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Challenges in the diagnosis of *Clostridium difficile* Infection

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Clostridium difficile is an increasingly prevalent infectious disease, with a significantly rising incidence and mortality [Clin Microbiol Infect 2006;12:2–18]. Recent studies have demonstrated increased sensitivity of diagnosis with the adoption of real-time polymerase chain reaction (PCR) testing [Clin Infect Dis 2011 Oct;53(7):81–90, Clin Infect Dis 2007 Nov 1;45(9):1152–60]. As a result of the increased sensitivity of testing, transition to PCR based assays has correlated with an increased incidence of *C. difficile* infection (CDI) [Infect Control Hosp Epidemiol. 2013 Oct;34(10):1055–61]. Previously defined risk factors for the development of CDI have included known risk factors for the development of healthcare-associated diarrhea including the use of feeding tubes and administration of laxatives [Infect Control Hosp Epidemiol 2011 Apr;32(4):360–6, J Clin Microbiol 2006 August; 44(8): 2785–2791]. Asymptomatic colonization with *C. difficile* also occurs, and estimates are as high as 25% in healthcare settings [Clin Infect Dis 1994;18(2):181–7] rising to nearly 50% during outbreaks [Clin Infect Dis 2007 Oct 15;45(8):992–8]. Therefore, the contribution of increased sensitivity of detection combined with high-rates of asymptomatic colonization, with an inability to differentiate healthcare-associated diarrhea secondary to pathogenic *C. difficile* from alternative causes, likely combine to account for some of the increased trends in detection of CDI.

In a large observational study, Planche *et al.* describe the differences in clinical outcomes with respect to two reference methods for the diagnosis of CDI. In addition, using four commercially available testing methods, individual assays and algorithms were compared against both reference methods in an attempt to define the optimum method of diagnosis with respect to patient-centered outcomes.

The study was performed through the diagnostic laboratories of four major teaching hospitals in the United Kingdom's National Health Service. Following a standard protocol, all unformed stool samples from hospitalized patients aged 2 and from community members aged 65 were submitted for *C. difficile* testing, regardless of requests for testing from the primary service.

Reference testing methods were performed on all clinical isolates. Cytotoxigenic culture was performed as a reference method in order to detect *C. difficile* strains in stool samples. Following the growth of *C. difficile* strains in anaerobic culture, the ability for the individual isolates to produce toxin was assessed using the culture supernatant in a cell cytotoxin assay. The cell cytotoxin assay was the second reference method of choice, in which supernatant from dilute fecal samples (or from anaerobic culture as part of the cytotoxigenic culture) was added to a Vero cell monolayer to assess for cell rounding. Therefore, the reference method of choice individually assessed for the presence of *C. difficile* within clinical isolates (cytotoxigenic culture) and for the direct detection of toxin (cell cytotoxin assay).

Overall, 12,420 samples were collected from 10,186 individual patients. In order to determine differences in clinical outcomes with respect to the reference method chosen, clinical outcome data was available from 6,522 inpatient episodes including blood test results obtained within three days of stool sample collection and 30-day all-cause mortality. Among inpatient episodes, when compared against the two available reference methods, 435 were positive by cell cytotoxin assay (fecal detection of toxin), 207 were positive by cytotoxigenic culture and negative by cytotoxin assay (detection of toxigenic *C. difficile* without in vitro detection of toxin) and 5880 isolates were negative by either reference method for a total prevalence of CDI of 9.8% in the submitted inpatient samples.

Adding clinical outcome data to the above reference methods, direct detection of fecal toxin by cell cytotoxin assay associated with a statistically significant elevation in white blood cell count compared to detection of toxigenic *C. difficile* by cytotoxigenic culture or negative results from both reference methods ($12.4 [8.9] \times 10^9/L$ versus $10.1 [5.8] \times 10^9/L$ [$P = .0004$] and $9.9 [10.7] \times 10^9/L$ [$P < .0001$]). In addition, 30-day all-cause mortality was significantly increased when toxin was detected in fecal samples by the cell cytotoxin assay as compared to detection by cytotoxigenic culture or negative results from either reference methods (16.6% versus 9.7% [$P = .044$] and 8.6% [$P < .001$]). In a multivariable regression model, detection of fecal toxin by cytotoxin assay as compared to negative results on both references methods resulted in a significant association with 30-day all-cause mortality (odds ratio 1.61, 95% confidence interval 1.12–2.31).

