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Clinical significance of direct cytotoxicity and toxigenic culture in *Clostridium difficile* infection

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ABSTRACT

Background: *Clostridium difficile* infection (CDI) is the leading cause of hospital-acquired diarrhoea in developed countries. Although an optimal diagnosis is crucial, laboratory diagnostics remain challenging. Currently, the reference methods are direct cytotoxicity assay and toxigenic culture; however there is controversy in the interpretation of discordant results of these tests.
Objective: The aim of our study was to determine the clinical significance of detecting *C. difficile* only by toxigenic culture with a negative direct cytotoxicity assay.
Methods: We conducted a prospective study in which patients aged >2 years with CDI were enrolled and monitored at least 2 months after their last episode. Samples were tested by both cytotoxicity assay and toxigenic culture.
Results: During the 6-month study period, we identified 169 episodes meeting CDI criteria that had been tested by both assays, out of which 115 were positive for both cytotoxicity assay and toxigenic culture, and 54 CDI episodes (31.9%) were positive only by toxigenic culture. Overall, patients median age was 71.3, 50.9% were male and the most frequent underlying disease was malignancy. The comparison of CDI episodes positive for both assays and by toxigenic culture only revealed the following, respectively: mild CDI (77.4% vs 94.4%; $p = 0.008$), severe CDI (21.7% vs 5.6%; $p = 0.008$), severe complicated (0.9% vs 0.0%; $p = 1.000$), pseudomembranous colitis (1.7% vs 1.9% $p = 1.000$), recurrence (17.4% vs 14.8%; $p = 0.825$), overall mortality (8.7% vs 7.4%; $p = 1.000$) and CDI related mortality (2.6% vs 0%; $p = 0.552$).
Conclusion: CDI episodes positive by cytotoxicity assay were more severe than those positive only by toxigenic culture, however there were a significant proportion of CDI cases (31.9%) that would have been missed if only cytotoxicity had been considered as clinically significant for CDI treatment, including severe CDI cases. Our data suggest that a positive test by toxigenic culture with a negative result for cytotoxicity should not be interpreted as colonization.

1. Introduction

Clostridium difficile infection (CDI) is the leading cause of hospital-acquired diarrhea in developed countries and is responsible for increased morbidity and mortality and prolonged hospital stay [1–3]. An optimal diagnosis is crucial for infection control and CDI treatment. Traditionally, the gold standard in CDI diagnosis has been direct cytotoxicity on stools, which consists in observing the cytopathic effect of *C. difficile* toxins. Toxigenic culture, is nowadays been considered as the gold standard by several authors [4–7] for its increased sensitivity over cytotoxicity. It is based on the detection of toxin production in the microorganism after isolation by culture.

However a significant proportion of patients present discrepancies between the results obtained by the two reference methods. Currently, there is controversy in the interpretation of discordant results of these laboratory tests [8–10]. These situations can pose

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great difficulty in clinical practice regarding management and treatment of patients. Identifying the clinical implications of these two methods is critical for infection control and treatment of CDI.

The aim of our study was to determine the clinical significance of detecting *C. difficile* by toxigenic culture while having a negative result for direct cytotoxicity.

2. Material and methods

2.1. Setting

Our institution is a large teaching hospital with 1550 beds. The clinical microbiology laboratory receives samples from patients hospitalized at our center and from all the outpatient institutions in our catchment area.

2.2. Design and study population

We conducted a prospective study in which patients aged >2 years with CDI (a positive test result for toxigenic *C. difficile* and >3 unformed stools/24 h or colonoscopic evidence of pseudomembranous colitis) were enrolled from January to June 2013 and monitored at least 2 months after their last episode (October 2013).

2.3. Definitions

A CDI episode was defined as the presence of a positive result for toxigenic *C. difficile* testing and the presence of diarrhea (≥3 unformed stools in 24 h) or colonoscopic findings demonstrating pseudomembranous colitis.

Severity of CDI was defined according to the guidelines of the European Society of Clinical Microbiology and Infectious Disease (ESCMID) [11].

CDI-associated mortality was defined as death not clearly attributable to other unrelated causes occurring within 10 days of the CDI diagnosis and/or due to well-known complications of CDI.

