Combination of culture, antigen and toxin detection, and cytotoxin neutralization assay for optimal *Clostridium difficile* diagnostic testing

Michelle J Alfa PhD\(^1,2\), Shadi Sepehri MD PhD\(^1\)

**BACKGROUND:** There has been a growing interest in developing an appropriate laboratory diagnostic algorithm for *Clostridium difficile*, mainly as a result of increases in both the number and severity of cases of *C. difficile* infection in the past decade. A *C. difficile* diagnostic algorithm is necessary because diagnostic kits, mostly for the detection of toxins A and B or glutamate dehydrogenase (GDH) antigen, are not sufficient as stand-alone assays for optimal diagnosis of *C. difficile* infection. In addition, conventional reference methods for *C. difficile* detection (e.g., toxigenic culture and cytotoxin neutralization [CTN] assays) are not routinely practiced in diagnostic laboratory settings.

**OBJECTIVE:** To review the four-step algorithm used at Diagnostic Services of Manitoba sites for the laboratory diagnosis of toxigenic *C. difficile*.

**RESULT:** One year of retrospective *C. difficile* data using the proposed algorithm was reported. Of 5695 stool samples tested, 9.1% (n=517) had toxigenic *C. difficile*. Sixty per cent (310 of 517) of toxigenic *C. difficile* stools were detected following the first two steps of the algorithm. CTN confirmation of GDH-positive, toxin A- and B-negative assays resulted in detection of an additional 37.7% (198 of 517) of toxigenic *C. difficile*. Culture of the third specimen, from patients who had two previous negative specimens, detected an additional 2.32% (12 of 517) of toxigenic *C. difficile* samples.

**DISCUSSION:** Using GDH antigen as the screening and toxin A and B as confirmatory test for *C. difficile*, 85% of specimens were reported negative or positive within 4 h. Without CTN confirmation for GDH antigen and toxin A and B discordant results, 37% (195 of 517) of toxigenic *C. difficile* stools would have been missed. Following the algorithm, culture was needed for only 2.72% of all specimens submitted for *C. difficile* testing.

**CONCLUSION:** The overview of the data illustrated the significance of each stage of this four-step *C. difficile* algorithm and emphasized the value of using CTN assay and culture as parts of an algorithm that ensures accurate diagnosis of toxigenic *C. difficile*.

**Key Words:** Laboratory diagnostic algorithm; Optimal laboratory diagnosis; Toxigenic *Clostridium difficile*.

**HISTORIQUE** : L’intérêt augmente envers l’élaboration d’un algorithme de diagnostic pertinent en laboratoire pour le *Clostridium difficile*, surtout en raison de l’augmentation du nombre et de la gravité des cas d’infection par le *C. difficile* depuis dix ans. Un tel algorithme diagnostique s’impose parce que les tâches diagnostiques, surtout pour détecter les toxines A et B ou l’antigène glutamate déshydrogénase (GDH), ne suffisent pas comme test unique pour assurer le diagnostic optimal de l’infection par le *C. difficile*. De plus, les méthodes de référence habituelles pour déceler le *C. difficile* (p. ex., culture toxigène et dosage de neutralisation des cytotoxines [NCT]) ne sont pas monnaie courante en laboratoire diagnostique.

**OBJECTIF** : Analyser l’algorithme en quatre étapes utilisé aux emplacements de services diagnostiques du Manitoba pour poser un diagnostic en laboratoire de *C. difficile* toxigène.

**RÉSULTAT** : Les chercheurs ont présenté les données rétrospectives sur le *C. difficile* obtenues à l’aide de l’algorithme proposé sur une période d’un an. Sur les 5 695 coprocultures testées, 9,1 % (n=517) présentaient un *C. difficile* toxigène. Soixante pour cent (310 sur 517) des coprocultures de *C. difficile* toxigène ont été décélées après les deux premières étapes de l’algorithme. La confirmation par NCT de dosages GDH positif, négatifs aux toxines A et B, a permis de décélérer 37,7 % (198 sur 517) d’autres cas de *C. difficile* toxigène. La culture du troisième échantillon, prélevé chez les patients dont les deux échantillons précédents étaient négatifs, a permis de décélérer 2,32 % (12 sur 517) d’échantillons de *C. difficile* toxigène.

