Application of Isothermal Helicase-Dependent Amplification with a Disposable Detection Device in a Simple Sensitive Stool Test for Toxigenic *Clostridium difficile*

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Enzyme immunoassays (EIAs) are commonly used for the diagnosis of cases of *Clostridium difficile*-associated diarrhea (CDAD). However, these EIAs have high false-negative rates, even in patients with severe clinical disease. We have developed an IsoAmp CDAD test using a simple and user-friendly procedure to identify toxigenic *C. difficile* in feces. After DNA extraction from fecal samples, both the conserved sequence of the 5′-end fragment of the *C. difficile tcdA* toxin gene and competitive amplification internal control sequence were amplified using helicase-dependent amplification. Amplification products were detected using a novel amplicon-containment detection device. The analytical sensitivity of the assay was 20 copies of *C. difficile* genomic DNA per reaction. Evaluation of the clinical sensitivity and specificity of the IsoAmp CDAD test versus an EIA method using a PCR method as the reference standard revealed 100% sensitivity and 100% specificity for the IsoAmp CDAD test compared with 90.9% sensitivity and 100% specificity for the EIA method. Because the IsoAmp CDAD test requires no expensive equipments for nucleic acid amplification or detection and can be performed on a random access basis, the test provides a practical alternative to immunoassays for the diagnosis of CDAD with improved sensitivity. *(J Mol Diagn 2008, 10:000–000; DOI: 10.2353/jmoldx.2008.080008)*

Clostridium difficile-associated diarrhea (CDAD) is the most common nosocomial infectious diarrhea usually associated with prior antibiotic therapy.[1,2] Laboratory diagnosis of *C. difficile* infection is based primarily on the detection of *C. difficile* toxin A (TcdA) and toxin B (TcdB) that are involved in the pathogenicity of the organism.[3–5] Although most pathogenic *C. difficile* strains produce both TcdA and TcdB (A+/B+), pathogenic strains producing TcdB only (A−/B+) have also been reported.[6] The majority of A−/B+ strains occur because of a ~1.8-kb deletion at the 3′ ends of their TcdA genes (*tcdA*). All known A−/B+ strains still contain *tcdA* gene encoding the glucosyltransferase domain on the N terminus of TcdA.[7–11]

The tissue culture cytotoxicity assay, which primarily detects TcdB, is sensitive,[12] but it is also time-consuming, requiring at least 24 hours for completion.[13] Enzyme immunoassay (EIA) for detection of TcdA, TcdB, or both is commonly used for rapid diagnosis of CDAD, but these EIAs are usually not as sensitive as the cytotoxicity assay.[14] Recently, nucleic acid amplification, in particular, real-time PCR for detection of *C. difficile* toxin genes has been reported in identification of pathogenic *C. difficile* strains with high sensitivity and specificity.[15–17] However, real-time PCR assay requires expensive instrumentation and well-trained personnel for operation. Development of a simple and easy-to-adapt test for accurate identification of pathogenic *C. difficile* in a timely fashion is highly desirable for hospital cost containment, patient management, and prompt epidemiological interventions.

Helicase-dependent amplification (HDA) is a unique isothermal nucleic acid amplification technique that relies on the use of a DNA helicase enzyme to unwind double-stranded DNA and RNA-DNA hybrid. Nucleic acid amplification through HDA is very robust, and the platform can be applied to both DNA and RNA pathogen amplification.[18,19] We report here the development of the IsoAmp CDAD test using HDA coupled with a simple disposable vertical-flow device, which is designed for instrument-free, cross-contamination-proof detection of amplicons, for rapid identification of toxigenic *C. difficile* in feces. This assay is based on the amplification of a conserved 5′-end fragment of *C. difficile tcdA* gene. A competitive amplification internal control (IC) is also included for monitoring the process of nucleic acid amplification and detection. Comparison of the clinical

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1

sensitivity and specificity of IsoAmp CDAD test with an EIA assay revealed that the isothermal nucleic acid test is more sensitive than the corresponding immunoassay.

Materials and Methods

Stool Specimens

The feces samples from patients with suspected CDAD were collected and frozen at −20°C within 24 hours of being received in the laboratory. The samples remained frozen until they were thawed for DNA extraction. These samples included liquid, soft, semiformed, and formed specimens.

