Performance Evaluation of the Luminex ARIES® C. difficile Assay in Comparison to Two Other Molecular Assays within a Multi-Hospital Health Care Center

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Abstract (224 words)

Clostridioides difficile infections (CDI) remain a serious issue in the U.S. Both fast and accurate diagnosis of CDI is paramount to achieve immediate infection control initiation, triaging and isolation, as well as appropriate antibiotic treatment. However, both, over- and under-diagnosis can lead to adverse patient outcomes, such as unnecessary administration of antibiotics or unwanted spread of spores in any hospital setting, respectively. In this prospective study, we evaluated the FDA-cleared ARIES® C. difficile Assay and compared its performance and workflow characteristics to the BD MAX Cdiff and Xpert C.difficile/Epi assays. Out of 302 samples tested, 55 (18.2%) samples were positive, and 234 (77.5%) samples were negative for C. difficile by all three testing methods. Comparison results showed a positive and negative percent agreement (PPA and NPA) between ARIES® and Xpert of 95.2% (PPA, 59/62) and 99.2% (NPA, 238/240), respectively. PPA and NPA between ARIES® and BD MAX were 91.8% (56/61) and 96.6% (230/238), respectively. Invalid rates were determined to be 2.6% for BD MAX, 1.0% for ARIES®, and 0% for Xpert. Hands-on time (HoT) and total turnaround time (TAT) varied considerably depending on sample number and instrument throughput. HoT ranged from 1.2 – 3.5 minutes per sample and TAT was 1 – 2.3 hours. Overall, the results demonstrated that the ARIES® assay is a rapid and sensitive method for the diagnosis of CDI in clinical laboratories.
Introduction

*Clostridioides difficile* is an anaerobic, fastidious, spore-forming, Gram-positive bacillus that causes hospital-acquired diarrhea and antibiotic-associated pseudomembranous colitis. The bacterium produces harmful exotoxins, toxin A (TcdA) and toxin B (TcdB), that damage the colonic mucosa and are associated with a more severe form of the disease. According to the Centers for Disease Control and Prevention (CDC), *C. difficile* infection (CDI) is one of the most common hospital-acquired infections and is responsible for approximately 15,000 deaths annually in the U.S. (1). It has been estimated that CDI-associated hospital stays have increased by 9.2% between 2011 and 2015, and the average cost per case for hospital-onset CDI is about $34,157, which is 1.5 times the cost of community-onset CDI (2, 3). The clinical signs and symptoms presented by CDI are highly nonspecific such as mild to moderate diarrhea, nausea, and abdominal cramps, and therefore it is difficult to distinguish patients with and without CDI (4). Several testing methods are currently available for the diagnosis of CDI such as stool and toxigenic culture, cell cytotoxicity neutralization assay (CCNA), glutamate dehydrogenase (GDH) assay, toxin detection by enzyme immunoassays (EIA), and toxin gene detection by nucleic acid amplification tests (NAATs).

NAATs for the detection of *C. difficile* have rapidly evolved over the years, and currently, more than a dozen U.S. Food and Drug Administration (FDA) approved NAATs are commercially available (5). Most of these assays detect both *tcdA* and *tcdB* genes for their corresponding toxins or the *tcdB* gene for TcdB only. Recent investigations show, TcdB is a more virulent toxin than TcdA in several cell types; however, detection of both *tcdA* and *tcdB* may be important as the individual contributions of the toxins in CDI pathogenesis remain ambiguous (6, 7).
The ARIES® C. difficile Assay (ARIES®) (Luminex Corporation, Austin, TX) is an automated NAAT for the qualitative detection of the tcdA and tcdB genes from stool samples from patients suspected of having CDI. The assay uses Luminex® Corporation’s MultiCode® chemistry in combination with the Luminex ARIES® System, an automated platform that performs nucleic acid extraction and purification, real-time PCR for the detection of target-specific DNA, and data analysis. The aim of this study was to evaluate the performance characteristics and workflow of the ARIES® C. difficile Assay as compared to the Xpert® C. difficile/Epi (Xpert) (Cepheid, Sunnyvale, CA) and the BD MAX™ Cdiff (BD MAX) (Becton, Dickinson, Franklin Lakes, NJ) assays using de-identified, remnant stool samples.

Materials and Methods

Samples: A total of 302 selected de-identified remnant stool samples in Cary-Blair submitted for routine clinical testing by Xpert were included in the study. Samples tested by the Xpert assay were de-identified and re-numbered to mask the identity of the subject to the operators and investigator. The comparator method’s result prior to study testing was not known to the operators.

