ORIGINAL ARTICLE

Prevalence and molecular epidemiology of *Clostridium difficile* infection in Thailand

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Abstract

Little is known about *Clostridium difficile* infection (CDI) in Asia generally, and specifically in Thailand. Given the high prevalence of inappropriate antibiotic usage in this region, CDI is likely to be common. This study investigated the prevalence and molecular epidemiology of CDI in Thailand. Stool specimens collected from inpatients with diarrhoea at Siriraj hospital in Bangkok ($n = 422$) were cultured on ChromID Cdiff agar and any presumptive *C. difficile* colonies were identified, PCR ribotyped and toxin profiled. As part of the routine *C. difficile* testing at Siriraj Hospital, 370 specimens also underwent testing with the BD MAX Cdiff assay to detect the presence of *tcdB*. With direct culture, 105 different isolates of *C. difficile* were recovered from 23.7% (100/422) of the stool specimens. The prevalence of toxigenic and nontoxigenic isolates was 9.2% (39/422) and 15.6% (66/422), respectively. Of the toxigenic isolates, 69.2% (27/39) and 30.8% (12/39) were *tcdA* and *tcdB* positive (A^+B^+), and A^-B^+, respectively; none contained binary toxin genes. The five most prevalent ribotypes (RTs) were 014/020 group (17/105), 010 (12/105), 017 (12/105), 039 (9/105) and 009 (6/105). Using toxigenic culture as the reference standard, the sensitivity, specificity, positive predictive value and negative predictive value of the BD MAX Cdiff assay were 68.6, 95.1, 63.2 and 96.1%, respectively. The high proportion of A^-B^+, RT 017 strains emphasises the need for diagnostic tests that detect either both toxins or just *tcdB*. Continued surveillance that involves stool culturing will allow molecular tracking and assist in elucidating the epidemiology of CDI in Thailand.

Keywords: BD MAX Cdiff assay, *Clostridium difficile*, epidemiology, PCR ribotype, prevalence, toxigenic culture
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Introduction

Clostridium difficile is an important aetiologic agent of antibiotic-associated infectious diarrhoea in the developed world [1]. The major virulence factor of *C. difficile* is the production of toxins A and B [1]. Individuals infected with a toxigenic strain may remain asymptomatic, or they may develop mild to severe diarrhoea, colitis and, in rare cases, pseudomembranous colitis [1]. The traditional risk factors associated with *C. difficile* infection (CDI) include advanced age, recent hospitalization and previous disruptions to the gut microbiome by agents such as antibiotics [1].

Interest in CDI increased exponentially after highly publicized outbreaks of infection in North America with *C. difficile* PCR ribotype (RT) 027 in the early 2000s [2] and the emergence of RT 078 in 2007 [3]. The increased morbidity and mortality observed sparked major public health concerns, which continue today [2]. In addition to toxins A and B, RTs 027 and 078 produce a third toxin, binary toxin, the role of which in disease pathogenesis remains controversial [4]. One important difference between infection with RTs 027 and 078 is that the latter often affects individuals who lack traditional risk factors [3]. RT 078 is associated with community-associated

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infection, which stimulated speculation regarding the possible source of infection outside healthcare facilities [3]. A recent study reported RT 078 strains isolated from humans and pigs to be indistinguishable via whole genome sequencing, suggesting that a zoonosis or anthroponosis may be occurring [5]. Additionally, food and environmental contamination have also been suggested as possible sources of infection [6].

In order to monitor the prevalence and molecular characteristics of *C. difficile* and to elucidate the epidemiology of CDI, continued surveillance is essential. Currently, most *C. difficile* research has been done in North America, Europe and Australia, and limited data are available for Asian countries, such as Thailand [7]. Several early prevalence studies conducted in Thailand used toxin A enzyme immunoassay (EIA) as the sole method of detection [7]. Given the recently reported high prevalence of a *tcdA*-negative, *tcdB*-positive (A^-B^+) RT 017 (41.5%) in Thailand and the low sensitivity of EIA [8,9], the widespread use of toxin A EIA alone is likely to have led to an underestimation of the true prevalence. Toxigenic culture, which is a current reference standard for *C. difficile* detection, is rarely performed these days because of time and cost constraints, while the use of PCR-based techniques has increased over the years [7]. In addition to a lack of an optimal detection method, the most recent prevalence study was conducted between 2010 and 2011 [10] and the most recent molecular epidemiologic study between 2006 and 2008 [9].