Recurrence (R-CDI) was defined as return of symptoms and positive stool sample separated from the former by between 15 and 60 days, after recovery from a previous episode (at least 3 days without diarrhea and clinical improvement). Episodes occurring more than 60 days after the previous one were not considered recurrences but new episodes that were not linked to the previous one.

2.4. Laboratory procedure

For each CDI episode only one sample was considered. Samples were tested by both direct cytotoxicity assay (C) and toxigenic culture (TC). For the cytotoxicity assay, MRC-5 cell line was used according to the procedure described in the Anaerobic Bacteriology Manual (Wadsworth, sixth edition) [12].

Toxigenic culture was performed in *Clostridium* selective agar medium (bioMérieux), and plates were incubated under anaerobic conditions at 35ºC–37 °C for 48 h. Following incubation, colony morphotypes compatible with *C. difficile* were selected with the help of a binocular magnifying glass. Identification of colonies suspected of being toxigenic *C. difficile* (TCD) was confirmed using an immunochromatographic system (C Diff Quik-Chek Complete assay, TechLab, Blacksburg, Virginia) and the MRC-5 cell line cytotoxicity test. All samples underwent toxigenic culture in *Clostridium* selective agar medium (bioMérieux), and plates were incubated under anaerobic conditions at 35ºC–37 °C for 48 h.

Direct cytotoxicity testing of the sample was performed using the MRC-5 cell line according to the procedure described previously [13].

A positive result for toxigenic *C. difficile* was regarded as any sample with a positive result by any of the reference techniques (toxigenic culture or direct cytotoxicity in stool).

Additionally, rapid tests were performed on all samples. The rapid detection test consisted of a two-step diagnostic algorithm based on a first immunochromatographic antigen detection of glutamate deshidrogenase (GDH) and toxins A/B simultaneously (C Diff Quik-Chek Complete assay, TechLab, Blacksburg, VA) and secondly if a stool specimen was positive for GDH but negative for toxins A/B it was tested by a real-time PCR of the B toxin gene (Xpert™ *C. difficile* Assay, GeneXpert, Cepheid, Sunnyvale, CA). Tests were performed according to the manufacturer's instructions.

Isolates were characterized using PCR-ribotyping [14]. Phylogenetic analysis of ribotyping profiles was conducted using the unweighted pair group method with arithmetic mean (UPGMA) and Dice coefficients (Bionumerics 5.0). Ribotypes were named using the international designation.

2.5. Data collection

Patient information was collected directly at the bedside by one of the investigators. All information was registered in a protocol. Demographic data included age, sex, hospital department or outpatient clinic at the time of diagnosis, and history of hospital admissions. Data on underlying conditions were recorded using the McCabe and Jackson score for underlying diseases; comorbid conditions were classified according to the Charlson index[19, 20]. Risk factors for CDI during the month prior to the diagnosis of CDI were recorded (e.g., previous antibiotics and proton-pump inhibitors, use of nasogastric tube, mechanical ventilation, chemotherapy, dialysis, inflammatory bowel disease and surgery). We also recorded history of previous CDI episodes.

The clinical data recorded for the CDI episode were days of diarrhea, presence of abdominal pain, abdominal distension, fever, hypotension, toxic megacolon, pseudomembranous colitis, and severity of CDI episode according to the criteria of the ESCMID. Antibiotic treatment for CDI episode and outcomes were also recorded (need for admission to the ICU, need for surgery for the CDI episode, recurrence, mortality, and CDI-associated mortality).

2.6. Data analysis

Data were analyzed using SPSS 18.0 (SPSS Inc, Chicago, Illinois, USA). Qualitative variables appear with their frequency distribution. Quantitative variables are expressed as the median and interquartile range (IQR). Groups were compared using the Fisher exact test for categorical variables and the Mann–Whitney or *t* test for continuous variables.

2.7. Ethical issues

This study was approved by the Hospital General Universitario Gregorio Marañón Ethics Committee.

3. Results

During the 6-month study period, we identified 169 episodes from 169 patients meeting CDI criteria (>3 unformed stools/24 h or colonoscopic evidence of pseudomembranous colitis) that had been tested by both assays.

Out of which, 115 were positive for both direct cytotoxicity assay and toxigenic culture and 54 CDI episodes (31.9%) were positive only by toxigenic culture. There were no episodes positive only by direct cytotoxicity.