PROSPECTIVE EVALUATION OF A NOVEL TWO-STEP PROTOCOL FOR SCREENING OF *CLOSTRIDIUM DIFFICILE* INFECTION IN HOSPITALIZED ADULT PATIENTS

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Abstract. *Clostridium difficile* infection (CDI) is one of the most common nosocomial infections in Thailand and worldwide. The clinical spectrum ranges from annoying diarrhea to severe life-threatening disease. Enzyme-linked immunofluorescent assay for cytotoxins A/B (cytotoxins A/B ELFA), which has been widely used in our institute, generally is considered as having low sensitivity for diagnosis of CDI. The study was a prospective evaluation of a novel two-step diagnostic algorithm, in which the first step involved concurrent cytotoxins A/B ELFA and enzyme immunoassay for glutamate dehydrogenase (GDH EIA) for CDI, followed by PCR assay of *tcdA* and *tcdB* in samples with discordant results. Of the 91 adult patients (37 males and 54 females, mean age of 60.0 ± 19.5 years) with suspected CDI hospitalized at King Chulalongkorn Memorial Hospital, Bangkok, Thailand from December 2012 to February 2013, 22 were diagnosed with CDI by the gold standard PCR test for *tcdA* and *tcdB*, among whom 21 were positive by GDH EIA, accounting for a sensitivity of 95%. Of the 69 patients without CDI, GDH EIA was negative in 46 patients, accounting for a specificity of 67%. The positive predictive value (PPV), negative predictive value (NPV) and accuracy of GDH EIA was 48%, 98% and 74%, respectively, whereas sensitivity, specificity, PPV, NPV, and accuracy of cytotoxins A/B ELFA was 73%, 96%, 84%, 92% and 92%, respectively. Some 30% of specimens required the more expensive PCR assay. However, this two-step protocol detected 20% more patients with CDI than the currently used cytotoxins A/B ELFA method.

Keywords: *Clostridium difficile*, diagnostic test, enzyme immunoassay, glutamate dehydrogenase, screening test

INTRODUCTION

Clostridium difficile is one of the most common causes of antibiotic-associated diarrhea (AAD), accounting for 15%-25% of AAD globally (Bartlett, 2002), and the prevalence of *C. difficile* infection (CDI) has increased worldwide (Bartlett, 2002; Pepin *et al*, 2005; Bauer *et al*, 2011; McDonald *et al*, 2005). In the United States the prevalence of CDI has increased from 31/100,000 in 1996 to 61/100,000 population in 2003 (McDonald *et al*, 2006); in Canada the prevalence has increased

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from 35.6/100,000 in 1991 to 156.3/100,000 in 2003, with accompanying increased severity (Pepin *et al*, 2004); and in Europe the occurrence of CDI has increased from 2.45/10,000 in 2005 to 4.1/10,000 patient-day in 2008 (Bauer *et al*, 2011). The prevalence of CDI in Thailand ranges from 12%-18% (Wongwanich *et al*, 2003; Thipmontree *et al*, 2011). However, to the best of our knowledge, there have been no studies on the prevalence of CDI in various hospitals in Thailand.

CDI has high mortality and morbidity rates unless prompt diagnosis and appropriate treatment are made. There are both invasive and non-invasive diagnostic methods of diagnosis. The widely used non-invasive enzyme-linked immunofluorescent assay for cytotoxins A/B (cytotoxins A/B ELFA) is generally considered to have low sensitivity but high specificity for diagnosis of CDI (Bartlett, 2009, 2010). The enzyme immunoassay for glutamate dehydrogenase (GDH EIA), another non-invasive test, has high sensitivity but less specificity as it can detect both toxigenic and nontoxigenic strains of *C. difficult* (Bartlett, 2009, 2010). Cytotoxic culture and cytotoxic cell neutralization assay (CCNA) are time-consuming and require skilled technicians to perform (Bartlett, 2009, 2010). The molecular methods are more expensive to perform and require specialized equipment (Wilkins and Lyerly, 2003; Barlett, 2010; Cohen *et al*, 2010; Kufelnicka and Kirn, 2011).

Cytotoxins A/B ELFA is the only diagnostic test for CDI most hospitals in Thailand, including our institute, and GDH EIA has never been used in Thailand. However, PCR-based method has been employed to detect CDI in Thailand (Wongwanich *et al*, 2003). Thus, this study determine the diagnostic performance of GDH EIA for CDI, in comparison with cytotoxins A/B, culture and PCR method. In addition, a novel two-step protocol for screening of *Clostridium difficile* infection, involving concurrent GDH EIA and cytotoxin A/B ELFA in the first step followed by PCR in the second step for samples with discorclant results in the first step.

