time PCR detection of nucleic acid sequences, and data analysis. The ARIES® *C. difficile* Assay detects the *C. difficile* toxin A gene (*tcdA*) and toxin B gene (*tcdB*). The performance of the ARIES® *C. difficile* Assay was compared to direct and enriched toxigenic culture as the reference comparator. Subsequently, we compared the agreement of the ARIES® *C. difficile* Assay with results of the other NAAT tests routinely used at the clinical sites, including the Xpert *C. difficile*/*Epi* assay and BD Max C Difficile assay that detect the *tcdB* gene by real-time PCR, and the FilmArray® Gastrointestinal (GI) Panel which detects both *tcdA* and *tcdB*. ARIES® results were also compared to the combined GDH/toxin EIA algorithm that was in use at one of the clinical sites.

2. Material and methods

2.1. Institutional and ethics review

The present study was conducted under waiver of informed consent and Institutional Review Board or Research Ethics Board approval at all participating clinical institutions.

2.2. Clinical specimen collection and processing

A total of 1021 unique stool specimens from pediatric or adult patients were submitted for *C. difficile* testing between October 2016 and February 2017. Of these, 984 met the inclusion/exclusion criteria. All included specimens were unpreserved, unformed (liquid or soft) stools with a Bristol Stool Scale score of 5, 6, or 7 and had been submitted for *C. difficile* testing at 4 geographically diverse clinical institutions located in the United States (Tricore Reference Laboratories, Albuquerque, NM; Indiana University, Indianapolis, IN; Tampa General Hospital, Tampa, FL and Ohio State University, Columbus, OH). All clinical specimens (raw stool) were transported to the institutions' clinical laboratories in a refrigerated or room temperature state (2 °C to 25 °C) and processed for toxigenic *C. difficile* testing as per their standard procedures as described below (Standard of Care *C. difficile* Testing). Any leftover stool that met the study eligibility criteria was deidentified, blinded, and assigned a unique clinical study number by an individual at the site who was not directly involved in testing. Multiple aliquots were then generated and placed into sterile, leak-proof containers. The time from collection to preparation of aliquots was ≤24 h. One stool aliquot was placed into an Anaerobic Tissue Transport Medium (ATTM; Anaerobe Systems, Morgan Hill, CA) tube and stored at room temperature (20–25 °C) until sent to a centralized reference laboratory (Microbiology Specialists, Inc., Houston, TX) for reference toxigenic culture testing. A second aliquot was placed in a cryovial and kept at the clinical laboratory for ARIES® *C. difficile* Assay testing. This aliquot was stored refrigerated (2 to 8 °C) if tested with the ARIES® *C. difficile* Assay within 36 h of collection. If testing with the ARIES® *C. difficile* Assay could not be performed within this time frame, the specimen aliquot was frozen (−65 to −95 °C) until testing. All specimens were tested by the direct CCNA and enhanced toxigenic culture reference standard within 72 h of collection. Two additional specimen aliquots were placed in cryovials and stored frozen (−65 to −95 °C) at the clinical laboratories for potential retesting or discordant analysis.

