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TECHNICAL ADVANCE

Application of Nuclear Magnetic Resonance to Detect Toxigenic *Clostridium difficile* from Stool Specimens

A Proof of Concept

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We evaluated the performance of an early prototype core molecular mirroring nuclear magnetic resonance detection platform (Mentor-100) to detect toxigenic *Clostridium difficile* from stool. This technology uses customized nanoparticles bound to target specific oligonucleotide probes that form binaries in the presence of nucleic acid from the target microorganism. Liquid patient stool specimens were seeded with *C. difficile* or other *Clostridium* species to determine the analytical sensitivity and specificity. Samples underwent nucleic acid extraction and target amplification with probes conjugated with iron nanoparticles. Signal from nuclear magnetic resonance spin–spin relaxation time was measured to detect the presence or absence of toxigenic *C. difficile*. The limit of detection was <180 colony forming units per reaction of toxigenic *C. difficile*. No cross-reactivity was observed with nontoxigenic *C. difficile*, *Clostridium sordellii*, *Clostridium perfringens*, *Bacillus subtilis*, or *Paenibacillus polymyxa* at 10^8 colony forming units/mL. Correlation studies using frozen stool samples yielded a sensitivity of 88.4% (61 of 69) and a specificity of 87.0% (40 of 46) as compared with a commercial PCR assay for *C. difficile*. The area under the curve in the receiver operating characteristic curve analysis was 0.922. The prototype molecular mirroring platform showed promising performance for pathogen detection from clinical specimens. The platform design has the potential to offer a novel, low-cost alternative to currently available nucleic acid–based tests. *(J Mol Diagn 2017, 19: 230–235; http://dx.doi.org/10.1016/j.jmoldx.2016.09.012)*

Accurate and prompt identification of pathogens from complex biological matrices at points of need are important for both clinical medicine and public health biosurveillance. Nuclear magnetic resonance (NMR) nanotechnology is a next-generation diagnostic method that can detect nucleic acids or antigens of microorganisms with high sensitivity and specificity. NMR technology has the potential to become mainstream in the clinical diagnostic space.[1–3]

A novel NMR technology has been developed with the potential to detect a variety of targets from different matrices [Mentor-100 (M100); Menon Biosensors, Inc., San Diego, CA]. Its core molecular mirroring NMR technology uses iron nanoparticles coated with target-specific biomarkers to bind to target molecules such as nucleic acids. For analyte-specific DNA detection, nucleic acid is amplified and then reacted with magnetic nanoparticle biomarkers. DNA–nanoparticle complexes are detected by NMR using M100. The NMR system generates a signal by measuring

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NMR spin–spin relaxation time (T_2), which is correlated with the presence or absence of microorganism DNA (US patent publication number US20130059293A1).

The M100 initially was funded by the US Department of Homeland Security and Department of Defense for biodefense applications, specifically for autonomous and continuous environmental monitoring of pathogens at key public facilities such as hospitals, railway stations, airports, and federal buildings. For this purpose, the system has been tested and validated in the field at the Boston Metro railway (Boston, MA) and the Edgewood Chemical Biological Center (US Army, Aberdeen Proving Ground, MD) to detect *Bacillus anthracis*, *Bacillus thuringiensis*, *Yersinia pestis*, and *Francisella tularensis* in aerosol and water samples in the presence of environmental interferents such as soil or sewage. The system showed 100% accuracy in blind sample detection of these targets during validation studies.

We evaluated the prototype M100 system to detect toxigenic *Clostridium difficile* from stool in its first clinical application study. *C. difficile* is a Gram-positive, anaerobic, spore-forming *Bacillus* that is responsible for 15% to 20% of antibiotic-related cases of diarrhea and nearly all cases of antibiotic-associated pseudomembranous colitis.[4] Elderly and hospitalized patients who have been on antibiotic therapy are at the highest risk for infection by *C. difficile*, but persons outside the health care environment are developing *C. difficile* infection with increasing frequency.[5] In this proof-of-concept study, our objective was to show the ability of the M100 NMR-based method to detect *C. difficile* in the complex matrix of a stool specimen, thus challenging its capabilities.

