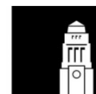

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1 **Ultrasensitive *Clostridioides difficile* Toxin Testing for Higher Diagnostic Accuracy**

2 Johanna Sandlund[1], Kerrie Davies[2], Mark H. Wilcox[2]

3

4 [1]Fluxus, Inc., Santa Clara, CA, USA

5 [2]Healthcare Associated Infections Research Group, Leeds Teaching Hospitals NHS Trust and

6 University of Leeds, Leeds, UK

7

8

9 **Corresponding author:**

10 Mark H. Wilcox

11 Microbiology, Old Medical School, Leeds General Infirmary, Leeds General Infirmary, Leeds LS1

12 3EX, UK

13 Phone: Tel +44 113 392 6818

14 Fax: +44 113 392 2696

15 Email: mark.wilcox@nhs.net

16

17 **Keywords:** *Clostridioides difficile*; *Clostridium difficile*; toxin; ultrasensitive; diarrhea

18 **ABSTRACT**

19 Currently available diagnostic tests for *Clostridioides difficile* infection (CDI) lack specificity or
20 sensitivity, which has led to guideline recommendations for multistep testing algorithms.
21 Ultrasensitive assays for detection of *C. difficile* toxins provide measurements of disease-
22 specific markers at very low concentrations. These assays may show improved accuracy
23 compared to current testing methods and offer a potential standalone solution for CDI
24 diagnosis, although large studies of clinical performance and accuracy are lacking.

25 *Clostridioides difficile* causes nosocomial and community-acquired gastroenteritis and is the
26 most common pathogen responsible for healthcare-associated infections (1, 2). *C. difficile*
27 infection (CDI), ranging in severity from mild to life-threatening diarrhea and colitis, is
28 associated with high morbidity, mortality, and costs, and so has been identified as a key
29 challenge (3, 4).

30 Currently available diagnostic tools for CDI are not optimized in terms of accuracy and/or
31 turnaround time, leading to recommendations for complex (typically algorithmic) testing
32 solutions. Early data suggest that ultrasensitive *C. difficile* toxin assays could offer a new
33 perspective on the laboratory diagnosis of CDI.

34

35 **CDI and the Diagnostic Landscape**

36 *C. difficile* is an anaerobic, Gram-positive bacillus that exists in a dormant spore-form and in a
37 vegetative form with toxin-producing capability (5). CDI is a toxin-mediated disease and two
38 exotoxins – the enterotoxin toxin A (TcdA) and the cytotoxin toxin B (TcdB) – cause diarrhea
39 and inflammation by cytopathic and cytotoxic effects (6). The majority of toxigenic strains can
40 produce both toxins, and strains predominantly producing either of the toxins have been shown
41 to cause disease (7, 8). Non-toxigenic strains are not pathogenic, and individuals can carry
42 toxigenic and toxin-producing *C. difficile* without having CDI (colonization) (6, 9).

43 Crucially, both *C. difficile* colonization and diarrhea – the cardinal symptom of CDI – are
44 common in at-risk populations and both outnumber CDI in most patient populations making it
45 imperative to have a clinically specific test. The prevalence of spores in hospitals and long-term

46 care facilities is relatively high. While 2-3% of healthy adults in the general population are
47 colonized with *C. difficile*, this rate can be up to 25% in hospitalized patients (10). Meanwhile,
48 12-32% of hospital in-patients have diarrhea, increasing to 80% in high-risk groups such as
49 transplant patients (11). A recent large UK study found that on average 1 in 20 hospitalized
50 patients develop diarrhea each day (12). In 85% of these patients with hospital-onset diarrhea,
51 a median of three possible causes for their symptoms could be identified (12). Thus, the
52 potential for non-infectious causes of diarrhea often appears to be underplayed. Taking these
53 rates, alongside the fact that the great majority of fecal samples submitted for testing for CDI
54 are found to be negative, it is clear that accurate clinical diagnosis of CDI is not possible without
55 the appropriate use of laboratory diagnostics, and vice versa, laboratory diagnostics cannot be
56 used accurately without first using appropriate clinical assessment. Unfortunately, however,
57 the wide range of laboratory tests for *C. difficile* vary considerably, not only in terms of the
58 targets used, but also with regard to assay sensitivity and specificity (13–15).