2.3. ARIES® C. difficile Assay testing

The ARIES® *C. difficile* Assay (Luminex Corporation, Austin, TX) was performed on stool specimens at each participating clinical laboratory according to manufacturer's instructions. Prior to each assay run, unpreserved, raw stool samples were preprocessed using a provided ARIES® Stool Resuspension Kit. Briefly, 800 μL ARIES® Stool Resuspension Buffer was added to a 2-mL ARIES® Stool Resuspension Tube. Using a swab, the primary stool sample (approximately 160 μL or mg) was added to the 2-mL ARIES® Stool Resuspension Tube containing the ARIES® Stool Resuspension Buffer then vortexed for 15 s and centrifuged for 30 s. A total of 200 μL of the preprocessed stool was then pipetted into the sample chamber of an ARIES® *C. difficile* Assay cassette. Following loading of the sample, the cassette was placed into an ARIES® magazine then inserted into an ARIES® System which automatically associates the preloaded ARIES® *C. difficile* Assay program with the cassette. Once a run is started, a sample processing control (SPC) is automatically added to the sample chamber of the cassette to control for sample lysis, recovery of extracted nucleic acid, detection of inhibitory substances, and confirmation of PCR reagent integrity. Sample and SPC lysis, as well as isolation and purification of nucleic acids, are automated within the ARIES® System and the *C. difficile* Assay cassette. Purified nucleic acids are automatically transferred to the cassette's PCR tube that contains lyophilized *C. difficile* Master Mix for the PCR amplification step. The *C. difficile* Master Mix contains primers specific to *tcdA*, *tcdB*, and the SPC sequence. Total assay time, including extraction and PCR cycling, takes approximately 2 h. Following completion of each run, ARIES® *C. difficile* Assay results were reported as toxigenic *C. difficile* positive, negative, or invalid. Results are reported as positive when *tcdA*, *tcdB*, or both are positive, and the SPC is valid but individual target results are available in the detailed report. Any sample generating an invalid result was retested once with the assay.

2.4. Toxigenic culture

Direct and enriched toxigenic culture on all specimens was performed by Microbiology Specialists Inc. (Houston, TX). Stool specimens were plated on Cycloserine-Cefoxitin-Fructose Agar (CCFA) media and incubated at 35 °C for up to 2 days in an anaerobic environment (this is the direct culture plate;– CCFA-D). An aliquot of the stool was also inoculated in TAL (taurocholate) broth and incubated for 2 days at 35 °C in an anaerobic environment. Enriched TAL broth specimens were then cultured on CCFA plates at 35 °C in an anaerobic environment for up to 2 days (this is the enriched culture plate; CCFA-E). CCFA-D and CFFA-E plates were examined for colonies with morphology and characteristics consistent with *C. difficile*. If no presumptive *C. difficile* colonies were observed on the CCFA-D and CCFA-E plates, then no further testing was performed and the specimen was interpreted as “negative” by reference culture. Suspected isolated *C. difficile* colonies on CCFA-D and CCFA-E plates were further assessed by aerotolerance, vancomycin susceptibility, gas–liquid chromatography (GLC), and cytotoxicity testing. Briefly, single isolated colonies were plated onto a chocolate agar (CHOC) plate at a 35 °C aerobic environment (CO_2) and a blood agar plate (BAP) at 35 °C anaerobic environment for 2 days and examined for growth. Following 2 days of growth on the BAP plate, a single well-isolated colony was inoculated in chopped meat carbohydrate broth (CMC) and incubated anaerobically for another 2 days at 35 °C prior to GLC and cytotoxicity testing. A BAP check plate inoculated from the CMC broth was used for vancomycin susceptibility testing and to confirm colony purity. GLC results were reported as isocaproic acid, isovaleric acid, and isobutyric acid peaks present for a positive toxigenic *C. difficile* sample or isocaproic acid, isovaleric acid, and isobutyric acid peaks absent for a negative toxigenic *C. difficile* sample. Isolates were further characterized by cytotoxicity testing. The organism grown in CMC broth was used in a CCNA cytotoxicity assay with the *C. difficile* Toxin Detection System and MRC-5 cells from Quidel (San Diego, CA) and performed according to the manufacturer's instructions.

2.5. Discordant analysis with bidirectional sequencing

Any discordant specimens where the ARIES® *C. difficile* Assay results were different from toxigenic culture results were assessed by bidirectional sequencing using analytically validated primers that