Materials and Methods

Patient Specimens and Bacterial Isolates

For analytical sensitivity and specificity studies, fresh unpreserved liquid stool specimens were verified to be negative for toxigenic *C. difficile* with a commercial FDA-cleared PCR assay to detect *C. difficile* and the NAP1 epidemic strain (Xpert *C. difficile*/Epi PCR assay; Cepheid, Sunnyvale, CA), which was performed per routine clinical care at Keck Medical Center of the University of Southern California (Los Angeles, CA). Liquid, as opposed to semisolid or solid, stool samples were chosen to ensure the accuracy of organism concentrations to be used in seeded studies, and to reflect diarrheal samples that typically accompany requests for toxigenic *C. difficile* work-up. For correlation studies, we tested patient samples that had been tested by the Xpert *C. difficile*/Epi assay per routine clinical care and then stored at −70°C from January 2014 to August 2015.

Seeded experiments used either type strains or bacterial isolates identified to the species level by the clinical microbiology laboratory. Toxigenic *C. difficile* strains included ATCC strain 9689 (Manassas, VA), CDC strain 2006023 (Atlanta, GA), and an in-house strain isolated from a stool specimen that was positive on Xpert *C. difficile*/Epi PCR assay. Culture isolation of the specimen was performed anaerobically using cycloserine–cefoxitin fructose agar plates (Hardy Diagnostics, Santa Maria, CA). Identification was made based on the characteristic yellow spreading colonies, Gram stain result, and positive result of the isolate by the Xpert *C. difficile*/NAP1 PCR assay for the *tcdB* gene. Other types of strains used included nontoxigenic *C. difficile* (ATCC strain 70057 and BAA strain 1801), *Bacillus subtilis* NRS 231 (ATCC 6633), and *Paenibacillus polymyxa* (ATCC 842). *Clostridium* species not *C. difficile* were identified in-house by phenotypic methods (RapID ANA II; Remel, Lenexa, KS) for testing performed during routine clinical care. *B. subtilis* NRS 231 served as the internal control target for each sample tested. Discarded packed red blood cells from a single donor unit were obtained for interfering substance studies.

The study protocol was approved by the Institutional Review Board of the University of Southern California.

Nucleic Acid Extraction

B. subtilis [10^7 colony forming units (CFU)/mL] internal control was seeded into each sample before extraction. Nucleic acid was extracted using the Menon-Universal Sample Preparation protocol: 750 μL lysis buffer and 10 μL proteinase K (1 mg/mL) were added to approximately 75 to 125 mg of stool, heated at 95°C for 5 minutes, and frozen at −80°C for 15 minutes. Then, samples were heated at 95°C for 5 minutes and centrifuged at 21,100 × *g* for 5 minutes. The supernatant of each sample was collected and mixed with a solution containing 300 μL molecular grade water, 354 μL ammonium acetate (2.5 mol/L), and 400 μL chloroform. After 5 minutes of centrifugation at 21,100 × *g*, the upper phase was collected and precipitated with isopropanol for 1 minute at room temperature. Tubes then were centrifuged for 5 minutes, the supernatant was discarded, and the pellet was washed with 900 μL of 75% ethanol solution. After repeating the centrifugation step and discarding the supernatant, the pellet was resuspended in 75 μL of 0.1× Tris-ethylenediaminetetraacetic acid buffer. The sample then was heated for 10 minutes at 95°C to dissolve the pellet. The elution was used as the template for the Biosensor PCR.

