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MINIREVIEW

Ultrasensitive Detection and Quantification of Toxins for Optimized Diagnosis of *Clostridium difficile* Infection

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Recently developed ultrasensitive and quantitative methods for detection of *Clostridium difficile* toxins provide new tools for diagnosis and, potentially, for management of *C. difficile* infection (CDI). Compared to methods that detect toxigenic organism, ultrasensitive toxin detection may allow diagnosis of CDI with increased clinical specificity, without sacrificing clinical sensitivity; measurement of toxin levels may also provide information relevant to disease prognosis. This minireview provides an overview of these new toxin detection technologies and considers what these new tools might add to the field.

Clostridium difficile is a significant nosocomial and community-acquired pathogen in adults and children, associated with disease ranging from mild diarrhea to severe pseudomembranous colitis resulting in colectomy and even death (1, 2). Transmission is primarily by person-to-person spread of spores (fecal-oral route), and prevention of transmission is significantly complicated by the high prevalence of asymptomatic colonization with *C. difficile* (e.g., between 5% and 50% in adult inpatient facilities [1]). Since the turn of the millennium, rates of *C. difficile* infection (CDI) have increased globally, concomitant with increased rates of severe clinical presentations and worsened clinical outcomes (1, 3). A recent U.S. prevalence survey of health care-associated infections (HAI) (4) found that *C. difficile* was the most commonly reported pathogen, causing 12.1% of HAI. Despite available therapies, treatment failure and relapse are common (1).

C. difficile isolates can be either nontoxigenic or toxigenic (producing toxins A and B); nontoxigenic strains are not considered to be pathogenic. Exposure to antibiotics increases the risk of CDI by disrupting the normal bowel flora and allowing the opportunistic proliferation of toxigenic *C. difficile*. These high-molecular-mass protein exotoxins (308 and 270 kDa, respectively) are immunologically and biologically distinct; depending on the experimental system used, the activity of each has been described in the literature as proinflammatory, cytotoxic, and enterotoxic (5, 6). Most strains produce both toxins A and B, though a minority of disease-causing strains produce toxin B only (see, e.g., reference 7). Toxins A and B are the primary virulence factors contributing to the pathogenesis of CDI (6, 8, 9), and the genes for these toxins (*tcdA* and *tcdB*) are colocated in a pathogenicity locus in toxigenic strains (5, 10). Importantly, these genes are under complex regulatory control and expression of toxin proteins is impacted by numerous environmental factors, including temperature, carbon source/amino acid availability, and antibiotic concentration (10, 11). A recent paper also provided evidence for regulation of toxin production by a quorum sensing system, with toxin synthesis being absent at low bacterial concentrations (12). While each of the two toxins has been shown to be independently capable of causing disease, the relative contributions of the two toxin proteins to disease remain unclear (see, e.g., references 6, 8, 13, and 14), in part due to differences in experimental systems (animals versus humans, purified toxins versus natural infection) and clinical contexts (adults versus children). In short, many complex and important questions remain regarding these toxins and the overall pathogenesis of CDI—questions that could begin to be addressed with a tool with which to sensitively detect and separately quantify toxins A and B in stool.

CURRENT DIAGNOSTIC STRATEGIES AND THEIR LIMITATIONS

Because toxin is necessary for disease, qualitative enzyme immunoassays (EIAs) that detect these toxins in stool were for many years the mainstay of diagnosis, used by more than 90% of U.S. laboratories (1). However, these assays are significantly limited in sensitivity (52% to 75% versus toxigenic culture [TC; see below] [15, 16]). In contrast, the assays have high (96% to 98%) specificity versus TC (15, 16). Attempts have been made to increase sensitivity by combining detection of a more sensitive but less specific target, glutamate dehydrogenase (GDH), with detection of toxin; however, this test must be followed by nucleic acid amplification testing (NAAT; see below) to resolve discordant results (GDH positive [GDH^+]/toxin negative [$toxin^-$]), increasing cost and time to results (5).

The test historically used as the laboratory gold standard, TC (in which *C. difficile* is cultured from stool and isolates are tested for cytotoxin production by cytotoxicity assay [3, 5]), has limited utility for clinical diagnosis. TC methods are slow (requiring 72 to 96 h), nonstandardized, and unsuitable for routine clinical testing. An additional limitation lies in the fact that TC examines toxin production *in vitro*, which may not reflect the strain's production of toxins in the highly variable *in vivo* environment. Notably, Akerlund et al. (17) demonstrated no correlation between fecal

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toxin levels and toxin yields *in vitro* for given isolates or between *in vitro* yields and disease severity.