were directed against different *tcdA* and *tcdB* regions than those targeted by the ARIES® *C. difficile* Assay (unpublished). Sequencings assays were developed and validated according to U.S. FDA guidance and requirements and were designed to have an LoD of approximately 5 times less than that of the ARIES assay. Sequencing was performed by the Applied Genomics and Sequencing Laboratory (Luminex Corporation, Toronto, ON) with personnel blinded to all prior results. Discordant specimens were subjected to bidirectional sequencing using M13 forward and reverse primers and the Sanger dideoxy sequencing method to retrieve DNA sequences. A 10-μL loopful (approximately 100–150 mg) or 100-μL stool was added to 1 mL of easyMAG® Lysis Buffer (bioMérieux, Inc., Durham, NC) in aN SK38 bead lysis tube (Bertin Corp., Rockville, MD), vortexed for 5 min, held at room temperature for 10 min, and centrifuged for 2 min. A total of 850 μL of the pretreated stool was extracted by the bioMérieux NUCLISENS® easyMAG (Specific B protocol, version 2.0.1) and eluted in 50 μL, and 5 μL of the extracted nucleic acids was subjected to PCR using the QIAGEN HotStarTaq Plus PCR Kit (Qiagen, Hilden, Germany). Amplicons were then treated with Exonuclease I and Shrimp Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, MA) in order to remove unincorporated primers and deoxynucleoside triphosphates (dNTPs) left over from the PCR reactions. Dye-labeled terminator cycle sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, Walthman, MA). Any unincorporated dye was removed using the BigDye Xterminator® Purification Kit (ThermoFisher). Sample electrophoresis and sequencing analysis were performed on the *3730xl* Analyzer (ThermoFisher) using the *3730xl* Data Collection software (v 3.1.1) and Sequencing Analysis software (v 5.4). Sequences that 1) were at least 200 bases in length, 2) had a PHRED score greater than or equal to 20 for at least 90% of the bases, and 3) contained fewer than 5% ambiguous base calls were considered for further analysis using BLAST (NCBI). Acceptable matches to BLAST reference sequences were those with greater than 95% query coverage and identity and an Expected Value (E-Value) less than 10^{-30} when compared to the reference sequence.

2.6. Standard of care C. difficile testing

Clinical stool specimens collected at 3 of the 4 clinical sites were tested by the BD MAX C Difficile assay (BD Diagnostics, San Diego, CA), Xpert *C. difficile/Epi (*Cepheid, Sunnyvale, CA*)*, or the FilmArray GI Panel (BioFire Diagnostics, Salt Lake City, UT) as part of the standard of care (SOC). For specimens tested by FilmArray GI, raw stool is received by the laboratory and an aliquot transferred to Cary-Blair transport medium for BioFire testing. At 1 site, positive results by BD MAX C Difficile, Xpert *C. difficile/Epi*, and/or FilmArray GI Panel were confirmed by culture, with the exception of 5 positive specimens that were not confirmed. One institution used a composite diagnostic algorithm consisting of *C. DIFF QUIK CHEK COMPLETE®* test (Alere/Abbott, Abbott Park, IL) followed by confirmatory testing of positive GDH antigen or positive toxin only results by BD MAX C Difficile assay. BD MAX C Difficile, Xpert *C. difficile/Epi*, FilmArray GI Panel, and *C. DIFF QUIK CHEK COMPLETE* tests were performed on stool specimens as according to the manufacturers' instructions.

2.7. Data collection and analysis

Diagnostic sensitivity and specificity of the ARIES® *C. difficile* Assay for toxigenic *C. difficile* were assessed against both direct and enriched toxigenic culture as the reference standard. Subsequently, the agreement of the ARIES® *C. difficile* Assay against the standard molecular or immunoassay methods for *C. difficile* testing routinely used at each participating institution was calculated since all specimens tested by the ARIES *C. difficile* Assay had also been tested by the SOC methods at each site. Sensitivity or PPA was calculated by dividing the total number of "true-positive" ARIES® *C. difficile* Assay results (TP) by the sum of the TP and "false-negative" (FN) ARIES® *C. difficile* Assay results. Specificity or NPA was calculated by dividing the total number of "true-negative" ARIES® *C. difficile* Assay results (TN) by the sum of the TN and "false-positive" (FP) ARIES® *C. difficile* Assay results. An ARIES® *C. difficile* Assay result was considered to be a TP or TN result only if it agreed with the reference method. Confidence intervals of 95% were calculated by SAS Proc Freq using the F distribution method given in Collett (1991) and also described by Leemis and Trivedi (1996) (Collett, 1991; Leemis and Trivedi, 1996). Results from discordant bidirectional sequencing analysis were not used in any of the assay performance calculations.