DNA Extraction and Sample Preparation

Genomic DNAs from strains listed in Table 1 were either purchased from American Type Culture Collection (ATCC) (Manassas, VA) or purified by using a QIAGEN Genomic-tip 20/G kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's instructions. Stool DNAs used in the analytical sensitivity test were extracted each from 200 mg of CDAD-negative fecal sample and eluted into 200 μl of elution buffer by using a QIAamp DNA Stool mini kit (Qiagen, Inc.) according to the manufacturer's instructions. Stool DNAs used in the clinical sensitivity and specificity tests and PCR assay were extracted by a NucliSENS easyMAG automated sample preparation system (bioMérieux, Inc., Durham, NC) after pretreatment using a modification of the technique described by Trujillo et al.[20]

Oligonucleotides and IC Plasmid for HDA

Multiple-sequence alignment of the *tcdA* gene of *C. difficile* was performed by analyzing the first 2-kb *tcdA* gene coding sequences at the 5′ end that are available from public databases with the Megalign program of Lasergene (DNASTAR, Inc., Madison, WI). A conserved region was chosen to design the HDA primer pair BioCDTAF1 and CDTAR1, and the *tcdA* gene probe CDTAP3FI with the help of Primer3 program (*http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi*). A 41-bp randomly selected IC sequence was used for designing the IC probe 22S2ICDig. The IC plasmid was made by cloning this IC sequence flanked by sequences of *tcdA* gene primers BioCDTAF1 and CDTAR1 into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA). The melting temperatures of the primers and the probes were selected at 57 to 62°C and 48 to 53°C, respectively, to suit HDA and cassette detection from the nearest neighbor calculation using Oligonucleotide Properties Calculator program (*http://www.basic.northwestern.edu/biotools/oligocalc. html*). The specificity of the primers and probes were analyzed with BLASTN (*http://www.ncbi.nlm.nih.gov/blast/Blast.cgi*). Primer BioCDTAF1 was labeled with biotin at its 5′ end. Probes CDTAP3FI and 22S2ICDig were labeled with fluorescein isothiocyanate (FITC) and digoxigenin (DIG) at their 3′ ends, respectively. All oligonucleotides were purchased from Operon Biotechnologies, Inc. (Huntsville, AL). The HDA primers and probes used in this study are listed in Table 2.

HDA

HDA was performed using either purified genomic DNA or total DNA extracted from fecal samples as template. HDA conditions and reagent concentrations were optimized to obtain the final parameters described hereafter. HDA was set up using an IsoAmp Rapid C. diff Detection kit (BioHelix Corporation, Beverly, MA). To generate biotin-labeled single-stranded amplicon for probe hybridization, asymmetric HDA was performed. In brief, 25 μl of Mix A containing DNA template and 5000 copies of IC plasmid was mixed with 21.5 μl of IsoAmp CDAD Reac-

Table 1.   Bacterial Strains Used in This Study

Species (strain number)	Toxinotype	TcdA/B production	IsoAmp test
C. difficile (ATCC 9689)*	0	TcdA and TcdB	Positive
C. difficile (CF1p)[†‡]	VIII	TcdB	Positive
C. difficile (CF2)[†‡]	VIII	TcdB	Positive
C. difficile (CF4)[†‡]	ND[§]	TcdB	Positive
C. difficile (8864)[†‡]	X	TcdB	Positive
C. difficile (ATCC 43255)[†]	0	TcdA and TcdB	Positive
C. difficile (ATCC 700057)[†]	NA[¶]	Negative	Negative
C. novyi (ATCC 19402)[†]	NA[¶]	Negative	Negative
C. perfringens (ATCC 13124)[†]	NA[¶]	Negative	Negative
C. septicum (ATCC 12464)[†]	NA[¶]	Negative	Negative
C. innocuum (ATCC 14501)[†]	NA[¶]	Negative	Negative
C. sordellii (ATCC 9714)[†]	NA[¶]	Negative	Negative
E. coli (strain: K-12)[†]	NA[¶]	Negative	Negative
S. flexneri (ATCC 700930)*	NA[¶]	Negative	Negative
S. enterica (ATCC 700720)*	NA[¶]	Negative	Negative
C. jejuni (ATCC 700819)*	NA[¶]	Negative	Negative
V. cholerae (ATCC 39315)*	NA[¶]	Negative	Negative

*Genomic DNAs were purchased from ATCC.
[†]Genomic DNAs were purified using a Qiagen Genomic-tip 20/G kit.
[‡]A−/B+ *C. difficile* strains provided by Dale Gerding (Hines Veterans Affairs Hospital).
[§]ND, not determined.
[¶]NA, not applicable.