59 The presence of toxins in a fecal sample better correlates with CDI severity and outcome of
60 disease than the presence of only toxins gene(s) does, i.e. toxigenic organisms with the capacity
61 to produce toxins (16, 17). However, conventional toxin enzyme immunoassays (EIAs) have
62 poor sensitivity and can miss CDI cases. In efforts to increase the sensitivity of toxin EIAs,
63 testing with such assays is often performed in combination with glutamate dehydrogenase
64 (GDH), a *C. difficile*-specific antigen that does not, however, differentiate between toxigenic and
65 non-toxigenic *C. difficile* (13).

66 Nucleic acid amplification tests (NAATs) detecting toxin gene(s) were introduced a decade ago
67 and, although associated with higher costs, they offered a sensitive and rapid solution. NAATs

68 detect toxigenic organisms but not the presence of free toxins, and therefore cannot
69 differentiate between colonization and disease and so have poor clinical specificity (9, 16, 17);
70 i.e., in the largest study of its type to date, the positive predictive value of NAAT for CDI was
71 54% (17). Institutions have reported up to a 67% increase in reported CDI rates after adopting
72 NAATs (18, 19). Use of NAATs, therefore, has considerable potential to result in overdiagnosis,
73 and overtreatment, of CDI (16, 17), which could cause harm to patients and represent an large
74 burden on healthcare providers.

75 The cell cytotoxicity neutralization assay (CCNA) and toxigenic culture (TC) are the reference
76 methods for detection of free toxins and toxigenic organisms, respectively, but these are labor
77 intensive, subjective, and have long turnaround times (13, 20). Regulatory studies require
78 comparison of toxin assays with CCNA, an assay that is known to have issues with sensitivity
79 and reproducibility (13, 20, 21).

80 Based on these diagnostic challenges, testing with multistep algorithms is currently
81 recommended, with the goal of increasing diagnostic accuracy by combining clinically sensitive
82 and specific methods, such as NAAT followed by a toxin EIA or a GDH/toxin EIA arbitrated by
83 NAAT, although this is associated with longer time to diagnosis (15, 22). An additional problem
84 is that there is not universal agreement on a case definition of CDI. The Infectious Diseases
85 Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA) guidelines
86 define CDI by the presence of symptoms (usually diarrhea) and a stool test positive for either
87 free toxins (toxin EIA or CCNA) or toxigenic *C. difficile* (NAAT or TC) (22), while the European
88 Society of Clinical Microbiology and Infectious Diseases (ESCMID) guidelines do not agree on
89 using NAATs alone and also require the exclusion of non-CDI-related causes of diarrhea for

90 diagnosis (15, 23). At the heart of this clinical conundrum is the desire for accurate diagnosis of
91 CDI, and yet the absence of a perfect solution based on currently available laboratory assays
92 means that new options are needed.
93

94 **Ultrasensitive Toxin Tests**

95 Since CDI is a toxin-mediated disease, there is a need for a more sensitive toxin assay that
96 better correlates with disease without missing cases. Two ultrasensitive and rapid assays for
97 TcdA and TcdB have recently been described/reported, both with limits of detection (LoDs) at
98 approximately 1 pg/mL per toxin (24–26). Single-molecule array (Simoa®) technology
99 (Quanterix, Inc., Lexington, MA, USA) is based on capture and labeling of single protein
100 molecules on paramagnetic beads and their detection in arrays of femtoliter-sized wells (24).
101 Single Molecule Counting Technology® (formerly Singulex, Inc., Alameda, CA, USA), performed
102 on the Clarity system, utilizes a paramagnetic microparticle-based immunoassay that uses
103 single-photon fluorescence detection for analyte measurement (25). A third technology
104 detecting *C. difficile* toxins, MultiPath™ (First Light Diagnostics, Chelmsford, MA, USA), uses
105 non-magnified digital imaging to enumerate microscopic fluorescent particles bound to
106 molecular targets (27). The analytical sensitivity of the MultiPath assay is significantly lower
107 than the other two (TcdB LoD 45 pg/mL; TcdA LoD not published) (27).