Four commercially available assays were then compared against either reference method in order to develop testing algorithms using commonly applied clinical detection methods: two for direct toxin detection (enzyme immunoassays [EIA]) and two for organism detection (a glutamate dehydrogenase [GDH] EIA and a nucleic acid amplification test [NAT] assay). In the training dataset no individual assay reached an adequate level of sensitivity or specificity (defined as $> 90\%$ sensitivity and $> 99.5\%$ specificity). Subsequently two-stage algorithms were compared with individual assays with improved area under receiver operator characteristics curve (AUROC). Combining data from a training and testing data set yielded

differences in the optimum algorithms based on reference method chosen. In order to reproduce cytotoxigenic culture results, a combination of a GDH EIA and NAT assay yielded a sensitivity of 91.5% and specificity of 98%. In order to reproduce the results of the cytotoxin assay as the reference method, a toxin EIA with either a GDH EIA or NAT assay yielded similar performance characteristics (sensitivity 81.8% and 82.9%, specificity 99.5% and 99.6%, respectively).

Given the demonstrated differences in clinical outcomes based on the choice of reference method, Planche *et al.* proposed the term, “potential *C. difficile* excretor” for patients with a positive cytotoxigenic culture but with negative fecal cytotoxin assay, therefore representing patients harboring potentially virulent isolates, without the detection of fecal secreted toxin. Utilizing additional clinical data available for a small subset, 64/143 potential *C. difficile* excretors did not have a chart diagnosis of diarrhea, and in the remaining 78 cases, the median duration of diarrhea was 2 days suggesting a benign clinical course among patients labeled potential *C. difficile* excretor.

Comment

The rising incidence of CDI [Clin Microbiol Infect. 2006;12:2–18] with the availability of two reference diagnostic methods, in addition to multiple targeted commercial assays, has led to controversy regarding accurate diagnosis of CDI; what constitutes clinically relevant active infection versus passive colonization with toxigenic isolates? Little data focusing on patient oriented outcomes and disease severity with respect to the diagnosis by either reference method or commercial assay exists to guide clinical decision-making.

Compounding the dilemma of accurate CDI detection are the variable targets of interest, including the presence of toxin, the presence of toxin-harboring isolates, and the presence of a common surface antigen GDH. Previously established reference standards including cell cytotoxin assay and cytotoxigenic culture have been largely replaced with more cost-effective and rapid PCR and EIA based assays. Virulent *C. difficile* organisms carry a pathogenicity locus and encode for toxins TcdA and TcdB which can disrupt the actin cytoskeleton of host cells resulting in cell death, although the exact contribution of either toxin to clinical virulence is a matter of active investigation [Nature 2009 Apr 30;458(7242): 1176–9, J Infect Dis 2014 Jan 1;209(1):9–11]. Further complicating the matter is the increased recognition of the presence of asymptomatic carriage of toxigenic isolates [J Hosp Infect 2013 Oct;85(2):155–8]. Differentiating diarrhea in asymptomatic carriers harboring toxigenic strains as a result from CDI versus alternative causes of healthcare-associated diarrhea cannot be performed on clinical grounds alone. Thus, at present there is no true gold standard for diagnosis of clinical disease due to *C. difficile*, and accurate diagnosis requires practitioners to integrate diagnostic test results with the appropriate clinical findings.

Given the variable targets for the diagnosis of CDI, as well as differences in test characteristics, it is not surprising that there is a lack of consensus on the optimal diagnostic paradigm. EIA based toxin detection methods are approximately 10–20% less sensitive than the reference cytotoxin assay [J Antimicrob Chemother. 1998 May;41 Suppl C:29–40].

