ABSTRACT

Clostridium difficile is a major cause of nosocomial antibiotic-associated infectious diarrhea and pseudomembranous colitis. Detection of C. difficile by anaerobic bacterial culture and/or cytotoxicity assays has been largely replaced by rapid enzyme immunoassays (EIA). However, due to the lack of sensitivity of stool EIA, we developed a multiplex real-time PCR assay targeting the C. difficile toxin genes tcdB. Stool samples from hospitalized pediatric patients suspected of having C. difficile-associated disease were prospectively collected. Three testing modalities were evaluated, including enriched culture, cepheid Xpert and real-time PCR (tcdB) on stool samples performed with tcdB gene-specific primers and hydrolysis probes. A total of 150 de-identified clinical specimen were analyzed. The sensitivities of stool real-time PCR were 95% against cepheid Xpert C. difficile and 93% against enriched culture respectively, with a specificity of 97% and 94%. The lower limit of detection of the stool real-time PCR was 0.5 cFU/ml of per reaction for tcdB. Direct detection of C. difficile toxin genes in stool samples by real-time PCR showed performance comparable to enriched culture. Real-time PCR of DNA from stool samples is a rapid and cost-effective diagnostic modality for patients that should facilitate appropriate patient management. (Int J Biomed Sci 2016; 12 (3): 83-88)

Keywords: Clostridium difficile; real-time PCR; enzyme immunoassays; tcdB

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INTRODUCTION

_Clostridium difficile_, a Gram-positive spore-forming bacillus, is the most common identifiable etiologic agent of antibiotic-associated diarrhea (13, 18). Initially described as a member of the commensal microbiota of neonates, _C. difficile_ was identified as a causal agent of antibiotic-associated diarrhea in the 1970s (3, 11). The clinical presentation of _C. difficile_-associated disease (CDAD) can range from asymptomatic carriage in the gastrointestinal tract, mild diarrhea, and potentially fatal pseudomembranous colitis (13, 18). Symptoms occur secondary to the production of two exotoxins, toxin A and toxin B, which disrupt the integrity of the colonic mucosa (24).

Alarming changes in the epidemiology of CDAD, including an increase in both the incidence and severity of the disease, have highlighted concerns about patterns of _C. difficile_ infection (17, 18, 19, 21). Analysis of U.S. hospital discharge data revealed that the national rates of CDAD doubled from 2000 to 2003 (17). In 2004, the Centers for Disease Control and Prevention reported that the mortality rate related to CDAD increased from 5.7 deaths per million individuals in 1999 to 23.7 deaths per million individuals (21). In addition to the profound morbidity and mortality, CDAD is also generating a substantial economic burden, with estimates ranging from $1.3 million to more than $3 billion annually (8, 16, 20). Due to the formidable impact of CDAD on the U.S. health care system, rapid and accurate diagnosis is essential for the timely enactment of infection control and treatment measures.

The changing epidemiology of _C. difficile_ infections in the pediatric population is a serious concern. While benign neonatal colonization with toxigenic _C. difficile_ is a well-documented phenomenon, recent studies have suggested an increased incidence of CDAD in children (2, 14, 22, 25). A large study encompassing data collected from 22 children’s hospitals in the United States reported an increased prevalence of CDAD in children, including infants (increased by 53% from 2001 to 2006, with 26% of patients with CDAD ≤1 year of age) (14). Utilizing CDAD data from the Agency for Healthcare and Research Quality, a similar study noted that the highest number of CDAD hospitalizations occurred in patients ≤1 year of age (25).

Initial strategies to detect _C. difficile_ consisted of anaerobic stool sample culture, usually with cycloserine-cefoxitin-fructose agar (CCFA) or a similar medium with or without a pretreatment alcohol shock step (7). Although this modality was quite sensitive and specific for detecting _C. difficile_, it took up to 5 days to confirm a negative culture and it did not discriminate between toxigenic and nontoxigenic isolates without further testing strategies. Furthermore, colonies with indeterminate colony characteristics were tested with L-proline-aminopeptidase (PRO Disc) or other biochemical tests to ensure the accurate identification of _C. difficile_ (9, 10). The development of the cell culture cytotoxicity assay circumvented stool sample culture by observing cytopathic effects of toxin B directly on cultured cells (4, 6). The cell culture cytotoxicity assay requires a neutralization step for specificity and maintenance of toxin-susceptible mammalian cell lines, and it takes 48 to 72 h to perform the assay (1, 5). Rapid antigen detection assays, consisting of common antigen testing (glutamate dehydrogenase) and toxin immunoassays, have largely replaced culture and the cytotoxic assay; however, neither type has the desired sensitivity or specificity to reliably confirm or rule out CDAD without the need for either serial testing or subsequent testing modalities. Therefore, real-time PCR is being investigated as the preferred diagnostic modality due to its rapid turnaround time and track record of superior sensitivity and specificity.