An alternative reference standard that detects toxin directly in stool filtrate is the cell culture cytotoxicity assay, which detects characteristic cell rounding in the presence of functional toxin. This qualitative, subjective assay is approximately 86% sensitive compared to TC (16) and primarily detects toxin B, which is far more potent than toxin A in this assay (18); specificity of cytotoxicity is confirmed by neutralizing antitoxin antibodies. Like TC, this assay is slow (requiring 24 to 48 h of incubation), nonstandardized, and not widely used for clinical testing. However, a recent United Kingdom-based study (19) compared TC with cytotoxicity testing on more than 12,000 specimens and correlated results with clinical data. While positive cytotoxicity assay results correlated with increased mortality, the combination of positive TC and negative cytotoxicity assay results did not, indicating that the actual presence of toxin (and not just the presence of toxigenic *C. difficile*) was of primary importance. The authors concluded that "detection of toxin is an essential step in the diagnosis of *C. difficile* infection" and proposed a new diagnostic category of "*C. difficile* excretor" (TC positive but cytotoxicity assay negative) to characterize patients without CDI but with possible colonization.

Given the suboptimal sensitivity of EIAs and the complexities and delayed turnaround times of the cytotoxicity assay (and TC), many laboratories have turned to NAAT for detection of the *tcdA* and *tcdB* genes, with its potential for high sensitivity and short turnaround time (despite potentially higher expense). However, despite relatively high sensitivity and specificity versus TC (90% and 96%, respectively, in a large comparison study [20]), the use of NAAT (like that of TC) is confounded by its inability to distinguish disease from colonization (5, 21). The problem remains that positive NAAT results indicate the presence of organisms capable of producing toxin—not whether (or at what levels) they are actually producing it *in vivo*. While this information may be optimal for determining the need for infection control measures, it is not necessarily optimal for deciding whether or not *C. difficile* is the cause of the patient's symptoms (22), and patients should be selected appropriately for testing with this highly sensitive method.

IS IT PREFERABLE TO DETECT TOXINS OR TOXIGENIC ORGANISMS?

Whether detection of toxins (EIA, cytotoxicity assay) or detection of toxigenic organisms (NAAT, TC) has higher clinical utility for diagnosis of CDI clearly remains controversial. Arguing for the higher utility of toxin detection, multiple studies comparing the clinical features of patients with different test outcomes have demonstrated that NAAT-positive, toxin-negative patients have milder symptoms than NAAT-positive, toxin-positive patients (see, e.g., references 22, 23, and 24), and others have shown that toxin-positive patients have higher mortality than toxin-negative patients (see, e.g., references 19, 22, 25, 26, and 27). Further arguing for the clinical utility of toxin detection, disease severity has been correlated to stool toxin levels in some preliminary studies (17, 19, 25, 28, 29), suggesting that the ability to quantify toxin levels in stool could potentially be clinically valuable to predict disease and treatment outcomes and in identifying those who need aggressive therapy. Recent data (30) indicate that toxins also may be detectable in blood in some individuals with CDI, providing another potential use for an ultrasensitive toxin detection tool. However, arguing against the utility of toxin detection (and potentially against ultrasensitive detection, in particular), it must be noted that multiple studies have detected toxin in the stool of some asymptomatic individuals (see, e.g., references 31, 32, and 33) and that even after effective therapy (i.e., with clinical improvement) toxin may remain detectable in stool in some patients (see, e.g., references 34 and 35). Clearly, while toxin is necessary for clinical disease, it is not sufficient, in that toxin can be present in stool in the absence of symptoms; the impact of host immunity on detectable toxin levels also remains unclear.

NOVEL APPROACHES TO ULTRASENSITIVE TOXIN DETECTION

Given the limitations of existing diagnostic testing and the building body of evidence that detection of toxins (rather than toxigenic organisms) has the highest clinical utility, the field would seem to be poised for a simple toxin detection test that combines high analytical sensitivity with the clinical specificity of toxin detection. In considering the potential utility of ultrasensitive assay technologies, an important initial question is what analytical sensitivity should be targeted for assay development. The current analytical limits of detection (LODs) for some of the highest-performing EIAs (16) range from 0.8 to 2.5 ng/ml, i.e., ~1 ng/ml, in stool (36, 37). Ryder et al. (28; discussed below) have described a cell-based assay for quantification of toxin B in stool and calculated toxin concentrations down to as low as 30 pg/ml. Their data indicated that almost half of the toxin-positive specimens in their study would not be detected by EIAs with LODs of ~1 ng/ml. Conventional cytotoxicity assays have demonstrated analytical LODs far below those of EIA for detection of toxin B in buffer (e.g., 1.5 pg/ml [38]), but achievable LODs for detection of toxins in stool samples appear to be higher (29, 39). Older literature (40) states that "1 pg of toxin B is sufficient to cause rounding of the cells" in this assay format, but how this corresponds to an actual concentration of toxin in stool is unclear.

