JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

A multi-laboratory comparison of two molecular methods for the detection of toxigenic *Clostridium difficile*

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Abstract

Introduction: Diarrheal disease due to toxigenic *Clostridium difficile* (CD) accounts for an increased number of hospitalizations and deaths each year. Published guidelines recommend reflex testing of CD antigen-positive samples to molecular testing or testing samples directly by a molecular assay. This multicenter study was designed to compare the accuracy of two different molecular methods targeting different CD genes: Xpert *C. difficile* Epi RUO RT-PCR assay (XPCR) which targets toxin B (Cepheid, Sunnyvale, CA) and a laboratory-developed PCR (LDPCR) which targets mutations in the *tcd*C regulatory gene.
Methodology: Two molecular methods for toxigenic CD detection, the Xpert *C. difficile* Epi RUO RT-PCR assay (XPCR) [Cepheid, Sunnyvale, CA] and a laboratory-developed PCR assay (LDPCR) were compared to a consensus gold standard (CGS) or toxigenic culture (TC) as the reference method. A subset of specimens was subjected to additional molecular characterization of toxigenic CD.
Results: Both molecular methods were >90% sensitive for CD detection. Discordant results were noted when molecular test results were compared to non-molecular methods. Supplemental molecular characterization illustrated inherent difficulties in comparisons using different molecular methods for CD.
Conclusion: Laboratories may consider using multiple CD detection methods or combinations of methods, including molecular detection for rapid and accurate diagnosis of CD, as driven by best practices for the respective healthcare environment. Laboratories must be aware of intrinsic differences when comparing performance characteristics of different molecular assays.

Key words: C. difficile diagnostics; molecular testing; *tcdC* gene.

J Infect Dev Ctries 2016; 10(1):062-067. doi:10.3855/jidc.6634

(Received 28 January 2015 – Accepted 07 August 2015)

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Introduction

The prevalence of diarrheal disease due to toxigenic *Clostridium difficile* (CD) has become a major concern in hospitals and other healthcare facilities. CD-associated disease (CDAD) has a significant negative impact on infection control, antibiotic utilization, and overall healthcare costs [1-5]. Increased morbidity and mortality and decreased infection control highlight the importance of identifying toxigenic CD by the clinical microbiology laboratory in a timely and cost-effective manner.

Two cytotoxins, A and B, contribute to pathogenicity in CD disease via disruption of the host cell cytoskeleton. The *tcdA* and *tcdB* genes, that code for toxin A and toxin B respectively, and the putative *tcd*C downstream-negative regulatory gene are found as part of a pathogenicity locus (PaLoc) located within the CD genome. There are two genes (*cdt*A and *cdt*B) outside the PaLoc that code for a binary toxin. Detection of both binary toxin genes and deletions in the *tcdC* regulatory gene have been proposed as a potential indicator of hypervirulent CD strains [6].

This multicenter CD study was performed to determine the accuracy of two real-time PCR (RT-PCR) molecular assays compared to a consensus gold standard (CGS) using toxin enzyme immunoassay (EIA), immunochromatographic lateral flow (ICLF), and toxigenic culture (TC). Additional molecular and sequencing assays were also compared as well. Studies were approved by the respective institutional review boards when required.

Materials and Methods

A total of 360 unique, non-duplicated unformed stool specimens were used in this study for CD toxin (CDT) detection using a molecular method. Of these, 263 and 97 samples were submitted to Baptist Health

(BH Lab #1) and Mayo Clinic in Florida (MCF Lab #2), respectively.

Samples were initially screened at each laboratory using the ImmunoCard TOXINS A&B (EIA), *C. difficile* Tox A/B II (EIA) (Meridian Bioscience, Cincinnati, OH), or C. DIFF QC COMPLETE (Wampole/TECHLAB) immunochromatographic lateral-flow (ICLF) tests, to detect CD glutamate dehydrogenase (GDH)+CD toxins A/B (Table 1). All assays were performed according to manufacturers' recommendations.

Xpert *C. difficile* Epi RUO RT-PCR assay (XPCR) was performed on 263 samples collected at Lab #1 using single-use disposable cartridges for extraction, amplification, and detection of the target sequences and a sample processing control to determine adequate processing of target bacteria and monitoring for presence of inhibitors. Although the Xpert test detects genes coding for CD toxB, binary toxin, and *tcdC* deletion at bp 117 for specific callout of the 027/NAP1/B1 strain, the overall result available within one hour was based on the PCR result for toxB gene.