Biosensor PCR

Target DNA was amplified by a rapid 20-minute thermocycling reaction. Biotinylated primers targeted the *C. difficile tcdA* gene [M1685 (forward): 5′-AGCGGAAATGGTAGAAATGTTGTAG-3′, M1686 (reverse): 5′-ATAAAGTCACTTCCTTCTGTAGACC-3′] or the *B. subtilis rpoB* gene [M1683 (forward): 5′-CAGCATCGTAGCTCGTTTCC-3′, M1684 (reverse): 5′-AAATAACAGCGGCACCAGAG-3′]. Separate PCR reaction tubes were designated for internal control (*B. subtilis*) and target (toxigenic *C. difficile*) amplifications for

each sample. PCR master mix for target amplification contained 0.3 μmol/L of the respective biotinylated primers, 2 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 5 μL polymerase buffer, 5 μL template, and 2.5 U DNA polymerase in a total of 25 μL. PCR cycling conditions were as follows: 40 seconds at 98°C, 42 cycles of 6 seconds at 98°C, 5 seconds at 58°C, and 1 second at 72°C, and a 4°C hold (Mastercycler thermal cycler; Eppendorf, Hauppauge, NY).

Nanoparticles and NMR Detection System

The molecular mirroring technology uses magnetic nanoparticles conjugated with oligonucleotide probes that mirror the target's DNA. T_2 signal measurements were taken on the M100 NMR system, a small desktop instrument with computerized output. In the presence of the target, a biotin-streptavidin-nanoparticle binary complex is formed, serving as the basis for signal detection. After PCR amplification, a 100-μL mixture containing 18 μL PCR product, streptavidin-coated iron beads (Ademtech, Pessac, France) at a final concentration of 2 ng/μL, and phosphate-buffered saline was measured to obtain the baseline NMR T_2 signal. The tubes were placed in the Menon Incubator for 15 minutes where nanoparticles formed binaries leading to signal amplification. The mixture then was measured again in the NMR system (final T_2 signal). The resulting ΔT_2 was obtained by subtracting the baseline signal from each final T_2 measurement, and was used for the plots and statistics. All measurements, that is, baseline and final T_2, were performed in duplicate and averages of duplicates were used for calculations. When no target was present, uniform distribution of nanoparticles occurred, resulting in lower ΔT_2. When target DNA was present, binary complexes of nanoparticles were formed, resulting in higher ΔT_2.

Positive (toxigenic *C. difficile* cells) and negative (water) controls were included for each batch of specimens starting with the nucleic acid extraction step. Between 1 and 24 samples could be tested in one batch. The NMR system used in this study is described in the US Patent Application Publications (US 2013/0059293).

Analytical Sensitivity Studies

Three strains of toxigenic *C. difficile* (ATCC 9689, CDC 2006023, and an in-house NAP1 strain) each were seeded into three liquid stool samples from different patients. Organism concentration calculations were based on a 1.0 McFarland standard (approximately 3.0×10^8 CFU/mL) of *C. difficile* organisms from solid media growth suspended in sterile saline. Serial dilutions then were made in sterile saline to achieve target test concentrations. Concentrations tested were as follows: 10^4, 10^5, and 10^6 colony forming units CFU/mL, corresponding, respectively, to 18, 180, and 1800 CFU/reaction. Each concentration for each strain and stool sample was performed in triplicate for a total of 81 reactions. Positive and negative controls were included with each batch of testing. In addition, unseeded patient stool samples were included to assess carryover contamination; each of the three negative, native stool samples were tested in triplicate and positioned between every three positive seeded samples.

Analytical Specificity Studies

Organism concentrations to be used in analytical specificity studies were prepared starting with a 4.0 McFarland standard suspension (approximately 1.2×10^9 CFU/mL) in sterile saline of the bacterial colonies to be tested. Suspension concentrations were adjusted further in saline, and then seeded to liquid stool samples to a final concentration of 10^8 CFU/mL. Strains tested included two nontoxigenic *C. difficile* strains and one each of *Clostridium sordellii*, *Clostridium. perfringens*, *B. subtilis*, and *P. polymyxa*. An additional sample was seeded with both 10^8 CFU/mL of nontoxigenic *C. difficile* (BAA-1801) and 10^4 CFU/mL of toxigenic *C. difficile* (ATCC 9689). Each experiment was performed in triplicate, with positive and negative controls included for each batch of testing. We also assessed for interference by human blood with appropriate internal control or T_2 signal detection by seeding and testing two toxigenic *C. difficile*—negative liquid stool specimens seeded with 5% (vol/vol) donor blood.

