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ARTICLE

Evaluation of three enzyme immunoassays and a loop-mediated isothermal amplification test for the laboratory diagnosis of *Clostridium difficile* infection

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Abstract The laboratory diagnosis of *Clostridium difficile* infection (CDI) consists of the detection of toxigenic *Clostridium difficile*, and/or its toxins A or B in stool preferably in a two-step algorithm. In a prospective study, we compared the performance of three toxin enzyme immunoassays (EIAs)—ImmunoCard Toxins A & B, Premier Toxins A & B and C. diff Quik Chek Complete, which combines a toxins test and a glutamate dehydrogenase (GDH) antigen EIA in one device —and the loop-mediated isothermal amplification assay Illumigene *C. difficile*. In total 986 stool samples were analyzed. Compared with toxigenic culture as the gold standard, sensitivities, specificities, PPV and NPV values of the toxin EIAs were 41.1–54.8 %, 98.9–100 %, 75.0–100 % and 95.5–96.5 % respectively, of the Illumigene assay 93.3 %, 99.7 %, 95.8 % and 99.5 %. Illumigene assays performed significantly better for non-014/020 PCR-ribotypes than for *C. difficile* isolates belonging to 014/020. Discrepant analysis of three culture-negative, but Illumigene-positive samples, revealed the presence of toxin genes using real-time PCRs. In addition to the GDH EIA (NPV of 99.8 %), the performance of Illumigene allows this test to be introduced as a first screening test for CDI- or as a confirmation test for GDH -positive samples, although the initial invalid Illumigene result of 4.4 % is a point of concern.

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Introduction

Clostridium difficile infection (CDI), caused by toxigenic *Clostridium difficile* (*C. difficile*), is a life-threatening disease that in the last decade has become more prevalent, not only as a healthcare-associated, but also as a community-acquired infection [10, 24]. The emergence of hypervirulent strains over the years has contributed to the severity of episodes of CDI [2, 18]. Rapid and accurate diagnosing of CDI is of importance to clinical outcome, effective infection control, and epidemiology. The laboratory diagnosis consists of the detection of toxigenic *C. difficile*, and/or its toxins A or B in stool, for which a variety of methods are available, each with its own qualities and limitations [17]. Selective stool culture for *C. difficile* has a high sensitivity but has a long turnaround time. A glutamate dehydrogenase (GDH) antigen assay can be used as a rapid screening for the presence of *C. difficile* in stool, but as with culture, the toxigenicity of the detected strain must be confirmed. The elaborate cell cytotoxin assay for toxin detection has been replaced by (rapid) enzyme immunoassays (EIAs), that generally tend to have low sensitivities [6]. Molecular methods, including commercial amplification assays, have been developed to detect *C. difficile* toxin genes (*tcdA*, *tcdB*) with high sensitivities and specificities in comparison with toxigenic cultures and are increasingly implemented in routine diagnostics [28]. From this range of assays each laboratory must compose its own appropriate diagnostic algorithm, taking into account test characteristics, turnaround time, workload and costs.

In a prospective study, we compared the performance of three toxin EIAs, a GDH antigen EIA and a new commercial toxin (A) gene amplification assay, the Illumigene *C. difficile* assay. The Illumigene test is a recently marketed assay that uses loop-mediated isothermal amplification (LAMP) to detect a 204-bp region of the conserved 5′sequence of the *C. difficile tcdA* gene [21]. All test results were compared with toxigenic culture as the "gold standard."

Materials and methods

Patients and samples

The study was conducted at the Laboratory of Clinical Microbiology and Infectious Diseases of the Isala klinieken, in Zwolle, The Netherlands. Every year, our laboratory processes approximately 3,300 stool samples to diagnose CDI, of which approximately 7 % are positive for either *C. difficile* free toxin or for toxigenic *C. difficile* culture. Based on the reported results of 70–80 % sensitivity of the three toxin EIAs included in our study, we calculated that approximately 1,000 samples were needed to determine a specificity of 99 % with a 95 % confidence interval of 10 % width [4, 9].

From October 2010 to February 2011, all unformed stool samples sent to our laboratory from patients with diarrhea, preferably those known to have CDI-associated symptoms or risk factors such as the recent use of antibiotics, were included in the study. We aimed to include both hospitalized and nonhospitalized patients.