Table 2. Oligonucleotides Used in This Study

Primer/probe	Target gene	Oligonucleotide sequence (5′→3′)	Amplicon size (bp)
BioCDTAF1	*tcdA*	Bio-GATGTTGATATGCTTCCAGGTATTCAC*	
CDTAR1	*tcdA*	TATCATTTCCCAACGGTCTAGTCCAAT	
CDTAP3FI	*tcdA*	AGAGCTAGGTCTAGATATTGTTT-FITC	*tcdA*:90
22S2ICDig	IC plasmid	ACGTCGTCTCTTACCGATTAAC-DIG	IC:95

*Bio, biotin.

tion Mix and 3.5 μl of IsoAmp Enzyme Mix in a 200-μl PCR reaction tube to provide a final concentration of 20 mmol/L Tris-HCl (pH 8.8 at 25°C), 10 mmol/L KCl, 40 mmol/L NaCl, 4 mmol/L MgSO$_4$, 400 μmol/L of each of the four deoxynucleoside triphosphates, 3 mmol/L dATP, 200 nmol/L *tcdA* gene primer BioCDTAF1, 100 nmol/L *tcdA* gene primer CDTAR1, 40 nmol/L *tcdA* gene probe CDTAP3FI, and 40 nmol/L IC probe 22S2ICDig in the HDA reaction. After a 60-minute incubation at 65°C in a water bath or heat block, the reaction tube was directly placed into the BioHelix Express Strip (BESt) Cassette (BioHelix Corporation) for amplicon detection.

BESt Cassette Detection

The dual-labeled probe-amplicon products generated from the asymmetric HDA were detected using a Type II BESt Cassette that is designed to detect two amplicons with the test (T) line for capturing FITC-labeled *tcdA* gene probe-amplicon and the control (C) line for capturing DIG-labeled IC probe-amplicon. The biotin labels in each amplicon attract streptavidin-conjugated color particles for visualization, and the test results are shown as a colored line visible by the naked eye. In brief, the HDA reaction tube was placed in an amplicon cartridge of a Type II BESt Cassette immediately after HDA. The cartridge was then closed and inserted into the detection chamber. The handle of the detection chamber was closed to start amplicon detection. The assay result was read by eye to score from the detection window of the chamber after 10 to 15 minutes. A positive read was scored when the T line or both the T line and the C line were visible through the detection window of the cassette. A negative read was scored when only the C line was displayed. The assay was regarded as invalid when neither the T line nor the C line was displayed.

Analytical Sensitivity and Specificity Tests

The bacterial strains used in the analytical sensitivity and specificity tests were listed in Table 1. The analytical sensitivity of the IsoAmp Rapid CDAD Test kit was evaluated by spiking genomic DNA of a representative A+/B+ *C. difficile* strain (ATCC 9689) and four A−/B+ *C. difficile* strains (CF1p, CF2, CF4, and 8864) provided by Dale Gerding at Hines Veterans Affairs Hospital into a pooled-DNA extracted from six CDAD-negative fecal samples collected at Beth Israel Deaconess Medical Center. Ten and 20 copies of *C. difficile* genomic DNA were first mixed with 2 μl of the pooled stool DNA, respectively. The spiked stool DNA was then used as template in the HDA and BESt Cassette de-

