Jpn. J. Infect. Dis., 65, 326-329, 2012

Short Communication

A Superior Test for Diagnosis of *Clostridium difficile*-Associated Diarrhea in Resource-Limited Settings

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(Received February 13, 2012. Accepted May 7, 2012)

SUMMARY: In this prospective cohort study, we investigated the prevalence of *Clostridium difficile*-associated diarrhea (CDAD) in adult patients with nosocomial diarrhea by performing enzyme immunoassay (EIA) for detecting toxins A and B and polymerase chain reaction (PCR) for detecting the presence of the *tcdB* gene in stool samples. We determined the factors associated with CDAD, and the treatment outcome of CDAD from May 2010 to January 2011. A total of 175 stool samples were tested by EIA and PCR. In total, 26.9% patients tested positive for *C. difficile*: 12.6% by EIA and 24.0% by PCR. The kappa coefficient and total agreement of both the tests were 0.46 and 83.2%, respectively. Onset of diarrhea after antibiotic administration for 10 days or more (OR, 2.71; 95% CI, 1.14–6.44; $P = 0.024$) and leukocyte count $>15{,}000$ cells/mm^3 (OR, 3.12; 95% CI, 1.24–7.88; $P = 0.016$) were significantly associated with occurrence of CDAD. The non-response rate to CDAD treatment was 24.1%, and the all-cause mortality rate was 31.9% in the CDAD group as against 35.9% in the non-CDAD group ($P = 0.721$). In our study, the performance of direct PCR of stool samples for detecting *tcdB* was better, with the number of positive results for stool toxins A and B being twofold higher than that in the case of EIA. Patients who have diarrhea after receiving antibiotics for 10 days or more or those who have a leukocyte count of $>15{,}000$ cells/mm^3 should be investigated for CDAD.

The increase in the incidence, severity, and mortality rates of *Clostridium difficile* infection (CDI) is a matter of serious concern (1,2). The reported prevalence of CDI differs across hospitals and depends on detection methods (3). In Thailand, the reported prevalence of *Clostridium difficile*-associated diarrhea (CDAD) has varied from 5% to 25% (4–7). The prevalence of CDAD has probably been underestimated because the widely used enzyme immunoassay (EIA) for detecting stool *C. difficile* (8) has low sensitivity. This study aims to investigate the prevalence of CDAD in patients with nosocomial diarrhea using different methods, including EIA and direct polymerase chain reaction (PCR) of stool samples for the *tcdB* gene, and to determine factors associated with and the treatment outcome in CDAD.

This prospective cohort study was conducted from May 2010 through January 2011 at Ramathibodi Hospital, a 1,000-bed tertiary-care university hospital. The patients included in this study were adult patients (age, ≥ 15 years) who were admitted to the medicine ward and developed diarrhea during their hospitalization (8). Patients whose stool samples were not available for EIA or direct PCR were excluded from the study. In this study, CDAD was defined as diarrhea with the presence of *C. difficile* toxins in stools, as detected by EIA, or positive PCR results for *tcdB*. This study was approved by the institutional review board.

The clinical records of patients were reviewed for demographics, clinical characteristics, laboratory investigations, the treatment received, and its outcomes.

EIA was performed using the *C. difficile* Tox A&B kit (VIDAS®; bioMérieux, Marcy l'Etoile, France). Equivocal values were interpreted as negative results in our study.

PCR was undertaken for detecting the *tcdB* gene. Genomic bacterial DNA was isolated directly from stool samples and was used as a template for PCR; amplification was achieved using in-house primers that targeted a highly conserved region of the *tcdB* gene, which is present in most toxigenic *C. difficile* strains (9). The sequence of the forward primer was 5′-GAAGATTT AGGAAATGAAGAAGGTGA-3′ and that of the reverse primer was 5′-AACCACTATATTCAACTGC TTGTCC-3′. The PCR conditions were as follows: initial denaturation at 92°C for 5 min, followed by 30 cycles of 92°C for 30 s, 55°C for 30 s, and 68°C for 60 s. After the final cycle, the samples were heated at 68°C for 5 min and cooled to 4°C. The PCR products were then analyzed by performing agarose gel electrophoresis.