Assays and testing protocol

Each stool sample was cultured upon arrival. On the same day two rapid EIAs were performed simultaneously: the horizontal flow EIA ImmunoCard Toxins A & B (Meridian Bioscience, Cincinnati, OH, USA), and the membrane EIA C. diff Quik Chek Complete (TechLab, Blacksburg, VA, USA), which combines toxins A and B with a GDH test in one device. Once a day we performed the breakaway micro wells EIA Premier Toxins A & B (Meridian Bioscience) and the toxin gene amplification assay Illumigene *C. difficile* (Meridian Bioscience), which both require batch testing. For samples received during weekends or public holidays all testing was completed on the following working day. All assays were performed according to the manufacturers' instructions and all results were interpreted by two technicians independently. The Premier EIA results were read with an automated microplate reader (Awareness Technology, Palm City, FL, USA). In between testing, the stool samples were stored at 4 °C. After testing the samples were frozen at −80 °C.

Toxigenic culture and ribotyping

Culture was performed according to our standard laboratory protocol. To select for clostridial spores all stool samples were pre-treated in a bench alcohol shock procedure, in which approximately 1.5-g or 1-mL stool sample is incubated with 100 % ethanol for 30 min. Culture was then performed on a selective *C. difficile* agar medium containing moxalactam and norfloxacin (CDMN; MediaProducts, Groningen, The Netherlands), which was incubated for 48 h under anaerobic conditions [1]. Identification of isolates recognized by their characteristic odor and colony morphology was based on the results of Gram staining and a specific somatic antigen latex agglutination test (Microgen *C. difficile* Rapid Test, Microgen Bioproducts, Camberley, UK). All isolates were confirmed to be toxigenic *C. difficile* at the reference laboratory of the Leiden University Medical Center (LUMC) in Leiden, The Netherlands, using PCRs for the detection of the *C. difficile*-specific GDH gene (*gluD*) [22] and the *tcdA* and *tcdB* genes, as previously described [13, 14]. The LUMC performed PCR ribotyping on all strains as previously described [3].

Analysis of discrepant results and data analysis

Because PCR was expected to have the highest sensitivity of all methods applied, real-time PCR was the method of choice for the discrepancy analysis for samples with a negative culture and a positive Illumigene test and if the Illumigene result was repeatedly "invalid". For discrepant analysis, a new DNA extract from a frozen aliquot of the original stool sample was tested with two real-time PCRs for the detection of *tcdA* (PCRA) and *tcdB* (PCRB) respectively. For both PCRs DNA was extracted as follows: 200 μg stool was vortexed with 800 μ lysis buffer for 5 min, spun down for 2 min at 10,000 *g* and subsequently frozen at −20 °C for 90 min. After thawing, 200 μL of the supernatant was used for automated DNA extraction on the NucliSense easyMAG platform (bioMérieux, Marcy l'Etoile, France). Phocine Herpes virus (PhHV) was added prior to the extraction as an internal control for inhibition and extraction [20]. Of a total 100 μL nucleic acid eluate, 10 μL was added to a 20-μl reaction mix containing 2x TaqMan® Gene Expression Master Mix (Life Technologies, Carlsbad, CA, USA) and oligonucleotides, for the duplex real-time detection of *tcdA* and PhHV. Previously published primers and probes were used for the PCRA [16], with one minor adaptation in the *tcdA* forward primer which now was: 5′-TTG TAT GGA TAG GTG GAG AAG TCA G-3′. VIC was attached to the minor groove binder probes as a fluorophore. The PCRB was performed as previously described [31].

A sample was considered "true positive" if the toxigenic culture (TC) was positive. The sensitivity, specificity, positive and negative predictive values of each toxin EIA, the

Illumigene assay and the GDH EIA were calculated against this gold standard. The C. diff Quik Chek GDH EIA was also compared with positive cultures of *C. difficile* including nontoxin-producing isolates. Additionally, we calculated whether the toxin(gene) tests' sensitivities varied among the different ribotypes.

Results

In total 986 stool samples were collected from hospitalized patients (n=318), outpatients (n=83), general practice patients (n=538), and nursing home residents (n=47). In total, 73 samples (7.4 %) were positive for toxigenic culture, from hospitalized patients (34; 10.7 %), outpatients (6; 7.2 %), general practice patients (23; 4.3 %), and nursing home residents (10; 21.3 %).