MATERIALS AND METHODS

Patients

A prospective study was carried out in 91 adult patients with suspected CDI, who were hospitalized at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, from December 2012 to February 2013. Inclusion criteria included all hospitalized patients > 18 years of age who had acute diarrhea and suspected CDI. Diarrhea is defined as the passage of three or more unformed stools within 24 or fewer consecutive hours (Cohen *et al*, 2010). Suspected CDI is defined as diarrhea unattributable to any other causes. The severity of suspected CDI was categorized into a) mild or moderate [without leukocytosis (white blood cell counts ≥15,000/μl and serum creatinine <1.5 folds of premorbid level)], b) severe (leukocytosis or serum creatinine level of ≥1.5 folds of premorbid level), and c) severe, complicated [with hypotension, shock, ileus, or megacolon (Cohen *et al*, 2010]. Each patient was interviewed and examined by one of the investigators. The medical records of these patients were reviewed. All data including epidemiology, clinical features, laboratory investigations including the results of all 4 diagnostic tests for CDI, treatment, and outcome at 30 days after treatment were analyzed.

The study was approved by the Institutional Review Board (IRB number 039/55). All patients gave written informed consent for authorization to col-

lect their data and clinical specimens prior to enrollment into the study program.

Diagnostic tests for CDI

GDH EIA. Premier™ *C. difficile* GDH (Meridian Bioscience, Cincinnati, OH) was used for GDH detection according to the manufacturer's instructions. Stool specimens were stored at -30°C, and were tested for GDH EIA within 2 months after collection. The results were recorded spectrophotometrically (DKSH, Bangkok, Thailand) at 450 nm, with absorbance < 0.200 considered as negative. If the frequency of low positive results (OD between 0.200 and 0.250) was greater than 5% of the specimens tested, measurements of the samples were repeated.

Cytotoxins A/B ELFA. *C. difficile* Toxin A & B ELFA (VIDAS® bioMérieux, Lyon, France) was performed on the day of collection according to the manufacturer's instructions. Fluorescence was measured at emission 450 nm (excitation at 370 nm) twice. The relative fluorescence value < 0.13, ≥ 0.13 but < 0.37, and ≥ 0.37 is defined as negative, equivocal, and positive, respectively.

***C. difficile* culture.** *C. difficile* culture was performed as previously described (Bishara *et al*, 2011). In brief, stool specimens were incubated for 30 minutes with 95% ethanol at room temperature, then cultured on phenylethyl alcohol agar (Becton, Dickinson, Le Pont de Claix, France) under anaerobic conditions at 37°C for 48 hours, followed by subculturing on Brucella agar (Becton, Dickinson, Le Pont de Claix, France). *C. difficile* isolates were identified by colony morphology and API 20A (BioMérieux, Marcy I'Étole, France).

PCR detection of *tcdA* and *tcdB*

DNA was extracted from stool using ExiPrep™ Bacteria Genomic DNA Kit (Bioneer, Alameda, CA). PCR was performed as previously described (Kato *et al*, 1998; Lemee *et al*, 2004) in a final volume of 25 μl containing 10% glycerol. One μM each primer, 200 μM each dNTP, and 0.5 U *Taq* DNA polymerase in 1X buffer (10 mM Tris HCl, 50 mM KCl, 2.5 mM $MgCl_2$). The *tcdA*-specific primers used were *today-F* (5'-AGATTCCTATATTTACATGACAATAT-3') and *tcdA-R* (5'GTATCAGGCATAAAGTAATATACTTT-3') and those for *tcdB* NK104 (5'-GTGTAGCAATGAAAGTCCAAGTTTACGC-3') and NK105 (5'-CACTTAGCT-3'). The *tcdB*-specific primers were designed from the conserved 5' region of *tcdB* and generated a 160-bp fragment. The *tcdA*-specific primers and *tcdA-R* were designed to flank the smallest of the three deletions in the 3' region of *tcdA* characterized in A-B+ variant strains, and generated a 369-bp fragment for A+B+ strains and a 110-bp fragment for A-B+ strains. Thermocycling was conducted on a GeneAmp PCR System 9700 thermal cycler, (Applied Biosystems Foster City, CA). Amplicons were resolved by electrophoresis in 2% agarose gel and stained with ethidium bromide.

Statistical analysis

Assuming the prevalence of CDI was 20% in our institute and that the sensitivity of GDH EIA was 95% (Fenner *et al*, 2008), a sample size of 91 patients was required, based on alpha and beta error of 0.05 and 0.10, respectively. Pearson chi-square test was used to compare categorical variables. Univariate analysis was performed to identify statistically significant difference between the 2 groups. Two-tailed *p*-value < 0.05 is considered statistically significant. SPSS software version 17 (SPSS, Armonk, NY) was used for the analyses.

