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Title: Molecular methods in the diagnosis of *Clostridium difficile* infections: an update

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Abstract/Summary:

Nucleic acid amplification techniques (NAATs) represent a major advance in the diagnosis of *C. difficile* infection (CDI). This review analyzes the different options available for a molecular diagnosis of CDI, as well as the strengths and weaknesses of NAATs. The performances of seven commercial NAATs are compared (BD GeneOhm Cdiff, Illumigene *C. difficile*, Xpert *C. difficile*, BD Max Cdiff, Portrait Toxigenic *C. difficile*, Progastro Cd, Seeplex Diarrhea ACE). The sensitivity and the rapidity of NAATs are excellent: additional efforts should focus on the discrimination between infection and colonization. Reporting the DNA load of toxigenic *C. difficile* in the stool sample may represent a solution. Diagnostic algorithms combining immunoassays and NAATs could also improve the specificity and reduce the global cost of this analysis.

Keywords:

Toxigenic culture, PCR, Inhibitor rate, Turnaround time, Clinical severity

Abbreviations:

CDI: *Clostridium difficile* infection

EIA: Enzyme immunoassay

CCNA: Cell culture neutralization assay

TC: Toxigenic culture

GDH: Glutamate dehydrogenase

NAATs: Nucleic acid amplification techniques

IBD: Inflammatory bowel disease

INTRODUCTION

Clostridium difficile infection (CDI) is a leading cause of nosocomial diarrhea. CDI is a life-threatening disease, associated with a 30-day mortality ranging from 3% to 30% [1]. CDI is defined by the presence of compatible symptoms (diarrhea or ileus) and the detection of toxigenic *C. difficile* (or its toxins) [2]. A positive laboratory test is not sufficient by itself, and should always be interpreted in the light of clinical data [3].

CURRENT METHODS AVAILABLE FOR CDI DIAGNOSIS

Two types of tests can be used for CDI diagnosis:

- detection of *C. difficile* toxins A and B: by enzyme immunoassays (EIA) or cell culture neutralization assay (CCNA).
- detection of toxigenic *C. difficile* strains: by toxigenic culture (TC) or nucleic acids amplification techniques (NAATs).

Enzyme immunoassays (EIA) detecting *C. difficile* toxins (TcdA and TcdB) are the most frequently employed tests. They are convenient to use, they provide a quick result for a low cost, and their specificity is good. The major drawback of EIA is their lack of sensitivity, ranging from 40 to 60% compared to TC [4,5]. Therefore, EIA detecting toxins cannot be recommended as a single test. Other immunoenzymatic tests detect the presence of glutamate dehydrogenase (GDH), an enzyme produced by *C. difficile*. GDH assays have a high negative predictive value, but they also detect non-toxigenic strains. Thus, GDH-positive results must be confirmed by another method. TC has a better sensitivity and specificity than GDH, but takes at least two days to complete. A quick diagnosis is needed to initiate treatment and infection control procedures.

Nucleic acids amplification techniques (NAATs) were developed to address these specific concerns. Sensitivity and specificity of NAATs are as good as the TC reference standard, and they provide a result in a few hours (1-3 hours). In recent published guidelines, NAATs are finally recognized as a superior method for the diagnosis of CDI [6]. The present review describes the different options available for a molecular diagnosis of CDI and analyze the strengths and weaknesses of NAATs.

GOLD STANDARD: CCNA OR TC?

New diagnostic assays must be compared against a reference method to evaluate their performances. The reference choice will alter the performances of the technique under evaluation. Two gold standards exist for CDI diagnosis: CCNA and TC. CCNA consist of incubation of the filtered stool sample onto a layer of cells sensitive to *C. difficile* toxins. Stools samples producing a specific cytopathic effect are considered positive. TC consists of inoculation of the stool sample into a selective medium, incubated anaerobically for 2 days. Then, the isolates of *C. difficile* recovered are tested for their ability to produce toxins.

The sensitivity of CCNA is only 67-86% compared to TC [7-9]. Thus, more stool samples are considered positive using TC than using CCNA, but that does not necessarily means TC is a better reference method. Indeed, CCNA detects the toxins present in stool samples but TC only evaluates the ability of *C. difficile* strains to produce toxins *in vitro*. The presence of free toxins in stool samples assessed by CCNA is thought to be more specific of CDI [10]. TC also detects patients colonized by a toxigenic strain of *C. difficile*, without any symptoms.

Pseudomembranous colitis diagnosed by sigmoidoscopy is pathognomonic of CDI [11]. The role of flexible sigmoidoscopy in CDI diagnosis was evaluated in 2003 [12]. CCNA was

positive in only 52% (29/56) of the CDI cases diagnosed by flexible sigmoidoscopy. These results need to be nuanced: 73 patients were excluded because CCNA was already known to be positive before sigmoidoscopy. Still, if all of these 73 patients had pseudomembranous colitis (best case scenario for CCNA), the sensitivity of CCNA would have been 79% (102/129). Among the 26 CCNA negative samples from the patients with pseudomembranous colitis, 9 were available for further testing. TC was positive in all of these cases (9/9). Another study evaluated the performances of CCNA and TC against a reference standard of clinically significant diarrhea and detection of toxigenic *C. difficile* [13]. CCNA had a lower sensitivity than TC (62.9% and 100%, respectively) but its specificity was not significantly different (93.9% and 92.2%). Thus, CCNA is not sensitive enough for the diagnosis of CDI: almost all the recent studies use TC as a reference standard. It also makes sense to compare NAATs to TC, because both of these techniques detect toxigenic strains of *C. difficile* in stool samples, and not free toxins.