3. Results and discussion

CDI has increased in frequency and severity in recent years due to the evolution of both the organism with emergence of hypervirulent strains, and host factors, such as advanced age, comorbidities, and recent healthcare exposure (DePestel and Aronoff, 2013). The lab diagnosis of *C. difficile* presents distinct challenges as there is an ongoing debate on the best testing methods that detect CDI and disease. While NAATs have become the predominant method used for diagnosis of CDI in the U.S., since they detect toxin genes and not the toxins, published literature has suggested that they lack clinical specificity and inflate CDI rates (Fang, Polage, and Wilcox, 2017). In 2016, Crobach et al. reported a meta-analysis that found that EIAs for toxin A/B were the most specific tests but GDH EIAs and NAATs were more sensitive, and thus, ESCMID recommends testing be performed according to a 2-step algorithm with NAAT or GDH EIA as the initial test followed by toxin EIA for specimens positive by the initial test (Crobach, 2016). IDSA guidelines reported in 2017 recommend a multistep algorithm (GDH plus toxin +/− arbitration by NAAT or NAAT + toxin) when preagreed institutional criteria for patient stool submission are lacking but NAAT alone or stool toxin test as part of a multistep algorithm when sample submission criteria are in place. Nonetheless, rapid and accurate diagnosis of CDI is essential both for improving outcomes of patients with CDI and for reducing nosocomial transmission in health care facilities.

The goal of the present study was to compare the diagnostic accuracy of the ARIES® *C. difficile* Assay to that of a reference standard consisting of direct and enriched toxigenic culture using residual specimens from 4 geographically diverse labs within the United States. A total of 984 prospectively collected unique, unformed stool specimens from pediatric or adult patients submitted for *C. difficile* testing that met the inclusion criteria were included in this study. Of these, 472 (48%) were collected from males and 512 (52%) were from females. Five specimens were from pediatric subjects at least 1 year of age but less than 12 years of age (0.5%), and 27 specimens were collected from individuals between the age of 12 and 21 (2.7%). Adults between the ages of 22 and 59 represented 47.0% of the study cohort (N = 462), while the remaining specimens were from subjects 60 years or older (N = 490; 49.8%). Approximately half of the specimens were collected from individuals who were hospitalized, presented to the institutions' emergency departments, or were residents of long-term care facilities (N = 505; 51.3%). Outpatients represented 48.7% of the study population.

ARIES® *C. difficile* Assay runs were performed at each clinical site on deidentified stool specimens that were either kept refrigerated (2–8 °C) for up to 36 h prior to testing (N = 875; 88.9%) or stored frozen (−70 °C) for up to 30 days prior to testing (N = 109; 11.1%). Of the 984 stool specimens tested, 28 generated invalid ARIES® *C. difficile* Assay results on the first attempt due to either run failure or instrument error (28/984; 2.8%). An additional 15 specimens were rerun by ARIES® *C. difficile* Assay because of either sample mix-up (N = 5) or improper sample storage or processing (N = 10). Thirty-eight of the 43 specimens that were rerun generated valid ARIES® *C. difficile* Assay results (i.e., positive or negative) after repeat testing. Five specimens remained invalid by ARIES® assay upon rerun. Four of these were negative and 1 was positive by toxigenic culture. A total of 17.1% (168/984) of unformed

stool specimens were reported as positive for toxigenic *C. difficile* by the ARIES® *C. difficile* Assay. By contrast, 10.8% ($N = 105$) and 15.0% ($N = 147$) of the specimens tested were positive by direct and direct plus enriched toxigenic culture, respectively.