Data were analyzed using the SPSS version 16.0 for Windows (SPSS Inc., Chicago, Ill., USA). Patients were categorized into two groups on the basis of their CDAD status. The 95% confidence interval (CI) for CDAD prevalence was calculated using a Microsoft Excel statistical function. Chi-square or Fisher's exact tests were used for comparing categorical variables. Student's *t* test and Mann-Whitney U tests were used to compare the means and medians of continuous varia-

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bles. Binary logistic regression analyses were performed for multivariate analysis to determine the factors associated with CDAD cases. Factors with a *P*-value of less than 0.1 as determined by univariate analysis were included in the multivariate analysis model, except the correlated factors. Odds ratios (ORs) and their 95% CI were estimated. A *P*-value of less than 0.05 was considered statistically significant.

Our study cohort consisted of 175 patients with 180 episodes of diarrhea. Of these, 4 episodes were excluded because of the absence of clinical records and 1 was excluded because the patient's stool sample was missing. Thus, 175 episodes in 168 patients were included for analysis. The mean age of the patients was 57.9 ± 19.1 years, and 51.4% patients were male. There were no differences in age and gender between the CDAD and non-CDAD groups. The median time from admission to onset of diarrhea was significantly longer in the CDAD patients than in the non-CDAD patients (15 days versus 9 days, $P = 0.04$). Underlying diseases were found in 91.4% of patients, with no significant difference between the 2 groups. Proton pump inhibitors were administered before the onset of diarrhea in 76.6% and 61.7% of CDAD patients and non-CDAD patients ($P = 0.07$), respectively. In addition, antibiotics were administered before the onset of diarrhea to 78.7% and 85.2% of CDAD patients and non-CDAD patients, respectively ($P = 0.36$). There was no difference between the groups with respect to whether each class of antibiotics was administered as a single agent or in combination with 2 or more antibiotics. The median time from the administration of antibiotics to the onset of diarrhea was significantly longer in CDAD patients than that in non-CDAD patients (14 days versus 8 days; $P = 0.003$); after receiving antibiotics for 10 days or more, 64.9% of CDAD patients developed diarrhea as against 34.6% of non-CDAD patients ($P = 0.036$).

Stool toxins A and B as detected by EIA were presented in 12.6% (95% CI, 8.0–18.4%) of patients, while *tcdB* as detected by direct PCR was presented in 24% (95% CI, 17.9–31%) of patients. In total, 26.9% (95% CI, 20.4–30.1%) patients were found to be positive for *C. difficile* (Fig. 1). The kappa coefficient between direct PCR of stool samples for *tcdB* and EIA for stool toxins A and B was 0.46 (95% CI, 0.29–0.62). The total agreement of direct PCR for stool samples and stool EIA was 83.2%. The median leukocyte count at the onset of diarrhea was higher in the CDAD group than in the non-CDAD group (10,315 cells/mm^3 versus 8,195 cells/mm^3; $P = 0.089$). The number of patients who had a leukocyte count higher than 15,000 cells/mm^3 was significantly greater in the CDAD group than in the non-CDAD group (34.8% versus 19.5%; $P = 0.044$). By multivariate analysis, diarrhea onset after 10 days or more of antibiotic administration (OR, 2.71; 95% CI, 1.14–6.44; $P = 0.024$) and a leukocyte count of $>15{,}000$ cells/mm^3 (OR, 3.12; 95% CI, 1.24–7.88; $P = 0.016$) were significantly associated with CDAD (Table 1). Of 26 patients who underwent proctoscopy or colonoscopy, 6 patients exhibited pseudomembranous colitis (PMC). In 2 out of the 6 PMC patients, the results of direct PCR of stool samples were positive but those of toxin EIA were negative. The PMC cases were pathologically confirmed to be cases of cytomegalovirus