A team led by Yi-Wei Tang, in collaboration with ACEA Biosciences (San Diego), has developed a real-time cellular analysis (RTCA) assay for detection of functional *C. difficile* toxin B directly from stool. This assay applies samples to cultured HS27 cells dispensed in microwells with imbedded electrodes and then measures cell status over time in "cell index" (CI) units, based on changes in electrode impedance with cytotoxic effects (changes in cell number, morphology, and spreading). The first generation of this assay (28) had a LOD for toxin B of 0.2 ng/ml and took >48 h to run. A second-generation assay ("RTCA2" [29]) incorporates a front-end sample processing step ("immunomagnetic separation enrichment process") in which toxin B is first captured from diluted stool supernatant via magnetic beads coated with toxin B-specific monoclonal antibodies (described as "nonneutralizing"); after elution, captured toxins are inoculated to cultured HS27 cells as described above. The cells are precultured (with CI monitoring, establishing a baseline CI) for 18 to 24 h on the specialized plates prior to addition of sample eluates, after which incubation of up to 36 h (with monitoring at 5-min intervals) is required for CI measurements. The time point at which the normalized cell index (nCI) has dropped by 30% is considered a "positive time step" (PST) which in turn is used to calculate toxin concentrations in

samples by comparison to a standard curve generated with buffer spiked with purified toxin B (List Biological Laboratories [Campbell, CA]), also taken through the immunocapture procedure. The calculated LOD for RTCA2 for detection of toxin in stool was 0.12 ng/ml (29). Among the 51 specimens that tested positive by RTCA2 during assay validation, the mean PST ranged from 1.43 to 35.85 h; the total turnaround time was approximately 60 h. Sensitivity and specificity of the RTCA2 versus quantitative toxigenic culture (qTC) were 96.2% and 99.7%, respectively, which were similar to the performance of NAAT (Xpert *C. difficile* assay [Cepheid, Sunnyvale, CA]) versus qTC (100.0% and 99.7%, respectively).

The authors (29) also performed a retrospective chart review to evaluate CDI severity (broken into 6 clinical categories, including no CDI) in study cohort patients. In the 51 patients whose stools tested positive by RTCA2, comparisons of measured toxin B concentrations across the 5 represented severity score groups indicated a correlation between toxin concentration and clinical CDI severity ($R^2 = 0.427$, $P = 0.002$), though no significant correlation between clinical CDI severity and threshold cycle (C_T) values (Xpert) or toxigenic *C. difficile* bacterial loads by qTC were observed. Notably, 14/51 patients were determined not to have CDI (severity group 1); the mean stool toxin B concentration in this group as measured by RTCA2 was 2.22 ng/ml (median, 1.59 ng/ml). The fact that this measured concentration is above the LOD of standard EIA, in combination with the very high sensitivity of the RTCA2 assay versus qTC and NAAT in this study (see above), might raise some uncertainty about the accuracy of the assay's calibration curve. However, given that this assay methodology measures concentrations of functional toxin rather than total toxin as detected by immunoassay, it is difficult to directly compare the LODs of the two types of assays. Measuring functional, and thus biologically relevant, toxin is one advantage of this type of assay. However, it should be noted here that cytotoxicity assays by definition measure only one aspect of toxin function and furthermore predominantly detect toxin B, thus potentially underestimating the contributions of toxin A to disease. The authors note the potential disadvantages of prolonged turnaround time and complexity of the RTCA2 assay and are working to improve both. Importantly, differential immunodetection of toxin B from highly virulent strains of *C. difficile* has recently been demonstrated (41), making it imperative that the front-end immunocapture step of this assay be performed with antibodies that have been shown to detect toxin B from all clinically relevant *C. difficile* strains.