Tables 1 and 2 summarize the performance characteristics of the ARIES® *C. difficile* Assay when compared to direct and direct plus enriched toxigenic culture, respectively. PPA of ARIES® *C. difficile* Assay against direct toxigenic culture was 98.1% (103/105; 95% confidence interval [CI], 93.3–99.5%). Compared to direct and enriched toxigenic culture, the sensitivity of the ARIES® *C. difficile* Assay was 90.5% (133/147; 95% CI, 84.6–94.2%). NPA for the ARIES® *C. difficile* Assay against direct toxigenic culture was 92.6% (809/874, 95% CI, 90.6–94.1%), and specificity against direct and enriched toxigenic culture was 95.8% (797/832, 95% CI, 94.2–97.0%). Fig. 1 shows the breakdown of ARIES results vs. that of direct and enriched toxigenic culture. Forty-six hospitalized patients and 55 outpatients were positive by direct toxigenic culture, and 69 hospitalized patients and 74 outpatients were positive by direct and enriched toxigenic culture. The ARIES® *C. difficile* Assay also tested positive for 95.7% (44/46) of hospitalized and 100% (55/55) of outpatients that tested positive by direct toxigenic culture. As compared to the direct and enriched toxigenic culture results, 87% (60/69) of hospitalized and 93.2% (69/74) of outpatients also tested positive by the ARIES® assay. The performance is comparable to other FDA-approved NAATs ($n = 19$) for the direct detection of *C. difficile* toxin genes. The reported sensitivities/PPAs of these assays (against direct and combined direct and enriched toxigenic culture) range from 86.1% to 99.0% and 80.5% to 95.2%, respectively, and the specificity/NPA values range from 87.6% to 97.0% and 92.5% to 98.9% (Nucleic Acid Based Tests, 2018). Thus, the performance among the NAAT platforms is generally comparable, although a single trial comparing them all has yet to be performed.

Table 3 shows the outcome of discordant analysis by bidirectional sequencing analysis using analytically validated primers that targeted genomic *tcdA* and *tcdB* regions distinct from that of the ARIES® *C. difficile* Assay. Two clinical specimens positive by direct toxigenic culture generated false-negative results with the ARIES® assay. One of these specimens was also *C. difficile* negative by bidirectional sequencing. However, since these specimens grew in culture, these were considered false negative by the ARIES® assay. When compared to direct and enriched toxigenic culture, 14 clinical specimens generated false-negative results by the ARIES® Assay. Of these, 13 specimens tested *C. difficile* negative by bidirectional sequencing. Of the 65 ARIES® *C. difficile* Assay positive specimens that were negative by direct toxigenic culture (i.e., ARIES® false positive), 30 were positive by enriched toxigenic culture. Eighteen of 30 ARIES® positive/enriched toxigenic culture positive/direct toxigenic culture negative, and 15 of 35 ARIES® positive/culture negative specimens were also positive by bidirectional sequencing. In all, 33 of the 65 ARIES positive/direct toxigenic culture negative specimens were also positive by bidirectional sequencing. Overall, discordant analysis by PCR/bidirectional sequencing agreed with the ARIES® test results as 13 of 14 ARIES® negative/culture positive specimens were also negative by bidirectional sequencing. ARIES Ct values for tcdA and tcdB were approximately 2 Ct lower for specimens that were positive by sequencing vs. negative by sequencing, and approximately 2–6 Ct lower for specimens that were positive by culture vs. negative by culture (data not shown). It is expected that a nucleic acid amplification assay would detect fewer numbers of organisms than could be recovered by culture.

Table 1
Comparison of the ARIES® *C. difficile* Assay to direct toxigenic *C. difficile* culture only.

ARIES® *C. difficile* Assay	Direct toxigenic culture		
	Positive	Negative	Total
Positive	103	65	168
Negative	2	809	811
Total	105	874	979[a]
		95% CI	
PPA	98.1%	93.3–99.5%	
NPA	92.6%	90.6–94.1%	

[a] Five specimens generated invalid results by the ARIES® *C. difficile* Assay after allowable rerun. Four of these were negative and 1 was positive by the reference method. All 5 of these specimens were excluded from the device performance calculations.