RESULTS

Diagnostic tests for CDI

Of 91 patients with suspected CDI,

there were 22 (24%) CDI cases diagnosed by the gold standard PCR method for *tcdA* and *tcdB*. The results were classified into 3 groups: A+B+ (positive for both *tcdA* and *tcdB*), *n* = 15 (16%), AdelB+ (positive *tcdB* and deleted *tcdA*), *n* = 5 (5%), and A-B+ (only *tcdB* positive), *n* = 2 (2%).

GDH EIA was positive in 21 patients, accounting for the sensitivity of 95% [95% confidence interval (CI): 0.91-0.99] and negative in 46 patients, thereby with specificity of 67% (95% CI: 0.57-0.77). The positive predictive value (PPV), negative predictive value (NPV), and accuracy was 48% (95%CI: 0.43-0.50), 98% (95% CI: 0.97-0.99), and 74% (95%CI: 0.68-0.80), respectively. Sensitivity, specificity, PPV, NPV, and accuracy of cytotoxins A/B ELFA was 73% (95% CI: 0.68-0.78), 96% (95%CI: 0.94-0.98), 84% (95% CI: 0.80-0.88), 92% (95% CI: 0.89-0.95), and 92% (95% CI: 0.89-0.95), respectively. *C. difficile* culture results were positive in 16 patients, thereby having a sensitivity of 82% (95% CI: 0.78-0.86) and specificity of 88% (95% CI: 0.85-0.91).

Patients' characteristics

There were 91 patients with suspected CDI (37 males and 54 females) with a mean age of 60.0 ± 19.5 years, of whom 22 had CDI as diagnosed by the gold standard PCR method for *tcdA* and *tcdB* (Table 1). Eighty-eight patients had underlying diseases, including malignancy (*n* = 45) [hematologic (*n* = 27) and non-hematologic (*n* = 18)], organ failure (*n* = 44), and organ transplant [solid organ (n = 7) and bone marrow (*n* = 1)]. There is no significant association among underlying diseases and CDI.

Risk factors for CDI

The most well-known drugs at risk of CDI are antibiotics, acid-neutralizing agents and chemotherapeutic agents (Bartlett, 2009, 2010). Eighty-eight patients (97%) had received antibiotics within 3 months and more than 3 days before developing diarrhea, accounting for 109 prescriptions (Table 2). There were 24 (22%) and 85 (78%) patients with and without CDI, respectively. The most frequently prescribed drug was beta-lactam antibiotics (90%), of which 22 (92%) and 76 (89%) were for patients with and without CDI, respectively ($p < 0.05$) (Table 2). There were 55 prescriptions of immunosuppressive drugs: 8 (14%) and 47 (85%) for patients with and without CDI, respectively (Table 2). Of 53 prescriptions for acid-neutralizing agents, 11 (92%) and 31 (76%) patients with and without CDI, respectively received proton-pumps inhibitors (PPIs), and 1 (8%) and 7 (17%) with and without CDI, respectively received ranitidine, a H2 receptor antagonist (H2RA) (Table 2).

Clinical characteristics

All patients, except one, had mild or moderate severity, 22 (24%) and 69 (76%) with and without CDI, respectively (Table 3). Only among patients (*n* = 57) with watery diarrhea is there a significant difference between those (*n* = 9; 41%) with and without (*n* = 48; 70%) CDI ($p = 0.02$), similarly with those (*n* = 34) with inflammatory diarrhea (13, 59%) and 21 (30%) with and without CDI, respectively, $p = 0.02$).

Laboratory investigations

Among patients with leukocytosis (*n* = 11, with rising of serum creatinine of >1.5 mg/dl (*n* = 1) and with stool white blood cells of ≥10 cells/high power field (*n* = 13), only in the latter is there a significant difference between those (*n* = 6; 27%) with and without (*n* = 7; 10%) CDI ($p = 0.046$) (Table 4).

Treatment and clinical outcome

Of 22 patients with CDI, 15 (71%) were treated with metronidazole and 1 (5%)

Table 1
Baseline characteristics of patients examined for CDI.

