

Diagnostic algorithm using a sensitive broth culture method for detection of *Clostridium difficile* toxin from stool samples

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P Bayardelle. Diagnostic algorithm using a sensitive broth culture method for detection of *Clostridium difficile* toxin from stool samples. *Can J Infect Dis Med Microbiol* 2009;20(4):e135-e138.

BACKGROUND: The two-step glutamate dehydrogenase antigen-cytotoxicity neutralization assay algorithm has been found to be reliable for the diagnosis of toxigenic *Clostridium difficile*. However, the high sensitivity of the screening method is compromised by the relative low sensitivity of the second step, the direct cytotoxin neutralization assay (DCNA) using a fecal filtrate. The objective of the present study was to compare the DCNA with an indirect cytotoxin neutralization assay (ICNA).

METHODS: For ICNA, the cytotoxin B of *C difficile* was obtained from a broth culture of the stools and neutralized according to a standard cytotoxin assay using MRC-5 fibroblast cells.

RESULTS: A total of 923 stool specimens from adults were tested during a three-month period from June to August 2008. The prevalence of toxigenic *C difficile* was 13.5%. The sensitivity of the two-step algorithm was 88%. With the ICNA, 12% toxigenic *C difficile* were detected that were missed by DCNA.

CONCLUSIONS: The use of broth for the ICNA is convenient, and results in increased sensitivity of detection of toxigenic *C difficile*. It can be implemented in routine diagnosis.

Key Words: Cytotoxin assay; GDH; Toxigenic *C difficile*

Clostridium difficile infection (CDI) is a frequent cause of nosocomial diarrhea (1,2) and is implicated in 15% to 25% of patients with antibiotic-associated diarrhea (2). The clinical course ranges from mild diarrhea to fulminant colitis and toxic megacolon (1). Morbidity and mortality have increased with the emergence of the hypervirulent strain of *C difficile*, which is characterized by restriction-endonuclease analysis, pulsed-field gel electrophoresis and polymerase chain reaction ribotyping as group BI, North American pulsed-field gel electrophoresis type 1 and polymerase reaction ribotype 027 (BI/NAP1/027) (3-5). In a recent study reported from the province of Quebec, the mortality rate of CDI in Quebec was 6.9% (6).

In North America, enzyme immunoassay (EIA) is the most commonly used method for the diagnosis of CDI, given its rapid turn-around time, simplicity and low cost. It can detect 100 pg to 1000 pg of toxin while cytotoxin assay may detect as little as 10 pg of toxin (1). Several EIAs are commercially available. They detect toxin A or toxins A and B in stool specimens. These methods are rapid and convenient. Although their specificity is usually good, they display poor sensitivity,

Un algorithme diagnostique faisant appel à un bouillon de culture sensible pour déceler la toxine du *Clostridium difficile* dans des coprocultures

HISTORIQUE : Il est établi que l'algorithme de l'épreuve de neutralisation de la cytotoxicité à l'antigène glutamate déshydrogénase en deux étapes est fiable pour diagnostiquer le *Clostridium difficile* toxicogénique. Cependant, la haute sensibilité de la méthode de dépistage est compromise par la sensibilité relativement faible de la deuxième étape, l'épreuve de neutralisation directe de la cytotoxine (ÉNDC) au moyen d'un filtre fécal. La présente étude visait à comparer l'ÉNDC à une épreuve de neutralisation indirecte de la cytotoxine (ÉNIC).

MÉTHODOLOGIE : Avec l'ÉNIC, on a prélevé la cytotoxine B du *C difficile* dans un bouillon de culture des selles et on l'a neutralisée conformément à une épreuve de cytotoxine standard, au moyen de cellules fibroblastiques MRC-5.

RÉSULTATS : Au total, on a testé 923 coprocultures d'adultes sur une période de trois mois, soit de juin à août 2008. La prévalence de *C difficile* toxicogénique était de 13,5 %. La sensibilité de l'algorithme à deux étapes s'élevait à 88 %. Avec l'ÉNIC, on a décelé 12 % de cas de *C difficile* toxicogénique non dépistés par l'ÉNDC.