Many methods have been described to culture *C. difficile* from stool samples and to assess its ability to produce toxins. In the first step, the stool can be treated by heat or alcohol to kill vegetative bacteria and select the hardy spores of *C. difficile*. Then, stool samples are inoculated onto an agar plate (direct plating) or into a selective broth (enriched TC) for enrichment before plating. Recently, 27 culture methods were evaluated for the recovery of low concentrations of *C. difficile* from stools [14]. Enriched TC methods provided the highest recovery rates, especially using cycloserin-cefoxitin mannitol broth after heat-shock or ethanol-shock. After a *C. difficile* strain is isolated, it is possible to determine if the strain is toxigenic with CCNA or NAATs. EIA should not be used directly on *C. difficile* strains, because results are not reproducible [15].

Studies using different reference methods (TC or CCNA) are not comparable. But even in studies using TC as a unique reference method, comparison is problematic. Indeed, TC is not standardized. The performances of NAATs should be interpreted differently if the gold standard is TC (direct plating) or enriched TC (selective broth). The sensitivity of the evaluated NAATs would appear higher compared to direct plating.

NAATS: IN-HOUSE ASSAYS

In-house NAATs detecting toxigenic *C. difficile* directly from stool samples were developed 20 years ago [16]. One of the principal difficulties encountered was the presence of PCR inhibitors in feces. Using a basic DNA extraction procedure (boiling of the stool followed by centrifugation), 30% of the positive samples failed to yield PCR products [17]. Thus, the extraction procedure has to be optimized specifically for feces. Depending on the DNA extraction method, PCR sensitivity to detect toxigenic *C. difficile* varies from 63 to 100% [18].

The *tcdB* gene (coding for TcdB) is often chosen as NAATs' target, because toxin B is produced by all the toxigenic strains of *C. difficile*. TcdB is also sufficient for the virulence of *C. difficile* in the hamster model of infection [19,20]. The *tcdA* gene (coding for TcdA) is less frequently used because 3.3% of the toxigenic strains from Europe do not produce toxin A [21]. The prevalence of TcdA⁻/TcdB⁺ strains is higher (27.0%) in Korea [22]. Nevertheless, a conserved region of the *tcdA* gene still exists in almost all the toxigenic strains, even in the strains deficient for toxin A production [23]. Only one *C. difficile* strain isolated from piglets presents a complete deletion of *tcdA* [24].

In-house NAATs present a good diagnostic accuracy. Peterson *et al.* reported a sensitivity of 93.3% and a specificity of 97.4% for their real-time PCR assay [25]. Multiplex in-house NAATs

were also developed to detect toxigenic *C. difficile* and report a presumptive identification of PCR-ribotype 027 [26]. Compared to enriched TC, the sensitivity and specificity were 86.6% and 97.4%, respectively. The presumptive identification of PCR-ribotype 027 could not be evaluated, because this PCR-ribotype was not detected during the study.

Even if in-house NAATs display good sensitivity and specificity, they also present some disadvantages. They require expertise in molecular biology and cannot be carried out immediately 7 days a week. In-house assays also require a method validation to comply with clinical laboratory accreditation standards [27]. Commercial molecular assays were developed to resolve these shortcomings.

NAATS: COMMERCIAL ASSAYS

BD GeneOhm Cdiff

The first and most extensively evaluated commercial assay developed is the BD GeneOhm Cdiff (BD Diagnostics, La Jolla, CA). This assay targets the *tcdB* gene. Extraction of DNA is manual: the specimen is submitted to mechanical lysis, then to thermal lysis. Addition of one freeze-thaw cycle can reduce the effects of some PCR inhibitors. Master mix has to be reconstituted immediately before use (3 hours of stability). To detect PCR inhibiting samples, an internal control is already included in the reaction mix. DNA is amplified using the SmartCycler System (Cepheid, Sunnyvale, CA). Fourteen clinical samples and 2 controls can be tested in each run. The turn-around-time (extraction and PCR) is about 120 min, including 45 min hands-on time. The use of frozen master mix reduces hands-on time by 15%, even if it is not recommended by the manufacturer. Sensitivity, specificity and inhibitor rates are similar with frozen or freshly reconstituted master mix [28].

Compared to TC, reported sensitivities and specificities of the BD GeneOhm range from 82.1% to 97.7% (median: 95.0%) and from 90.6% to 99.7% (median: 98.4%), respectively (Table 1). Invalid result rates reported in the same studies range from 0% to 6.4% (median: 0.9%) [4,7,8,29,30]. False-positive results due to a defective SmartCycler module have been reported once by Louie *et al.* [31]. These authors recommend that users request access to amplification curves. Although the software provides automatic interpretation, verification of the amplification curves by the laboratory staff may add another level of security.

Illumigene C. difficile

The *illumigene C. difficile* (Meridian Bioscience, Cincinnati, OH) assay uses loop-mediated isothermal DNA amplification (LAMP) to target a conserved region of the *tcdA* gene. This assay can also detect toxigenic strains deficient for toxin A. Extraction of DNA is manual: the specimen is diluted and subjected to thermal lysis. Diluent contains formalin-treated *Staphylococcus aureus*, acting as an internal control of amplification. Specimen are then processed through two chambers: one with primers specific to toxigenic *C. difficile* and the other for *S. aureus*. Positive and negative controls must be tested for each new kit lot. DNA is amplified using the *illumipro-10* (Meridian Bioscience, Cincinnati, OH) under isothermal conditions (65°C). DNA amplification forms magnesium pyrophosphate, which increases the turbidity of the reaction solution. Then, optical detection determines whether a sample is positive. The turn-around-time is about 60 min.