Investigators from the Feng laboratory (18) developed a cell-based immunocytotoxicity assay similarly based on a real-time cell electronic sensing system (xCELLigence; Roche Applied Science, Indianapolis, IN), with readouts in CI units. These investigators uniquely utilized a mouse monoclonal antibody (A1H3) against toxin A to enhance its cytotoxic effect on mRG1-1 cells (an engineered CHO cell line expressing murine FcγRI-α chain [18]) attached to the bottom of microelectrode-embedded microplate wells. Using this method, the team achieved a sensitivity for toxin A of 0.1 to 1 pg/ml in buffer and also managed to detect toxin activity in a small number of porcine stool samples (the LOD for toxin detection in stool was not reported). Overall turnaround time for this assay was as short as 3 to 4 h, achieved by adding freshly thawed mRG1-1 cells (from cryopreservation) together with toxins to the microplate wells and thus avoiding a prolonged preculture step.

An alternative immunoassay approach to ultrasensitive toxin detection has recently been developed based on single-molecule array (Simoa) technology (39). Simoa technology (Quanterix; Lexington, MA), also known as "digital enzyme-linked immunosorbent assay (ELISA)," is based on efficient capture, labeling, and detection of single protein molecules on paramagnetic beads in arrays of femtoliter-sized wells; in terms of achievable LODs, digital ELISA is typically 1,000-fold more sensitive than conventional ELISA (42). Beth Israel Deaconess Medical Center investigators, in collaboration with Quanterix, developed digital ELISAs for toxins A and B and validated the assays using both culture filtrates prepared from a panel of clinical *C. difficile* strains (representing the most common strains in circulation) and adult clinical stool specimens submitted to the hospital laboratory for routine testing (NAAT) for *C. difficile*. The digital ELISAs detected toxins A and B produced by all of the strains in the panel and detected native toxins in stool with LODs of 0.45 pg/ml (toxin A) and 1.50 pg/ml (toxin B), respectively, as calibrated against purified native toxins spiked into NAAT-negative stool samples. Total assay time was 69 min, and sample processing prior to testing was minimal (dilution and filtration); assays were performed on an automated platform ("HD-1").

For validation of the digital ELISAs, 149 clinical stool samples (previously tested by NAAT [illumigene, Meridian Bioscience, Inc.]) were tested by TC (followed by restriction endonuclease analysis [REA] typing of any *C. difficile* isolates), cytotoxicity assay, and the digital ELISAs in parallel. A clinical cutoff for positive results for each digital ELISA was established by averaging the Simoa signal values (average enzymes per bead, or AEB) for "true negative" samples (negative by NAAT, TC, and cytotoxicity assay), plus 3 standard deviations of that mean. The calculated assay cutoffs were 29.4 pg/ml (toxin A) and 23.3 pg/ml (toxin B), respectively; with these cutoffs, the specificities of the digital ELISA in the true-negative group were 96% and 98%, respectively. Toxin concentrations in clinical samples as measured by digital ELISAs spanned a >4-log dynamic range (Fig. 1). As expected, despite the low LOD for the toxin B digital ELISA, 16/65 (25%) samples that were positive by NAAT and 14/63 (22%) samples that were positive by TC were negative by the toxin B digital ELISA, consistent with the presence of organism (but minimal or no toxin) in those samples. However, 34/34 (100%) samples positive by cytotoxicity assay were positive by the toxin B digital ELISA (Fig. 1). There were four samples which were positive by TC and cytotoxicity assay but negative by toxin A digital ELISA; REA typing of isolates obtained from TC confirmed that all four were REA type CF (known to produce toxin B but not toxin A). Mean toxin levels (toxin [A], toxin [B], or toxin [A plus B]) in the 5 subjects with CDI-attributable severe outcomes were higher (1.7-fold, 1.5-fold, and 1.6-fold, respectively) than mean toxin levels in the 68 subjects without CDI-attributable severe outcomes, though these trends did not reach statistical significance ($P = 0.10$, 0.18, and 0.08, respectively). Notably, the sensitivity of the toxin B digital ELISA versus TC (78% [39]) was much lower than the sensitivity reported for the RTCA2 assay versus quantitative TC (96.2% [29]), despite the digital ELISA having a significantly lower reported LOD than the RTCA2 as detailed above. Both toxin B digital ELISA and RTCA2 had high specificity versus TC (97% and 99.7%, respectively), and in both studies, NAAT and TC results were tightly correlated, suggesting similar performances of the TC in both