**EXPOSÉ** : En utilisant l’antigène GDH comme outil de dépistage et les toxines A et B comme test de confirmation de l’infection à *C. difficile*, 85 % des échantillons étaient négatifs ou positifs dans un délai de quatre heures. Sans confirmation par NCT des résultats discordants de l’antigène GDH et des toxines A et B, 37 % (195 sur 517) des coprocultures de *C. difficile* toxigène n’auraient pas été décélées. Une fois l’algorithme effectué, il n’a fallu effectuer une culture que pour 2,72 % de tous les échantillons soumis en vue d’un test de dépistage du *C. difficile*.

**CONCLUSION** : L’appréciation des données fait ressortir l’importance de chaque étape de l’algorithme de *C. difficile* en quatre étapes et la valeur du dosage de NCT et de la culture dans le cadre de l’algorithme pour assurer un diagnostic précis de *C. difficile* toxigène.

*Clostridium difficile* is an anaerobic spore-forming pathogen and the leading cause of hospital-acquired diarrhea (1). The incidence and severity of *C. difficile* infection (CDI) has increased drastically over the past decade, emphasizing the need for optimal laboratory diagnosis of CDI (2). The accurate and rapid diagnosis of CDI not only has a positive effect on patient care but is the key element in undertaking effective infection control measures that will ultimately reduce the likelihood of spread of this *C. difficile* (3).

Despite recent advances in clinical microbiology diagnostics, the accurate laboratory testing for toxigenic *C. difficile* has remained challenging (1,4). Traditional *C. difficile* tests, such as toxigenic culture and cytotoxin neutralization (CTN) assays, have been identified as the gold standard methods for clinical diagnosis of *C. difficile*. However, these techniques are time consuming, labour-intensive and, at times, technically challenging (1,4,5). In recent years, simpler and quicker methods have been developed for the diagnosis of toxigenic *C. difficile*. Direct detection of toxins A and B and/or glutamate dehydrogenase (GDH) antigen have become the routine testing practice in many laboratories (6,7). However, a number of studies have revealed insufficiencies of these rapid methods as the sole assay for the detection of...
C difficile (4,7,8). Assays that detect toxins A and B are usually highly specific, but lack sensitivity, in contrast with GDH antigen detection tests that are sensitive but not specific (1,9). Recent studies support the application of a multistep testing algorithm (2,4,5,7,10-12). Most research groups recommend the use of a sensitive assay such as GDH antigen as a screening tool followed by a more specific test such as toxin detection, culture or nucleic acid amplification test (NAAT) as the confirmatory assay (1,6,8,13).

It is evident that none of the C difficile detection assays when used alone are 100% sensitive and specific (5,13), thereby supporting the necessity for a multistep C difficile diagnostic algorithm. Here, we introduce a four-step algorithm for clinical detection of C difficile that we have used in our diagnostic laboratories since 2008. Analysis of one year of data supports the value of this four-step algorithm as the optimal approach to diagnosis of CDI.

COMMON C DIFFICILE DIAGNOSTIC TESTING

The number and severity of CDI cases have increased significantly during the past decade (2,10,14). The increase in severity of CDI has largely been associated with the emergence of novel hypervirulent strains of C difficile, particularly ribotype 027/NAP1 (also referred to as North American pulsortype 1 or toxintype III) (2,15). Moreover, less-than-optimal laboratory practices for C difficile diagnosis may have also contributed to the spread of this organism. According to survey data from the College of American Pathology proficiency testing, as of 2008, 89% of American laboratories applied toxin A and B and/or GDH antigen assays for C difficile diagnostic testing whereas only 1% of laboratories used CTN assays (5).

Stool culture and CTN assays

Stool culture to detect toxigenic C difficile (toxigenic culture) is one of the most sensitive methods (1) and has been suggested as the gold standard assay by many groups (2,3,5). However, the culture process is time consuming (up to five days) and is not widely performed in North American diagnostic laboratories (5,9,11,16). Similarly, CTN assay has long been used as a reference method of C difficile identification because of its high specificity and high positive predictive value (2,3,7).

Although the CTN assay is more specific than toxigenic culture and the results are available within 48 h, the assay is technically complex, requires a significant amount of hands-on time and, therefore, is performed in only approximately 1% of diagnostic laboratories (5,15).