Patient's characteristics		CDI	
		Positive (n = 22)	Negative (n = 69)
Age, years (mean ± SD)		60.1 ± 21.5	59.9 ± 18.9
Sex:	Male	9	28
	Female	13	41
Duration of admission before developing diarrhea, days (median ± 1QR)		14 ± 16	15 ± 19
Underlying diseases		22	66
Malignancy:	Total	9	36
	Hematologic malignancy	6	21
	Acute myeloid leukemia	2	11
	Chronic myeloid leukemia	0	1
	Chronic lymphocytic leukemia	0	1
	Non-Hodgkin's lymphoma	4	5
	Hodgkin's lymphoma	0	1
	Multiple myeloma	0	1
	Acute myelofibrosis	0	1
	Solid organ malignancy	3	15
	Lung cancer	2	3
	Colonic cancer	0	3
	Hepatocellular carcinoma	0	3
	Cholangiocarcinoma	1	1
	Pancreatic cancer	0	1
	Cervical cancer	0	1
	Breast cancer	0	1
	Medulloblastoma	0	1
	Thymoma	0	1
Transplant:	Total	0	8
	Bone marrow transplant	0	1
	Kidney transplant	0	5
	Liver transplant	0	2
Organ failure:	Total	12	32
	End-stage renal disease	3	7
	Ischemic heart disease	2	5
	Chronic lung disease	2	2
	Cirrhosis	0	2
	Diabetes mellitus	2	15
	Myelodysplastic syndrome	2	1
	Thalassemia disease	1	0
Others:	Hypertension	2	12
	HIV	2	2
	Systemic lupus erythematosus	0	2

Table 2
Types of antibiotics, chemotherapeutic and acid-neutralizing agents prescribed to patients in the study.

Antibiotics[a]		CDI		
		Positive (%) (n = 24)	Negative (%) (n = 85)	
Beta-lactam		22 (92)	76 (89)	
Beta-lactam/beta-lactamase inhibitor[d]		4 (17)	35 (41)	
Piperacillin/tazobactam		1	20	
Cefoperazone/sulbactam		1	6	
Ampicillin/sulbactam		2	8	
Amoxicillin/clavulanic acid		0	1	
Cephalosporin		10 (42)	17 (20)	
Ceftazidime		6	14	
Ceftriaxone		4	3	
Carbapenem		8 (33)	24 (28)	
Meropenem		4	18	
Imipenem		4	4	
Ertapenem		0	2	
Quinolone (ciprofloxacin)		0	2 (2)	
Macrolide (azithromycin)		0	1 (1)	
Sulfonamide (TMP/SMX)		0	1 (1)	
Colistin		1 (4)	4 (5)	
Aminoglycoside (gentamicin)		1 (4)	0	
Fosfomycin		0	1 (1)	
Duration of antibiotics used before developing diarrhea, days (median ± 1 QR)		12 ± 9	13 ± 8	1 ± 8
Chemotherapeutic agents[b]		**CDI**		
		Positive (%) (n = 8)	Negative (%) (n = 47)	
Cell cycle-specific:	Total	7 (87)	43 (91)	
	Antimetabolyte	1	14	
	Antibiotic, anthracycline	4	23	
	Vinca alkaloid	2	6	
Non-cell cycle-nonspecific:	Alkylating agent	0	4	
Retinoids		1 (100)	0	
Duration of chemotherapeutic agents used before developing diarrhea, days (median ± 1 QR)		11 ± 13	14 ± 28	14 ± 19
Acid-neutralizing agents[c]		**CDI**		
		Positive (%) (n = 12)	Negative (%) (n = 41)	
Proton pump inhibitor:	Total	11 (92)	31 (76)	
	Omeprazole	9	25	
	Pantoprazole	2	3	
	Esomeprazole	0	3	
	Lansoprazole	0	3	
H2 blocker:	Ranitidine	1 (8)	7 (17)	
Duration of acid-neutralizing agents used before developing diarrhea, days (median ± 1 QR)		4 ± 15	6 ± 39	

Each patient might use more than one type of: [a]antibiotics, [b]chemotherapeutic agents, [c]acid-neutralizing agents, [d]$p<0.05$.

Table 3
Signs and symptoms and correlation with CDI of patients in the study.

Sign and symptom		CDI	
		Positive (n = 22)	Negative (n = 69)
Systemic symptom:	Fever	12 (54.5)	26 (37.7)
Local symptom:	Abdominal pain	11 (50.0)	29 (42.0)
	Generalized pain	8	18
	Periumbilical pain	3	9
	Right lower quadrant pain	0	2
	Diarrhea	22 (100)	69 (100)
	Watery diarrhea[a]	9 (40.9)	48 (69.6)
	Inflammatory diarrhea[a]	13 (59.1)	21 (30.4)
	Mucous	10	19
	Mucous/bloody	2	2
	Bloody	1	0

[a]p = 0.02.

received vancomycin. The only one patient with severe symptoms received both oral vancomycin (500 mg every 6 hours) and intravenous metronidazole (500 mg every 8 hours) (Table 5).

