CLINICAL EVALUATION OF A *C. DIFFICILE* PCR ASSAY BASED ON INTEGRATED CAPILLARY ELECTROPHORESIS METHOD

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Abstract

Background: *Clostridium difficile* infection (CDI) is one of the most prevalent causes of healthcare associated infections (HAI). Clinical manifestations of CDI can range from mild diarrhea to a life-threatening pseudomembraneous colitis. CDI usually develops after treatment with antibiotics. CDI is a toxin-mediated intestinal disease generally associated with toxin B which is a major virulence factor that is encoded by the *tcdB* gene. We evaluated the newly developed ICEPlex *C. difficile* Assay (PrimeraDx, Mansfield, MA) to establish its usability and utility in clinical lab settings.

Materials& Methods: The study was performed on one hundred and ninety-nine fresh stool samples. Nucleic acids for testing were extracted using NucliSens easyMAG™ (bioMerieux, Durham, NC.) The ICEPlex *C. difficile* Assay is a qualitative molecular diagnostic test for the detection of toxigenic *C. difficile* nucleic acids. The test is based on Primera Dx's proprietary technology enabling multiplex real-time PCR. The ICEPlex system integrates PCR amplification with capillary electrophoresis (CE) detection of amplified product. CE injections from the same reaction are performed over successive PCR cycles to enable collection of real-time PCR data. Oligonucleotide primers are designed to produce PCR products with unique CE mobility enabling detection of multiple targets, controls and calibrators in the same PCR reaction volume. The ICEPlex *C. difficile* Assay was designed to target three independent regions on the *tcdB* gene for expanded sensitivity and inclusivity. Each PCR reaction is internally controlled for performance by internal control and three Calibration standards. The ICEPlex system tests 48samples per run (half 96-well plate).

Results and Conclusion: Thirty-eight samples were reported positive by the ICEPlex *C. difficile* Assay, providing positive rate of 19%. The ICEPlex *C.difficile* Assay is comparable to our current molecular method, BD GeneOhm C.diff test in clinical utility. The ICEPlex instrument has the capability of testing 48 samples compared to 16 for the Smart Cycler; therefore it is better suited for high-volume laboratories. The ICEPlex also has the potential to yield quantitative results and to include a significant degree of multiplexing for testing other stool pathogens.

Technology

The ICEPlex System is a fully automated real time PCR platform that combines an amplication module (thermocycler) and a detection module (a capillary electrophoresis cartridge, two solid state lasers with excitation maximum at 488 nm and 639 nm and a spectrophotometer with CCD camera). All ICEPlex System reagents are kept on board of the platform enabling an easy consumable maintenance (Figure 1).

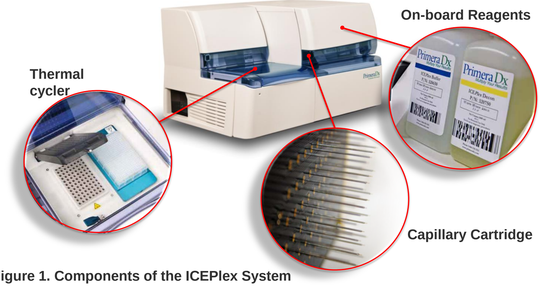

Figure 1. Components of the ICEPlex System

The ICEPlex System generates fluorescently labeled PCR products (amplicons) which are separated based on their different sizes by capillary gel electrophoresis (CE). Amounts of the fluorescent amplicons are monitored in real time by system's software that converts the fluorescent signal into amplification curves and calculates cycle thresholds (C_ts) for all PCR targets. The combination of PCR and CE enables simultaneous detection and quantification of multiplex targets in 48 individual reactions in the same manner as traditional real-time PCR methods (Figure 2).

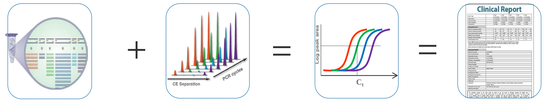

Figure 2. Multiplex real-time PCR detection on the ICEPlex System

Introduction

C.Difficile infection is a toxin-mediated intestinal disease. *C.Difficile* Toxin B is generally considered the major virulent factor associated with *C.difficile* infection. Some strains of *C.Difficile* are capable of producing additional toxins such as Toxin A and a binary toxin. However, the roles of these two toxins in *C.difficile* infection have not been established yet. The ICEPlex *C.difficile* Assay is a molecular diagnostic test for qualitative detection of the toxigenic *C.difficile* nucleic acid isolated and purified from liquid or soft stool specimens, obtained from symptomatic patients. The assay targets the *C.difficile* toxin B gene (*tcdB*). The ICEPlex *C.difficile* Assay has been developed for use on the Primera Dx ICEPlex System.

Materials and methods

Primer and assay design: Oligonucleotide primers in the ICEPlex *C.difficile* Assay were designed to generate PCR amplicons with different CE motilities, enabling simultaneous detection of multiple DNA targets in a single reaction. Each individual test includes three targets sites of the *tcdB* gene, Internal Control and three Calibration Controls that produce ~~have three~~ different amplicon sizes. The ICEPlex System software uses the Calibration Controls as size standards to align and assign the different CE peaks, which correspond to the different target amplicons. The Calibration Controls are added to each PCR reaction and could also be utilized for quantification of DNA targets of interest.