Compared to TC, reported sensitivities and specificities of the *illumigene* range from 73.0% to 98.1% (median: 93.3%) and from 91.5% to 100% (median: 99.7%), respectively (Table 1). It should be noted that one study reports a significantly lower sensitivity, without a clear explanation [32]. Invalid result rates range from 0.8% to 4.3% (median: 2.6%) [9,33].

Xpert C. difficile

The Xpert *C. difficile* (Cepheid, Sunnyvale, CA) uses disposable unitary cartridges, which contain all the reagents necessary to perform multiplex real-time PCR. This assay targets *tcdB* (toxin B), *cdt* (binary toxin), and *tcdC*Δ117 (a deletion frequently associated with PCR-ribotype 027). Hands-on time is minimal, because DNA extraction and amplification are completely automatized. The turn-around time is about 1 hour. Each module can process one sample independently from the others. Cartridges contain an internal control (spores of *Bacillus globigii*).

Compared to TC, reported sensitivities and specificities of the Xpert *C. difficile* range from 93.5% to 100% (median: 100%) and from 91.7% to 99.2% (median: 95.5%), respectively (Table 1). Invalid result rates reported in the same studies range from 0.9% to 13.4% (median: 3.7%) [34-36].

Besides detecting toxigenic *C. difficile*, the Xpert *C. difficile* assay reports presumptive identification of PCR-ribotype 027 (positive for *cdt* and *tcdC*Δ117). PCR-ribotype 027 strains are highly virulent. Notably, the production of binary toxin is associated with an increased mortality [37]. However, false positives have been reported for the presumptive identification of PCR-ribotype 027 using the Xpert assay [38]. This problem seems to arise with strains that possess *cdt* and present a point mutation at the position 117 of *tcdC* instead of the typical single-base deletion.

BD MAX Cdiff

The BD MAX Cdiff (BD Diagnostics, La Jolla, CA) allows the amplification of the *tcdB* gene by real-time PCR. A stool specimen is diluted in a sample buffer tube and associated with a

specific bar code. This assay is entirely automated, resulting in minimal hands-on time. An enzymatic extraction is performed with achromopeptidase, then DNA is captured on magnetic beads. DNA is eluted by heat, and added to a lyophilized reagent mix. PCR reactions are performed in microfluidic chambers, allowing fast temperature variations and short thermocycling times. A plasmid containing a synthetic target DNA sequence is included in each extraction tube, acting as an internal control. Twenty-four samples can be processed for each run. The turn-around-time is about 100 min (70 min extraction, 30 min of DNA amplification).

Compared to TC, reported sensitivities and specificities of the BD MAX Cdiff range from 90.5% to 97.7% and from 97.9% to 99.7%, respectively (Table 1). Invalid result rates range from 1.8% to 3.8% in the first run, but decrease to 0-0.4% when the test is repeated [39,40].

Portrait Toxigenic C. difficile

The Portrait Toxigenic *C. difficile* assay (Great Basin, Salt Lake City, UT) uses isothermal helicase-dependent amplification (HDA) of *tcdB* with blocked primers [41]. Extraction, amplification and detection are automated. DNA is continuously amplified at 65°C, using a helicase for denaturation. Then, biotin-labelled amplified DNA is bound to capture probes on a silicon chip and revealed by horseradish peroxidase. Each test contains an internal control (*S. aureus nuc* gene), a hybridization control and a detection control.

Buchan *et al.* reported a sensitivity of 98.2% and a specificity of 92.8% against TC [42]. Invalid result rate was 4.6% in the first run, but dropped to 0.5% after the test was repeated. This assay does not require thermal cycling nor real-time fluorescence detection, thus limiting the cost of instrument and reagents.

ProGastro Cd

The ProGastro Cd assay (Hologic Gen-Probe Inc., San Diego, CA) is a real-time PCR diagnostic test targeting the *tcdB* gene. Stool samples are diluted in S.T.A.R. buffer (Roche Diagnostics Corporation, Indianapolis, IN), then DNA is extracted using the NucliSENS easyMAG System (bioMérieux Inc., Durham, NC). DNA amplification is performed in a SmartCycler. Internal control is included for each specimen. Positive and negative controls must be tested in each run. The turn-around-time is about 3 hours, which is higher than other molecular assays.

Performances of the ProGastro Cd in comparison to TC are variable. Stamper *et al.* found a low sensitivity (77.3%) and a high specificity (99.2%) [43], whereas Selvaraju *et al.* reported an excellent sensitivity (100%) and a lower specificity (93.4%) [29]. These discordant results may be partly explained because Stamper *et al.* used enriched TC, a more sensitive reference method. These studies report an invalid result rate between 0 and 1.8% [29,43].

Seeplex Diarrhea ACE

The Seeplex Diarrhea-B1 ACE (Seegene Diagnostics, Seoul, South Korea) detects simultaneously toxigenic *C. difficile* (*tcdB*), along with *Salmonella* spp., *Shigella* spp., *Vibrio* spp., and *Campylobacter* spp. by multiplex PCR. This assay uses dual priming oligonucleotides (DPO), consisting of two priming regions bound by a linker [44]. DPO increases the specificity of DNA extension, which is important for multiplex PCR. DNA extraction is not included and must be performed with a kit from another manufacturer. After DNA amplification, PCR products are submitted to capillary electrophoresis. The turn-around time is about 4 hours.

The Seeplex has a sensitivity of 90% and a specificity of 97.1% against TC [45]. False negative results occurred mainly for the lowest concentrations of *C. difficile* in stool samples (assessed by semi-quantitative culture).