Nine (43%), 6 (29%), and 6 (29%) patients with mild or moderate severity received appropriate treatment with discontinuation of the prescribed drug, appropriate treatment without discontinuation the prescribed drug, and no treatment, respectively (Table 5). Of 9 patients in the first category, 5 were cured, 1 had persistent diarrhea, 1 had a relapse, and 2 died. Of 6 patients in the second category, 4 were cured, 1 had persistent diarrhea and 1 died. The single patient with severe illness eventually succumbed to the illness.

DISCUSSION

Using PCR-based assays, three groups of CDI (A+B+, AdelB+ and A-B+), but not *tcdA* alone. This may indicate a true negative or the false negative finding probably due to the limited number of the organism in the stool specimens. A previous study in Thailand has shown that all patients (574 stool specimens) but one had CDI-positive PCR test for both *tcdA* and *tcdB* on direct stool examination (Wongwanich *et al*, 2003). The prevalence of *C. difficile* with positive *tcdB* but negative *tcdA* has been reported in Japan (Komatsu *et al*, 2003) and Korea (Kim *et al*, 2008). On the other hand, the prevalence of *C. difficile* with *tcdB* but not *tcdA* is < 3% in the United Kingdom, France, and the United States (Drudy *et al*, 2007; Cohen *et al*, 2010).

Sensitivity and specificity of GDH EIA for CDI were consistent with a previous study (Fenner *et al*, 2008). However, cytotoxins A/B ELFA that has been used as the sole assay for diagnosis of CDI in our Institute had lower sensitivity but higher specificity. Eastwood *et al* (2009) have reported sensitivity and specificity of VIDAS® *C. difficile* Toxin A & B assay kit (bioMérieux, Lyon, France) of 80% and

Table 4
Laboratory investigations and correlation with CDI of patients in the study.

Laboratory investigation		CDI	
		Positive (%) (n = 22)	Negative (%) (n = 69)
White cell count:	Neutropenia	4 (18)	12 (17)
	Normal	15 (68)	49 (71)
	Leukocytosis	3 (14)	8 (12)
Creatinine:	Relative stability	17 (77)	61 (88)
	Rising of >1.5 mg/dl	1 (4)	0
	ESRD on hemodialysis	4 (18)	8 (12)
Stool white cells[a]:	< 10/HPF	16 (73)	62 (90)
	≥ 10/HPF	6 (27)	7 (10)
Stool red cells:	Present	19 (86)	66 (96)
	Absent	3 (14)	3 (4)

[a]p = 0.046. ESRD, end-stage renal disease; HPF, high power field.

Table 5
Clinical outcome as regards appropriateness of treatment for CDI.

Severity	Appropriateness of treatment	Discontinuatiion of offending drug	Outcome			
			Cured (%) (n = 14)	Persistence (%) (n = 2)	Recurrence (%) (n = 1)	Mortality (%) (n = 5)
Mild/moderate	Yes	Yes	5 (36)	1 (50)	1 (100)	2 (40)
		No	4 (28)	1 (50)	0	1 (20)
	No	Yes	0	0	0	0
		No	5 (36)	0	0	1 (20)
Severe	Yes	Yes	0	0	0	0
		No	0	0	0	1 (20)
	No	Unsure	0	0	0	0

97.36%, respectively. In the present study, sensitivity was only 73%. The discrepancy is probably due to differences in the gold standard test used (PCR in the present study and cytotoxigenic culture in the other study), to strains of *C. difficile* used, or to amounts of the organism in each stool specimen (VIDAS® *C. difficile* Toxin A & B assay detects toxin A at a level of ≥ 3 ng/ml and toxin B at ≥ 1 ng/ml).

In the present study, sensitivity and specificity of *C. difficile* culture was 82% and 88%, respectively. A recent study has shown that the sensitivity of Brucella agar supplemented with 1 mg/ml, vitamin K1 and 5 mg/ml hemin for culturing anaero-

bic organisms including *C. difficile* is > 95% (Roe *et al*, 2002). The presence of 0.01%-0.1% sodium taurocholate is associated with significantly higher recovery (96%-100%) of *C. difficile*, equal to cycloserine-cefoxitin-fructose agar (CCFA) (Nerandzic and Donskey, 2009).

We propose a two-step diagnostic algorithm for detecting CDI. In the first step, GDH EIA and cytotoxins A/B ELFA are performed concurrently, the results being reported within 3 hours. For discordant results, PCR detection of *tcdA* and *tcdB* will be performed in the second step, the results being available within 6-8 hours. In our hands, 30% of specimens required the more expensive PCR assay. However, this two-step procedure detected 20% more patients with CDI than the currently used assay.

