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Evaluation of *Clostridium difficile* Fecal Load and Limit of Detection during a Prospective Comparison of Two Molecular Tests, the illumigene *C. difficile* and Xpert *C. difficile*/Epi Tests

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In a large prospective comparison, the illumigene test detected *Clostridium difficile* in 98% of toxin-positive and 58% of toxin-negative samples confirmed positive by other methods. The Xpert was uniformly sensitive. Most samples with discrepant results had *C. difficile* concentrations below the illumigene limit of detection. The significance of low-level *C. difficile* detection needs investigation.

The incidence and severity of *Clostridium difficile* infection (CDI) have increased dramatically in North America and Europe over the last decade (1). In the United States, rates of CDI are now at an all-time high (2). With this change in incidence, the performance of *C. difficile* testing has been reexamined and there has been a movement toward tests that detect *C. difficile* antigens or DNA directly versus tests that detect *C. difficile* toxins (3). As a group, *C. difficile* detection tests (e.g., PCR, loop-mediated amplification [LAMP], glutamate dehydrogenase immunoassay) are more sensitive than toxin tests, but the reported sensitivities still vary for unclear reasons. For example, the published sensitivity of the illumigene *C. difficile* assay ranges from 81 to 98% (4–11), leaving laboratories with uncertainty about which test to use and what performance to expect at their own institution.

To address this issue, we evaluated the fecal *C. difficile* DNA load of positive samples as part of a large, prospective comparison of two FDA-approved nucleic acid amplification tests (NAATs) for *C. difficile*, the illumigene *C. difficile* test and the Xpert *C. difficile*/Epi test, with toxigenic culture. When the clinical results and fecal *C. difficile* load comparisons suggested a genuine difference in the sensitivity of these two NAATs, we evaluated the inoculum size and analytical limit of detection (LOD) of both assays to further investigate the causes and significance of discrepant results.

Study population and test methods. Consecutive diarrheal stool samples submitted for *C. difficile* testing from adult inpatients ≥ 72 h after admission between January and October 2011 were included in the study; nonconforming stool samples were rejected. Each sample was tested for toxigenic *C. difficile* by three tests, (i) an illumigene *C. difficile* LAMP assay (Meridian Bioscience), (ii) an Xpert *C. difficile*/Epi real-time PCR assay (Cepheid), and (iii) a toxigenic culture. In addition, the fecal toxin status of each sample was determined by a toxin immunoassay (Premier *C. difficile* Toxins A & B; Meridian Bioscience) performed on all samples and a cytotoxicity assay (Wampole *C. difficile* Tox-B [TechLab]; MHRF cells [Diagnostic Hybrids]) performed on immunoassay-negative, *C. difficile*-positive samples from frozen aliquots when available (55/61; 90.2%) (12). All tests except cytotoxicity were performed daily on fresh stool by following the manufacturer's instructions. For toxigenic culture, 0.5 ml stool was mixed 1:1 with 95% EtOH for 10 min and a swab was used to

inoculate a prereduced agar medium (cycloserine cefoxitin fructose agar supplemented with taurocholate [CCFA-ST]; Remel). Cultures were incubated anaerobically at 37°C for 3 days, and suspicious colonies were identified by colony and Gram stain morphology, odor, production of L-proline aminopeptidase, and fluorescence under long-range UV light. *In vitro* toxin production of isolates was confirmed by cytotoxicity testing of isolates grown in chopped meat broth (Remel) at 48 h (13). Samples that were negative with the initial EtOH shock culture but positive by either of the NAATs were cultured in enrichment broth (cycloserine cefoxitin mannitol broth with taurocholate lysozyme cysteine [CCMB-TAL]; Anaerobe Systems) from a frozen aliquot ($n = 10$) (14). The ability of the illumigene assay to detect toxigenic *C. difficile* isolates recovered from culture of illumigene-negative samples was tested directly using a 100- μ l volume of a 4 McFarland suspension from fresh subculture.

Fecal load, inoculum size, and LOD methods. The fecal *C. difficile* DNA load of positive samples was calculated from the Xpert *tcdB* gene PCR cycle number at the endpoint of detection using standard curves performed with each lot of test cartridges as previously described (12). The typical inoculum size of each test was evaluated by weighing five replicate samplings of one soft, one loose, and one watery stool sample using the sample collection brush (illumigene) or swab (Copan) by following the manufacturer's instructions. For the Xpert assay, our practice was to dip the swab fully and wipe any excess on the container wall. The LOD of each NAAT was determined by replicate testing of a 100- μ l volume from serial dilutions (10-fold and 2-fold) of a 24-hour culture of *C. difficile* ATCC 43255 (VPI 10463), with the concentra-

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TABLE 1 Number of samples called positive by each test and overall sensitivity and specificity

Reference classification ^a	Total no. of samples	No. (% ^b) of samples testing positive with each test or % sensitivity/specificity (95% CI)		
		Toxigenic culture	illumigene <i>C. difficile</i>	Xpert <i>C. difficile</i> /Epi
Toxin positive/ <i>C. difficile</i> positive	64	62 (96.9)	63 (98.4)	64 (100.0)
Toxin negative/ <i>C. difficile</i> positive	36	32 (88.9)	21 (58.3)	36 (100.0)
<i>C. difficile</i> negative	468	0 (0.0)	0 (0.0)	6 (1.3)
Sensitivity	100	94 (89.4–98.7)	84 (76.8–91.2)	100 (95.4–100)
Specificity	468	100 (99.0–100)	100 (99.0–100)	98.7 (97.7–99.7)

^a ≥ 2 tests positive.