Table 2
Comparison of the ARIES® *C. difficile* Assay to direct and enriched toxigenic *C. difficile* culture.

ARIES® *C. difficile* Assay	Direct and enriched toxigenic culture		
	Positive	Negative	Total
Positive	133	35	168
Negative	14	797	811
Total	147	832	979[a]
		95% CI	
Sensitivity	90.5%	84.6–94.2%	
Specificity	95.8%	94.2–97.0%	

[a] Five specimens generated invalid results by the ARIES® *C. difficile* Assay after allowable re-run. Four of these were negative and 1 was positive by the reference method. All 5 of these specimens were excluded from the device performance calculations.

Subsequently, we also compared the results of the ARIES® *C. difficile* Assay to the various SOC molecular or immunoassay *C. difficile* testing methods being performed at the testing sites since the same specimens tested by the ARIES® *C. difficile* Assay were also tested according to the routine methods. These results are summarized in Table 4. When compared to BD MAX C Difficile, Xpert *C. difficile/Epi*, and FilmArray GI Panel, the PPA values of the ARIES® *C. difficile* Assay were 100% *(38/38;* 95% CI, 90.8–100%), 95.6% (65/68; 95% CI, 87.8–98.5%), and 90% (9/10; 95% CI 59.6–98.2%), respectively. All specimens that were reported as negative by the ARIES® *C. difficile* Assay but positive by Xpert *C. difficile/Epi* ($N = 3$) or FilmArray GI Panel ($N = 1$) tested negative by both direct and enriched toxigenic culture. NPA of the ARIES® *C. difficile* Assay was 99.1% (215/217; 95% CI, 96.7–99.8%) against BD MAX C Difficile, 97.4% (375/385; 95% CI, 95.3–98.6%) as compared to Xpert *C. difficile/Epi*, and 100% versus the FilmArray GI Panel (53/53; 95% CI, 93.2–100%). All specimens with false-positive results by the ARIES® *C. difficile* Assay ($N = 12$) were also negative by direct and enriched toxigenic culture.

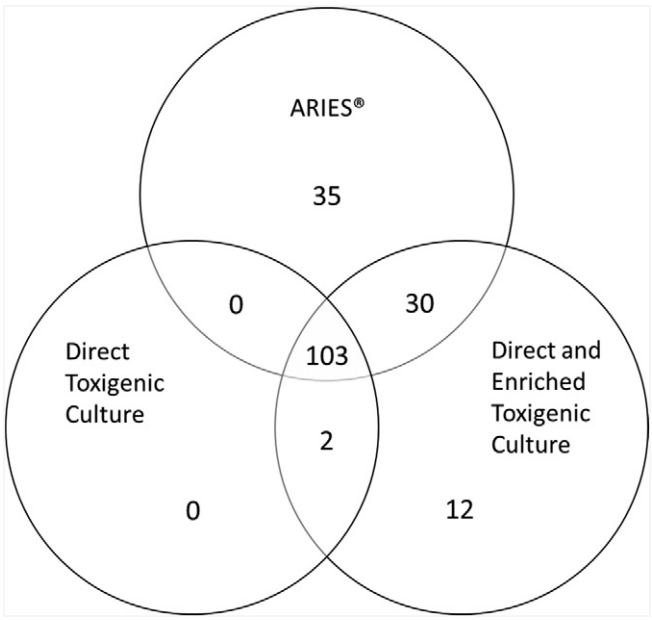

Fig. 1. Venn diagram of ARIES positive, direct toxigenic culture positive, and direct and enriched toxigenic culture positive specimens. Diagram shows the distribution of specimens positive for *C. difficile* by the test and comparator methods from this study. One specimen that was repeatedly invalid by the ARIES® assay but was positive by direct and enriched toxigenic culture was excluded from analysis.