The Seeplex allows simultaneous detection of *C. difficile* and other enteropathogenic bacteria. Infections due to *Salmonella*, *Shigella*, *Vibrio* and *Campylobacter* are usually community-acquired. The recovery rate of these bacteria in nosocomial diarrhea is low, about 1% [46]. Nosocomial outbreaks caused by these bacteria are possible but rare in developed countries. However, immunosuppressed patients may develop nosocomial diarrhea due to reactivation of one of these enteropathogenic bacteria, especially under cancer chemotherapy [47]. Therefore, this assay seems mainly interesting to explore either community-acquired diarrhea or nosocomial diarrhea in immunosuppressed patients.

Overview

Performances of the different commercial molecular assays are summarized in Table 1. Only studies where TC or enriched TC was performed as a reference method on all the samples are included.

Pooled sensitivities for BD GeneOhm, illumigene, and Xpert *C. difficile* assays were calculated using Meta-DiSc (Figure 1), including only studies where TC (without enrichment) was performed as a reference method on all the samples. We did not present estimates of pooled specificity because: (i) the reference method (TC) is not very specific of CDI, (ii) sometimes NAATs can be more sensitive than TC without enrichment.

Pooled sensitivity was 93% for BD GeneOhm (95% confidence interval [CI], 89%-95%), 92% for illumigene (95% CI, 88%-94%) and 99% for Xpert *C. difficile* (95% CI, 97%-100%). The

Xpert *C. difficile* presented the highest pooled sensitivity, compared to TC without enrichment. However, the additional cases detected by the Xpert *C. difficile* could also correspond to asymptomatic colonization [48]. Further studies are needed to assess the clinical value of a positive NAAT result in case of a low concentration of *C. difficile*.

Cost per test, hands-on time and turnaround time are also important characteristics for the comparison of diagnostic assays (Table 2). Cost per test is minimal (\$25) for the Portrait Toxigenic *C. difficile* and the Progastro Cd. Hands-on time is minimal (10 min) for the three fully automated assays (Xpert *C. difficile*, BD MAX Cdiff, and Portrait *C. difficile*) and for the *illumigene C. difficile*. Turn-around time is minimal (<60 min) for the Xpert *C. difficile* and the *illumigene C. difficile*. Two assays also use multiplex PCR to provide additional information (Xpert *C. difficile* and Seeplex Diarrhea ACE).

ROLE OF DIAGNOSTIC ALGORITHMS

GDH: a first-line test

Glutamate dehydrogenase (GDH) is an enzyme produced in large amounts by *C. difficile* isolates. Thus, EIA detecting GDH present a higher sensitivity than EIA detecting toxins [49]. Eastwood *et al.* estimated GDH sensitivity at 87.6% compared to TC [8]. The negative predictive value of GDH is high (> 98%) [8], but positive results must be confirmed by another test. Therefore, GDH is often used as the first step of diagnostic algorithms.

Impact of strain type on GDH

The sensitivity of GDH seems to depend on *C. difficile* PCR-ribotype. GDH sensitivity was evaluated at 90.9% on PCR-ribotype 027 isolates, but dropped to 69.4% on non-027 isolates, compared to enriched TC [34]. In the same study, NAATs sensitivity of the Xpert *C. difficile* kit

was similar on 027 isolates (90.9%) and on non-027 isolates (91.7%). However, this part of the study involved only 47 positive stool samples, and the authors combined the results of 2 different GDH algorithms. More recently, an *in vitro* study reported that the production of GDH was highly conserved among *C. difficile* ribotypes [50], but the authors worked directly with *C. difficile* strains and not with clinical stool samples. Depending on the local epidemiology, algorithms using GDH as a first line test may have a lower sensitivity than previously admitted.

Two-steps algorithms

To lessen the cost of NAATs, Swindells *et al.* proposed a two-steps diagnostic algorithm [51]. First, both GDH and toxins are detected by EIA with the C. Diff Quik Chek Complete (TechLab, Blacksburg, VA). Three possibilities may occur: (i) the sample is negative for GDH: CDI is ruled out; (ii) the sample is positive for both GDH and toxin production: CDI is likely; (iii) the sample is positive for GDH only: NAAT must be performed. If NAAT is negative, CDI is ruled out. If NAAT is positive, CDI is probable.

The value of this algorithm was investigated by Culbreath *et al.* [52]. In their institution, this algorithm was 56% cheaper than applying the Xpert *C. difficile* systematically. This algorithm also presents the advantage to individualize a subgroup of patients with free toxins in the stools. For some authors, the presence of free toxin assessed by EIA is more specific of the disease than the presence of toxigenic *C. difficile* assessed by NAATs [10].

The GDH sensitivity is lower than NAATs, and some positive cases will be missed by the diagnostic algorithms. This is well illustrated by the application of Bayes' theorem to diagnostic tests [53]. The post-test probability represents the probability that a particular patient has the disease after the result of a diagnostic test is known. It depends on the

diagnostic test performances and on the pre-test probability. The pre-test probability is estimated from the patient clinical data and the local prevalence of the disease. Peterson *et al.* evaluated an EIA detecting toxins and GDH (C. Diff Quik Chek Complete) as well as a commercial NAATs (BD GeneOhm) on 1000 samples, compared to enriched TC [4]. The post-test probability of CDI after a negative EIA, GDH, or NAATs was estimated using the results from their study (Figure 2). NAATs are the best assays to rule out CDI. Thus, some laboratories routinely use diagnostic algorithms, but they also allow physicians to directly request NAATs in case of a high suspicion of CDI [52].

Globally, two-steps algorithms constitute an excellent alternative to the exclusive use of NAATs. Indeed, these algorithms provide additional information (EIA is performed on all the stool samples to detect free toxins), associated with a relatively low cost.