Toxigenic strains of _C. difficile_ contain a 19.6-kb pathogenicity locus (PaLoc) that includes five contiguous chromosomal genes responsible for the development of CDAD—_tcdABCDE_ (24). _tcdA_ and _tcdB_ encode exotoxins A (enterotoxin) and B (cytotoxin), respectively; _tcdC_ and _tcdD_ encode negative and positive regulators, respectively, that control the level of toxin production; and _tcdE_ is purported to encode a holin-like protein thought to facilitate toxin release from the bacterial cell wall (24). Because toxins A and/or B are implicated in CDAD and genetic diversity of the PaLoc has been reported (23), we developed and clinically validated one hydrolysis probe real-time PCR assays targeting the _tcdB_ genes (12, 15, 24). While the molecular methods utilized by this assay were not novel, the application of molecular testing for _C. difficile_ infection is unique when the stool sample could be tested directly without nuclear acid extraction. This will greatly facilitate quick testing of _C. difficile_ in clinical setting.

MATERIALS AND METHOD

_C. difficile_ Strains: The following strains were used for LoD study: _C. difficile_ ATCC 43255 (ZeptoMetrix), _C. difficile_ NAP1A (ZeptoMetrix).

Extraction, Real-time PCR Amplification and Detection: Lab developed _C. difficile_ Direct Kit contains all reagents for on-board extraction and real-time PCR amplification.
Fifty μL of C. difficile Direct reaction mix was loaded into the reaction port and 50 μL of sample was directly loaded into the sample port on the Amplification cell. All testing was performed using real time PCR. Assay time is about 60 minutes.

Limit of Detection (LoD): The LoD for each C. difficile stock was determined as the lowest concentration with ≥95% detection in negative stool matrix.

Reproducibility: Thirty-six replicates of the following contrived panel in negative stool matrix were tested: C. difficile Low Positive (ATCC 43255), C. difficile Medium Positive (ATCC 43255), C. difficile Low Positive (NAP1A), C. difficile Medium Positive (NAP1A). Low Positive was defined as 1X LoD; medium positive was defined as 3X LoD.

Positive and Negative Agreement: A panel of 150 de-identified clinical specimens was evaluated using the Lab developed C. difficile Direct assay. Lab developed test results were compared to Cepheid Xpert C. difficile and enriched culture results.

Cross-Reactivity: The cross-reactivity panel of 126 different organisms consisted of industry equivalent 106 CFU/mL of bacteria or 105 TCID50/mL of virus in negative stool matrix.

Inhibition/interference: The interference panel was contrived with the ATCC 43255 or NAP1A strain at 4-fold the LoD concentration. Each substance was spiked into the C. difficile contrived stool samples and tested using Lab developed C. difficile Direct.

RESULTS

Limit of Detection: C. difficile ATCC 43255 LoD was 0.5 CFU/mL. C. difficile NAP1A strain LoD was 1.6 CFU/mL in stool matrix (Table 1).

C. difficile Reproducibility: For ATCC 43255 and NAP1A medium- and low-contrived panels, C. difficile strains were detected in 100% of replicates. Standard deviations were <0.99. Percent coefficients of variation were <3.3 (Table 2).

Table 1. C. difficile Limit of Detection

| Bacterial Strain | (LoD) Concentration | Detection Rate | Average Ct | Maximum Ct | Minimum Ct |
|------------------|----------------------|----------------|------------|------------|------------|
| ATCC 43255       | 0.5 CFU/mL           | 95% (19/20)    | 39.1       | 41         | 38.5       |
| NAP1A            | 1.3 CFU/mL           | 100% (20/20)   | 39.1       | 42.0       | 38.4       |