108 None of these ultrasensitive toxin assays are currently commercially available, but efforts
109 towards regulatory approval are ongoing as well as assay development using other
110 ultrasensitive technologies. For comparison, the toxin assay with the lowest claimed LOD

111 currently on the market is *C. Diff Quik Chek Complete*® (TechLab, Inc., Blacksburg, VA, USA),
112 which detects levels of TcdA at ≥630 pg/mL, TcdB at ≥160 pg/mL, and GDH at ≥800 pg/mL (28),
113 (although there is evidence that at least one assay (*C. DIFFICILE TOX A/B II*, Techlab, Inc.) is
114 more sensitive for toxin detection (29)). As a result, ultrasensitive toxin tests have been shown
115 to have 27.0%–39.4% higher analytical sensitivity than toxin EIAs when using CCNA as a
116 reference method (21, 26, 30, 31). In a prospective, multicenter study on 2,000 patients
117 samples, the Clarity assay had 96.3% positive agreement (PA; "sensitivity") and 93.0% negative
118 agreement (NA; "specificity") with CCNA (although this was after discrepant analysis), while a
119 toxin EIA (*C. Diff Quik Chek Complete*) had 59.8% PA (21). Simoa reported 88.0-84.8%
120 sensitivity and 83.9-84.0% specificity for toxin A, 95.5-100% sensitivity and 83.3-87.0%
121 specificity for toxin B, and 95.5% sensitivity and 79.3% specificity for both toxins combined
122 when compared to CCNA (24, 26). MultiPath technology showed 97.0% sensitivity and 98.3%
123 specificity for a TcdB assay when compared to CCNA (27), although this was in an unblinded
124 training set.

125 Data suggest that ultrasensitive toxin assays could provide increased clinical specificity
126 compared with NAAT and increased sensitivity compared with toxin EIAs (7–9, 21, 24–26, 26,
127 30–37), and with overall higher accuracy than multistep algorithms (30, 33), and the studies
128 have also revealed limitations with other methods such as the risk of missing cases using assay
129 that only detect toxin B or its gene, the poor reproducibility of CCNA, and the suboptimal
130 performance of NAAT cycle thresholds for prediction of toxins (7, 8, 21, 37). In a study where
131 results from a GDH/toxin EIA, NAAT, and an ultrasensitive toxin assay were compared to those
132 for CCNA, sensitivity and specificity for an individual assay and an algorithm (combining a

133 clinically sensitive and specific test) ending with the same method were identical (30). The
134 sensitivity and specificity for NAAT and for an algorithm, where discordant GDH/toxin EIA
135 results were arbitrated by NAAT, were both 97.0% and 79.0%, respectively; for the toxin EIA
136 and an algorithm where NAAT-positive samples were tested with toxin EIA, they were both
137 57.6% and 100%, respectively, when compared to CCNA. In a prospective multicenter study,
138 samples were tested with the same assays as above and algorithms did not improve accuracy
139 over single-assay testing (21). Combining a sensitive and a specific test allows negatives to be
140 screened out (using the first test; either NAAT or GDH) and can provide additional information
141 over and above a single test result (for example, if the patient is a potential *C. difficile* carrier).
142 However, combining tests means that the ultimate sensitivity of the algorithm is a product of
143 the sensitivities of each test (which therefore is lower than the sensitivity of an individual test),
144 and the second test (either NAAT or a toxin test) drives the detection performance of the full
145 algorithm (21, 30).