Other considerations: biosafety level

C. difficile is a biosafety level 2 (BSL-2) pathogen. Laboratory-acquired infections have been described, especially due to PCR-ribotype 027 strains [54]. Laboratory personnel working on stool samples should use class II biosafety cabinets. Multi-step algorithms may expose laboratory workers to a greater risk of infection, even though this risk is probably low.

TARGET POPULATION

The asymptomatic colonization rate is about 1.9% in healthy adults [55]. However, this rate can be as high as 51% for residents of long term care facilities [56]. Therefore, it is important to test only diarrheal stool samples, especially using a sensitive test like NAATs.

CDI testing rate / CDI incidence

CDI is an underdiagnosed disease. In a Spanish study, 118 laboratories sent all the diarrheal stool samples they had received on a single day to a reference laboratory [57]. TC was performed by the reference laboratory on all the samples, regardless of the diagnosis requested by clinicians. Toxigenic *C. difficile* was recovered by the reference laboratory for 45 samples:

- 31% (14/45) were successfully diagnosed by the participating laboratories.
- 20% (9/45) were diagnosed as false-negative by the participating laboratories. In fact, 83% of the participating laboratories used EIA, which has a low sensitivity.
- 49% (22/45) were not tested at all for *C. difficile* presence by the participating laboratories because it was not requested by clinicians.

CDI rates are principally influenced by the frequency of the laboratory tests carried out to detect toxigenic *C. difficile*. Across European countries, the testing rate for CDI varies from 3 to 141 per 10,000 patients-days [21]. Countries from Northern Europe have a higher incidence rate of CDI, but they also have a higher testing rate. To optimize the detection of CDI, all the cases of unexplained diarrhea should be tested.

Changing epidemiology of CDI

The three major risk factors for CDI are exposure to antibiotics, exposure to healthcare facilities and older age [58]. Recently, increased rates of infection were observed in patients previously thought to be at low risk for CDI [59]. Community-acquired CDI has become more prevalent the last 10 years, and represent now 20-59% of all the CDI cases [60-63]. Moreover, peripartum women may be at risk for severe CDI, even if they are younger than typical patients suffering from CDI [64]. Caesarean section seems to be an additional risk factor, probably due to antimicrobial prophylaxis [65].

The epidemiology of the PCR-ribotypes responsible for CDI is changing in European hospitals. Between 2005 and 2008, the prevalence of the PCR-ribotype 078 increased from 3% to 8%, whereas the prevalence of the PCR-ribotype 027 slightly dropped from 6% to 5% [21,66]. This is important because the genotype of *C. difficile* strains seems to be correlated with the severity of the disease. In a large study including 2222 adults suffering from CDI, Walker *et al.* explored the relationship between strain-type and mortality [67]. The 14-day mortality was highest for PCR-ribotypes 078 (25%) and 027 (20%), compared to an overall mortality of 13% ($p < 0.0001$). Inclusion of a large number of CDI cases was necessary to detect a difference between genotypes with sufficient power [68].

Repeated NAATs testing

After a positive result, NAATs should not be repeated. NAATs can remain positive for weeks after an episode of CDI. The only “test of cure” is the resolution of the diarrhea. Moreover, infection control precautions should only be maintained for the duration of diarrhea, irrespective of laboratory results [2].

After a negative result, the post-test probability of CDI is low because NAATs possess a high negative predictive value. When NAATs are repeated within 14 days, negative results are confirmed in 97.5% of the cases [69]. Repeated testing generates additional costs, which are not justified in light of this low diagnostic yield.

Caution to Interpret test in children and IBD patients

The rate of colonization by toxigenic *C. difficile* is high in children under the age of 2 years, dropping progressively from 38% (1 to 12 months) to 28% (12 to 24 months) [70]. Several hypotheses have been proposed to explain why *C. difficile* does not cause disease in infants.

In particular, this could be due to a diminished number of toxin receptors on the intestinal mucosal surface, or to their immaturity [71]. Although uncommon, it is possible for children to suffer from CDI. That is why the American Academy of Pediatrics published recommendations for the diagnosis of CDI in children [72]. Children younger than 1 year old should not be tested for CDI, except if they have Hirschsprung disease or during an outbreak situation. Between 1 and 2-year of life, positive *C. difficile* tests are difficult to interpret (“possible CDI”). After 3-year of life, positive *C. difficile* tests indicate probable CDI.

Carriage of toxigenic *C. difficile* is also significantly higher in inflammatory bowel diseases (IBD) patients (8.2%, n=122) compared to healthy adults (1.0%, n=99) ($p=0.02$) [73]. Ulcerative colitis patients present a higher carriage rate of toxigenic *C. difficile* (9.4%) than Crohn’s disease patients (6.9%). PCR-ribotyping suggests that these strains are probably community-acquired. None of these patients presented a clinical episode of CDI during the 6 months following the study [73]. Thus, clinical interpretation of positive NAATs for IBD patients is particularly difficult. Since the mortality of CDI is higher in IBD patients [74], recent guidelines recommend that all the IBD patients who are hospitalized for a disease flare should be tested for CDI [6].

DISCREPANCIES TOXIN / NAAT

Clinical severity

The higher sensitivity of NAATs compared to EIA has been criticized because the additional cases detected only by NAATs could also correspond to asymptomatic colonization. Humphries *et al.* recently evaluated the sensitivity of EIA and NAATs stratified by the severity of CDI, compared to TC [75]. The severity of CDI was evaluated based on age, albumin level,

white blood cell count, admission to the intensive care unit, and presence of pseudomembranous colitis. EIA was more sensitive in patients with severe CDI (58%) than in patients with mild disease (49%), even though this difference was not statistically significant ($p=0.31$). NAATs (Illumigene) presented the same sensitivity in patients with mild or severe CDI (98%). In other words, 42% of the severe CDI cases would have been missed for EIA against only 2% for NAATs. Thus, the additional cases detected only by NAATs also involve severe disease.