The laboratory-developed PCR (LDPCR) used in Lab #2 was performed on the 97 specimens with discordant EIA and ICLF results. Detection of the *tcdC* gene was based on methodology described by Sloan *et al* [7]. Briefly, a swab saturated with fecal material was expressed into 1.0 mL of sterile water. Two hundred µL of the solution was inoculated into a DNA extraction cartridge (Total nucleic acid isolation kit; MagNA Pure Compact, Roche Diagnostics, Mannheim, Germany). Detection and amplification of PCR products were performed on the LightCycler 2.0 (Roche Diagnostics, Indianapolis, IN). Fifteen µL of master mix containing DNA hybridization probes (*Taq* DNA polymerase, buffer, dNTPs, dUTP, 10 mM $MgCl_2$) and 5 µL of extracted DNA were combined, and the mixture was placed into the LightCycler. The cycling parameters were: 95°C for 10 minutes, amplification with 45 cycles, 10 seconds at 95°C, 10 seconds at 55°C, and 15 seconds at 72°C. Product was detected by melting curve analysis: 0 seconds at 95°C, 20 seconds at 59°C, and 20 seconds at 45°C. Positive and negative DNA plasmid controls were included with each run.

Specimens were excluded from analysis at each laboratory if they produced repeat indeterminate results or control failure with a molecular method. Fifty specimens with discordant results after EIA, ICLF, and molecular testing at the respective investigational site were submitted to the other laboratory for molecular testing used at that site (*i.e.* XPCR or LDPCR).

In addition, a total of 50 discordant specimens from both sites with sufficient volume, previously frozen at ≥ -70°C, were submitted to a reference laboratory for cytotoxic culture (TC) using an ETOH shocked CCFA culture/broth amplification-cytotoxic assay (TECHLAB, Inc. Blacksburg, USA). The final result for each sample was interpreted as true positive (consensus gold standard [CGS]) if at least two CD toxin assays performed on a given sample were positive, or if the TC for that sample was positive.

Twenty-seven discrepant samples of the 50 samples tested by both molecular assays were also referred to Mayo Medical Laboratories, Rochester, MN for repeat LDPCR by the method described by Sloan *et al* [7]. In addition, DNA sequencing of the *tcdC* gene by either analysis of the sequence encompassing the *tcdC* deletion [8] or by sequencing of the full-length *tcdC* gene [6] was performed according to established protocols. Finally, conventional PCR for the detection of CD binary toxin by the method of Terhes *et al* was performed [9]. An analysis of reagent cost and observation of laboratory workflow was performed for molecular tests performed in laboratories 1 and 2 (Table 2).

Results

Of the 263 evaluable specimens received by Lab #1, 188 (71.5%) provided concordant results (118

Table 1. Number of specimens and laboratory assays performed by each investigator site.

	Lab #1 (Number of Tests)	*Lab #2 (Number of Tests)*
EIA ImmunoCard Toxins A&B	263	NP
EIA *C. difficile* TOX A/B II	NP	537
ICLF	263	537
XPCR	263	NP
LDPCR	NP	97

EIA = enzyme immunoassay; ICLF = immunochromatographic lateral-flow; XPCR = Xpert C.dfficile Epi RUO RT-PCR; LDPCR = laboratory-developed PCR; NP = not performed.

Table 2. Analysis of laboratory test time and reagent cost for molecular assays (2015 information).

Molecular Test	Time to Perform (minutes)	Reagent Cost per test	Agreement compared to GCS
XPCR	47	$37.50	Positive: 121/138 (87.6%) Negative: 125/125 (100%) Total: 246 /263 (93.5%)
LDPCR	150	$11.83	Positive: 35/38 (92.1%) Negative: 57/59 (96.6%) Total: 92 /97 (94.8%)

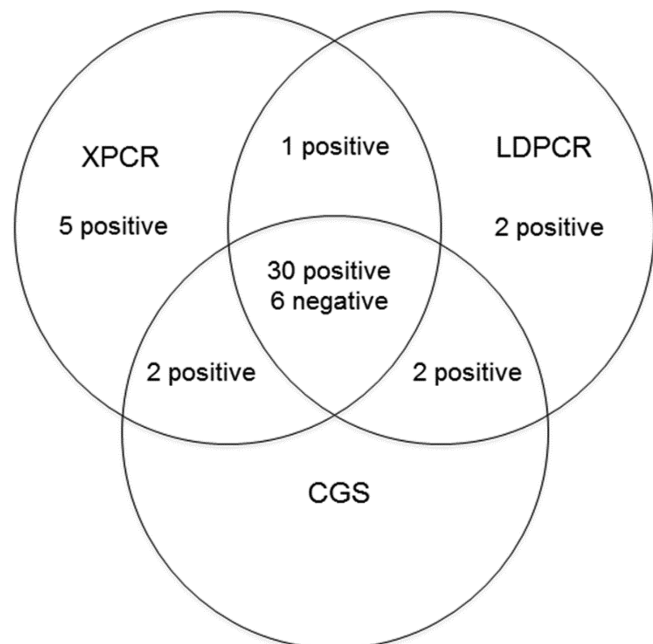
negative, 70 positive), and 75 (28.5%) gave discordant toxin A&B EIA, ICLF, and XPCR results. Of the 75 specimens giving discordant results, 51 (68.0%) were confirmed to be positive by the CGS and/or TC while the remaining samples were negative. All of the concordant (70) and CGS/TC (51) positive specimens (121 total true positives) were positive by the Xpert *C. difficile* RT-PCR for *tcdB* (toxin B); 53 of the 121 specimens (43.8%) were flagged for specific callout of the epidemic 027-NAP1-B1 strain.