Study Design: This prospective comparison study was performed at Northwell Health Laboratories (Lake Success, New York, USA) under an IRB-approved protocol (# 17-08-269-03). De-identified remnant stool samples submitted for routine clinical testing by the Xpert assay occurred between July 1st and October 15th, 2017. The samples were subsequently tested by the ARIES® and the BD MAX assays. Any disagreement between all three NAAT assays was further investigated by toxin gene sequencing.
Molecular Testing: All assays were performed according to the individual manufacturer’s instructions. For ARIES®, unpreserved, raw stool samples were pre-processed using the ARIES® Stool Resuspension Kit. 800 μL of ARIES® Stool Resuspension Buffer was added to a 2 mL ARIES® Stool Resuspension Tube. The primary stool sample (~160 μL) was added using the ARIES® Stool Resuspension Swab. The tube was vortexed for 15 seconds, and centrifuged at 2000 x g for 30 seconds. A total of 200 μL of the pre-processed stool was pipetted into the sample chamber of the ARIES® C. difficile Assay cassette. The cassette was then placed into an ARIES® magazine which was inserted into an ARIES® System. Internal scanning automatically associates the preloaded ARIES® C. difficile Assay program with the cassette. Once the run is initiated, nucleic acid from the sample is extracted, purified, and amplified automatically within the ARIES® System and the C. difficile Assay cassette. Total assay time, including extraction and PCR cycling is approximately 2 hours. Following completion, the results were reported as either toxigenic C. difficile positive, negative, or invalid. None of the samples underwent a freeze-thaw cycle and all samples remaining after testing were frozen at < -70º C for discordant analysis and/or confirmatory testing as needed.

Discordant Analysis: All samples that showed disagreement with any of the three NAAT assays were further investigated using an alternative PCR with subsequent bidirectional sequencing. A 10 μL loopful (approximately 100-150 mg) of the stool sample was pretreated in 1 mL of easyMAG® Lysis Buffer (bioMérieux, Inc., Durham, NC), 850 μL of the pretreated stool was extracted by the bioMérieux NUCLISENS® easyMAG, and 5 μL of the extracted nucleic acids were subjected to singleplex PCR. Bidirectional sequencing was performed using M13 tagged forward and reverse primers that targeted different regions of tcdA and tcdB than the ARIES® C. difficile Assay. Following PCR, unincorporated primers and deoxynucleoside triphosphates
(dNTPs) were removed by treatment with Exonuclease I and Shrimp Alkaline Phosphatase (Thermo Fisher Scientific, Waltham, MA). The BigDye® Terminator v3.1 Cycle Sequencing Kit (ThermoFisher, Walthman, MA) was used to perform dye-labeled terminator cycle sequencing with detection on the 3730xl Analyser (ThermoFisher). BLAST analysis (NCBI) was conducted for sequences that were at least 200 bases in length, had a PHRED score greater than or equal to 20 for at least 90% of the bases, and contained fewer than 5% ambiguous base calls. Sequences with greater than 95% query coverage and identity, and an Expected Value (E-Value) less than $10^{-30}$ when compared to the reference sequence were considered positive. A weak positive result may be obtained if the sequence that was generated was specific to the target, but was only 100-199 nucleotides or was only detected in one direction.

**Time and Motion Analysis:** The workflow analysis of the Xpert, BD MAX, and ARIES® assays was conducted on one single day. Workflow analysis was performed for different run sizes (one, six, and twelve samples) to mimic potential different work-processes and volumes at other institutions. The following parameters were included: 1) hands-on time (HoT), including manual steps such as sample set up, initiation of the instrument, post-run tasks, and result reporting; and 2) total turnaround time (TAT) including HoT and hands-off or automation time (AuT). The time required for the different phases of the assay were recorded by two observers independent of performing the assay. All assays were performed by experienced medical technologists who were familiar with the instruments and had prior training on the assay procedures.

**Statistical Analysis:** Results were analyzed to determine the positive percent agreement (PPA) \[
\frac{\text{True Positive}}{\text{True Positive} + \text{False Negative}}\], negative percent agreement (NPA) \[
\frac{\text{True Negative}}{\text{True Negative} + \text{False Positive}}\] and the overall percent agreement for ARIES® with each of the comparator platforms. True positivity/negativity was determined as two of three
positives/negatives by any assay. Confidence intervals (CI) of 95% including continuity correction were calculated according to the procedure outlined by E. B. Wilson and described by Robert Newcombe (8, 9).