146 Multiple comparisons between ultrasensitive toxin testing and standard-of-care algorithms
147 have been performed. In a study from Stanford University on 311 samples, Clarity had 97.7%
148 sensitivity and 100% specificity compared with an algorithm utilizing NAAT followed by toxin
149 testing using EIA and CCNA (when EIA negative) (25). Over 1,000 samples were tested in a
150 German study and the ultrasensitive toxin assay showed improved accuracy compared to an
151 algorithm utilizing a GDH/toxin EIA reflexed to NAAT (33). Depending on the comparison test
152 algorithm result, the Clarity assay had high agreement in a study where 211 samples were
153 tested with GDH/toxin EIA and reflexed to a semi-quantitative CCNA (which is more sensitive
154 than conventional CCNA) (38). In a study from Mayo Clinic on nearly 500 patients, an

155 ultrasensitive toxin assay had 91.0% sensitivity and 99.1% specificity compared with an

156 algorithm where a GDH/toxin EIA reflexed to NAAT (no laboratory reference method utilized)

157 (34). In a UK study, Clarity had high PA with a toxin EIA (96.9%) and PA with multistep

158 algorithms ending with toxin EIA (95.8-100%), and high NA with NAAT (89.9%) and a multistep

159 algorithm ending with NAAT (91.7%); the low NA and PA compared with toxin EIA (49.6%) and

160 NAAT (69.4%), respectively, reflected the poor sensitivity of current toxin EIAs and low

161 specificity of NAAT (35). These data suggest that ultrasensitive toxin assays could offer an

162 alternative to conventional testing, including toxin-based algorithms, but further larger studies,

163 in particular with outcome data, are needed to fully understand the clinical meaning of

164 discordant results.

165

166 **Correlation with Disease: What Specificity Can Be Achieved?**

167 Host-response factors play an important role in development of CDI and asymptomatic

168 individuals can have toxins present in stool, something that has been described previously (39–

169 42). This has also been observed when using an ultrasensitive toxin assay (Simoa), where the

170 presence of toxin or toxin gene could not differentiate an individual with CDI from one with

171 asymptomatic carriage, both as determined by NAAT (9). Toxin concentrations, however, were

172 higher in CDI patients than in carriers, but only when CDI was diagnosed by toxin detection

173 (cutoff 20 pg/mL), which made the authors conclude that toxin detection is more clinically

174 relevant than detection of the toxin gene (9). Indeed, defining CDI/asymptomatic carriage on

175 NAAT may have added confusion to this study.

176 It is well established that CDI is a clinical diagnosis, i.e. that no test can be used to rule in
177 disease. Instead of implying that a highly imperfect test (NAAT) can only be replaced by a
178 perfect test, the relevant questions to focus on are: how much could ultrasensitive toxin assays
179 improve the diagnostic accuracy and clinical specificity compared with NAATs, and what
180 positive predictive value (PPV) is achievable? In a recent US study, nearly 300 patients were
181 tested with NAAT and the Clarity assay, with discordant samples tested with CCNA and results
182 correlated with disease severity and outcome (32). Among the NAAT+/Clarity- patients, nearly
183 70% had a non-CDI-related cause of diarrhea, compared with less than 22% of NAAT+/Clarity+
184 patients – a threefold difference. If using one of the guideline CDI case definitions (23), the
185 ultrasensitive toxin test thereby achieved 97.4% clinical specificity and 78.1% PPV, while NAAT
186 had 89.0% clinical specificity and 54.7% PPV (32), although larger studies are needed. The
187 obvious counterargument is that using a CDI case definition that does not include an
188 assessment of non-CDI-related causes of diarrhea would change the specificities to the higher.
189 However, he NAAT overdiagnosis rate and a PPV of just over 50% – a statistician's term for "a
190 flip of a coin" – may be unacceptable to many clinicians when interpreting laboratory tests.
191 Presence of toxins also correlated with outcome; CDI relapse only occurred in Clarity+ patients
192 (12.5% of Clarity+ patients), and NAAT+/Clarity+ patients had longer length of stay compared to
193 NAAT+/Clarity- patients (14.2 versus 7.6 days), although this was not statistically different.