Table 3
ARIES® *C. difficile* Assay and toxigenic culture discordant analysis.

Number of specimens	ARIES® result	Direct culture result	Enriched culture result	Sequencing result	Site SOC result
ARIES® result verified					
17	+	−	+	+	+
12	+	−	−	+	+
12	−	−	+	−	−
3	+	−	−	+	−
1	+	−	+	+	−
1	−	+	+	−	−
ARIES® result not verified					
12[a]	+	−	−	−	−
11	+	−	+	−	+
8[b]	+	−	−	−	+
1	+	−	+	−	−
1	−	+	+	+	+

[a] Three of these specimens were *tcdA*+ *tcdB*− by the ARIES® assay.
[b] One of these specimens was *tcdA*+ *tcdB*− by the ARIES® assay.

However, 1 of 2 and 2 of 10 ARIES® false-positive specimens (as compared to BD MAX C Difficile and Xpert *C. difficile/Epi*, respectively) were also positive for *C. difficile* by bidirectional sequencing, thereby confirming the ARIES® *C. difficile* Assay results.

At 1 institution, ARIES® *C. difficile* results were compared to a standard diagnostic algorithm consisting of the *C. DIFF QUIK CHEK COMPLETE* test followed by confirmatory testing of positive GDH antigen only or toxin only samples by the BD MAX C Difficile assay (Table 4). PPA and NPA of ARIES® *C. difficile* Assay versus this testing algorithm were 82.9% (34/41; 95% CI, 68.7–91.5%) and 94.5% (156/165; 95% CI, 90.0–97.1%), respectively. Seven ARIES® *C.difficile* negative specimens were positive for GDH only and confirmed positive by the BD MAX C Difficile assay. Six of the 7 ARIES® *C. difficile* Assay negative specimens were also negative by both direct and enriched toxigenic culture. One ARIES® false-negative specimen was positive by both direct and enriched toxigenic culture and by bidirectional sequencing. In addition, 6 of the 9 ARIES® *C. difficile* Assay positive specimens that were negative by the SOC diagnostic method used at the site (i.e., ARIES® false positive) were confirmed as positive by both direct and enriched toxigenic culture. Three ARIES® false-positive specimens were negative by both direct and enriched toxigenic culture and by bidirectional sequencing. While, in the comparison to the SOC test results, agreement with ARIES® was high, the lower PPA (82.9%) and NPA (94.5%) when compared with this diagnostic algorithm may be due to variability in the performance of the GDH test that may also be impacted by the types or strains circulating (Tenover et al., 2010; Lee et al., 2014). The latter undermines the reliability of the GDH as a screening test based on strains encountered.

In a review of the pathogenic properties of *C. difficile* toxins A and B, Di Bella et al. discuss how clinical isolates from CDI patients express either both toxins or TcdB alone and cases due to A+/B− strains have not been identified, suggesting TcdB is required for CDI pathogenesis in humans (Di Bella et al., 2016). However, the 2 toxins have been shown to have similar enzymatic activities (Voth and Ballard, 2005; Davies et al., 2011), and the difference in cytopathogenicity is due to differences in the efficiency of binding or delivery into the target cell (Pruitt and Lacy, 2012). The enzymatic activity of TcdA may yet be important in the pathogenesis of *C. difficile* since it inactivates proteins that regulate cell-cell junctions and therefore could be important for disruption of intestinal epithelium integrity (Di Bella et al., 2016). In the current study, 6 of the 168 specimens positive by the ARIES® *C. difficile* Assay were *tcdA*+/*tcdB*−. Of these, 4 were negative by both direct and enriched toxigenic culture, and 3 of these 4 were also negative by Xpert *C. difficile/Epi* (which targets *tcdB*). However, 1 specimen was positive by Xpert *C. difficile/Epi*. One of the 6 ARIES *tcdA*+/*tcdB*− specimens was positive by both direct and enriched toxigenic culture and by Xpert *C. difficile/Epi*. Finally, 1 of the 6 ARIES *tcdA*+/*tcdB*− specimens was positive by enriched toxigenic culture and negative by the C. DIFF QUIK CHEK COMPLETE diagnostic algorithm used at the site. We speculate therefore that the samples that tested *tcdA*+/*tcdB*− by the ARIES® assay vs. the results of the other test methods may be due to differences in limits of detection between the various testing methods.