Of the 97 specimens tested by LDPCR in Lab #2, 62 (63.9%) provided concordant results (51 negative, 11 positive), and 35 specimens (36.1%) presented discordant results between toxin A/B EIA, ICLF, and LDPCR. Of the 35 specimens with discordant results, 27 (77.1%) were positive and eight (22.8%) were negative by LDPCR. In addition, 24 of the 35 specimens (68.6%) were positive and six (17.1%) were negative by the GCS/TC. The remaining five specimens, which were LDPCR positive (three specimens) or negative (two specimens), did not agree with the GCS/TC result. A total of 92 (94.8%) specimens tested by LDPCR agreed with the CGS/TC results. A time and reagent cost analysis of XPCR vs. LDPCR indicated significant differences in the laboratory time needed to perform the test. There was also a significant difference in test cost. However, overall agreement of both molecular methods for detection of CD as compared to the GCS was virtually the same; XPCR – 93.5%, LDPCR – 94.8% (Table 2)

A total of 50 samples were tested by both XPCR and LDPCR. Two samples produced invalid results with XPCR and were removed from the molecular comparison study. When comparing the XPCR and LDPCR qualitative results, 37 of 48 samples (77.1%) were concordant (31 positive and six negative) and 11 samples were discordant. Forty of 48 results agreed with the CGS; one XPCR/LDPCR positive did not agree with the GCS (Figure 1). Since both molecular assays target the *tcd*C gene, the *tcd*C results from the 48 samples were also compared. Nine were XPCR, LDPCR, and *tcd*C gene positive and nine were negative. The thirty remaining samples gave discordant results.

Among the specimens tested by both CD molecular methods, 27 specimens had sufficient volume to submit to a collaborating laboratory for *tcd*C gene sequencing and binary toxin conventional PCR. Eleven of 27 were concordant positive and four were concordant negative by Xpert, LDPCR, and *tcd*C gene sequencing. Two specimens were invalid by Xpert. Among discordants, five samples were CD positive by both LDPCR and Xpert but were negative by *tcd*C sequence, the remaining five samples gave discordant Xpert and LDPCR results (Table 3). Five specimens with presumptive callout for the NAP-1 epidemic strain by the XPCR were *tcd*C positive by sequencing, and two were also binary toxin positive by conventional PCR.

Figure 1. Venn diagram for CD specimens tested by XPCR and LCPCR as compared to the CGS (N=48). Five specimens positive by XPCR and 2 specimens positive by LCPCR did not agree with the corresponding molecular method for CD or the CGS. A single specimen positive for CD by both molecular methods did not agree with CGS.



Toxigenic culture and sequencing results were available for 16 out of the 27 specimens. Eight of 16 TC results were concordant with LDPCR/ XPCR/ sequencing (seven positive and one negative). Seven TC positive were LDPCR/XPCR/sequencing negative, and one was TC negative but positive by LDPCR/XPCR/sequencing (Table 3).

Discussion

Results from this study and others [10-13] highlight the accuracy of RT-PCR as a rapid method for detection of toxigenic strains of CD. A laboratory-developed PCR assay, as well as an RUO method (now commercially available) demonstrated a strong agreement with their CGS or TC. However, upon comparing the results from these two PCR assays, only 37 of 48 samples (77.1%) were concordant (31 positive and six negative). Even though both target the *tcd*C gene, positive callout for the Xpert result is based on a toxB target, while the LDPCR is based on detection of specific deletions in the *tcd*C gene.

When comparing the specific *tcd*C PCR results, there was only a 37.5% (18/48) agreement. It is important to note that the Xpert *tcdC* PCR specifically targets the nucleotide deletion at bp 117 of the *tcdC* gene, whereas the LDPCR method specifically detects deletions at bp positions 18 and 39. LDPCR does not detect the same deletion as the Xpert RT-PCR assay. This factor most likely accounted for the disagreement between the two *tcdC* methods and highlights the potential difference in results that may be obtained when using different molecular assays. Clinical correlation studies would, therefore, be important in determining the accuracy of an assay prior to implementation.