Correlation Studies

To explore the feasibility of using clinical samples on the M100 assay, 118 specimens from a frozen repository with a mix of positive and negative results for toxigenic *C. difficile* by the Xpert *C. difficile*/Epi PCR assay were selected for testing. Results from the M100 system were compared with those of the Xpert *C. difficile*/Epi PCR assay to determine sensitivity, specificity, and the receiver operating characteristics curve. Positive and negative controls were included with each batch of testing.

Results

T_2 Signal Measurements

The average time for amplification and T_2 signals by this manual method was approximately 45 minutes per sample. Based on the analytical sensitivity and specificity studies, the cut-off ΔT_2 value for positive results was determined empirically to be 100 ms for both target analyte and internal control. Positive ΔT_2 values for toxigenic *C. difficile* in the seeded studies ranged from 100.5 to 843 ms, whereas negative controls (stool samples negative for toxigenic *C. difficile* by Xpert PCR) yielded ΔT_2 values of −15.5 to 88 ms (Figure 1). Internal control ΔT_2 values during the analytical sensitivity and specificity studies ranged from 128.5 to 1243 ms.

Analytical Sensitivity Studies

At 10^4 CFU/mL (18 CFU/reaction), there were 2 internal control failures that were excluded from further analysis,

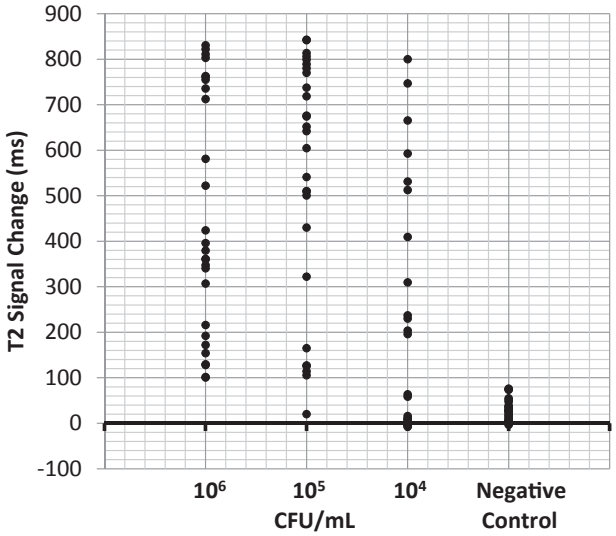

Figure 1 T_2 signal change values for concentrations of toxigenic *Clostridium difficile* used to assess analytical sensitivity. A T_2 signal change cut-off value of >100 ms was determined empirically to distinguish positive from negative test results. CFU, colony forming units.

and 11 of the 25 remaining samples (44.0%) were positive for toxigenic *C. difficile*. At 10^5 CFU/mL (180 CFU/reaction), 26 of 27 (96.3%) samples were positive by the M100 system. At 10^6 CFU/mL (1800 CFU/reaction), 27 of 27 (100%) samples were positive (Figure 1). Considering the lowest concentration at which the target is detected reproducibly (>95%), the limit of detection of the M100 system for toxigenic *C. difficile* was close to 180 CFU/reaction, and between 18 and 180 CFU/reaction. Overall, 2 internal control failures occurred in the 81 reactions (2.5%). All triplicate tests of the negative unseeded stool samples (total, 9 samples) yielded negative results on the M100 system. The assay set-up was intended to yield qualitative results and the T_2 signal was not observed to correlate with the target concentration.

Analytical Specificity Studies

No cross-reactivity was observed with nontoxigenic *C. difficile*, non–*C. difficile* clostridial species, *B. subtilis*, or *P. polymyxa* in any of the triplicate tests (overall $n = 18$). No interference was noted in the two stool samples seeded with red blood cells, both of which tested negative. ΔT_2 values ranged from −15.5 to 82 ms. The M100 system was able to detect all three replicates of the sample with 10^4 CFU/mL toxigenic *C. difficile* (ATCC 9689) and 10^8 CFU/mL nontoxigenic *C. difficile*, with ΔT_2 values of 197 to 358 ms. There were no internal control failures.