Antigen and toxin assays

Using toxin A and B immunoassays as a stand-alone diagnostic test for C difficile has been a routine practice in many laboratories in recent years (1,4,10-12). Toxin detection assays are easy to perform and allow the same-day reporting of the results. However, recent studies proved these tests have suboptimal sensitivities (as low as 38% reported by Ticehurst et al [7]) and suggested toxin enzyme immunoassays alone are no longer adequate for the proper diagnosis of C difficile (4,7,8,10,12,17).

A sensitive GDH antigen assay that is now widely used is based on the detection of cell wall protein GDH, which is produced in much higher amounts than toxins A and B (5). The GDH antigen can be produced by other normal gastrointestinal microorganisms, and so is not totally specific for C difficile (5). Moreover, the GDH assay, similar to toxigenic culture, is unable to differentiate toxigenic and nontoxigenic strains (6). The high sensitivity and very high positive predictive value (5-8,11,12,16) are properties that make GDH an optimal assay for C difficile screening (1,8). While negative GDH results can be reliably reported as negative for C difficile without further testing (7), the positive GDH results need to be further confirmed by a more specific assay to rule out the false-positive cases as well as nontoxigenic C difficile strains (5-7).

NAAT

Recently, the use of NAAT for diagnosis of CDI has been recommended as a more sensitive diagnostic test (18,19). Both polymerase chain reaction as well as isothermal amplification assays are available. There have been concerns with detection of asymptomatically carried spores giving false positives in these assays. Furthermore, cost has been a major implementation issue for the amplification-based test kits.

C DIFFICILE DIAGNOSTIC ALGORITHMS

Two-step algorithms

A two-step algorithm for CDI diagnosis, including GDH screening followed by a toxin detection assay, was proposed many years ago (6,20). According to this algorithm, GDH-negative results were reported as negative for C difficile without further testing, while GDH-positive specimens were tested for toxin A and B. The presence of GDH, as well as A and B antigens, confirmed the toxigenic C difficile diagnosis (14). Following this two-step algorithm, 80% to 90% of results are reported positive or negative in <4 h (5,10,16). A study by Gilligan (11) demonstrated that applying this two-step algorithm enhanced the detection of toxigenic C difficile by 40% compared with toxin A and B immunoassays alone. Another two-step algorithm was proposed by John Hopkins Hospital laboratories (Baltimore, USA), which included primary GDH screening followed by CTN assay for confirmation of GDH-positive results (7). Applying the latter algorithm, 75% to 80% of specimens were reported negative for C difficile on the same day (GDH negative). However, the results of the remaining 20% to 25% (GDH positive) were pending for 48 h until the final reading of CTN assay was available (7). The authors reported that using such an algorithm reduced their laboratories’ workload and expenses by 81.5% and 61%, respectively (7). The European study by Planche et al (13) also supported the use of a CTN assay as a second step in C difficile diagnosis algorithm and argued that the reduced cases of CDI in hospitals, reduced burden on infection control and savings in antibiotic costs will offset the additional laboratory expenditure of CTN assay. A study by Wern et al (12) suggested the combination of toxin A and B assay followed by fecal lactoferrin test as the second step of the algorithm, because they believed the correlation of lactoferrin and inflammation was a better indicator of the clinical state of the CDI.

Multistep diagnostic algorithms

GDH antigen testing and toxin A and B detection are suggested as the first and second steps of the C difficile specimen processing algorithm, respectively (Figure 1). As the third step in the C difficile diagnosis algorithm, and to resolve discordant antigen tests (i.e., the GDH-positive, toxin A and B-negative cases), the CTN assay is recommended (Figure 1). The results showed that without performing a confirmatory test for stools with discordant antigen results, 37.7% (195 of 517) of toxigenic C difficile stools would be missed (Figure 2). Similar to these findings, Schmidt and Gilligan (5) reported that by applying a two-step algorithm, approximately 25% of true positives would be overlooked and emphasized the use of a third confirmatory assay for GDH-positive, toxin A- and B-negative specimens. According to Fenner et al (10), GDH-positive, toxin A- and B-negative specimens may represent false-positive GDH cases, nontoxigenic C difficile isolates or toxigenic strains missed by the toxin antigen assays. They recommended using stool culture to resolve these discordant specimens (10). Schmidt and Gilligan (5,11), however, suggested three possible options for the third confirmatory test; namely, toxigenic culture, CTN or NAAT, and discussed the advantages and drawbacks of each method. Overall, all three proposed confirmatory assays proved to be complex, labour-intensive and costly, and were not feasible for smaller community-based hospitals. The authors suggested that in such settings, laboratories must perform the two-step algorithm and send the discordant samples to reference laboratories for subsequent confirmation (5).