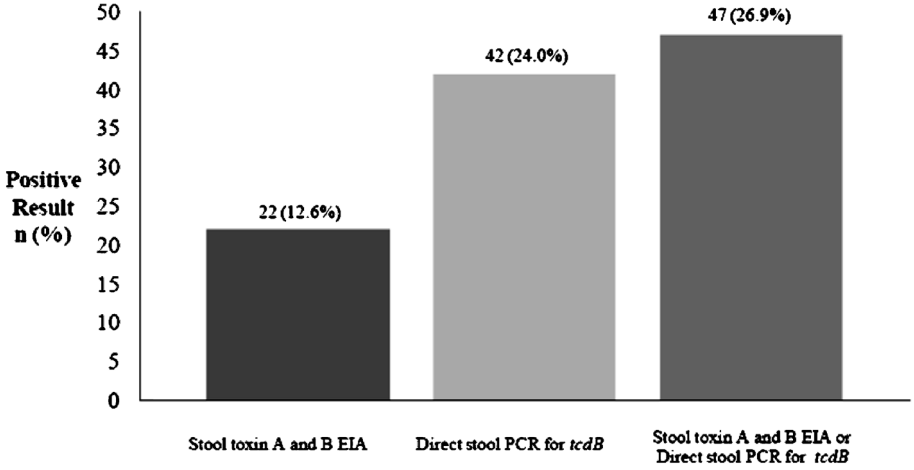

Fig. 1. Positive test for *Clostridium difficile*-associated diarrhea based on detection method ($n = 175$).

Table 1. Univariate and multivariate analyses of factors associated with CDAD cases

Factor	Crude OR	95% CI	*P*	Adjusted OR	95% CI	*P*
Onset after admission ≥48 h	4.37	1.46–13.05	0.008	2.95	0.74–11.75	0.126
Heart diseases	1.94	0.90–4.19	0.093	1.81	0.79–4.15	0.164
Diarrhea onset after ≥10 days of antibiotic administration	2.39	1.10–5.17	0.028	2.71	1.14–6.44	0.024
Proton pump inhibitors	2.03	0.95–4.36	0.069	1.07	0.42–2.73	0.891
Steroid	1.96	0.97–3.95	0.062	1.63	0.67–3.93	0.278
Leukocyte count >15,000 cells/mm^3	2.20	1.04–4.64	0.039	3.12	1.24–7.88	0.016

CDAD, *Clostridium difficile* associated-diarrhea; OR, odds ratio; CI, confidence interval.

colitis in 2 patients, and in 1 of these patients, the results of direct PCR of stool samples were positive but those of toxin EIA were negative. In the case of the other 2 patients, both direct PCR of stool samples and toxin EIA led to negative results. In the case of 1 patient for whom computed tomography indicated CDAD, the results of direct PCR of stool samples were positive but those for toxin EIA were negative.

Thirty-six of 47 episodes in the CDAD group and 45 of 128 episodes in the non-CDAD group were treated using anti-*C. difficile* therapy. In the CDAD group, the median (interquartile range [IQR]) time from treatment to cessation of diarrhea in 27 patients who received oral metronidazole and in 9 patients who received oral vancomycin was 5 (2–8) days and 2 (1–10) days, respectively. Furthermore, 75.9% of the diarrheal episodes improved within 10 days after treatment. The all-cause mortality rate was 31.9% in the CDAD group and 35.9% in the non-CDAD group ($P = 0.721$). *C. difficile*-related mortality rate was 6.4%.

In Thailand, the prevalence of CDAD as determined on the basis of EIAs and direct PCR of stool samples has been reported to be 7.1–8.7% (5,7) and 8.4% (7), respectively. The prevalence of CDAD as determined by either of the 2 tests in our study was higher than that reported previously. This might imply that the incidence of CDAD in Thailand is on a rise over time, as indicated by studies from other countries (1,10).