tection system described above. A total of 12 replicates for the A+/B+ strain and 3 replicates for each of the 4 A−/B+ strains were performed in the analytical sensitivity test. The analytical specificity of the IsoAmp CDAD test was evaluated by testing a panel of 12 bacterial species including 1 nontoxigenic *C. difficile* strain (ATCC 700057); 5 other *Clostridium* species: *C. novyi* (ATCC 19402), *C. perfringens* (ATCC 13124), *C. septicum* (ATCC 12464), *C. innocuum* (ATCC 14501), and *C. sordellii* (ATCC 9714); and 5 bacterial species involved in food poisoning and diarrhea: *Escherichia coli* (strain K-12), *Shigella flexneri* (ATCC 700930), *Salmonella enterica* (ATCC 700720), *Campylobacter jejuni* (ATCC 700819), and *Vibrio cholerae* (ATCC 39315). A toxigenic *C. difficile* strain (ATCC 43255) was also included in the specificity test and served as a positive control. Five nanograms (representing about 10^6 genome copies) of genomic DNA of each of these 12 bacterial species was used in the HDA and BESt Cassette detection system. The analytical specificity test was performed in triplicate.

Clinical Sensitivity and Specificity Tests

A convenience sample of 101 stool specimens collected at Vanderbilt University Medical Center from patients with suspected CDAD consisting of 60 positive and 41 negative specimens determined by the EIA assay were used in the IsoAmp CDAD test to evaluate its clinical sensitivity and specificity. Five microliters of each extracted stool DNA was used in the HDA and BESt Cassette assay. When an invalid read was scored, the stool DNA was sequentially diluted by 2.5-, 10-, and 100-fold and reapplied to the HDA and BESt Cassette until a positive or a negative read was obtained. The clinical sensitivity and specificity tests were performed in a blinded procedure in which the blinded stool DNAs extracted at Vanderbilt University Medical Center were sent to BioHelix Corporation to perform the IsoAmp CDAD test. The IsoAmp CDAD test results were then reported back to Vanderbilt University Medical Center and compared with the test results from the EIA assay and the PCR assay.

EIA Assay

Diarrhea stool specimens were tested for *C. difficile* using the Premier Toxins A&B Enzyme Immunoassay for the detection of *C. difficile* toxin A and toxin B in stool specimens (Meridian Bioscience, Inc., Cincinnati, OH) according to the manufacturer's instructions. This is a qualitative test that uses monoclonal and polyclonal antibodies to directly detect both A and B toxins in stool samples.

Results were interpreted via visual reading and reported as positive or negative.

PCR Assay

DNAs extracted from the abovementioned specimens were used in a PCR assay to detect the *tcdC* gene of the *C. difficile* pathogenicity locus. Briefly, multiple-sequence alignment was used to identify consensus sequences in the *tcdC* gene for primer development. Primer Express Software (version 3; Applied Biosystems, Foster City, CA) was used to design forward and reverse primers that amplify a 178 or 196 bp fragment (forward primer, 5′-CAAATTGTCTGATGCTGAACC-3; reverse primer, 5′-TCAGATGTTCTAGCTAATTGGTCA-3′).[21] The 5′ end of the forward primer was labeled with 6-carboxyfluorescein for fragment analysis by capillary electrophoresis on the ABI 3730xl automated sequencer, and data were analyzed using GeneMapper 4.0 software. Positive specimens demonstrated fragment lengths of 178 or 196 bp, dependent on *C. difficile* strain. Negative specimens demonstrated no amplification.

Results

Development of the IsoAmp CDAD Test

An asymmetric HDA assay with an IC was developed to amplify a conserved region among toxigenic *C. difficile* isolates. The excess primer of *tcdA* gene was labeled with biotin for binding the streptavidin-conjugated color particles, and the *tcdA* probe was labeled with FITC for the capture at the T line on the vertical-flow DNA detection strip (Figure 1A). The asymmetric HDA of *C. difficile* was performed in the presence of an IC and an IC probe, which was labeled with DIG for the capture at the C line on the DNA detection strip, to monitor the performance of HDA and the subsequent BESt Cassette detection (Figure 1A). More biotin labeled strands were produced than unlabeled complementary strands in the asymmetric HDA, and thus no denaturation was required to make the biotin strand available for binding by the FITC- or the DIG-labeled probe. The concentrations of the primers, the probes, and the amount of IC were optimized to achieve the best performance of asymmetric HDA and BESt Cassette detection.