For 43 samples the initial Illumigene result was "invalid" (failed). When these were repeated, 38 samples were negative, one was positive and from this sample a toxigenic PCR-ribotype 002 was cultured, and four samples were again invalid. These four samples, negative in all other tests including PCRA and PCRB, were excluded from the analysis of the Illumigene results.

Table 1 shows the test results and characteristics of toxin (gene) and GDH tests compared with TC. All toxin assays performed in an inferior manner to the Illumigene assay and had sensitivities ranging from 41.1 to 54.8 %. The C. diff Quik Chek toxin test was the most sensitive of the three toxin detection assays and performed better than the ImmunoCard, which had the same sensitivity as the Premier EIA. The Quik Chek toxin detection was the only assay that showed no false-positive results in relation to the gold standard and hence had a specificity of 100 %.

Of the three samples that were culture-negative, but Illumigene positive, two were positive in both PCRA and PCRB. Of these, one was positive in the GDH test. For all three samples the toxin detection assays were negative.

In total, 86 *gluD* PCR confirmed *C. difficile* isolates, both toxigenic (84.9 %) and nontoxigenic (15.1 %) were cultured. Of 86 *C. difficile* culture-positive samples, 82 were positive in the C. diff Quik Chek GDH assay. Eleven stool samples were GDH-positive, but culture negative, resulting in a sensitivity, specificity, PPV and NPV of the GDH assay compared with positive culture (for both toxigenic and nontoxigenic strains) of 95.4 %,98.8 %, 88.2 %, and 99.6 % respectively. Compared with the gold standard, the GDH assay was false-negative for two samples, resulting in a NPV of CDI of 99.8 % (Table 1).

Positive TC, comprising 73 isolates, yielded a total of 23 different PCR ribotypes, of which the most prevalent were 014/020 (17.8 %), 078 (13.7 %), and 265 (13.7 %). To five strains no ribotype could be assigned at the reference laboratory. The Illumigene assay was positive for 10 of the 13 ribotype 014/020 strains (76.9 %), which was significantly more than the toxin tests detected (23.1–30.8 %, P=0.047, Fisher's exact test). However, the Illumigene assay was less sensitive in detecting ribotype 014/020 than for non-014/020, including the nontypable strains (P=0.037). The GDH test was positive for all 014/020 isolates. For the other ribotypes the numbers were too small to calculate the statistical significance of differences among the assay results.

Discussion

In this study, the sensitivities ranged between 40 and 55 % for the toxin EIAs and was 93.2 % for the Illumigene toxin

Table 1 Comparison of toxin EIA, Illumigene and GDH EIA results with toxigenic culture results

Assay	Result	Toxigenic culture result		% Sensitivity (95 % CI)	% Specificity (95 % CI)	% PPV (95 % CI)	% NPV (95 % CI)
		Number positive	Number negative				
IC	Positive	30	9	41.1 (30.5–52.6)	99.0 (98.1–99.5)	76.9 (61.7–87.4)	95.5 (93.9–96.6)
	Negative	43	904				
QCT	Positive	40	0	54.8 (43.4–65.7)	100 (99.6–100)	100 (91.2–100)	96.5 (95.1–97.5)
	Negative	33	913				
PT	Positive	30	10	41.1 (30.5–52.6)	98.9 (98.0–99.4)	75.0 (59.8–85.8)	95.5 (93.9–96.6)
	Negative	43	903				
IL	Positive	68	3	93.2 (85.0–97.0)	99.7 (99.0–99.9)	95.8 (88.3–98.6)	99.5 (98.8–99.8)
	Negative	5	906				
GDH	Positive	71	22	97.3 (90.6–99.3)	97.6 (96.4–98.4)	76.3 (66.8–83.8)	99.8 (99.2–99.9)
	Negative	2	891				

CI confidence interval, PPV positive predictive value, NPV negative predictive value, IC ImmunoCard Toxins A&B, QCT C. diff Quik Chek toxin assay, PT Premier Toxins A&B EIA assay, IL Illumigene *C. difficile* amplification assay, GDH C. diff Quik Chek GDH assay

gene assay. The C. diff Quik Chek toxin test showed the highest sensitivity (54.8 %) and was 100 % specific. The GDH component of the C. diff Quik Chek assay showed a high sensitivity in detecting all samples positive for *C. difficile* including non-toxigenic strains and a high NPV (99.8 %) for CDI.