Kit components of the ICEPlex C.difficile Assay:

The ICEPlex *C.difficile* Assay contains sufficient reagents to perform 100 tests and includes the following components: 2X PCR buffer, 25X Primer Mix – a mixture of FAM-, TYE-labeled and unlabeled oligonucleotide primers specific to the *tcdB* gene of the *C.difficile*, Internal Control and Calibration Controls, PCR enzyme, 25X Calibrators Mix: a mixture of Calibration control templates, Internal Control: a non-target nucleic acid that is co-extracted and co-amplified with the *tcdB* targets. The Internal Control is added to monitor the extraction efficiency, integrity of the reagents and the presence of PCR inhibitors in patient samples, 10X Injection Buffer: a salt solution that is diluted 1:10 and it is used to filled the unused wells of the PCR plate, Positive Control: a solution of the DNA templates that are used as External Positive Controls

Test procedures: The workflow of the ICEPlex *C.difficile* Assay is described in the Figure 3.
One hundred microliters of liquid or soft raw stool specimen were diluted and fixed in 400 µl of S.T.A.R. buffer (Roche Diagnostics, Indianapolis, ID). After the samples were spun down, 200 µl of the clarified lysate was transferred in clean tubes. Ten microliters of the Internal Control were added to easyMAG extraction cartridge. Eighty microliters of the clarified lysate, Negative Control and Known Positive Samples were added to the corresponding ports of the extraction cartridges and processed for nucleic acid extraction on the easyMAG extraction system following manufacturer's instructions. The ICEPlex *C.difficile* Assay PCR master mix was assembled in clean area and 40 µl of it was aliquoted in the wells of 96 well PCR plate. Ten microliters from the extracted patient specimen, Positive Control and Negative Control were also added to the corresponded wells. All PCR reactions were overlaid with a drop of mineral oil and the PCR plate was loaded on the ICEPlex System.

The fully automated multiplex ICEPlex detection System processed extracts from the patient samples in less than 4 hours and generated result reports. Multiplex capability of the ICEPlex system enabled simultaneous detection of the *tcdB* target, Calibration and Internal Controls in single PCR reaction (Figure 4).
Same Clinical specimens were also tested in parallel in the BD Gene Ohm ™C.difficile Assay on SmartCycler® system with Dx Software (Cepheid, Sunnyvale, CA), following manufacturer's instructions.

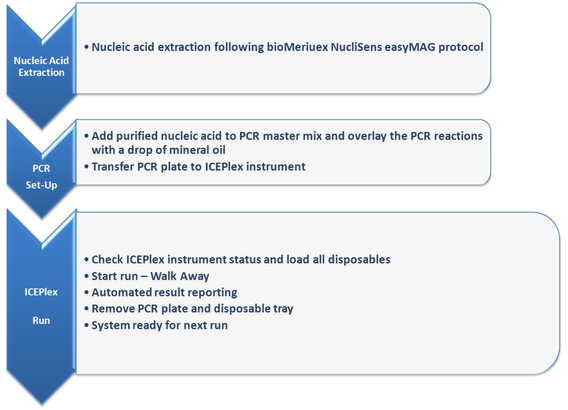

Figure 3. ICEPlex *C.difficile* Assay workflow

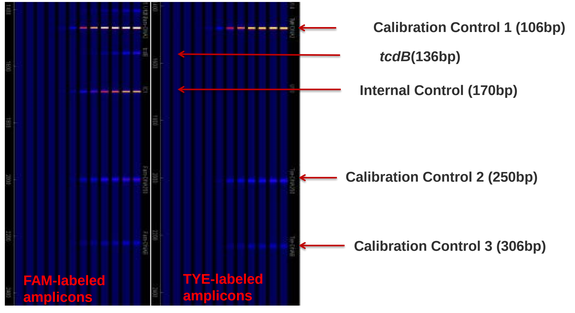

Figure 4. The Multiplex ICEPlex *C.difficile* Assay can detect *tcdB* target, Calibration and Internal Control in a single PCR reaction.

Results

One hundred and ninety-nine patient specimen were tested in the ICEPlex *C.difficile* Assay on ICEPlex system and in in the BD Gene Ohm ™C.difficile Assay on SmartCycler® starter system .Thirty three specimen were positive and one hundred fifty nine were negative by both methods (Table 1). The Positive Predictive Value was 84.6. The Negative Predictive Value was 99.4.

	BD Gene Ohm ™C.difficile Assay	
ICEPlex *C.difficile* Assay	Positive	Negative
Positive	33	6
Negative	1	159

Table 1. Results from testing of 199 patient specimen in ICEPlex *C.difficile* Assay and in BD GeneOhm™ C.difficile Assay

The sensitivity of the ICEPlex *C.difficile* Assay when measured against BD GeneOhm™ C.difficile Assay was 97.06%, while the specificity was 96.36%.

Conclusions

1. The ICEPlex system is capable of detecting and quantifying simultaneously multiple nucleic acid targets, which makes it an attractive platform for developing of pathogen detection assays.
2. The fully automated ICEPlex instrument can process up to 48 samples in less than 4 hours, which is an important feature for high-throughput laboratories.
3. Embedded Internal/extraction control in the ICEPlex *C.difficile* Assay enables monitoring of the extraction efficiency, integrity of the PCR reagents and the presence of PCR inhibitors.
4. The sensitivity and specificity of the ICEPlex *C.difficile* Assay are comparable to those of BD GeneOhm test currently used in our diagnostic lab. Additionally the ICEPlex system has throughput of 48 samples in a single run.

The PrimeraDx ICEPlex system and *C. difficile* Assay have not been approved by the FDA for IVD. This information is for demonstration purposes only.