A Type II BESt Cassette was used for end-point analysis of the HDA products. The self-contained cassette comprises two individual components: i) an amplicon cartridge that holds the running buffer and a single 0.2-ml thin-wall PCR tube containing the amplified product, and ii) the detection chamber, which houses the amplicon cartridge and a vertical-flow DNA detection strip embedded into the cassette. The DNA detection strip is coated with an anti-FITC antibody and an anti-digoxigenin antibody that captures the corresponding labeled probe and serves as the T line and the C line, respectively, in the assay. A razor blade and a plastic pin lodged at the bottom of the detection chamber cut open the PCR tube and the running buffer bulb when the handle of the de-
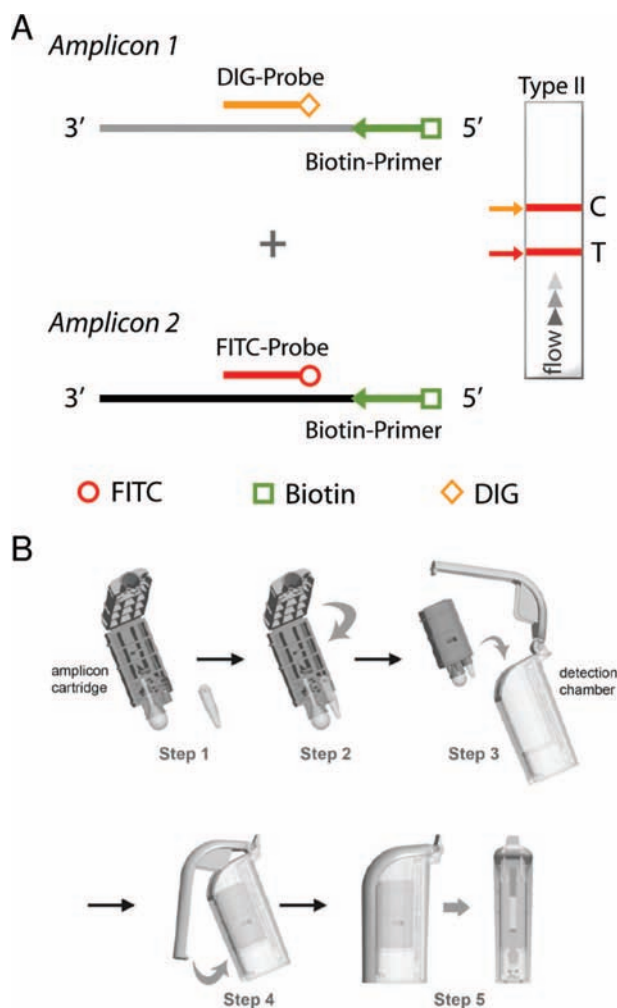

Figure 1. Schematic diagram of the mechanisms for IsoAmp CDAD test. **A:** Using a biotin-labeled excess primer, a 3′-FITC-labeled *tcdA* gene probe and a 3′-DIG-labeled IC probe can bind to the biotin-labeled single-stranded DNA generated from the asymmetric HDA reaction in the amplification of *C. difficile* toxin gene *tcdA* and an IC. These probe-target hybrids then bind to streptavidin-conjugated color particles and are captured by an anti-FITC and an anti-DIG antibody coated on the DNA detection strip. **B:** The procedure of using a BESt Cassette for amplicon detection. The amplification reaction vessel is placed in an amplicon cartridge (step 1); the cartridge is closed to immobilize the reaction vessel (step 2); the amplicon cartridge is inserted into the detection chamber (step 3); the handle of the detection chamber is closed to seal the vessel into the chamber and to cut open the running buffer reservoir and the reaction vessel (step 4); and after 10 to 15 minutes, the detection window of the chamber is read by eye to score the assay result (step 5).

tection chamber is closed. The mixture flows through a fiberglass paper connected with the DNA detection strip that is attached with a fiberglass pad preloaded with streptavidin-conjugated color particles for color visualization. The detection strip can be read 10 to 15 minutes after the reaction tube is placed into the cassette. The procedure of using BESt Cassette for amplicon detection is described in Figure 1B.