In this large study, we included stool samples from different patient groups with varying prevalences of CDI and *C. difficile* PCR ribotypes. To avoid discordant results between the toxin tests owing to toxin degradation, all EIAs were performed as soon as possible after sample arrival. Between tests, samples were kept at 4 °C, to prevent a decrease in toxin level [11].

Rapid EIAs for the detection of toxins A and B have short turnaround times (approximately 30 min for a single test with membrane or flow EIA, approximately 90 min for a batch of tests with a well-type EIA), are easy to perform but have low sensitivities [6]. For the C. diff Quik Chek toxin assay a sensitivity range of 60–80 % has been determined against both toxigenic culture and PCR-based gold standards [9, 15, 23, 25, 27]. For the ImmunoCard assay known sensitivities are 48 % and 80.8 % compared with TC, for the Premier EIA sensitivity ranges between 48 and 70 % [9, 23, 26, 31]. Overall, the sensitivities we determined were lower than those found by others. For all tests, the published specificities range between 93 and 100 %, which is similar to our findings. In our study, the C. diff Quik Chek toxin test showed a PPV of 100 %. The PPVs for the Premier EIA and the ImmunoCard assay in our study with an overall CDI prevalence of 7.4 % (75.0 and 76.9 % respectively, with rather wide confidence intervals) were consistent with those previously calculated at prevalence rates of 5–10 % (56–73 % and 72–84 % respectively) [6].

For the Illumigene *C. difficile* amplification assay, we found high sensitivity (93.2 %) and specificity (99.7 %), similar to those determined previously [7, 8, 19]. Moreover, the Illumigene assay detected two positive samples that were culture-false-negative. The assay takes approximately 60 min to test 10 stool samples. The actual isothermal DNA amplification takes place in a closed test device with two separate chambers in the Meridian illumipro-10 Incubator/Reader. The assay can be performed in any laboratory without special requirements such as separate pre- and post-PCR rooms, which are necessary for real-time PCR or other PCR-based techniques. However, specific training is required, because during performance filling the sample collection brush with just the right amount of stool and guarding the time between preparing the test device and putting it in the illumipro-10 appeared to be critical points to prevent an “invalid” result. The initial result was invalid for 43 samples (4.4 %), of which four could not be resolved by repeating the test. The one sample in the study that was positive when repeated after the initial invalid result was also PCRA and TC-positive.

In a multicenter study, toxin EIAs were significantly less sensitive in detecting specific *C. difficile* PCR-ribotypes (002, 027, and 106) than a commercial toxin gene amplification assay [29]. We found a statistically higher sensitivity of the Illumigene assay for ribotype 014/020 compared with the toxin EIAs, while at the same time Illumigene seemed to detect 014/020 strains less accurately than other ribotypes. Numbers were relatively small, however, and more research is needed to explore this. For all 014/020 strains the GDH test was positive.

With a CDI prevalence of 7.4 %, using a diagnostic protocol according to which every stool sample is cultured means that >90 % of the cultures will be *C. difficile* negative. The C. diff Quik Chek GDH assay showed a high NPV compared with TC that has been confirmed by others [5, 15, 23, 25, 27]. This suggests the implementation of a two-step algorithm with a GDH assay as a screening test, which will substantially save on workload and costs [12, 30]. Only GDH-positive samples need to be tested further, preferably using a highly sensitive and specific PCR-based method to detect toxin genes or toxigenic culture. A large, recently completed study in the UK (more than 12,000 samples tested) to which the Health Protection Agency contributed, summarized the preliminary findings to define an optimal two-step algorithm. A GDH test applied as screening test followed by a molecular test for confirmation revealed the most sensitive performance, whereas the algorithm with a toxin EIA as a screening and a molecular test as confirmation was more specific. These algorithms are currently under further evaluation and will be launched as guidelines in 2012. (http://www.hpa.org.uk/web/HPAweb&Page&HPAwebAutoListName/Page/1179745281238). Our findings indicate that the GDH component of the C. diff Quik Chek Complete and the Illumigene amplification can both be applied as a screening test in combination with a toxigenic culture as confirmation. An additional advantage will be the availability of *C. difficile* isolates for further molecular typing to detect spread and early outbreaks. The performance of the Illumigene amplification assay (NPV 99.5 %) allows the introduction of this test as a first screening, although a high rate of invalid results may delay a prompt and appropriate response to possible CDI.

Conflict of interest The authors declare that they have no conflict of interest.

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