^b Percentages are the number of samples positive by the test/the number of samples positive by the reference classification times 100.

tions confirmed by the average of triplicate colony counts on brucella agar.

Data and statistical methods. The first positive sample from each patient or first sample from negative patients was included; duplicate samples were excluded. Samples with ≥ 2 positive results (e.g., toxigenic culture, Xpert, illumigene, toxin by immunoassay or cytotoxicity) were considered to be confirmed as positive for toxigenic *C. difficile*. Sensitivity and specificity values and 95% confidence intervals (CIs) were calculated against the 2-test reference standard. Chi-square and Fisher's exact tests were used for categorical data.

Test results from clinical samples. Of the 693 samples tested, 568 samples and patients were included in the analysis. One hundred (17.6%) samples, including 64 (64%) fecal-toxin-positive samples and 36 (36%) toxin-negative samples, had toxigenic *C. difficile* detected by ≥ 2 methods. The number of positives detected by each method (e.g., toxigenic culture, illumigene *C. difficile*, Xpert *C. difficile*/Epi) and the calculated sensitivities and specificities are shown in Table 1. Overall, both NAATs were highly sensitive ($>98\%$) for *C. difficile* in fecal-toxin-positive samples, but the illumigene detected *C. difficile* in only 21/36 (58.3%) fecal-toxin-negative samples while the Xpert detected *C. difficile* in all 36 (100%) samples in this group (Table 1). The Xpert had 6 additional positive results that were not able to be confirmed by any other test. These were considered false positives.

Fecal *C. difficile* load, inoculum size, and LOD evaluations. Comparison of the fecal DNA concentrations of samples with *C. difficile* detected by the Xpert but missed by the illumigene showed that virtually all (15/16; 93.8%) were stools with no fecal toxins detected and with a relatively low concentration of *C. difficile* DNA (Fig. 1). For direct comparison, the median concentration of *C. difficile* DNA for the 84 samples detected by illumigene was 6.64 (95% CI, 6.24 to 6.70) \log_{10} *C. difficile tcdB* DNA copies/ml; the median concentration of the 16 illumigene-negative, Xpert-positive samples was 4.11 (95% CI, 3.82 to 4.60) \log_{10} *C. difficile tcdB* DNA copies/ml. To better understand why the illumigene missed the *C. difficile* DNA in these low-concentration samples, we examined the inoculum size and LOD of both tests. The mean inoculum weights differed slightly between the two tests (ratio of sample weights between the Xpert swab and the illumigene brush, 0.7 to 1.6) but not to a degree that would explain the sensitivity difference among toxin-negative samples. In contrast, the analytical

LOD of the two NAATs differed by 1.2 \log_{10} DNA copies for the VPI 10463 strain we tested (Fig. 1). The illumigene detected 9/9 replicates at 32,750 CFU/ml (4.52 \log_{10}) and 5/10 replicates at 16,375 CFU/ml (4.21 \log_{10}), yielding an LOD of 4.52 \log_{10} *C. difficile tcdB* DNA copies/ml. The Xpert detected 10/10 replicates at 2,047 CFU/ml (3.31 \log_{10}) and 0/5 replicates at 1,024 CFU/ml (3.01 \log_{10}), yielding an LOD of 3.31 \log_{10} *C. difficile tcdB* DNA copies/ml. Using these LODs, 9/16 (56.3%) illumigene-negative, Xpert-positive samples had *C. difficile* DNA concentrations below the illumigene LOD; 7/16 (43.8%) had concentrations just above the LOD (range, 0.19 to 1.11 \log_{10} above the LOD). To confirm that the illumigene assay can detect the *C. difficile* strains from samples with discrepant results, higher concentrations were tested directly from culture. Of the 15 available isolates, all were amplified and detected.