The total agreement between EIA and direct PCR of stool samples was 83.2%; however, the kappa coefficient showed moderate agreement ($\kappa = 0.46$). We found that the EIA and PCR tests yielded concordant negative results but discordant positive test results. Direct PCR for stool samples gave approximately 2 times higher positive cases than EIA of stool toxins. The limitation of the EIA test was its low sensitivity (75–83%) (11). *C. difficile* toxins are unstable and may be degraded within 2 h after collection (12). This may explain the low sensitivity of EIA. Direct PCR of stool has been shown to exhibit higher sensitivity (>90%) (13). Our results support a previous study which indicated that the performance of PCR is superior, thus offering clinical benefits in CDAD detection (14). However, molecular detection of *C. difficile* toxin genes may yield false-positive results for the DNA in the absence of toxin production; thus, positive PCR results must be carefully interpreted. One of the known risks of CDAD, among others, is antibiotic therapy. We also found that the duration of antibiotic administration was significantly longer in the CDAD group (14 days versus 8 days; $P = 0.003$), which is in accordance with previously reported findings (15), and thus unnecessarily prolonged treatment with antibiotics should be discouraged.

The significant laboratory finding in our study was that the CDAD group had a higher number of patients with a leukocyte count of >15,000 cells/mm^3 on the day of diarrhea onset. In an observational study, leukocytosis was found in 50% of patients with CDI (16). In another study on patients with unexplained leukocytosis, 58% of patinets had CDI (17).

Previous randomized-treatment trials showed that the symptoms of patients improved within 1 or 2 days after therapy, with a mean time of 3–6 days for diarrhea resolution (18). In our study, treatment with oral vancomycin resulted in shorter median time (IQR) to improvement of diarrhea than treatment with oral metronidazole (2 days versus 5 days). However, 4 of 5 patients for whom the treatment was changed from oral metronidazole to oral vancomycin showed improvement of diarrhea on the first day of switching therapy. It is difficult to conclude whether this was the late effect of oral metronidazole or the early effect of oral vancomycin. A relatively poor response to metronidazole was observed, with patients continuing to have symptoms of colitis for 10 days or more despite treatment (19). The overall nonresponse rate in the case of metronidazole and vancomycin in our study was 24.1%, which was comparable to the 22–29% in other studies (19,20).

The limitations of this study include the lack of another diagnostic test such as cell cytotoxicity or toxigenic *C. difficile* culture, because of which we could not identify the false positives or negatives in the cases of discordant test results. However, the analytical sensitivity and specificity of our PCR assay with *tcdA* + */tcdB* + *C. difficile*, *tcdA* − */tcdB* + *C. difficile* isolates, other *Clostridium* spp., and enteric bacterial organisms showed no false-positive or false-negative results. Furthermore, as previously described, the relatively small number of CDAD cases makes it difficult to identify the risk factors for CDAD.

In conclusion, the prevalence of CDAD infection has shown an increasing trend over time. Our direct PCR of stool for *tcdB* showed a higher number of positive results than the EIA results for stool toxins A and B. Diarrhea in patients who receive antibiotics for 10 days or more or those who have a high leukocyte count of >15,000 cells/mm^3 should alert clinicians to examine the patient for CDAD. The performance of the PCR test was superior, and PCR can serve as an effective diagnostic test for CDAD detection.

Acknowledgments The authors would like to thank Dr. Piriyaporn Chongtrakool and Ms. Supawan Boonsongin in the Division of Microbiology, Ms. Nissara Saengpiya in the Division of Immunology, Department of Pathology, Faculty of Medicine Ramathibodi Hospital, Mahidol University for their generous assistance in laboratory work.

This study was supported by Grant from Faculty of Medicine Ramathibodi Hospital and Faculty of Science, Mahidol University.

Parts of the results were presented at the 49th Annual Meeting of the Infectious Diseases Society of America (IDSA), October 20–23, 2011, Boston, MA, USA.

Conflict of interest None to declare.

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