Recently, the American Society for Microbiology published a guidance document for the laboratory detection of toxigenic C difficile (8). In this document, the confirmation of GDH screening by toxin A and B, CTN or NAAT assays followed by toxigenic culture or NAAT to resolve the GDH-positive, toxin A and B-negative was recommended.
Influence of volume of stool submitted

Many laboratories indicate that if the volume of stool submitted for C. difficile detection was insufficient, the sample would be rejected. However, there are no published studies that indicate what volume of stool is actually considered insufficient for appropriate C. difficile testing. Because of the concern regarding delayed diagnosis when specimens are rejected, it was opted to use toxigenic culture (after alcohol treatment) for testing stool samples <1 mL volume rather than rejecting the specimen.

Figure 1) The four-step Clostridium difficile specimen processing algorithm: C. difficile testing algorithm for up to two nonformed stool samples submitted from one patient (algorithm 1), and C. difficile testing algorithm for the third nonformed stool sample submitted from a patient with relevant clinical symptoms but two previously negative C. difficile results (algorithm 2). Algorithm 1 is for up to two stool samples of the same patient received on two separate days. If more than one sample was received on the same day, they were pooled and then tested according to the algorithm 1. If the volume of sample was insufficient (<1 mL) to perform proper testing according to algorithm 1, or if two separate specimens were negative and the third stool sample was received from the same patient, then algorithm 2 was followed. BAK Blood agar containing vitamin K and hemin; BHI Brain heart infusion broth; CDMN C. difficile moxalactam norfloxacin agar; CTN Cytotoxin neutralization; FAB Fastidious anaerobic broth; GDH Glutamate dehydrogenase; PCR Polymerase chain reaction; RT Room temperature

(8). The authors agree with Snell et al (6) and Reller et al (16), who recommended CTN as a third assay for resolving GDH-positive, toxin A- and B-negative specimens. Cytotoxins assays not only have high specificity, but are also able to distinguish toxigenic from nontoxigenic C. difficile and the results are highly correlated with the clinical presentation of CDI. An additional 24 h to 48 h is needed to finalize the CTN assay.

Toxigenic culture as part of multistep algorithms

There is no doubt that adding culture to the C. difficile testing algorithm has epidemiological and clinical advantages because the isolate will be available for typing and susceptibility studies (1,4,9). The documentation of C. difficile subtypes is essential during CDI outbreaks in hospitals (9). However, because the culture process is slow, culture is not an optimal method from the CDI management point of view. As a solution, Gerdinger (9) recognized Ticehurst et al’s two-step algorithm (7) that consisted of a GDH screening test followed by toxin A and B-negative (Neg) stools, 37.7% (195 of 517) toxigenic C. difficile would have been missed. If culture was not performed on the 3rd specimen, from patients who had two previous diarrheal samples submitted that were reported as negative, 2.32% (12 of 517) of toxigenic C. difficile would not have been diagnosed.

Because it is difficult to determine the volume of stool, a prospective study was performed in which 300 consecutive stool specimens submitted to the laboratory for C. difficile testing during March and April 2010 were weighed. Three millilitres of five different non-formed stool (NF-stool) specimens with different densities were weighed, and it was found that 1 mL of NF-stool weighs approximately 1.08 g (data not shown); thus, it was concluded that weight is a good indicator of NF-stool volume. The mean weight of NF-stools submitted for CDI diagnostic testing was 22.58 g (range 0.55 g to 108.28 g). Overall, 31 (10.3%) samples were positive for toxigenic C. difficile, and the mean (± SD) weight of the C. difficile-positive NF-stools (26.44±3.90 g) was not significantly different from the mean weight of C. difficile-negative NF-stools (22.13±1.21 g) using Student’s t-test (P=0.29, data not shown). A review of the data showed that for NF-stool specimens as little as 0.55 g, it was still possible to test according to algorithm 1 and reliably detect specimens that were positive for both GDH and toxins A and B (data not shown). However, toxigenic culture is suggested as an alternative step for occasions in which the laboratory technologist is unsure of sufficiency of stool volume (Figure 1). None of the 300 stool samples submitted in 2010 were rejected due to the insufficient volume.