194 Testing criteria for CDI, i.e. who and when to test, are subject to ongoing discussions.
195 IDSA/SHEA agrees on using NAAT alone if testing excludes stool specimens from patients
196 receiving laxatives and with less than three unformed stools in 24 hours (22). However, in the
197 study evaluating clinical specificity (32), the hospital had previously successfully implemented

198 stringent stool-submission criteria adherent with the IDSA/SHEA guidelines (43), but still
199 observed that two-thirds of NAAT+/toxin- patients had a non-CDI-related cause of diarrhea,
200 indicating that suggested guideline criteria on whom to test are ineffective. Conversely, lack of
201 clinical suspicion for testing, i.e. no test requested, can lead to under-diagnosis of CDI (44).
202 Given that both symptoms and colonization are common, the strategy of limiting testing to
203 those patients with higher disease probability and thereby achieving an increase of the NAAT
204 PPV to acceptable levels seems likely to be problematic and unsuccessful. A consistent finding is
205 that ~30%-50% of NAAT+ patients have *no* detectable toxin in stool, as measured by
206 ultrasensitive assays (21, 25, 30, 32, 35), indicating that these toxigenic bacteria are not
207 producing toxins, although some might argue that ultrasensitive toxin assays are not sensitive
208 enough. CDI is a toxin-mediated disease and the lack of toxin in NAAT+ samples therefore has
209 important implications for diagnostic accuracy and clinical specificity. Diagnosis by NAAT may
210 ultimately lead to use of unnecessary antibiotics and infection-control measures. Although CDI
211 is a clinical diagnosis, physicians often base treatment decisions on laboratory reports,
212 demonstrated by studies where all asymptomatically colonized patients (defined as individuals
213 without clinically significant diarrhea and with positive NAAT) (42, 45) and 95% of patients
214 tested inappropriately (43) were treated when NAAT was used. In addition, there are multiple
215 examples of clinical trials that have failed to meet their end-points when CDI diagnosis was
216 based on NAAT (46), indicating that NAAT does not accurately define disease.

217 As ultrasensitive, quantitative toxin assays become available, there is an interest in correlating
218 toxin concentration with disease, to improve severity assessment and guide treatment. Toxins
219 in patients with suspected CDI are detected in a wide range, up to 300 ng/mL (21, 24, 25).

220 Higher toxin concentrations have been reported in PCR ribotype 027 than in non-027 strains
221 (25), but there was no difference in toxin concentration between multiple non-027 ribotype
222 strains (35). Although a correlation between toxin concentrations and CDI severity has been
223 observed (47) and high concentrations have been reported in individual patients with severe
224 disease and ileus (32, 36), the lack of such a correlation has also been reported (9, 24, 41, 42).
225 Factors related to host response are important in disease progression (39, 40), and larger
226 observational and interventional studies are needed to understand the role of toxin
227 concentration and disease. A fundamental issue here, however, is that the fluid
228 content/volume of stool/diarrhea is variable in an individual, and so the concentration of toxin
229 measured at any particular time point could be markedly affected. While assay manufacturers
230 need to consider the clinical utility of providing quantitative versus qualitative reporting when
231 developing ultrasensitive toxin assays, at this point, a quantitative readout has not been shown
232 to provide additional value.

233

234 **Ruling Out Disease: What Sensitivity Is Needed?**

235 The ultrasensitive assays allow for quantification of *C. difficile* toxins, and assay developers are
236 challenged with optimizing analytical sensitivity and threshold for a qualitative readout. Simoa
237 and Single Molecule Counting Technology utilize cutoffs (evaluated compared to CCNA or assay
238 combinations including CCNA) between 12.0 pg/mL of the toxins combined to 29.4 pg/mL per
239 toxin (9, 21, 24–26). In a multicenter study on 2,000 samples, 33.1% (108/326) of Clarity+

240 samples were under 45 pg/mL and 17.8% (58/326) were under 20 pg/mL (21), indicating that an
241 LoD (27) or cutoff (9, 26) in that range may be too high and will lead to missed cases.