There are other versions of a two-step algorithm for CDI detection. Fenner *et al* (2008) employed GDH ELISA and cytotoxins A/B EIA in the first step, and if discordant results are obtained, then cytotoxigenic culture is performed, resulting in a sensitivity for diagnosis of CDI of 92% (based on the multiplex PCR as the gold standard method). Gilligan (2008) used GDH ELISA and tissue culture cytotoxicity neutralization assay in the first and second step, respectively, resulting in an enhanced ability to detect CDI by 40% over that using cytotoxins A/B EIA. A two-step algorithm consisting of GDH EIA and PCR amplification of *tcdB* in the first and second step respectively has a sensitivity and specificity of 94% and 99%, respectively (Goldenberg *et al*, 2010). However, inclusion of both GDH and cytotoxins A/B EIA in the first step improves sensitivity (98.7%) but not specificity (89.7%) (Culbreath *et al*, 2012), whereas Sharp *et al* (2010) using algorithm reported a sensitivity and specificity of 99.6% and 100%, respectively. In 2010, the clinical practice guidelines of the Society for Healthcare Epidemiology of America and the Infectious Diseases Society of America recommended the two-step algorithm [GDH EIA as the screening test, and cytotoxic culture or cytotoxic cell neutralization assay (CCNA) as the confirmatory test] for diagnosis of CDI in adults (Cohen *et al*, 2010).

In the present study, the overall prevalence of CDI at King Chulalongkorn Memorial Hospital, Bangkok, Thailand, was 24%, compared to a study in 2000-2001 at Siriraj Hospital, Mahidol University, Bangkok and other hospitals in Thailand that showed a prevalence of CDI, determined by PCR for *tcdA* and *tcdB*, is 18.64% (Wongwanich *et al*, 2003). However, a recent study from Siriraj Hospital reported the prevalence of CDI, determined by cytotoxins A/B EIA is reduced to 12.3% (Thipmontree *et al*, 2011). In the United States, the prevalence of CDI has doubled from 31/100,000 to 61/100,000 population from 1996 to 2003 (McDonald *et al*, 2006). In Europe, the prevalence of CDI in many countries ranges from 24.5/100,000 to 41/100,000 patient-days (Bauer *et al*, 2011). Possible explanations are discussed below.

In the present study, treatment with antibiotics was a more important risk factor compared to chemotherapeutic and acid-suppressive agent's treatment. Third-generation cephalosporin was the most frequently prescribed antibiotic associated with CDI, consistent with previous studies in our Institute and Siriraj Hospital (Pupaibool *et al*, 2008; Thipmontree *et al*, 2011), as well as in the United States and Europe (Thielman and Wilson, 2009; Bauer *et al*, 2011; Hensgens *et al*, 2012), possibly due to its broad antibacterial spectrum and high prescription rate (Thielman

and Wilson, 2009; Wilcox *et al*, 2012). We observed that the duration of antibiotic exposure before developing diarrhea (12 ± 9 days) is consistent with previous study (Hensgens *et al*, 2012), although Hensgens *et al* (2012) reported a longer duration (≥ 14 days). The discrepancy is probably due to the differences in demography, risk factors, and preexisting conditions.

The duration of chemotherapeutic agent exposure before developing diarrhea (11 ± 13 days) is similar to a study by de Blank *et al* (2013) who reported the highest risk of CDI is 8-14 days after an exposure to chemotherapeutic agent (Bartlett, 2009, 2010).

The high prevalence (23%) of CDI in patients with current and/or recent use of PPIs observed in this study is similarly to that (55.4%) of Pupaibool *et al* (2008) at the same institute. Thipmontree *et al* (2011) also have demonstrated an association between acid-neutralizing agents and CDI. Tleyjeh *et al* (2012) have confirmed the risk of CDI especially if PPIs or H2RAs are combined with antibiotics exposure within the first two weeks. Moreover, PPIs are associated with recurrent CDI (Kwok *et al*, 2012).

More than 80% of CDI patients had fever, and inflammatory diarrhea was noted in a higher number of patients with CDI. Due to the enterotoxic and cytotoxic effects of CD toxins A and B, patients with CDI tend to have colitis (Riegler *et al*, 1995; Sears and Kaper, 1996; Drudy *et al*, 2007).

The present study has a number of limitations. Firstly, the study was conducted in adult patients, most of whom hospitalized in Medicine Department, hence the results cannot be generalized to other groups of patients, but the diagnostic tests should not be affected by patients' status. Secondly, the gold standard test for diagnosis of CDI should be cytotoxigenic culture, but the present study employed PCR assay. However, currently PCR assay is accepted as the gold standard test (Kuehne *et al*, 2010; Larson *et al*, 2010). Thirdly, the in-house PCR assay has not been validated with the United States Food and Drug Administration (US FDA) approved commercial PCR assays (Bartlett, 2009, 2010). However, previous studies also used the modified Kato method (Lemee *et al*, 2004) as also used in the present study. We plan to validate our in-house PCR assay with those US FDA approved assays.