On the other hand, complication rates of nosocomial CDI were evaluated by Longtin *et al.* depending on the type of diagnostic test (NAATs versus EIA/CCNA) [76]. Complications were defined as all-cause mortality during the first 30 days, colectomy, admission to the intensive care unit or recurrence of CDI. As expected, more nosocomial cases of CDI were detected with NAATs (Illumigene) than with EIA/CCNA (85 versus 56, respectively). NAATs detected all the 23 cases who presented a complicated course of CDI, and EIA/CCNA detected almost all of these cases (22/23). The prevalence of complicated CDI was 39% for the CDI cases detected by NAATs and EIA/CCNA (22/56), and only 3% for the CDI cases detected by NAATs only (1/29). However, in this study the physicians had only access to the results of NAATs. It is possible that some of the cases detected by NAATs only would have developed complications if they had not received treatment for CDI.

EIA is probably more specific of complicated CDI than NAATs, but the cases detected by NAATs only can also correspond to a severe or complicated disease.

Quantitation / load: useful parameter?

The concentration of toxigenic *C. difficile* can be estimated by NAATs using standard curves. Leslie *et al.* reported the *tcdB* DNA load of samples positive with EIA or NAAT (Xpert *C. difficile*) [77]. The *tcdB* DNA load was 10 to 10,000 times higher for EIA-positive samples compared to EIA-negative NAAT-positive samples. Nevertheless, 48% of the additional cases detected only by NAATs also involve high *tcdB* DNA load (from 10^5 to 10^8 copies/mL). Further studies are needed to determine if the DNA load may separate asymptomatic colonization from infection. Reporting the concentration of toxigenic *C. difficile* to the clinicians may solve the problem of asymptomatic colonization detection by NAATs.

EXPERT COMMENTARY

Clostridium difficile is responsible for 10 to 25% of antibiotic associated diarrhea and for virtually all cases of pseudomembranous colitis. Rapid detection of toxigenic *C. difficile* is critical to begin optimal therapy of the patient and to interrupt transmission of *C. difficile* to other patients by applying infection control measures. Although toxigenic culture is the gold standard test to diagnose the infection, it is slow, laborious and requires 72-96 hours to give a reply to the clinician. Commercial EIA assays detecting *C. difficile* toxins show a low sensitivity ranging from 40 to 60% compared to toxigenic culture and cannot be recommended as single test.

NAATs represent a major advance in the diagnosis of CDI. Commercial assays are often easy to use and reduce hands-on time for technical staff, compared to in-house methods. However, the prescription of these tests must be strictly limited to diarrheal stool specimen (Bristol Stool Chart 5 to 7) in patients without laxatives, to avoid the detection of asymptomatic colonization. Moreover, these molecular tests are only useful for the initial diagnosis and do not permit to follow the evolution under treatment. Toxigenic *C. difficile*

DNA (*tcdB*) may remain positive for several weeks after the resolution of clinical symptoms. NAATs remain expensive but they can be integrated in a well thought algorithm, depending on the local epidemiology. Notably, the use of GDH as a first line test decreases the global cost. NAATs provide a quick and accurate diagnosis, allowing to limit the widespread diffusion of toxigenic *C. difficile* in our hospitals. The higher cost of NAATs may be counterbalanced by a decrease in healthcare-associated infections costs [78].

FIVE-YEAR VIEW

NAATs will probably be considered as the standard diagnostic test for CDI, either as a standalone test or included in an algorithm. The sensitivity and rapidity of NAATs are already excellent: additional efforts should focus on the prediction of the disease severity. NAATs results should always be interpreted in light of clinical data, but reporting the DNA load of the toxigenic *C. difficile* gene target may help to discriminate between colonization and infection. Moreover, reporting the PCR-ribotype strain type and presence of virulence factors could help to predict the severity of the disease. One assay (Xpert *C. difficile*) already reports the presumptive identification of PCR-ribotype 027 from stool samples, but other PCR-ribotypes seem to emerge as responsible for severe disease. Keeping up with the continuously evolving epidemiology of CDI represents a challenge for clinical laboratories.

Whole genome sequencing seems particularly promising to investigate epidemiological links between bacterial isolates [79]. It provides in-depth information about the spread of *C. difficile* in hospitals and communities, and lead to a better understanding of *C. difficile* transmission [80]. Since turnaround-time and costs decrease steadily, whole genome sequencing will probably become affordable for clinical laboratories within a few years.

Key issues:

- NAATs must only be performed on diarrheal stools (Bristol stool chart 5 to 7)
- NAATs combine excellent positive and negative predictive values, in contrast to EIA and GDH assays
- Qualitative NAATs cannot discriminate colonization from infection, but reporting the DNA load of toxigenic *C. difficile* using quantitative NAATs may add diagnostic value
- The only “test of cure” is the resolution of the diarrhea
- Positive *C. difficile* NAATs are difficult to interpret in children before 2-year of age and in inflammatory bowel disease patients
- Diagnostic algorithms reduce the cost of NAATs and individualize a subgroup of patients with free toxins in stools, which is more specific of the disease
- Even if NAATs are more expensive, this may be counterbalanced by a shorter technical working time and a decrease in costs due to healthcare-associated infections

Table 1. Performances of commercial NAATs against toxigenic culture (TC)