Table 2. Lab developed C. difficile Direct Quantitative Reproducibility

| Channel/Detector | Sample Name     | N  | Mean Ct | Between Instrument | Between Operator | Between Run | Within Run | Total |
|------------------|-----------------|----|---------|--------------------|------------------|-------------|------------|-------|
|                  |                 |    | SD      | %CV                | SD               | %CV         | SD         | %CV   |
| C. diff (FAM)    | Low Pos 43255   | 36 | 38.7    | 0                   | 0                | 0           | 0.87       | 2.6   | 0.94 | 2.7 |
|                  | Low Pos NAP1A   | 36 | 38.8    | 0                   | 0                | 0           | 0.76       | 1.8   | 0.74 | 1.9 |
|                  | Med Pos 43255   | 36 | 38      | 0.31               | 0.9              | 0           | 0.53       | 2     | 0.83 | 2.3 |
|                  | Med Pos NAP1A   | 36 | 37.2    | 0.16               | 0.2              | 0           | 0.58       | 1.7   | 0.52 | 1.2 |
|                  | Pos Control     | 36 | 31.2    | 0.27               | 0.5              | 0           | 0.37       | 1.6   | 0.38 | 1.6 |
| IC (Q670)        | Low Pos 43255   | 36 | 29.9    | 0                   | 0                | 0           | 0.87       | 3.1   | 0.99 | 3.1 |
|                  | Low Pos NAP1A   | 36 | 29.8    | 0.28               | 0.9              | 0           | 0.74       | 2.3   | 0.76 | 2.8 |
|                  | Med Pos 43255   | 36 | 29.6    | 0.26               | 0.5              | 0           | 0.62       | 2.3   | 0.69 | 2.4 |
|                  | Med Pos NAP1A   | 36 | 29.9    | 0.35               | 1.5              | 0           | 0.6        | 2.4   | 0.75 | 2.6 |
|                  | Negative        | 36 | 30.1    | 0.12               | 0.4              | 0           | 0.79       | 2.8   | 0.81 | 2.5 |
|                  | Pos Control     | 36 | 30.8    | 0.33               | 1                 | 0           | 0.7        | 2.2   | 0.76 | 2.9 |
C. difficile Positive and Negative Agreement: Results from Lab developed C. difficile Direct and Cepheid Xpert C. difficile were in agreement for 95% of positive specimens and 97% of negative specimens (Tables 3 and 4).

Results from Lab developed C. difficile Direct and enriched culture were in agreement for 93% of positive specimens and 94% of negative specimens (Tables 3 and 5).

Cross-Reactivity: No cross-reactivity was detected with the 126 pathogens tested (subset of representative strains listed in Table 6).

Substance Interference: No interference was detected with the substances tested (Table 7).

### Table 3. Lab developed C. difficile Direct Positive and Negative Agreement

|                      | Lab developed C. difficile Direct vs Cepheid Xpert C. difficile (n=150) | Lab developed C. difficile Direct vs Enriched Culture (n=110) |
|----------------------|------------------------------------------------------------------------|-------------------------------------------------------------|
| Positive Agreement (Sensitivity) | 95%                                                                   | 93%                                                        |
| Negative Agreement (Specificity)    | 97%                                                                   | 94%                                                        |

### Table 4. Lab developed C. difficile Direct Agreement with Cepheid Xpert C. difficile

|                      | Positive | Negative | Total |
|----------------------|----------|----------|-------|
| Lab developed C. difficile Direct | 32       | 3        | 35    |
|                      | 2        | 113      | 115   |
| Total                | 34       | 116      | 150   |

### Table 5. Lab developed C. difficile Direct Agreement with Enriched Toxigenic Culture

|                      | Positive | Negative | Total |
|----------------------|----------|----------|-------|
| Lab developed C. difficile Direct | 41       | 6        | 47    |
|                      | 3        | 100      | 103   |
| Total                | 44       | 106      | 150   |

### Table 6. C. difficile Cross-Reactivity Pathogens Tested in Stool Matrix (Representative Strains)

| Pathogen                          |
|-----------------------------------|
| Abiotrophia defectiva             |
| Acinetobacter baumannii           |
| Acinetobacter Iwofii              |
| Aeromonas hydrophila              |
| Alcaligenes faecalis subsp. Faecalis |
| Bifidobacterium longum            |
| Campylobacter coli                 |
| Campylobacter jejuni sub sp. jejuni |
| Candida albicans                  |
| Candida catenulate                |
| Clostridium bifermentans          |
| Clostridium bolteae               |
| Clostridium butyricum             |
| Clostridium chauvoei              |
| Clostridium fallax                |
| Clostridium ramosurn              |
| Clostridium scindens              |
| Clostridium septicum              |
| Clostridium tetani                |
| Clostridium difficile (non-toxigenic ATCC43593) |
| Desulfovibrio piper               |
| Edwardsiella tarda                |
| Eggerthellalenta                  |
| Enterobacter aerogenes            |
| Enterobacter cloacae              |
**CONCLUSION**

Lab developed *C. difficile* Direct can provide an option for simplified *C. difficile* testing on the real time PCR. This test was also comparable to both Xpert *C. difficile* and enriched toxigenic culture for identifying *C. difficile*.

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