Table 4
Comparison of the ARIES® *C. difficile* Assay and site standard of care results.

Comparator method	ARIES® result[a]				PPA	95% CI	NPA	95% CI
	True POS	False POS[b]	True NEG	False NEG[c]				
BD MAX C Difficile[d]	38	2	215	0	100%	90.8–100%	99.1%	96.7–99.8%
Xpert *C. difficile/Epi*[e,f]	65	10	375	3	95.6%	87.8–98.5%	97.4%	95.3–98.6%
FilmArray GI[g]	10	0	53	1	90.0%	59.6–98.2%	100%	93.2–100%
C. DIFF QUIK CHEK COMPLETE with confirmation of GDH or toxin only positives by BD MAX C Difficile[h]	34	9	156	7	82.9%	68.7–91.5%	94.5%	90.0–97.1%

[a] Five specimens generated invalid results by the ARIES® *C. difficile* Assay after allowable rerun. All of these specimens were negative by the SOC method(s) used at the sites.
[b] One of 2 and 2 of 10 ARIES® false-positive specimens (compared to BD MAX C diff and Xpert *C. difficile/Epi*, respectively) were positive for *C. difficile* by bidirectional sequencing. Six of the 9 ARIES® *C. difficile* Assay positive specimens that were negative by the *C. DIFF QUIK CHEK COMPLETE* diagnostic algorithm used at 1 of the sites were confirmed as positive by both direct and enriched toxigenic culture.
[c] All specimens that were reported as negative by ARIES® *C. difficile* Assay but positive by FilmArray GI Panel ($N = 1$) or Xpert *C. difficile/Epi* ($N = 3$) tested negative by both direct and enriched toxigenic culture. Six of the 7 ARIES® *C. difficile* Assay negative specimens that were positive by the *C. DIFF QUIK CHEK COMPLETE* diagnostic algorithm used at 1 of the sites were negative by both direct and enriched toxigenic culture. One ARIES® false-negative specimen was positive by both direct and enriched toxigenic culture and bidirectional sequencing.
[d] Indiana University, Indianapolis, IN, and Tampa General Hospital, Tampa, FL.
[e] The Ohio State University Wexner Medical Center, Columbus, OH, and Tampa General Hospital, Tampa, FL.
[f] Xpert *C. difficile/Epi* standard of care results were unavailable for 1 specimen.
[g] Tampa General Hospital, Tampa, FL.
[h] Tricore Reference Laboratories, Albuquerque, NM.

The comparison with other NAAT (and EIA) tests shows that the ARIES® *C. difficile* Assay is a sensitive and specific diagnostic tool for the detection of toxigenic *C. difficile* in stool specimens from subjects requiring CDI evaluation and is comparable to other commercial tests available. The *C. difficile* testing platforms and their ease of use vary considerably. Results can be obtained most rapidly and with less hands-on time and a simpler workflow by real-time PCR closed walk-away systems. The ARIES® *C. difficile* Assay requires only about 2.6 min/specimen total hands-on time and about 2 h of total turnaround time until results are available (Lawson et al., 2018). Studies have shown laboratory cost savings using strategies that incorporate NAAT or multistep algorithms (Culbreath et al., 2012; Schroeder et al., 2014), but more data are needed to demonstrate the impact of more sensitive NAATs on institutional costs and *C. difficile* transmission. NAATs should be used appropriately since they detect genes for toxin and not the toxin itself. Outcome studies and incorporation of patient data are necessary to ultimately determine the best diagnostic approach (McDonald et al., 2018).

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