Assay	Authors	Year	Gold Standard	Number	Sensitivity (%)	Specificity (%)
BD GeneOhm	Dubberke [13]	2011	TC	150	97.7	90.6
	Eastwood [8]	2009	TC	552	88.5	95.4
	Le Guern [40]	2012	TC	360	95.5	99.7
	Selvaraju [29]	2011	TC	200	89.6	96.7
	Shin [45]	2012	TC	243	95.7	96.5
	Swindells [51]	2010	TC	150	94.4	99.2
	Viala [30]	2012	TC	94	95.5	97.9
	Zidaric [35]	2011	TC	194	82.1	98.2
	Buchan [42]	2012	Enriched TC	169	97.4	98.5
	Stamper [7]	2008	Enriched TC	401	83.6	98.2
	Peterson [4]	2011	Enriched TC	1000	94.5	96.5
illumigene	Bruins [33]	2012	TC	986	93.2	99.7
	Dubberke [13]	2011	TC	150	95.5	91.5
	Viala [30]	2012	TC	94	86.7	100.0
	Walkty [32]	2013	TC	427	73.0	99.7
	Ylisiurua [81]	2013	TC	430	97.7	99.6
	Buchan [42]	2012	Enriched TC	96	93.3	95.1
	Lalande [9]	2011	Enriched TC	472	91.8	99.1
Xpert <i>C. difficile</i>	Dalpke [39]	2013	TC	448	97.3	97.9
	Dubberke [13]	2011	TC	150	100.0	93.4
	Goldenberg [82]	2010	TC	224	100.0	96.7
	Shin [83]	2012	TC	253	100.0	94.6
	Swindells [51]	2010	TC	150	100.0	99.2
	Tenover [34]	2010	TC	2296	98.8	90.8
	Viala [30]	2012	TC	94	97.8	97.9
	Zidaric [35]	2011	TC	178	96.4	97.3
	Buchan [42]	2012	Enriched TC	275	100.0	91.7
	Novak-Weekley [36]	2010	Enriched TC	428	94.4	96.3
	Tenover [34]	2010	Enriched TC	2296	93.5	94.0
BD Max	Dalpke [39]	2013	TC	448	90.5	97.9
	Le Guern [40]	2012	TC	360	97.7	99.7
Portrait	Buchan [42]	2012	Enriched	540	98.2	92.8
ProGastro	Selvaraju [29]	2011	TC	200	100.0	93.4
	Stamper [43]	2009	Enriched	280	77.3	99.2
SeeGene	Shin [45]	2012	TC	243	90.0	97.1

Table 2. Characteristics of commercial NAATs

Assay	Method	Target	DNA Extraction	HOT (min) ^a	TAT (min) ^a	Cost per test
BD GeneOhm Cdiff	RT-PCR	<i>tcdB</i>	Manual	45	120	\$47 ^b
<i>Illumigene C. difficile</i>	LAMP	<i>tcdA</i>	Manual	10	60	\$33 ^b
Xpert <i>C. difficile</i>	Multiplex RT-PCR	<i>tcdB, cdt, tcdCΔ117</i>	Automated	10	60	\$52 ^b
BD MAX Cdiff	RT-PCR	<i>tcdB</i>	Automated	10	100	\$43 ^b
Portrait <i>C. difficile</i>	HDA	<i>tcdB</i>	Automated	10	100	\$25
Progestro Cd	RT-PCR	<i>tcdB</i>	Automated (easyMAG)	45	180	\$25
Seeplex Diarrhea ACE	Multiplex PCR	<i>tcdB</i>	Manual	N/A	240	\$41 ^b