Limitations of this study include the small number of samples available for testing by both molecular methods and TC. In some cases, a limited specimen volume was available to perform binary toxin and *tcdC* PCR as well as sequencing of samples giving discordant results. There was also a difference in how each laboratory established their CGS. Lab #1 performed an EIA, ICLF, and XPCR on all samples from their institution, whereas Lab #2 performed an EIA and ICLF on all samples and reflexed the discordant specimens to their LDPCR assay. This may have skewed the LDPCR results to appear more favorable than if performed on all specimens and may have accounted for the relatively low concordance between assays. As demonstrated in other studies, use of the TC as a true gold standard may not be 100% sensitive or specific and may account for the instances where LCPCR did not agree with the GCS [14].

An advantage of the Xpert *C. difficile* Epi Assay is the detection of the NAP-1 strain for the benefit of epidemiology and infection control in addition to identifying the toxin B gene. Identification of the epidemic strain may also have implications for treatment in the future.

Limited data showed that three specimens containing a confirmed 117-bp deletion by full-length *tcdC* sequencing were also positive by the Xpert *tcdC* assay and were flagged by this assay to be a NAP-1 strain. These three samples were also positive by the LDPCR assay, which infers that there may have been an additional deletion at 18- or 39-bp. Other authors have indicated that a deletion at 18-bp may occur concurrently with a deletion at position 117 in toxin

Table 3. Discrepant RT-PCR results among specimens submitted for molecular sequencing analysis.

Specimen	LDPCR	XPCR ToxB	XPCR *tcd*C	Conventional PCR		XPCR Binary Toxin	Toxigenic Culture	CGS
				*tcd*C seq.	Binary Toxin			
211	POS	POS	NEG	NEG	NEG	POS	N/A	POS
229	POS	POS	NEG	NEG	NEG	NEG	POS	POS
232	POS	POS	POS	NEG	NEG	POS	POS	POS
234	POS	POS	POS	NEG	NEG	POS	POS	POS
253	POS	POS	NEG	NEG	NEG	NEG	POS	POS
238	POS	NEG	NEG	POS	POS	NEG	N/A	NEG
239	POS	NEG	NEG	NEG	NEG	NEG	NEG	NEG
244	POS	NEG	POS	NEG	NEG	NEG	POS	POS
252	NEG	POS	NEG	NEG	NEG	NEG	N/A	NEG
254	NEG	POS	POS	POS	POS	POS	POS	POS

LDPCR: Laboratory-developed PCR, XPCR: Xpert® *C. difficile* Epi RUO RT-PCR assay, CGS: Consensus Gold Standard, N/A: Result not available.

hyper-producing strains of CD [15].

Both XPCR and LDPCR demonstrated excellent overall accuracy in detection of CD. The XPCR requires less laboratory hands-on time with discrete results generated by the instrument software, but reagent costs are higher. The LDPCR requires additional laboratory-hands on time, plus technologist interpretation of results, but reagent costs are significantly lower. A careful consideration of advantages and disadvantages of molecular testing platforms in relation to individual laboratory workflow and test cost is strongly recommended.

This multicenter study evaluated the performance of two molecular methods, each targeting different genes for a positive interpretation of disease due to CD. Each institution appreciated excellent sensitivity with their respective molecular assay when compared to their established CGS and/or TC. However, when specimens presenting discordant results were submitted to the other site to test by the molecular method used at that laboratory, there was a low degree of concordance for the detection of CD, most likely reflecting either the difference in targets for a positive CD toxin result or the difference in the diagnostic algorithm used in the respective laboratories. The results of this study highlight the difference in molecular targets used to provide a laboratory diagnosis of CD. Laboratorians and clinical providers will want to carefully consider these diagnostic methods in order to determine the best approach for detection of CD disease.

Acknowledgements

C. DIFF QC CHEK COMPLETE kits were kindly supplied by Alere Inc, ImmunoCard Toxins A&B by Meridian BioScience, Inc., C. DIFFICILE TOX A/B II kits by Techlab/Wampole and *C.diff* Xpert RUO RT-PCR kits by Cepheid.

We are deeply appreciative to the following groups for their professional and technical support: Baptist Health/Baptist Medical Center Microbiology staff including Page McKitrick and Joan Abid; Mayo Clinic - Florida Microbiology laboratory; Mayo Clinic – Rochester, MN Division of Clinical Microbiology; Dr. Jon Rosenblatt , Dr. Robin Patel; Mary Goodykoontz and staff, TECHLAB for technical assistance. In addition, we would like to thank Cepheid Inc., Alere Inc., and Meridian Bioscience for their support of this study.

Author Contributions

DCH and DJH were responsible for study conception and design, study supervision, and writing/ revision of the manuscript. JA, LS, DM and DRW were responsible for data collection and analysis.

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Conflict of interests: No conflict of interests is declared.