To better understand the current epidemiology of CDI in Thailand, we investigated the prevalence and molecular characteristics of circulating Thai *C. difficile* strains and assessed the performance of the routine diagnostic procedure used at Siriraj Hospital to detect *C. difficile* in stool specimens.

Materials and Methods

Sample collection and transport

Between April and June 2015, as part of the routine pathology testing at the microbiology department at Siriraj Hospital, Bangkok, Thailand, a total of 422 nonrepeat diarrhoeal samples were collected. Of these, 370 and 52 specimens were submitted for *C. difficile* testing and non–*C. difficile*–related tests, respectively. All samples were obtained from patients aged ≥18 years who experienced clinically significant diarrhoea as defined by at least 3 diarrhoeal bowel movements in the prior 24 hours corresponding to Bristol stool chart grade 6–7, or a single diarrhoeal bowel movement corresponding to Bristol stool chart grade 6–7 and associated with abdominal pain and/or cramping. After routine *C. difficile* testing at Siriraj Hospital (described below), all diarrhoeal samples were sent to a *C. difficile* reference laboratory in Perth, Western Australia. Stools were maintained at ambient temperature during transportation.

Detection of *C. difficile* toxin B gene in stool specimens

All specimens submitted for *C. difficile* testing ($n = 370$) were subjected to *tcdB* detection using the BD MAX Cdiff assay (BD Diagnostic, Franklin Lakes, NJ, USA). This was done as per the manufacturer's instruction and as part of routine pathology testing at Siriraj Hospital.

Isolation and identification of *C. difficile*

At the *C. difficile* reference laboratory, stool specimens were cultured both directly on *C. difficile* ChromID agar (bioMérieux, Marcy l'Etoile, France) and in a cooked meat enrichment broth containing gentamicin (5 mg/L), cefoxitin (10 mg/L), cycloserine (200 mg/L) and taurocholate (0.1%) as previously described [11]. After 48 hours of incubation, to select for spores, 1 mL of each enrichment broth was added to equal volumes of 96% alcohol, left at room temperature for at least 60 minutes and then plated onto *C. difficile* ChromID agar. All plates were incubated in an anaerobic chamber (Don Whitley Scientific, Shipley, West Yorkshire, UK) at 37°C in an atmosphere containing 80% N_2, 10% CO_2, and 10% H_2. Putative *C. difficile* colonies were identified as described previously [11].

Molecular characterization of *C. difficile* isolates

All isolates were screened by in-house PCRs for the presence of *tcdA* and *tcdB*, and binary toxin genes (*cdtA* and *cdtB*) [11], and PCR ribotyping was performed as previously described [12]. Isolates that could not be identified with the reference library were designated with an internal nomenclature, prefixed with QX.

Results

Isolation of *C. difficile* from patients stool specimens

In total, 23.7% (100/422) of the specimens were positive via direct culture, yielding 105 isolates (four specimens contained more than one strain). The rest of the specimens (76.3%; 322/422) were negative by both direct and enrichment culture techniques (Table 1). The isolation rates for *C. difficile* from specimens routinely tested with the BD MAX Cdiff assay and those not tested were 24.1% (89/370) and 21.2% (11/52), respectively.