In conclusion, the present study is the first prospective study in Thailand, which compared all 4 diagnostic tests for CDI. In addition, we propose a two-step diagnostic algorithm that should improve sensitivity and specificity and be cost effectiveness in diagnosis of CDI.

ACKNOWLEDGEMENTS

We would like to thank Department of Microbiology, Faculty of Medicine, Chulalongkorn University, for the assistance in all laboratory works.

REFERENCES

Bartlett JG. Clinical practice. Antibiotic-associated diarrhea. *N Engl J Med* 2002; 346: 334-9.

Bartlett JG. *Clostridium difficile* infection: historic review. *Anaerobe* 2009; 15: 227-9.

Bartlett JG. Detection of *Clostridium difficile* infection. *Infect Control Hosp Epidemiol* 2010; 31 (suppl 1): S35-7.

Bauer MP, Notermans DW, van Benthem BH, *et al*. *Clostridium difficile* infection in Europe: a hospital-based survey. *Lancet* 2011; 377(9759): 63-73.

Bishara J, Goldberg E, Madar-Shapiro L, Behor

J, Samra Z. Molecular epidemiology of *Clostridium difficile* in a tertiary medical center in Israel: emergence of the polymerase chain reaction ribotype 027. *Isr Med Assoc J* 2011; 13: 338-41.

Cohen SH, Gerding DN, Johnson S, *et al*. Clinical practice guidelines for *Clostridium difficil*e infection in adults: 2010 update by the Society for Healthcare Epidemiology of America (SHEA) and the Infectious Diseases Society of America (IDSA). *Infect Control Hosp Epidemiol* 2010; 31: 431-55.

Culbreath K, Ager E, Nemeyer RJ, Kerr A, Gilligan PH. Evolution of testing algorithms at a university hospital for detection of *Clostridium difficile* infections. *J Clin Microbiol* 2012; 50: 3073-6.

de Blank P, Zaoutis T, Fisher B, Troxel A, Kim J, Aplenc R. Trends in *Clostridium difficile* infection and risk factors for hospital acquisition of *Clostridium difficile* among children with cancer. *J Pediatr* 2013; 163: 699-705.e1.

Drudy D, Fanning S, Kyne L. Toxin A-negative, toxin B-positive *Clostridium difficile*. *Int J Infect Dis* 2007; 11: 5-10.

Eastwood K, Else P, Charlett A, Wilcox M. Comparison of nine commercially available *Clostridium difficile* toxin detection assays, a real-time PCR assay for *C. difficile tcdB*, and a glutamate dehydrogenase detection assay to cytotoxin testing and cytotoxigenic culture methods. *J Clin Microbiol* 2009; 47: 3211-7.

Fenner L, Widmer AF, Goy G, Rudin S, Frei R. Rapid and reliable diagnostic algorithm for detection of *Clostridium difficile*. *J Clin Microbiol* 2008; 46: 328-30.

Gilligan PH. Is a two-step glutamate dehyrogenase antigen-cytotoxicity neutralization assay algorithm superior to the premier toxin A and B enzyme immunoassay for laboratory detection of *Clostridium difficile*? *J Clin Microbiol* 2008; 46: 1523-5.

Goldenberg SD, Cliff PR, Smith S, Milner M, French GL. Two-step glutamate dehydrogenase antigen real-time polymerase chain reaction assay for detection of toxigenic *Clostridium difficile*. *J Hosp Infect* 2010; 74: 48-54.

Hensgens MP, Goorhuis A, Dekkers OM, Kuijper EJ. Time interval of increased risk for *Clostridium difficile* infection after exposure to antibiotics. *J Antimicrob Chemother* 2012; 67: 742-8.

Kato H, Kato N, Watanabe K, *et al*. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. *J Clin Microbiol* 1998; 36: 2178-82.

Kim H, Riley TV, Kim M, *et al*. Increasing prevalence of toxin A-negative, toxin B-positive isolates of *Clostridium difficile* in Korea: impact on laboratory diagnosis. *J Clin Microbiol* 2008; 46: 1116-7.

Komatsu M, Kato H, Aihara M, *et al*. High frequency of antibiotic-associated diarrhea due to toxin A-negative, toxin B-positive *Clostridium difficile* in a hospital in Japan and risk factors for infection. *Eur J Clin Microbiol Infect Dis* 2003; 22: 525-9.