CONCLUSIONS : Le recours à un bouillon de culture pour l'ÉNIC est pratique, et il accroît la sensibilité de détection du *C difficile* toxicogénique. On peut l'adopter pour les diagnostics systématiques.

ranging between 38% and 76% (7,8). Even the widely used EIA tests for toxin A and B are only 70% to 80% as sensitive as the cell cytotoxicity assay (1). With a prevalence of toxin in stool samples of less than 10%, the positive predictive value of these assays is unacceptably low (less than 50% in some circumstances) and will vary depending on the assay and number of samples tested (9). Cytotoxin assay has a sensitivity of 60% to 85% but is less sensitive than culture of *C difficile* on blood agar (8,10-13). Selective enrichment broths including chopped meat broth with antibiotics have also been reported to be a sensitive technique for the detection of *C difficile* (10,14).

A new approach using a two-step algorithm including tests for enzyme glutamate dehydrogenase (GDH), a specific antigen of *C difficile*, and direct cytotoxin neutralization assay (DCNA), has been described. It reduces cell culture workload by 75% to 80% (7) but is less sensitive than culture of toxigenic *C difficile* (15).

The purpose of the present study was to compare the DCNA, the second step of the algorithm, with an indirect cytotoxin neutralization assay (ICNA). A fecal filtrate is the sample used for DCNA and a broth culture of stool is the sample for ICNA. The neutralization of the cytotoxin B of the

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stool broth culture was done according to our standard cytotoxin assay (16).

METHODS

Stool specimens

This study was done during a three-month period from June to August 2008. All liquid and unformed stool samples from adult symptomatic patients submitted to the laboratory with a request for *C difficile* toxin detection were studied seven days a week. Specimens were processed immediately or stored at 4°C for less than 24 h before assay.

GDH testing and cytotoxin assay

An EIA was used to detect the presence of GDH (*C. Diff* CHEK-60; Techlab, USA) and, if positive, the DCNA was performed on fecal filtrates. The tests were run according to the manufacturer's protocol, using a microplate assay with 96 wells containing immobilized polyclonal antibody against the GDH of *C difficile*. A spectrophotometer capable of reading dual wavelength at 450/620 nm was used as microplate reader. A test was considered positive if the optical density was 0.080 or greater and negative if the optical density was less than 0.080. GDH-positive specimens were further tested for cytotoxin B by DCNA as previously described (16). Fresh stools were diluted in phosphate buffered saline, vortexed and centrifuged. The supernatant was filtered and the 1:10 final dilution was inoculated in a 96-well microtitre plate containing the MRC-5 fibroblast cells. One specimen diluted at 1:10 was also inoculated with *C difficile* antitoxin. The plates were incubated at 37°C under 5% CO₂ and examined at 24 h, 48 h and 72 h for cytopathic effects characteristic of *C difficile* toxin B.

Culture for *C difficile*

Culture of *C difficile* in broth was done for GDH-positive stool samples. Fresh stool specimens (two to three drops of liquid stools or 0.2 g of unformed stools) were inoculated into chopped meat broth containing 20 µl/mL of cefoxitin and incubated at 37°C for 48 h. After incubation, the broth culture was transferred in a 5 mL conical tube, centrifuged at 2550 rpm for 30 min at 4°C and the supernatant filtered. The broth filtrate obtained was incubated with MRC-5 cells at 37°C under 5% CO₂. The filtrate was also inoculated with *C difficile* antitoxin. The cells were examined after 24 h and 48 h for cytopathic effects. A total of 254 stool samples were cultured in broth – 202 GDH-positive specimens and 52 GDH-negative controls.

Cycloserine-cefoxitin-fructose agar (CCFA) was inoculated with broth, producing toxin in MRC-5 cells, only when GDH was positive and DCNA was negative. The purpose of this approach was to confirm the presence of typical colonies of *C difficile* and their toxigenicity to validate this new approach with a standard method. The identification of *C difficile* colonies was based on the Gram stain (bacteria always Gram-positive) and the following properties: characteristic flat irregular morphology and horse barn odour, ground-glass appearance, yellow fluorescence with a Wood's lamp and positive for proline.

Toxigenicity testing on isolates of *C difficile*

Chopped meat broth was inoculated with five characteristic colonies of *C difficile* and incubated at 37°C during 48 h or until the turbidity was equivalent to a 0.5 McFarland standard. The method for obtaining the extract and to inoculate MRC-5 cells was the same as that described for stool samples.

Confirmation of true positive sample for toxigenic *C difficile*

For the purpose of the present study, a true positive sample was defined as a sample positive for GDH and detection of *C difficile* by broth culture followed by specific neutralization with antitoxin in MRC-5 cells.

