NEW DIAGNOSTIC METHODS

Comparison of the ToxA test with cytotoxicity assay and culture for the detection of Clostridium difficile-associated diarrhoeal disease

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Summary. Stool samples (355 from 350 patients) were examined in a new commercial assay, the ToxA test, for the rapid diagnosis of Clostridium difficile-associated diarrhoea. The results were compared with direct assay of cytotoxin in McCoy cell tissue culture, and detection of toxigenic C. difficile by culture and cytotoxin testing of isolates. Discordant results were resolved by consultation of clinical records. Test sensitivities were 84.6% for the ToxA test, 78.5% for the direct cytotoxicity assay and 95.4% for culture. The specificity was 100% for all the tests. The ToxA test is a rapid and reliable alternative for the detection of toxigenic C. difficile where tissue culture facilities are unavailable.

Introduction

Antibiotic-associated diarrhoea or pseudomembranous colitis results from overgrowth in the bowel of Clostridium difficile with production of two toxins—toxin A, a lethal enterotoxin that causes haemorrhage and fluid secretion, and the cytotoxic toxin B.

Confirmatory diagnosis of C. difficile-associated disease is best established by detecting the presence of the organism and toxin A or toxin B in stool samples. Toxin B can be detected by its cytopathic effect on tissue-culture cells and this is considered as the "gold standard" method for diagnosis of C. difficile diarrhoeal disease. However, the cytotoxin assay is costly, slow and requires some expertise. Alternative methods of toxin detection have been developed including latex agglutination, dot immunobinding, PCR, DNA probes and ELISA. In this study, a commercial monoclonal antibody (MAb)-based ELISA test for toxin A was evaluated.

Materials and methods

Specimens

Stool specimens (355) collected over a period of 2 months from 350 in-patients in Strasbourg University Hospital, France, were assayed for cytotoxin and cultured for C. difficile on receipt. Held at 4°C for toxin A testing (processed within 48 h), and then frozen at -80°C for further studies if required.

Isolation and toxigenicity testing (IT culture)

Stool specimens were inoculated onto cycloserine-cefoxitin-fructose agar (CCFA) and incubated anaerobically at 37°C for 48 h. Suspect colonies were identified by the Rapid 32A system (bioMérieux, France). C. difficile isolates were grown anaerobically for 48 h at 37°C in 2–3 ml of peptone-glucose-yeast (PGY) broth. The culture was centrifuged and the supernate was assayed for cytotoxin as described below.

Cytotoxin assay

Stool samples were suspended at c. 1 in 10 dilution in sterile double-distilled water and centrifuged for 30 min at 2500 g. The supernate was filtered through a 0.45-μm membrane filter (Millipore). The filtrate was added to a confluent monolayer of McCoy cells in the wells of a microtitration plate, and incubated for 24 h at 37°C in an atmosphere of CO₂ 5-5% in air. Positive cytotoxin activity, indicated by rounding of the cells, was confirmed by neutralisation with toxin B-specific antiserum.

ToxA test

The ToxA test is a new commercial assay from Biowhittaker (Fontenay-sous-bois, France).

Stool samples were suspended at c. 1 in 2 in diluent buffer and two drops (c. 100 μl) of the suspension were added to a microtitration well coated with toxin A-specific polyclonal antibody. A further drop of detection reagent (toxin A-specific MAb linked to horse-radish peroxidase) was added. Negative and positive controls were distributed into designated wells. If
present, toxin A binds to the detecting antibody and to the immobilised polyclonal antibody during incubation at 37°C for 2 h. Unbound material was removed by five washes with the supplied solution at room temperature. One drop each of substrate solution A (urea peroxide) and substrate solution B (tetramethylbenzidine) were added and the plates were incubated for 15 min at room temperature. Retention of the enzyme-antibody-antigen complex formed in the presence of toxin A was indicated by development of a yellow colour, the intensity of which was read at 450 nm in a Multiskan MCC/340 spectrophotometer. Optical density readings (ODs) were interpreted according to the manufacturer’s guidelines: wells yielding ODs < 0·1 were considered negative; ODs of 0·1–0·2 were considered equivocal, and re-tested; and wells yielding ODs > 0·2 were considered positive for toxin A.