Results

Analytical Performance

Of the 302 samples included in this study, 55 (18.2%) samples were positive, and 234 (77.5%) samples were negative for *C. difficile* by all three assays. A total of 62 (20.5%) samples were positive by Xpert, 61 (20.2%) samples were positive by ARIES®, and 56 (18.5%) samples were positive by BD MAX (Table 1). The PPA, NPA, and overall agreement between the ARIES® and Xpert assays were 95.2% (59/62; 95% CI, 85.6%-98.7%), 99.2% (238/240; 95% CI, 96.7%-99.9%), and 98.3% (297/302), respectively (Table 2). The PPA, NPA, and overall agreement between the ARIES® and BD MAX assays were 98.2% (55/56; 95% CI, 89.2%-99.9%), 97.5% (236/242; 95% CI, 94.4%-98.9%), and 97.7% (291/298), respectively (Table 3). Comparison between the BD MAX and Xpert assays revealed an overall agreement of 98.0% (292/298; 95% CI, 95.7%-99.3%), and PPA and NPA of 90.3% (56/62; 95% CI, 79.5%-96.0%), and 100.0% (236/236; 95% CI, 98.0%-100.0%), respectively.

Eight (2.6%) samples yielded invalid results on the initial run by BD MAX. Seven of these samples were available for retesting, and four of them generated a valid negative result upon repeat testing. Three (1%) invalid results were obtained by the ARIES® assay; however, upon retesting, valid negative results were obtained. No samples yielded an invalid result with the Xpert assay.
Discordant Analysis

A total of 13 (4.3%) samples yielded discrepant results where the three NAAT assays were not in agreement. One sample was not further tested and was eliminated from the discordant analysis. The disposition of the 12 remaining discordant samples and analysis is shown in Table 4. Three samples tested negative by both ARIES® and Xpert but produced an invalid result with BD MAX even after retesting. Two of these samples were also negative by bidirectional sequencing and one sample produced a weak positive sequencing result for tcdA. A sequencing result was considered weak positive when the sequence generated was specific to the target, but was only 100-199 nucleotides or was only detected in one direction. Two samples tested negative by both ARIES® and BD MAX, but generated a positive result with Xpert and bidirectional sequencing. One specimen tested positive by both Xpert and BD MAX, but generated a negative result with ARIES® (ARIES® false negative). This specimen was also positive by bidirectional sequencing. Two specimens resulted negative by both Xpert and BD MAX, but generated a positive result for tcdB by ARIES. Upon sequencing, only one of these two specimens confirmed as tcdB positive. Finally, four samples tested positive with ARIES® and Xpert but generated negative results with BD MAX. Three of these samples were also positive by bidirectional sequencing.

Time and Motion Data

TAT and HoT varied depending on sample number and instrument throughput. The total HoT for testing one sample was less than 4 min (range 0:01:59-0:03:30 [h:min:sec]) for all three assays (Figure 1). The HoT for testing one sample was lowest for BD MAX (0:01:59) and was highest for the ARIES® assay (0:03:30). However, as the number of samples increased to six and twelve, the Xpert assay required the highest total HoT (0:14:43 and 0:28:20, respectively).
whereas ARIES® was 0:10:51 and 0:20:08, and the BD MAX assay was fastest at 0:7:33 and 0:14:15. The total TAT for testing one, six, and twelve samples was highest for the ARIES® assay (2:00:51, 2:08:29, and 2:18:07) and lowest for the Xpert assay (0:59:22, 1:15:38, and 1:35:09), provided sufficient bays are installed in the Xpert instrument (Figure 2). The TAT for BD Max was in between at 1:53:22, 1:59:24, and 2:07:03, respectively, for testing one, six, and twelve samples.

Discussion

In this study, we compared the performance and workflow characteristics of three commercially available NAATs for *C. difficile* detection – the ARIES® *C. difficile* Assay, the Xpert *C. difficile/Epi* assay, and the BD MAX Cdiff assay. Three hundred and two stool samples were collected from patients suspected of having CDI, and 55 (18.2%) samples were identified as positive for *C. difficile* by all three molecular assays. Analytical results demonstrated that all three molecular assays had comparable sensitivity and specificity (>91% PPA and >98% NPA), and were in agreement for 289 (95.7%) of the samples. All three molecular platforms had a relatively low invalid rate (< 3%), and these invalid rates data were in agreement with previous studies (10, 11).