RESULTS

During the three-month period between June and August 2008, a total of 923 consecutive stool specimens received at the CHUM Notre-Dame Hospital were tested. Using the two-step algorithm, the prevalence of CDI was 13.5% (125 of 923) with DCNA. 202 (21.9%) specimens were GDH-positive and 721 (78.1%) GDH-negative. Of the GDH-positive specimens, 126 (62.4%) were DCNA positive and 76 (37.6%) were DCNA negative (Figure 1). Among the DCNA-negative specimens, 17 were positive by ICNA and 16 of 17 had colonies of *C difficile* on CCFA agar that were toxigenic when tested with MRC-5 cells. The sensitivity of DCNA-positive samples by both methods (DCNA and ICNA) in comparison with ICNA alone was 88% (125 of 142). The sensitivity and the specificity of the broth method were 100% (142 of 142) and 96.4%, respectively.

DISCUSSION

EIA is often used in North American and European hospitals (1,9). These tests are convenient and rapid but have a low sensitivity. The need to combine toxigenic culture with these tests was underlined in a study where 25.9% of CDI including three patients with pseudomembranous colitis would have been missed without culture. Chart reviews have confirmed that the toxigenic culture was clinically significant in those cases (17). More recently, the toxigenic culture was recommended to optimize the diagnosis of CDI when EIA was used (9,18). Chart reviews of the involved patients were not systematically carry out because it was not a purpose of the study to do so and the charts were held in three different locations. Among the additional 17 cases found by ICNA, the treatment they received could not be traced for three patients because they were not hospitalized. However, 11 of 14 patients (78.5%) did receive specific treatment meaning that the attending physicians considered that CDI was likely. Of these, 10 received vancomycin or metronidazole and one patient with a previously positive DCNA received cholestyramine.

The cytotoxicity assay has been considered the gold standard for the diagnosis of CDI. However, the workload associated with this method is considerable when the assay is performed on all stool samples. It also lacks sensitivity. Currently, the culture of *C difficile* followed by testing of the isolates for toxigenicity is considered the 'real' gold standard (11) and is recommended for evaluation of new diagnostic tools (11,15). It is used in routine practice in several hospitals (12,13) but the workload is heavy and its cost is high. The two-step algorithm has been used to avoid bacterial culture, reduce workload and decrease costs substantially. The two-step algorithm also reduces cell culture workload by 75% to 80% as reported in the literature (7). The EIA for GDH has a sensitivity of 93.4% to 95% (15) and a negative predictive value of 98.5% to 99.2% (19,20). The results of the negative specimens for GDH are available the same day the test is performed. For positive GDH specimens, cytotoxin assay is the next step. The

whole testing process is completed within three days (7). When using stool culture on CCFA and toxin testing on isolates of *C difficile* compared to the two-step algorithm, the sensitivity of the two-step approach was 77% and that of culture was 87% (15). An additional 23% of toxigenic *C difficile* was detected by Reller et al (15) with the combination of the two-step algorithm and culture followed by testing of isolate of *C difficile* for toxin.

However, the authors concluded that the two-step algorithm is the preferred approach to be used in routine testing because culture with cytotoxin assay required five to nine days. Because Reller et al (15) found that the GDH screen reliably identified 100% of culture-proven *C difficile* cases, and considering the workload and cost of the culture, the culture of all stools was not done in our study. Reller et al also found that neither the two-step algorithm nor culture was 100% sensitive when compared with combined testing that includes both culture and cytotoxicity neutralization assay testing. They viewed the combined testing as the composite gold standard (15).

The objective of the present study was to improve the second step of the two-step algorithm, comparing DCNA with a more sensitive method (ICNA) that was also applicable to routine clinical laboratory testing. However, to validate Reller et al's results in Notre-Dame microbiology laboratory, 101 consecutive GDH-liquid stool specimens were tested with ICNA. Only two were found to be positive by ICNA. The result confirms the high negative predictive value of GDH screening described by several authors. Because rare cases of false negative GDH may be found, if CDI is strongly suspected despite a negative GDH screening, a new test may be requested without the GDH screening method. Only DCNA and ICNA will be done. Rare cases of GDH-negative screen CDI have been found in Notre-Dame hospital using this approach.