Analytical Sensitivity and Specificity Tests

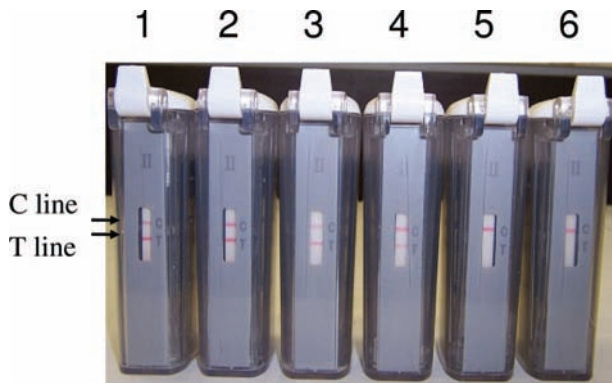Twelve of 12 IsoAmp CDAD assays of the A+/B+ *C. difficile* strain (ATCC 9689) and 3 of 3 of each of the 4

Figure 2. Determining the analytical sensitivity of IsoAmp CDAD test. Different amounts of genomic DNA of an A+/B+ *C. difficile* strain (ATCC 9689) mixed with 5×10^3 copies of IC plasmid and 2 μl of pooled stool DNA from CDAD-negative fecal samples were used as the template in the IsoAmp CDAD test. The amounts of *C. difficile* genomic DNA per reaction used in the test are as follows: 1, 200 copies; 2, 20 copies; 3, 10 copies; 4, 5 copies; 5, 2 copies; and 6, 0 copies.

A−/B+ *C. difficile* strains detected 20 copies of genomic DNA premixed with 2 μl of pooled DNA extracted from CDAD-negative stool samples. When the copy number in each reaction was reduced to 10, 6 of 12 samples of the A+/B+ *C. difficile* strain and 3, 2, 3, and 2 of 3 of the 4 A−/B+ *C. difficile* strains CF1p, CF2, CF4, and 8864 were tested positive, respectively. These test results suggest an analytical sensitivity of 20 copies of *C. difficile* per test for both the A+/B+ and A−/B+ strains. An example from the analytical sensitivity study of the IsoAmp CDAD test is shown in Figure 2.

The test was positive only for the toxigenic *C. difficile* strain and negative for all of the other 11 bacterial species used in the triplicate analytical specificity tests (Table 1). Notably, no cross-reactivity was found with the nontoxigenic *C. difficile* strain or *C. sordellii*, which carries the hemorrhagic toxin gene and the lethal toxin gene closely related to *C. difficile tcdA* gene and *tcdB* gene.[22] This indicated a high specificity of IsoAmp CDAD test for toxigenic *C. difficile* strain detection.

Clinical Sensitivity and Specificity Tests

Of the 101 fecal samples, 60 were scored positive for CDAD by the EIA assay, the PCR assay, and the IsoAmp CDAD test. Six were positive by both PCR and IsoAmp but negative by EIA. The other 35 were negative by all three techniques (Table 3). HDA was inhibited in 8 out of the 101 stool DNA samples tested, which resulted in invalid read in the IsoAmp CDAD test, when 5 μl of the extracted stool DNA was used in the assay. The inhibition was overcome when diluted stool DNA was reapplied in the test. Overall, using the PCR assay as a reference standard, the IsoAmp CDAD test demonstrated a sensitivity of 100%, a

specificity of 100%, and a diagnostic accuracy of 100%; whereas the EIA method showed a sensitivity of 90.9%, a specificity of 100%, and a diagnostic accuracy of 94.1% (Table 3). The IsoAmp CDAD test thus displayed a performance comparable with PCR assay and posted a higher sensitivity compared with EIA assay.

Discussion

In this study, we have developed a simple-to-perform nucleic acid test, the IsoAmp CDAD test, which can be easily adapted to different laboratory settings for the rapid identification of toxigenic *C. difficile* in feces. Total material cost for each IsoAmp *C. difficile* assay, including the HDA amplification reagents and the BESt Cassette, is about $7, which is less than or equivalent to the cost of most molecular diagnostic devices and reagents available in the market. Stool DNA can be extracted by an automatic sample preparation system like the NucliSENS easyMAG system (bioMérieux) or a manual kit like QIAamp DNA Stool mini kit (Qiagen).