Compared to PCR based tests, diagnosis by EIA may be missing up to 50% of cases [J Clin Microbiol. 2008 Jun;46(6):1996–2001, J Clin Microbiol. 2010 Jan;48(1):124–30]. A 2008 systemic review of six commercially available toxin EIA assays found a positive predictive value of <50% for some tests, which is not clinically acceptable [Lancet Infect Dis. 2008 Dec;8(12):777–84]. These findings were confirmed in a 2009 study comparing nine commercial toxin EIA assays to reference methods, finding a PPV ranging from 48.6% to 86.8% [J Clin Microbiol. 2009 Oct;47(10):3211–7]. Used in isolation, EIA based toxin detection methods have a widely variable, but overall low sensitivity to detect the presence of CDI. Combining EIA based detection of GDH and toxin A/B can, however, lead to negative predictive value of nearly 98% [Infect Dis Rep. 2011 May 5;3(1):e5]. This could increase the usefulness of combination EIA-based testing as the initial screening step in two-step algorithms, where a second method is used to adjudicate discordant results (i.e. GDH⁺/toxin⁻ or GDH⁻/toxin⁺).

PCR for toxin is more sensitive than EIA-based detection methods, when compared to a cytotoxin assay (92.2%) or a cytotoxigenic culture (88.5%) [J Clin Microbiol. 2009 Oct; 47(10):3211–7]. However, it is worth again noting that these molecular tests evaluate for the presence of isolates with the ability to produce toxin in vitro and not actual toxin, which is seminal in the pathogenesis of CDI. Thus, from a single PCR test, one is unable to differentiate colonization from symptomatic CDI. A diagnosis of CDI based on a single PCR detection of toxigenic *C. difficile* organisms can lead to inappropriate administration of antibiotics to patients who are asymptomatically colonized, with subsequent increased healthcare costs and preventable adverse outcomes, including disruption of the intestinal microbiome.

Relating testing differences to clinical outcomes will ultimately be required to guide clinical decision-making and treatment selection. A recent study by Baker et al. evaluated the clinical features of patients diagnosed with CDI by toxin EIA positivity compared to those diagnosed by PCR alone, while negative for toxin by EIA. The PCR positive/EIA negative group had a significantly lower 14-day all-cause mortality (11%; 95% confidence interval [CI]: 4–23%) compared to patients with both a positive PCR and toxin detection by EIA (37%; 95% CI: 19–59%; *P* = .01). Additionally, the positive toxin EIA was an independent predictor of death (odds ratio [OR]: 4.7, 95% CI: 1.4–15.4; *P* = 0.01) as well as prolonged diarrhea (OR: 8.6; CI: 2.9–25.6; *P* < .001 [J Hosp Infect. 2013 Aug;84(4):311–5]. This implicates that detection of toxin expression by EIA results in clinically meaningful outcomes with increased disease severity.

Planche *et al.* should be commended for conducting a large, well-done, systematic observational study demonstrating how clinical outcomes are related to detection of *C. difficile* toxin, and for further extending their study to include the development of algorithms using rapid testing methods best fit for either reference method. Furthermore, they describe a unique CDI diagnosis group, “potential *C. difficile* excretor,” based on the detection of a toxigenic isolate without the detection of expressed toxin. In doing so, they introduce the possibility for further therapeutic and natural history studies to determine the long-term clinical outcomes of the variable diagnosis groups. In addition, they highlight the need to

understand the strengths and limitations of the various testing methods when compared to two unique reference standards.

Given the complexity and limitations described with CDI testing, it is critically important to understand the methods used by the clinical laboratory performing the testing, and to not simplify the test result by treating it as a purely binary (positive/negative) variable. The proper interpretation of diagnostic tests requires an understanding of the testing characteristics and limitations. Further studies incorporating various testing methods with patient level data, including the use of infection specific biomarkers [PLoS One. 2013;8(3):e58265] may be of utility in unravelling the differentiation between asymptomatic carriage of toxigenic isolates and pathologic diarrhea caused by *C. difficile*.