Approximately 4% of the samples had discrepant results among the three molecular methods. One specimen tested negative by both ARIES® and Xpert, and was invalid on BD MAX, but generated a positive result with bidirectional sequencing. However, the sequencing assay was weakly positive only for *tcdA* (1 of 2 replicates, 1 primer set only), indicating that the pathogen concentration might have been below the limit of detection for ARIES®. The Xpert
assay can only detect tcdB and therefore generated a negative result for this sample. Additionally, since the BD MAX assay was repeatedly invalid for this specimen, sample inhibition may have also contributed to the discrepant results. Two samples tested positive by Xpert but generated a negative result by ARIES® and BD MAX. Bidirectional sequencing for these samples was also positive. However, the sequencing assay uses more of the specimen than ARIES®, undergoes a separate, off-board extraction, and is performed in singleplex, which may account for a higher sensitivity. Differences in the limits of detection (LOD) for the three assays might also be responsible for the discrepancies in the test results. According to the analytical performance data described in the U.S. FDA 510(k) summaries for these assays, the reported LODs of ARIES®, BD MAX and Xpert are 5.10 CFU/cassette, 265 CFU/loop, and 460 CFU/swab, respectively (https://www.accessdata.fda.gov). This suggests that ARIES® is the most sensitive of the three assays which could account for the ARIES® “false positive” results. However all three assays are highly sensitive and show very similar performance characteristics in this study.

Analysis of the time and motion data revealed that the BD MAX assay has the lowest HoT for testing one sample, followed by the Xpert and ARIES® assays. As the number of samples increased to six and twelve, the Xpert assay demonstrated the highest HoT, followed by ARIES® and BD MAX. The TAT was lowest for the Xpert assay, followed by BD MAX and ARIES®; however, TAT can differ considerably depending on the number of systems, modules or bays installed for each platform. The ARIES® and BD MAX systems include two bays or modules and can test 12 and 24 samples at a time, respectively. The Xpert system is available in 1, 2, 4, 16, 48, or 80-module configurations and therefore, the TAT could be considerable higher if fewer modules are installed than can accommodate the sample load. For the BD MAX assay,
the higher invalid rate compared to ARIES® and Xpert may further increase the TAT, if repeat testing is considered in the TAT calculations.

The diagnostic paradigm for *C. difficile* has evolved and changed in recent years but the optimal diagnostic method for CDI is still a controversial subject (12). Diagnosis and management of CDI is particularly challenging because of nonspecific clinical symptoms, high rates of asymptomatic colonization with both toxigenic and nontoxigenic strains of *C. difficile*, and the difficulties associated with identifying unstable exotoxin (13). According to the diagnostic guidelines published by the European Society of Clinical Microbiology and Infectious Diseases (ECSMID) and Infectious Diseases Society of America (IDSA), effective diagnosis of CDI requires both clinical presentation of the symptoms and confirmatory laboratory evidence of *C. difficile* exotoxin or toxin producing *C. difficile* strains in stool samples (14, 15). EIA assays for toxins A and B are rapid and relatively inexpensive, but have demonstrated poor sensitivity (47%-72%) when compared to molecular tests (16, 17). Additionally, no correlation has been observed between the active stool toxins measured by EIAs and disease severity in CDI (18). CCNA and toxigenic cultures are more sensitive than EIA; however, these assays are labor intensive and time-consuming and can have a turnaround time of up to 3 days (19). GDH assays have demonstrated >90% sensitivity and specificity when compared to culture and are commonly used as a screening test (20). However, since GDH is expressed by both toxigenic and nontoxigenic strains of *C. difficile*, confirmatory tests such as NAATs or toxin assays are required for positive results. NAATs have demonstrated higher sensitivity when compared to currently available toxin assays (92.2% - 97.7% vs. 42.3% - 82.8%), and the more rapid diagnosis can positively impact patient care by decreasing empirical therapy among patients without CDI, from 13.6% to 5.6%
Additionally, since NAATs can detect more toxigenic *C. difficile* cases, implementation of these assays can facilitate improved infection control measures, thereby reducing the overall infection rate of the clinical institution (23, 24). Although, most clinical laboratories have shifted from toxin assays to NAATs, the clinical utility of NAATs is still under debate for diagnosing CDI (12). Since NAATs target the toxin genes regardless of toxin production, a NAAT positive result does not always indicate clinical disease. Recent studies have demonstrated that relying exclusively on NAATs for the diagnosis of CDI can result in overdiagnosis, overtreatment, and an increase in healthcare costs (25, 26). To address this problem, current ECSMID guidelines recommends a multistep algorithm for diagnosing CDI that includes confirmation of a positive result by the first diagnostic test by one or two other confirmatory tests or reference methods (14). However, the latest guidelines from the IDSA recommend that NAATs can be used as a standalone diagnostic test in cases where there are pre-agreed institutional criteria for patient stool submission (15). Thus, as there is still lack of consensus regarding the best diagnostic method for CDI, clinical laboratories should develop and optimize their own diagnostic algorithm based on their patient population, CDI severity, and prevalence rate.