Correlation Studies

Of 118 stool specimens tested, 3 had internal control failures for an invalid rate of 2.5%. Receiver operating characteristics curve analysis yielded an area under the curve of 0.922 (Figure 2). The ΔT_2 cut-off value of 100 ms as determined earlier was judged to be acceptable for optimizing both sensitivity and specificity for clinical samples. Based on this cut-off value, of 69 Xpert *C. difficile*–positive specimens, 61 were positive and 8 were negative by the M100 assay, for a sensitivity of 88.4% (95% CI, 78.4% to 94.9%). Of 46 Xpert *C. difficile*–negative specimens, 40 were negative and 6 were positive by the M100 assay, yielding a specificity of 87.0% (95% CI, 73.7% to 95.1%). Overall concordance between the two assays was 87.8% (101/115).

Discussion

Molecular diagnostic methods used in clinical microbiology laboratories have evolved over past decades. Nucleic acid amplification–based commercial systems have become more commonplace in everyday testing as a result of sample-to-answer user friendliness. Even more recently there has been progression beyond nucleic acid amplification technologies, as exemplified by mass spectrometry–based organism identification and T_2 magnetic resonance for direct detection of pathogens in whole blood.[2,6] Advancements in clinical diagnostics continue to be important in providing creative alternatives to generate cost and time efficiencies, less environmental waste, and easier accessibility to new methods for small and large clinical laboratories alike.[7]

Application of NMR to the detection of biological substances has not fully developed its potential as an *in vitro* diagnostic method. The basic principle is to detect binary complexes of particles in the presence of target molecules (eg, nucleic acids) by use of superparamagnetic nanoparticles bound, for example, to an oligonucleotide.

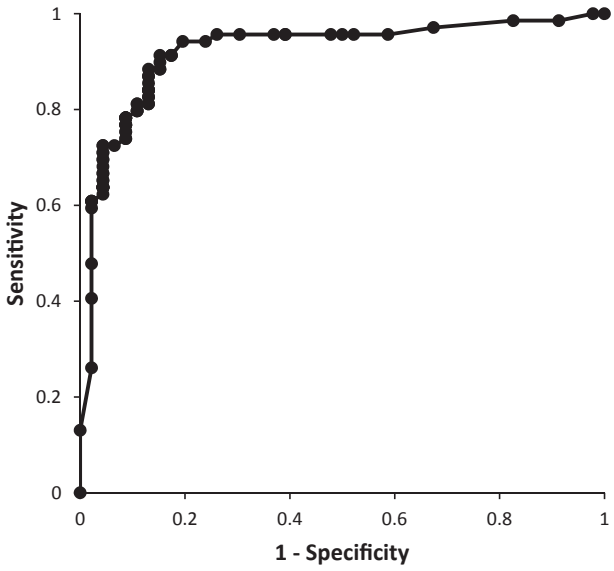

Figure 2 Receiver operating characteristics curve for the M100 *Clostridium difficile* assay based on results of 115 clinical samples compared with the Xpert *C. difficile*/Epi PCR assay. Area under the curve, 0.922.

T_2 relaxation times of surrounding water molecules, which are dependent on the presence or absence of target, are measured after application of a magnetic field. Surrounding water molecules number in the billions, thereby amplifying the magnetic resonance signal. This approach is able to detect concentrations of nucleic acid in the femtomolar range.[8] Magnetic nanoparticles have low reagent costs, maintain stability with storage, and are relatively nonhazardous to the environment. Furthermore, magnetic resonance has a high tolerance for sample background because it is not dependent on optical measurements and biological samples have very little magnetic background.[3,9]