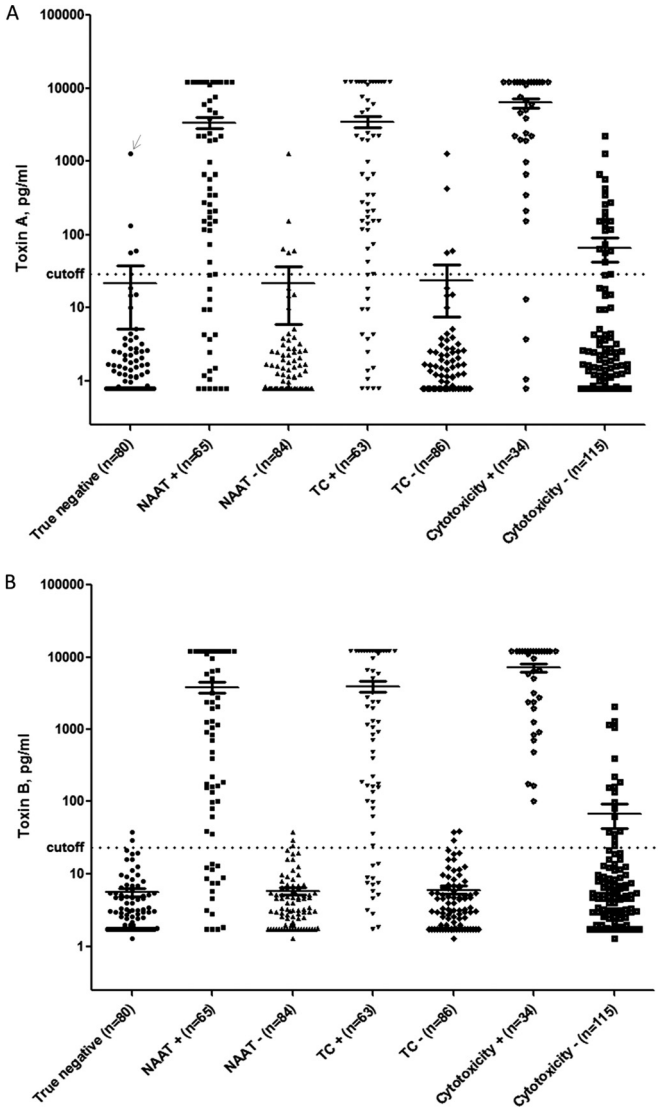

FIG 1 Toxin A and B digital ELISA results (39). Toxin A and B digital ELISA results for groups of samples testing positive versus negative on other assays (i.e., $NAAT^+$ versus $NAAT^-$, TC^+ versus TC^-, and $cytotoxicity^+$ versus $cytotoxicity^-$) are shown in Fig. 1A and B, respectively. Mean signals in each group are indicated by horizontal lines. The calculated clinical cutoffs for each digital ELISA (29.4 pg/ml for the toxin A assay and 23.3 pg/ml for the toxin B assay) are shown as dotted lines spanning each panel. The arrow in Fig. 1A indicates a sample that was excluded from calculation of the cutoff because it was an extreme outlier and substantially distorted the mean for that assay. NAAT, nucleic acid amplification testing; TC, toxigenic culture. (Reprinted from reference 39.)

studies. It is difficult to explain this discrepancy without a direct comparison of the two assays, given the different methodologies used for toxin detection, different standards used for assay calibration, and potential differences in sample handling and clinical cohorts studied.

Alternative immunoassay approaches to ultrasensitive toxin detection are currently in early development, including two based on sandwich-type electrochemical immunosensor methodology (43, 44). Preliminarily, both methods appear to be able to detect toxins in buffer with analytical LODs of ≤1 pg/ml; definition of LOD for toxin in stool samples and, ultimately, assay validation using well-characterized clinical samples will provide important detail as to the potential clinical utility of these approaches.

In summary, a tool capable of sensitive detection and quantification of *C. difficile* toxins in stool offers significant potential for improvements to the current paradigm for diagnosis of CDI. Compared to methods that detect toxigenic organism, ultrasensitive toxin detection may allow diagnosis of CDI with increased clinical specificity, without sacrificing clinical sensitivity. For such a tool to be suitable for clinical use, it must improve on the complexity and lengthy turnaround times limiting cytotoxicity assays, while also improving on the sensitivity limitations of currently available EIAs; optimally, the new tool will be rapid, robust, and simple to use. Future studies should focus on determining the clinical diagnostic and prognostic value of ultrasensitive detection and quantification of stool toxins (both A and B) in symptomatic patients, as well as the clinical significance of TC or NAAT positivity in the absence of detectable toxin. Optimization of clinical cutoffs for these ultrasensitive assays may be refined by analysis of toxin presence and quantity in asymptomatic hosts and the potential impact of host factors (particularly host antitoxin antibodies) on disease expression. If a direct and definitive correlation between toxin quantities and clinical course were shown, this new tool would have not only diagnostic but also prognostic value, allowing toxin measurements made at the time of diagnosis to influence management decisions—a rational yet entirely new direction for the field.

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