Figure 2) Schematic overview of clinical Clostridium difficile diagnostic testing outcomes from stool samples processed in Winnipeg, Manitoba, from January to December 2009 following the proposed four-step C. difficile specimen processing algorithm. *This total number does not include the six samples that were rejected due to either insufficient quantity (n=1) or previous positive C. difficile results (n=5). In total, 9.1% of all stools tested were positive for toxigenic C. difficile. Eighty per cent (4558 of 5695) of all specimens processed were reported negative for toxigenic C difficile after screening for glutamate dehydrogenase antigen (GDH Ag). Without cytotoxin neutralization (CTN) confirmation for GDH-positive (Pos) toxin A and B-negative (Neg) stools, 37.7% (195 of 517) toxigenic C. difficile would have been missed. If culture was not performed on the 3rd specimen, from patients who had two previous diarrheal samples submitted that were reported as negative, 2.32% (12 of 517) of toxigenic C difficile would not have been diagnosed.


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We recommend that culture be used as a fourth step of our algorithm for stool quantities <1 mL, and for highly suspicious CDI patients who have had two previous negative C. difficile test results following GDH, toxin A and B, and CTN steps (Figure 1). Doing et al. (4) collected up to three samples on separate days from patients with high suspicion of CDI when the first and second day toxin results were negative and have shown that testing sequential samples within three days with the same method hold no diagnostic value. Hence, we propose the use of toxigenic culture for patients with persistent diarrhea who have had two negative C. difficile stools (Figure 1). Using our algorithm, we cultured 2.72% (155 of 5696) of all stool samples submitted to the laboratory and detected 12 toxigenic C. difficile stools (Figure 2) that would otherwise have been missed.

Overall, 9.1% (517 of 5695) of all stools tested in our diagnosis services contained toxigenic C. difficile (Figure 2). Similar prevalence rates have been reported by European (8.6%) (12) and American (7.7%) (16) groups.

Role of NAAT in C. difficile testing
Recent studies have utilized molecular amplification of a portion of tcdB (gene encoding cytotoxin B) and found that this diagnostic approach to be a rapid and efficient method for either the confirmation of a positive screening test or to be used as a primary assay for C. difficile diagnosis (1,2,4,8,15). Such molecular techniques require a high level of technical expertise as well as special laboratory instruments and reagents and, as such, are costly compared with other antigen-based assays and are often inaccessible, particularly for smaller laboratories (4,16). Moreover, NAAT assays, when used alone, can only detect the presence of the toxin gene and not the toxin itself. The relevance of such positive results has yet to be fully clarified (15). Although more traditional tests such as toxigenic culture and cytotoxin assays are less expensive, molecular amplification is the only test that allows same-day reporting of the results while ensuring excellent correlation with toxigenic culture (4). Further investigations are warranted to determine how to optimally include molecular testing in the current four-step C. difficile diagnostic algorithm.

If one assumes that the cost ranges (supplies only) for the various tests are: antigen tests (GDH and toxin A and B); $7 to $10; toxigenic culture $8.50 to $11.50; enzyme immunoassay $12 to $14; and NAAT is $28 to $40, then for the four-step algorithm a crude cost estimate would be $51,657 to $73,722 for the specimens processed in Figure 2 compared with $91,016 to $112,250 for the same specimens if they were processed using a two-step algorithm based on enzyme immunoassay (GDH and toxin A and B performed concurrently) followed by NAAT for discordant antigen results.

CONCLUSION
The aim of the present study was to introduce our optimal four-step algorithm for C. difficile specimen processing and to report data from one year to demonstrate the value of following such an algorithm. Our data demonstrated that if only a two-step algorithm (GDH antigen for screening followed by toxin A and B confirmation) is used, more than one-third of toxigenic C. difficile infections would be missed. Our study highlights the need for a confirmatory test for discordant results where GDH was positive and toxin A and B antigens were negative and emphasizes the value of culturing the third specimen to optimize detection of toxigenic C. difficile isolates in patients with persistent diarrhea whose CDI diagnosis was not made by other C. difficile detection assays.

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