Kwok CS, Arthur AK, Anibueze CI, Singh S, Cavallazzi R, Loke YK. Risk of *Clostridium difficile* infection with acid suppressing drugs and antibiotics: meta-analysis. *Am J Gastroenterol* 2012; 107: 1011-9.

Kuehne SA, Cartman ST, Heap JT, Kelly ML, Cockayne A, Minton NP. The role of toxin A and toxin B in *Clostridium difficile* infection. *Nature* 2010; 467(7316): 711-3.

Kufelnicka AM, Kirn TJ. Effective utilization of evolving methods for the laboratory diagnosis of *Clostridium difficile* infection. *Clin Infect Dis* 2011; 52: 1451-7.

Larson AM, Fung AM, Fang FC. Evaluation of *tcdB* real-time PCR in a three-step diagnostic algorithm for detection of toxigenic *Clostridium difficile*. *J Clin Microbiol* 2010; 48: 124-30.

Lemee L, Dhalluin A, Testelin S, *et al*. Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (Toxin A), and *tcdB* (Toxin B) genes for toxigenic culture of *Clostridium difficile*. *J Clin Microbiol* 2004; 42: 5710-4.

McDonald LC, Killgore GE, Owens RC Jr, *et al*. An epidemic, toxin gene-variant strain of *Clostridium diffecile*. *N Engl J Med* 2005; 353 (23): 2433-41.

McDonald LC, Owings M, Jernigan DB. *Clostridium difficile* infection in patients discharged from US short-stay hospitals, 1996-2003. *Emerg Infect Dis* 2006; 12: 409-15.

Nerandzic MM, Donskey CJ. Effective and reduced-cost modified selective medium for isolation of *Clostridium difficile*. *J Clin Microbiol* 2009; 47: 397-400.

Pepin J, Saheb N, Coulombe MA, *et al*. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. *Clin Infect Dis* 2005; 41: 1254-60.

Pepin J, Valiquette L, Alary ME, *et al*. *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* 2004; 171: 466-72.

Pupaibool J, Khantipong M, Suankratay C. A study of *Clostridium difficile*-associated disease at King Chulalongkorn Memorial Hospital, Thailand. *J Med Assoc Thai* 2008; 91: 37-43.

Riegler M, Sedivy R, Pothoulakis C, *et al*. *Clostridium difficile* toxin B is more potent than toxin A in damaging human colonic epithelium in vitro. *J Clin Invest* 1995; 95: 2004-11.

Roe DE, Finegold SM, Citron DM, *et al*. Multilaboratory comparison of growth characteristics for anaerobes, using 5 different agar media. *Clin Infect Dis* 2002; 35 (suppl 1): S36-9.

Sears CL, Kaper JB. Enteric bacterial toxins: mechanisms of action and linkage to intestinal secretion. *Microbiol Rev* 1996; 60: 167-215.

Sharp SE, Ruden LO, Pohl JC, Hatcher PA, Jayne LM, Ivie WM. Evaluation of the C.Diff Quik Chek Complete Assay, a new glutamate dehydrogenase and A/B toxin combination lateral flow assay for use in rapid, simple diagnosis of *Clostridium difficile* disease. *J Clin Microbiol* 2010; 48: 2082-6.

Thielman NM, Wilson KH. Antibiotic-associated colitis. In: Mandell GL, Bennett JE, Dolin R, eds. Mandell: Mandell, Douglas, and Bennett's principles and practice of infectious diseases. 7th ed. Philadelphia: Elsevier, 2009: 1375-87.

Thipmontree W, Kiratisin P, Manatsathit S, Thamlikitkul V. Epidemiology of suspected *Clostridium difficile*-associated hospital-acquired diarrhea in hospitalized patients at Siriraj Hospital. *J Med Assoc Thai* 2011; 94 (suppl 1): S207-16.

Tleyjeh IM, Bin Abdulhak AA, Riaz M, *et al*. Association between proton pump inhibitor therapy and *Clostridium difficile* infection: a contemporary systematic review and meta-analysis. *PLOS One* 2012; 7: e50836.

Wilcox MH, Shetty N, Fawley WN, *et al*. Changing epidemiology of *Clostridium difficile* infection following the introduction of a national ribotyping-based surveillance scheme in England. *Clin Infect Dis* 2012; 55: 1056-63.

Wilkins TD, Lyerly DM. *Clostridium difficile* testing: after 20 years, still challenging. *J Clin Microbiol* 2003; 41: 531-4.

Wongwanich S, Rugdeekha S, Pongpech P, Dhiraputra C. Detection of *Clostridium difficile* toxin A and B genes from stool samples of Thai diarrheal patients by polymerase chain reaction technique. *J Med Assoc Thai* 2003; 86: 970-5.