Conclusions. Overall, we found a 16% sensitivity difference between the illumigene *C. difficile* LAMP assay and the Xpert *C. difficile*/Epi real-time PCR test in a large-scale, prospective comparison with toxigenic culture. When the fecal toxin status of samples was considered, the illumigene and Xpert tests performed

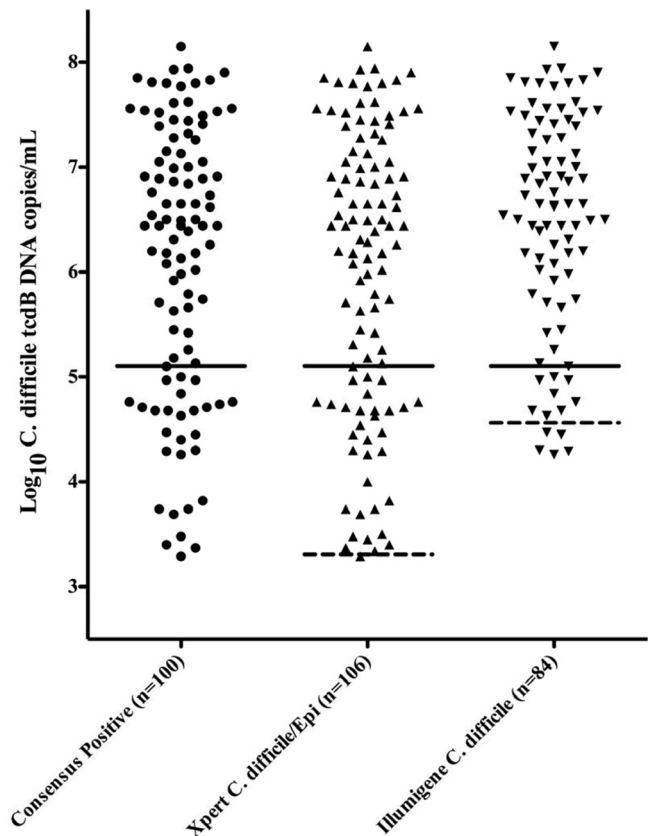


FIG 1 Fecal *C. difficile* concentrations of positive stool samples overall and detected by each test. Black circles (●) are consensus positive samples with toxigenic *C. difficile* detected by ≥ 2 tests. Upward-pointing triangles (▲) are all samples reported positive by the Xpert *C. difficile*/Epi test. Downward-pointing triangles (▼) are samples reported positive by the illumigene *C. difficile* test. Solid lines represent a 95% sensitivity cutoff for toxin detection (5.10 \log_{10} *C. difficile tcdB* DNA copies/ml) from reference 12. Above this line, 61/73 (83.6%) of samples were toxin positive. Below this line, 24/27 (88.9%) samples were toxin negative. Dashed lines indicate the Xpert and illumigene *C. difficile* DNA LOD values discussed in the text (Xpert, 3.31 \log_{10} *C. difficile tcdB* DNA copies/ml; illumigene, 4.52 \log_{10} *C. difficile tcdB* DNA copies/ml).

similarly and were both highly sensitive for toxin-positive samples, but the illumigene was much less sensitive with toxin-negative samples (58% for illumigene versus 100% for Xpert; $P < 0.001$). We suspected that this was due to the low concentration of *C. difficile* target DNA in these samples. To validate this hypothesis and our overall results, we compared the *C. difficile* concentrations of positive samples detected by both tests and positive samples missed by the illumigene and determined the LOD of both tests. These investigations confirmed that virtually all of the clinical samples with discrepant results had low *C. difficile* DNA concentrations that were near or below the illumigene LOD but above the Xpert LOD, as expected. The LOD thresholds we measured showed the illumigene LAMP assay to be less sensitive analytically than the Xpert real-time PCR test by about $1.2 \log_{10}$ DNA copies/ml and were in agreement with the LOD data provided in the manufacturer's package insert for each test (15, 16). Conversely, when *C. difficile* isolates from samples originally called negative by the illumigene were retested at a higher concentration from culture, all were amplified and detected. Together, these findings demonstrate a clinical sensitivity difference between the illumigene *C. difficile* assay and the Xpert *C. difficile*/Epi test at low *C. difficile* concentrations that appears to be due to a difference in the analytical sensitivity of these two NAATs and not due to nonamplification due to DNA sequence polymorphisms. Careful review of the instructions for these two tests seems to suggest that the difference may be due to the additional sample dilution steps included in the preanalytical processing with the illumigene assay (15). Two other studies have reported sensitivities similar to ours for the illumigene and Xpert assays, but the underlying causes were not investigated (10, 11). Others have reported higher sensitivities for the illumigene assay in the range of 92 to 98% sensitivity, but it is possible that some of these were biased by use of a less-sensitive reference method or inclusion of large numbers of toxin-positive samples in the study (4–9).

We believe that these results show conclusively that the illumigene *C. difficile* LAMP assay and the Xpert *C. difficile*/Epi test differ in their analytical and clinical sensitivity, in particular in their ability to detect low-concentration, toxin-negative, *C. difficile*-positive samples. However, since the clinical significance of fecal samples with a low bacterial load of *C. difficile* is unknown, these results suggest that more questions need to be answered. If these patients are simply carriers without clinically significant CDI, then the illumigene assay may be more accurate. Alternatively, if there is no safe level of *C. difficile* for either CDI or transmission, then the more-sensitive Xpert assay may be preferable.

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All other authors declare no conflicts of interest.

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