A conserved region found in pathogenic *C. difficile* strains was chosen as the target for HDA amplification in the presence of an IC with a scrambled sequence, using a water bath or heat block. The amplified product was then detected in a simple, self-contained disposable device, the BESt Cassette, without opening the amplification tube. The entire procedure, starting from specimen processing and ending at result readout, can be completed in less than 2.5 hours. Specific product detection was enabled by the inclusion of sequence-specific probes in the IsoAmp CDAD test. The probes can bind the amplified products from asymmetric HDA for direct detection on a DNA detection strip without the need for denaturation. Inclusion of an IC is especially useful in monitoring inhibition associated with nucleic acid amplification of the stool DNA. The IC plasmid can also be spiked directly into the stool specimens to monitor the performance of the whole assay process from sample preparation to detection. Application of BESt Cassette for amplicon detection in the IsoAmp CDAD test effectively eliminates the problem generated from postamplification contamination, which is associated with many nucleic acid amplification-based platforms, without the use of expensive instrument.

The toxigenic *C. difficile* loads in patients' feces vary significantly, in a range from about 10^7 to 10^4 colony forming units/g stool.[15] High detection sensitivity is thus critical in the success of nucleic acid amplification-based CDAD test. The IsoAmp CDAD test is highly sensitive with an analytical sensitivity around 20 copies of *C. difficile* genomic DNA per test, which is equivalent to about 10^4 colony forming units/g stool. This puts the analytical sen-

Table 3. Comparison of the IsoAmp and EIA Assays for Diagnosis of *C. difficile*-Associated Diarrhea

Test	PCR+/Test+	PCR+/Test−	PCR−/Test+	PCR−/Test−	Sensitivity (%)	Specificity (%)
EIA	60	6	0	35	90.9	100.0
IsoAmp	66	0	0	35	100.0	100.0

sitivity of IsoAmp CDAD test at the same level as real-time PCR assay, which is around 5×10^4 colony forming units/g stool.[15] In addition, IsoAmp CDAD test is highly specific to toxigenic *C. difficile* strains without any observed cross-reactivity with nontoxigenic *C. difficile*, other *Clostridium* species, or the food-borne pathogens tested. Moreover, the clinical sensitivity and specificity of the Iso-Amp CDAD test was as good as an in-house developed PCR assay with clinical sensitivity better than an EIA assay.

One of the major challenges for nucleic acid amplification-based CDAD tests is to overcome the amplification-inhibitory components found in fecal samples. About 7.9% (8 of 101) of stool DNA extracted from patients' feces by easyMAG displayed inhibition in the IsoAmp CDAD test when 5 μl of the extracted stool DNA was used. The inhibition rate decreased from 7.9 to 2.0% after a 10-fold dilution of these stool DNA samples, and no inhibition was detected after a 100-fold dilution of these stool DNA samples. Overall, the inhibition was overcome by a simple dilution of the stool DNA without affecting the clinical sensitivity and specificity in IsoAmp CDAD test compared with a PCR assay. However, dilution of the stool DNA reduces the analytical sensitivity of the test and also increases the time and the cost needed for the test. Sepharose 4B column has been previously reported to separate humic substance and DNA from environmental samples,[23] and its use in overcoming inhibition in stool DNA will be evaluated in the IsoAmp CDAD test. Inhibition in stool DNA appears also dependent on the methods used in DNA extraction. No inhibition in stool DNA prepared by a rapid DNA extraction kit (Infectio Diagnostic, Inc., Sainte-Foy, Quebec, Canada) was observed in a PCR assay.[15]

One potential limitation of the IsoAmp CDAD test is that only *tcdA* gene of toxigenic *C. difficile* was used for detection in the test. Although the conserved region used to amplify the *tcdA* gene was found in all published sequences of pathogenic *C. difficile* strains, including the ones producing TcdB only, we cannot exclude the possibility that pathogenic *C. difficile* strains with deletion of the complete *tcdA* gene and nonpathogenic strains containing portions of the *tcdA* gene but not expressing active toxins A or B may exist in nature, which would lead to false-negative and false-positive results, respectively, in the IsoAmp CDAD test.

In summary, the IsoAmp CDAD test does not require expensive instrumentation or expertise for nucleic acid amplification and detection and can be easily adapted to different laboratory settings. The development of this simple-to-perform, rapid, and accurate test for the identification of toxigenic *C. difficile* in feces by combination of an isothermal helicase-dependent amplification technology with a self-contained detection device will provide a valuable alternative to immunoassays and PCR-based tests for diagnosis of CDAD.

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