This study demonstrated that the ARIES® *C. difficile* Assay has comparable performance and workflow characteristics when compared to other established molecular CDI diagnostic tests. The ARIES® assay also provides wider flexibility with a unique mixture of batched and ‘sample-to-answer’ testing. It can be concluded that for the diagnosis of CDI, NAATs such as ARIES®, Xpert, or BD MAX can provide highly sensitive and specific results in a considerably shorter time when compared to other available non-molecular methods. However, future studies evaluating the different diagnostic algorithms involving NAATs are required to understand the complete potential of these assays in CDI diagnosis.
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Table 1. Analysis of test results for all samples using the ARIES®, Xpert and BD MAX assays

| Samples | ARIES® | Xpert | BD MAX |
|---------|--------|-------|--------|
| 55      | +      | +     | +      |
| 4       | +      | +     | -      |
| 2       | +      | -     | -      |
| 2       | -      | +     | -      |
| 1       | -      | +     | +      |
| 3       | -      | -     | Invalid* |
| 1       | -      | -     | Not determined† |
| 234     | -      | -     | -      |

*These specimens remained invalid after repeat testing.

†This specimen was invalid on initial testing and was not retested.

Table 2. Performance characteristics of the ARIES® assay compared to the Xpert assay.

| ARIES® | Xpert |
|--------|-------|
|        | Positive | Negative | Total |
| Positive | 59       | 2       | 61    |
| Negative | 3       | 238     | 241   |
| Total   | 62      | 240     | 302   |

95% CI

PPA 95.2% 85.6%-98.7%
Table 3. Performance characteristics of the ARIES® assay compared to the BD MAX assay.

| ARIES® | BD MAX |
|--------|--------|
|        | Positive | Negative | Total |
|        | 55       | 6        | 61    |
|        | 1        | 236      | 237   |
| Total  | 56       | 242      | 298   |

*95% CI*

PPA 98.2% 89.2%-99.9%
NPA 97.5% 94.4%-98.9%

*Four samples generated invalid results by BD MAX. Three of these samples remained invalid after repeat testing. One sample was not further tested. All four samples tested negative by the ARIES® and Xpert assays.

*Confidence Interval

Table 4. Results of discordant analysis for discrepant samples.

| Sample ID | ARIES® | Xpert | BD MAX | Bidirectional Sequencing | Final Call |
|-----------|--------|-------|--------|--------------------------|------------|
| 65        | -      | -     | Invalid| -                        | Negative   |
|     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|
| 423 | -   | -   | -   | -   | -   |
| 109 | -   | +   | -   | +   | tcdA, tcdB | Positive |
| 131 | +   | +   | tcdA, tcdB | Positive |
| 209 | tcdB | -   | -   | -   | Negative |
| 243 | tcdB | -   | -   | + (tcdB) | Positive |
| 232 | tcdA, tcdB | +   | -   | -   | Positive |
| 226 | tcdA, tcdB | +   | -   | -   | Positive |
| 117 | tcdB | +   | -   | + (tcdB) | Positive |
| 207 | tcdA | +   | -   | + (tcdA, tcdB) | Positive |

**Figure 1.** Comparison of Hands-on Time (HoT) for *C. difficile* Test Systems.

**Figure 2.** Comparison of Total Turnaround Time (TAT) for *C. difficile* Test Systems. The hands-on time (HoT) is shown in light-shaded bars and the automation time is shown in the dark-shaded bars.
Comparison of Hands-on Time (HoT) for *C. difficile* Test Systems

| Test Systems and Sample Numbers | Time in Minutes:Seconds |
|---------------------------------|-------------------------|
| ARIES (1 Sample)                | 03:30                   |
| Cepheid (1 Sample)              | 03:23                   |
| BD Max (1 Sample)               | 01:59                   |
| ARIES (6 Samples)               | 10:51                   |
| Cepheid (6 Samples)             | 14:43                   |
| BD Max (6 Samples)              | 07:33                   |
| ARIES (12 Samples)              | 20:08                   |
| Cepheid (12 Samples)            | 14:15                   |
| BD Max (12 Samples)             | 28:20                   |

Legend:
- Assay Set-Up
- Initiating Assay Run
- Post Run Tasks