LAMP: loop-mediated isothermal amplification

HDA: helicase-dependent amplification

HOT: hands-on time

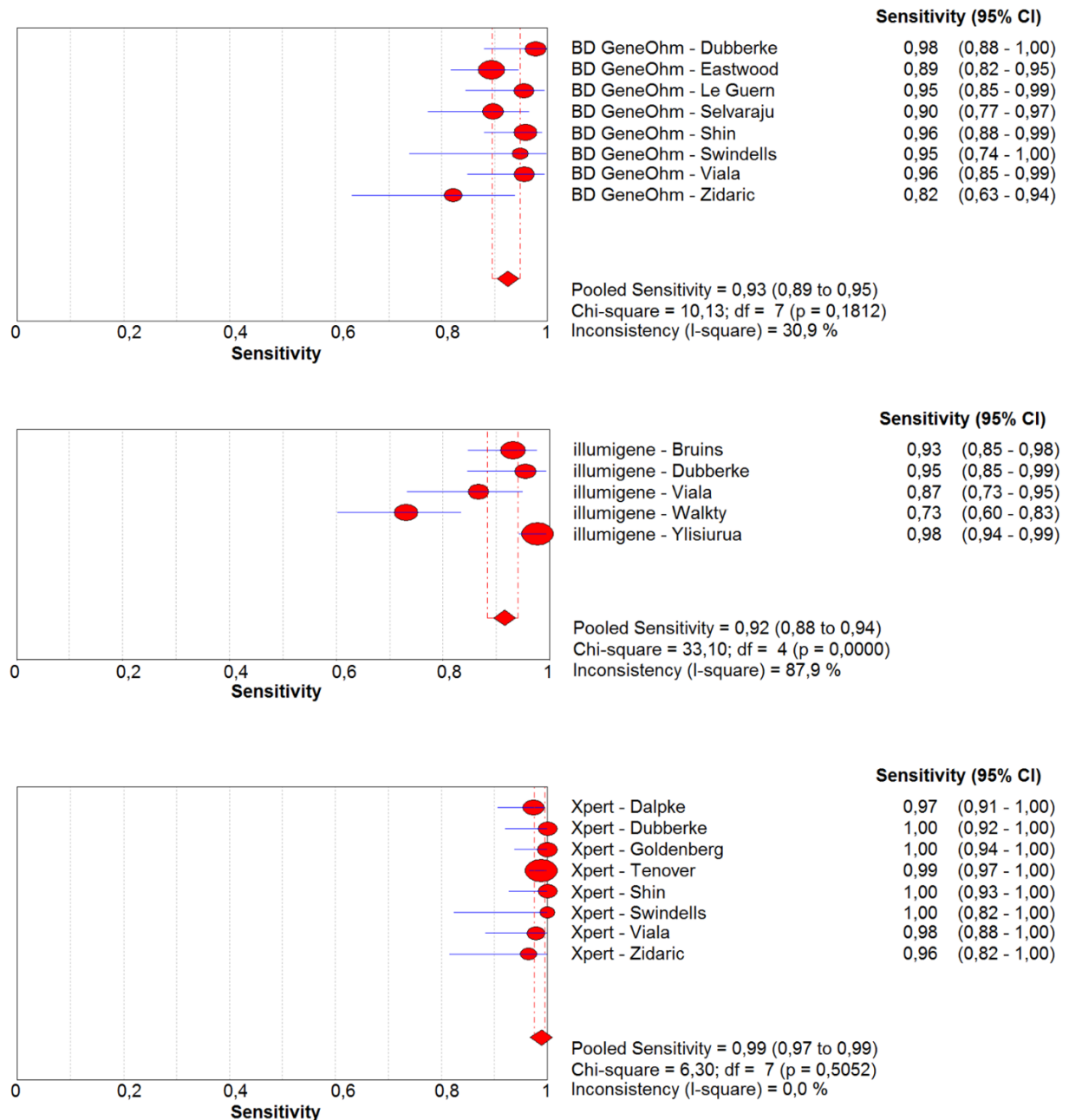
TAT: turn-around time

N/A: not available

^a: HOT and TAT for a batch of 5 samples

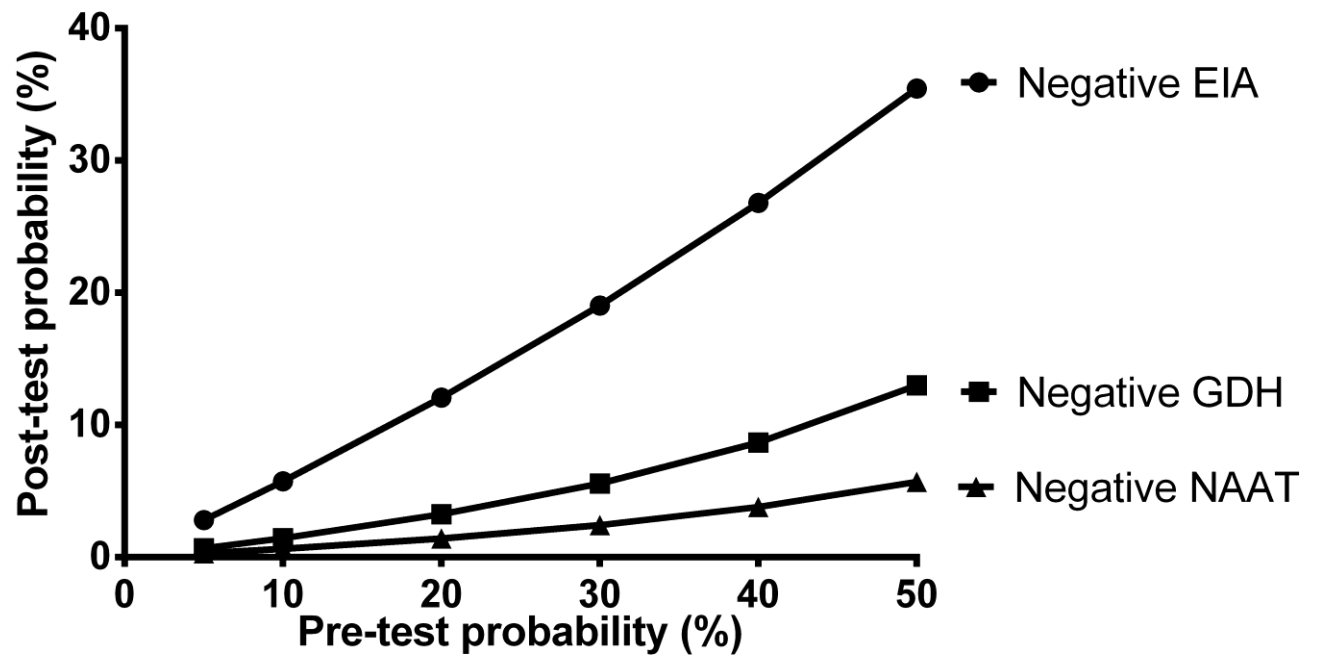
^b: these prices were obtained in euros, then converted to US dollars

Figure 1. Forest plot estimates of pooled sensitivities for three popular commercial NAATs compared to toxigenic culture (without enrichment).



Pooled sensitivities are presented for the BD GeneOhm Cdiff, the *illumigene C. difficile*, and the Xpert *C. difficile*, respectively (based only on studies where toxigenic without enrichment was performed on all the samples)

Figure 2. Post-test probability of CDI after a diagnostic test yield a negative result, using the results published by Peterson *et al.* [4]



EIA: Enzyme immunoassay (toxin component of C. Diff Quik Chek Complete)

GDH: Glutamate dehydrogenase (GDH component of C. Diff Quik Chek Complete)

NAAT: Nucleic acid amplification technique (BD GeneOhm Cdiff)

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