Clinical assessment

Clinical records were consulted for all specimens that gave discordant results in the three assays. The diagnosis of *C. difficile*-associated diarrhoea (CDAD) was considered correct if all of the following conditions were met: the patients had received an antibiotic during the previous 3 weeks; they had subsequently developed diarrhoea or liquid stools (more than three loose or watery stools a day, for at least 2 days); the diarrhoea had ceased after withdrawal of the antibiotic or after oral administration of vancomycin or metronidazole; and stool samples were positive in the IT culture, cytotoxin or ToxA assays.

Results

Of the 355 specimens tested, 47 (13·2%) gave positive results in all three assays; 290 (81·7%) gave negative results in all three assays; and 18 (5·1%) gave positive results in some, but not all assays (table I). Sixteen of the 18 patients in the latter group presented with diarrhoeal symptoms after antibiotic therapy, recovered after appropriate treatment or after withdrawal of antibiotics, and were judged to be positive for *C. difficile* (table I). The two remaining patients in this group had a diagnosis of pseudomembranous colitis confirmed by endoscopic examination; the results for one were ToxA-positive but cytotoxin- and IT culture-negative, for the other, ToxA- and IT culture-positive but cytotoxin-negative.

The ToxA test showed a sensitivity of 96% and a specificity of 98% when compared with the cytotoxin assay, and a sensitivity of 83·9% and a specificity of 99% when compared with IT culture. ToxA and cytotoxin results agreed for 97·7% of specimens, and ToxA and IT culture results agreed for 96·3% of specimens. Of the 65 specimens for patients considered to have CDAD, the ToxA test was positive for 55, the cytotoxin assay was positive for 51, and IT culture was positive for 62. No false positive results were found. All three tests showed a specificity of 100%; the ToxA test showed a greater sensitivity (84·6%) than the cytotoxin assay (78·5%), but a lower sensitivity than IT culture (95·4%; table II).

Six specimens yielded equivocal results on initial ToxA assay; repeat tests on these specimens yielded clear negative results. All ToxA-positive stools yielded further positive results on repeat testing after freezing at −80°C and thawing. Twenty-one patients (59%) harboured non-toxigenic *C. difficile* strains.

Discussion

The widespread use of broad-spectrum antibiotics has led to an ever increasing incidence of pseudomembranous colitis and antibiotic-associated diarrhoea. Approximately 10% of hospitalised patients harbour *C. difficile* (unpublished data, national sample survey; Biocodex, Montrouge, France). The presence of diarrhoea and a history of recent antimicrobial therapy is only suggestive of CDAD; laboratory tests are necessary to confirm the diagnosis. Although tissue-culture assay for cytotoxin is considered the “gold standard” method for confirmation, this is a costly, slow assay that requires some expertise. IT culture is also slow and isolates must be tested for toxigenicity because of the significant incidence of carriage of non-toxigenic strains; however, this was the most sensitive (95·4%) of the tests in this study.
The 13 instances of cytotoxin-negative, IT culture-positive stools and two instances of IT culture-negative and cytotoxin-positive stools may be explained by the problems inherent in sampling non-homogeneous specimens. Furthermore, the isolation of both cytotoxigenic and non-cytotoxigenic C. difficile strains from the same stool sample has been reported elsewhere. Clearly, this demonstrates the possibility that picking a single colony for the toxigenicity assay might yield a false negative result.

Two cytotoxin-negative specimens came from patients with pseudomembranous colitis, one of which was ToxA-positive, but IT culture-negative. The reason is unclear but this result could be consistent with the fact that toxin A may be more relevant in the pathology of CDAD. Confirmation of CDAD in multiple test systems increases detection rates, but only marginally, and at considerable cost and effort.

The results presented here (sensitivity 84.6%, specificity 100%) differ from those of a previous study comparing the ToxA test to cytotoxin assay without consultation of clinical records (sensitivity 99%, specificity 93%) and also differ from the values of sensitivity 93%, specificity 91% found in a study in which the same criteria as in this study were used for the diagnosis of CDAD. The discrepant sensitivities of the cytotoxin assay in different laboratories may be due to differences in methodology, particularly the sensitivity of the cell line, the age of the cells and specimen preparation. Similarly divergent results were found in studies with the Premier enzyme immunoassay for toxin A, where sensitivity estimates ranged from 40 to 92%, and specificity estimates from 96 to 99.5%.

We consider that the ToxA test is a valuable option for those laboratories without facilities for tissue culture. It combines speed (30 min of processing time and 2 h of incubation for 20 samples) and requires little technical expertise. The ToxA test has excellent specificity and very good sensitivity compared with the cytotoxicity assay and culture for C. difficile. This test can be used alone or combined with other tests.

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References