242 CCNA has an estimated LoD of 50–100 pg/mL (48), which is significantly higher than the
243 ultrasensitive assays' detection limits at 1 pg/mL. Indeed, the ultrasensitive assays detected
244 toxins in 22.7% of NAAT+/toxin EIA-/CCNA- (25) and in 41.3% of GDH+/CCNA-/NAAT+ (26)
245 samples, indicating that Simoa and Single Molecule Counting technologies are more sensitive
246 than CCNA and that their specificity therefore may be underestimated in direct comparisons
247 (21, 26). For less sensitive assays, such as the MultiPath assay that has an LoD in a similar range
248 as CCNA, an accuracy comparison will look more favorable (27). In the prospective, multicenter
249 study comparing Clarity with CCNA, samples with discrepant results were retested with CCNA
250 when the ultrasensitive toxin result agreed with that of at least one other comparator method
251 (GDH EIA, toxin EIA, or NAAT), and a different CCNA result was reported for as many as 42% of
252 retested samples (21); CCNAs (in which results are read by microscopy) require experienced
253 workers to optimize reproducibility. Lastly, CCNA is impacted by toxin stability and subjectivity
254 (13), something that has not been observed using automated ultrasensitive toxin detection (25,
255 26). The issues with CCNA reproducibility and sensitivity need to be considered when evaluating
256 new toxin tests. A comparison with TC would not solve this problem, as this method detects
257 toxigenic organisms only – similar to NAAT – and does not provide any information on toxin
258 production *in vivo*.

259 When establishing an optimized cutoff compared with CCNA, there is a risk of overestimating
260 an assay's clinical sensitivity, i.e. wanting to set the cutoff too high, although avoiding setting
261 the cutoff too low to avoid false negatives is also critical to avoid background signal and

262 maintain specificity. If toxin concentration does not correlate with disease severity (9, 24, 42),
263 there might be value in solely providing clinicians with information on toxin detection on the
264 lowest, reproducible level. In cases where NAAT+/toxin- patients were deemed to have CDI
265 when retrospectively reviewed by a clinical panel, toxin was present but under the assay's
266 cutoff (36), although it was not investigated further whether this was background signal.
267 Developers of ultrasensitive toxin assays may want to take this into account when optimizing
268 analytical sensitivity.

269

270 **Future of CDI Diagnostics: Back to Standalone Toxin Detection?**

271 Ultrasensitive toxin assays may improve the detection of CDI compared to current testing
272 methods. However, further studies are required so that recommendations can be formulated
273 on how best ultrasensitive assays, as they become commercially available, can be utilized in
274 clinical practice. In the US, the Centers for Disease Control and Prevention (CDC) and the
275 National Health Safety Network (NHSN) adjust the *C. difficile* laboratory-identified event (LabID-
276 CDI event) standardized infection ratio (SIR) – the primary measure used to track healthcare-
277 associated infections – based on the test used at the facility (NAAT, toxin EIA, or other), and, for
278 multistep algorithms, on the last test that is placed in the patient medical record (49). High SIRs
279 place financial and reputational burdens on healthcare providers, and the recognition that
280 testing methods impact incidence is important. There are concerns that the CDI SIR risk-
281 adjustment formula used by CDC and NHSN to take account of diagnostic method may not be
282 sufficient to account for the effects of those testing methods on reported CDI rates. An

283 unintended consequence here could be that hospitals decide on a testing method(s) based on a
284 desire to achieve lower LabID-CDI event rates and SIRs (50, 51). If tests with enhanced
285 sensitivity to detect CDI are to be adopted, a way to overcome the effects of increased
286 reporting will be needed.

287 Ultrasensitive *C. difficile* toxins assays provide detection of disease-mediating toxins at very low
288 concentrations. Better CDI diagnostics with higher PPVs could improve antibiotic-stewardship
289 efforts and has the potential to make infection-control practices more efficient. It is noteworthy
290 that CDI diagnosis has had several major shifts in the four decades since *C. difficile* was first
291 described as a human pathogen. Toxin detection by immunoassays supplanted culture and/or
292 cytotoxin detection-based methods, and these were followed by a rapid uptake of NAATs in
293 some countries. Implementation of standalone ultrasensitive toxin testing could offer a new
294 way forward in CDI diagnostics.

295

296 **Financial / Potential Conflicts of Interest**

297 JS is a former employee of Singulex, Inc. KD has received honoraria from Astellas Pharma
298 Europe, Cepheid Inc and Summit, and grant support from Alere, Astellas Pharma Europe,
299 bioMerieux, Pfizer, Sanofi-Pasteur and Techlab Inc. MHW has provided consultancy advice to
300 multiple CDI diagnostic companies, including Singulex, Inc, First Light, Cepheid, Alere, Meridian
301 and bioMerieux.

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