One advantage of the M100 system in contrast to other NMR-based approaches is that it uses a magnetic resonance imaging–type magnet and radiofrequency coil design to enable detection of large-volume samples in the presence of interferents. The magnetic fields allow the interaction between the nanoparticles, producing a signal within seconds and decreasing the time required for test results.[2,10] In addition, the technology is able to exploit a combination of multiple sensitivity multipliers including nucleic acid amplification and binary complex formations to result in a large T_2 change in the presence of target DNA, thus allowing for increased sensitivity. Based on work that has been performed on simulated samples and based on the theoretical potential of magnetic resonance by other investigators, it is probable that refinements to this method can extend the sensitivity limit of the M100 system in fecal samples. Another important potential of this system is that it can be designed to operate without disposable reagent cartridges, unlike most sample-to-answer systems used in clinical laboratories today. Prototype instruments have been engineered so that samples may be loaded directly and taken through the various processing steps via flushable and reusable fluidic channels. At the current estimation that hospitals generate 25.1 pounds of waste per staffed bed per day (Practice Green Health, *https://practicegreenhealth.org/topics/less-waste*, last accessed April 27, 2016), this new paradigm for molecular testing is essential to consider for reducing environmental impact and biohazardous waste disposal costs associated with laboratory testing. With the goal of a sample-to-answer system in mind, Menon optimized a nucleic acid extraction method for downstream PCR and NMR application that, although performed manually in our study, is the basis for the automated platform currently under development. Overall testing costs with such a design therefore are minimized because of conservation of raw materials, enhancing the already low reagent costs for this technology.

A *C. difficile* diagnosis relies greatly on clinical laboratory testing of stool specimens to determine the presence or absence of the toxigenic organism or its toxins.[11] Traditional methods such as cytotoxin neutralization assay or toxigenic culture require at least 48 hours for completion. Immunoassays for toxins A or B suffer from poor sensitivity and specificity and largely have been supplanted by other tests in the United States.[12] Nucleic acid amplification is considered an optimal single test for *C. difficile* infection, with a rapid turnaround time and high analytical sensitivity.[13] It offers the potential to reduce the detection time, thereby enabling the prompt initiation of antimicrobial treatment and implementation of infection control measures.[14] In this proof-of-concept study, we show that the M100 NMR bioassay's limit of detection for toxigenic *C. difficile* in stool specimens (<180 CFU/reaction) is similar to those claimed by Food and Drug Administration–cleared commercial systems, including the Xpert *C. difficile*/NAP1 assay (23 to 460 CFU/reaction) and the *illumigene C. difficile* (4 to 64 CFU/reaction; Meridian Biosciences, Cincinnati, OH). With our observed area under the curve of 0.922, correlation with the Xpert *C. difficile*/Epi assay was encouraging, considering that this study was the first time this NMR assay was challenged with a variety of clinical samples of a complex matrix. The specificity of 87.0% compared with the Xpert *C. difficile* PCR assay indicates the possibility of excess false-positive results; further optimization of this NMR bioassay may be required to minimize signal detection in the absence of target and will be explored in future iterations of the assay. We applied the NMR approach to detection of toxigenic *C. difficile* from stool, but the technology can be adapted readily to detect other clinically important pathogens from other biological matrices. Although the current study focused on detection of a single *C. difficile* gene target per reaction, the technology has multiplexing capabilities that were beyond the scope of this study. Multiplexing may be performed in the T_2 NMR method by performing final T_2 measurements in series after selective cleaving of target binaries.

We showed that the M100 NMR method had few issues with detection of toxigenic *C. difficile* in seeded liquid stool specimens, although the variety of patient specimens studied was limited and may be expanded in future studies. Internal pilot studies of this platform with other sample types and other analytes support the wide applicability of this method, with little background interference and a dynamic range of up to 8 log. Although configured in this study specifically for qualitative analysis, the M100 technology is capable of quantification by using T_2 signal intensity.[9] Further studies still are needed to show performance of the M100 system with a broader array of specimen types and pathogen targets. We believe that additional refinements to the method can enhance its analytical and clinical sensitivity further, but this initial study shows promise.

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