In the present study, we used the two-step algorithm and a broth culture of stool samples to detect toxigenic *C difficile*. Our results with GDH and DCNA are similar to those reported in the literature (7,15). The addition of broth culture increases the detection of toxigenic *C difficile* and is simple to do. No anaerobic apparatus or particular technical skill is needed. The incubation period is shorter and results are available within 72 h in the majority of the cases. As previously described for DCNA, the cytopathic effect of stool filtrates is observed as early as 4 h with DCNA in nearly 50% of the specimens (16). Although not specifically tested in our study with ICNA, the cytopathic effect may also be observed after 4 h because the amount of toxin is higher after broth culture. A reading made after 4 h may give definite results in more than one-half of the additional cases of patients with CDI, suggesting that results may be available within 52 h with ICNA.

The use of broth has already been shown to facilitate detection of *C difficile* (10,14). The time required for toxin detection in comparison with plate culture is reduced by 50% and the sensitivity is higher (10). To our knowledge, this method has not been proposed within the scope of the two-step algorithm. Our results show that sensitivity was increased as an additional 12% of toxigenic *C difficile* were found. GDH reporting is available the same day, DCNA within 24 h in 85% of cases and ICNA usually within 72 h (87.8% of cases) not exceeding 96 h. The final read of DCNA is 48 h. The presence of a toxin-producing *C difficile* strain in stool of a patient with diarrhea is

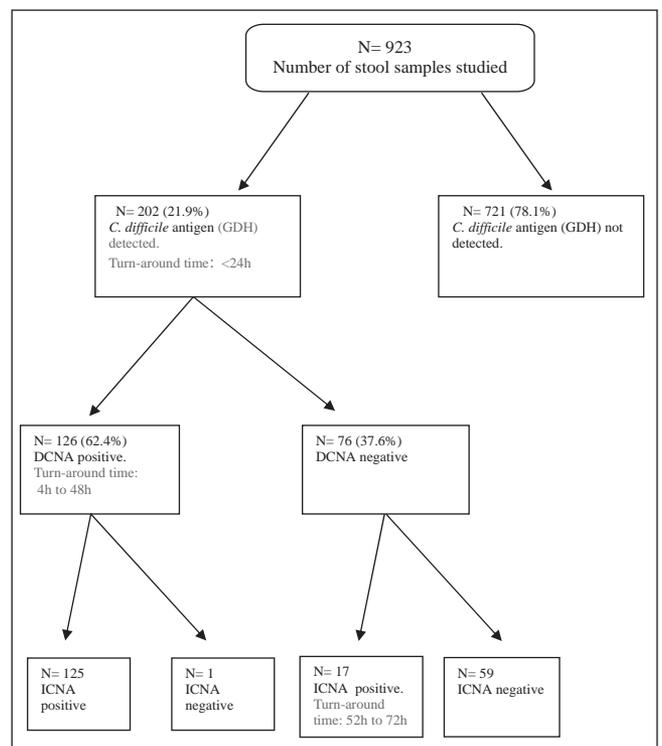


Figure 1) Detection of toxigenic *Clostridium difficile* in stool samples. DCNA Direct cytotoxin neutralization assay from fecal filtrate; GDH Glutamate dehydrogenase; ICNA Indirect cytotoxin neutralization assay using broth culture of stool samples

regarded as evidence of CDI if there is no alternative explanation for the diarrhea. Some physicians may consider that patient is colonized asymptomatically with a toxigenic strain of *C difficile* and that diarrhea is from other etiology. However, most clinicians would opt to treat such a patients for CDI (1).

CONCLUSION

The two-step algorithm is an interesting approach and the broth culture is useful to increase the sensitivity of detection of the toxigenic *C difficile* and can be implemented in routine diagnostic testing. It has been introduced as such in our hospital. The culture also offers the possibility of testing antimicrobial susceptibility and characterizing the strain of *C difficile* for epidemiological purposes. The subculture of the broth on agar plates was done in the present study to validate our results.

However it is not necessary to do such culture in routine practice. Only broth culture and specific neutralization assay are necessary. Subculture on agar plates may be done selectively for epidemiological purposes or for susceptibility testing.

ACKNOWLEDGEMENTS: The author thanks Muhammad Zafarullah, Research Centre, Centre hospitalier de l'Université de Montréal (CHUM) and Dr Michel Brazeau for reviewing this manuscript. He also thanks Sylvie Arel, Sylvie Bouchard, Josette Côté, Lise Garon, Loan Luu, Veronica Pinto and France Vigneault for their technical assistance. This study was presented in part at the 26th International Congress of Chemotherapy and Infection, Toronto, Ontario, June 18 to 21, 2009 (abstract P-12).

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