Toxin gene profiling and PCR ribotyping of *C. difficile* isolates

As shown in Table 1, the overall prevalence of toxigenic *C. difficile* was 9.2% (39/422). Of the toxigenic isolates

TABLE 1. Summary of the BD MAX Cdiff assay and toxigenic culture results

BD MAX	Culture	Toxin profile	Specimens, *n* (%)	Isolates, *n* (%)
Positive	Positive	$A^+B^+CDT^-$	16 (3.8)	16 (15.2)[a]
		$A^-B^+CDT^-$	8 (1.9)	8 (7.6)
		$A^-B^-CDT^-$	2 (0.5)	5 (4.8)[a]
Positive	Negative	—	12 (2.8)	—
Negative	Positive	$A^+B^+CDT^-$	7 (1.7)	7 (6.7)
		$A^-B^+CDT^-$	4 (0.9)	4 (3.8)
		$A^-B^-CDT^-$	52 (12.3)	54 (51.4)[b]
Negative	Negative	—	269 (63.7)	—
Untested	Positive	$A^+B^+CDT^-$	4 (0.9)	4 (3.8)
		$A^-B^-CDT^-$	7 (1.7)	7 (6.7)
Untested	Negative	—	41 (9.7)	—
Total			422	105

[a]One specimen contained two strains, one toxigenic and one nontoxigenic. Only the toxigenic isolate was included in the final calculation to evaluation of the BD MAX Cdiff assay performance. Additionally, another BD MAX–positive specimen contained three strains, all of which were nontoxigenic. Only one of the three was included in the final calculation.
[b]Two specimens contained two strains each.

TABLE 2. Summary of *Clostridium difficile* PCR ribotypes and toxin profiles

	Toxin profile			
PCR ribotype	*tcdA*	*tcdB*	*cdtA/B*	*n* (%)
014/020 group[a]	+/−	+/−	−	17 (16.2)
010	−	−	−	12 (11.4)
017	−	+	−	12 (11.4)
039	−	−	−	9 (8.6)
009	−	−	−	6 (5.7)
QX 002	−	−	−	5 (4.8)
QX 178	−	−	−	4 (3.8)
QX 514	−	−	−	3 (2.9)
QX 001	+	+	−	2 (1.9)
QX 083, QX 107, QX 190, QX 506, QX 511 and QX 524 (2 each)	−	−	−	12 (11.4)
005, 103, QX 026, QX 032, QX 102, QX 161, QX 176, QX 455 and QX 517 (1 each)	+	+	−	9 (8.6)
QX 011, QX 077, QX 078, QX 117, QX 138, QX 507, QX 508, QX 509, QX 510, QX 513, QX 515, QX 516, QX 522 and QX 523 (1 each)	−	−	−	14 (13.3)
			Total	105

[a]RT 014/020 group contains 16 $A^+B^+CDT^-$ isolates and one nontoxigenic isolate.

recovered, 69.2% (27/39) were A^+B^+, while 30.8% (12/39) were A^-B^+. None of the isolate was positive for the binary toxin genes *cdtA* or *cdtB*. Thus, the majority of isolates recovered did not carry any toxin genes and were therefore considered nontoxigenic (62.9%; 66/105).

The 38 RTs identified are shown in Table 2 along with their respective toxin profile. Of the 105 isolates, 55.2% (58/105) were assigned to internationally recognized RTs 005 ($n = 1$), 009 ($n = 6$), 010 ($n = 12$), 014/020 group ($n = 17$), 017 ($n = 12$), 039 ($n = 9$) and 103 ($n = 1$). The remaining 44.8% (47/105) of the isolates did not match any reference strains and were designated with an internal nomenclature (Table 2).

Assessment of BD MAX Cdiff assay performance

Of the 370 specimens tested with the BD MAX Cdiff assay, 38 (10.3%) and 332 (89.7%) were BD MAX positive and negative, respectively (Table 1). When compared against the toxigenic culture results, 79.2% (293/370) of the specimens were concordant.

The performance of BD MAX Cdiff assay was calculated after excluding the BD MAX negative specimens that yielded nontoxigenic isolates ($n = 52$). With toxigenic culture as a reference standard, the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of the BD MAX Cdiff assay, and their corresponding 95% confidence intervals (in parentheses) were 68.6% (50.7–83.1), 95.1% (91.9–97.3), 63.2% (46.0–78.2) and 96.1% (93.1–98.0), respectively.

Discussion

A major risk factor associated with CDI is prior exposure to antibiotics [13]. Given the healthcare system in Thailand, which allows drugstores to freely distribute oral antibiotics and patients to self-medicate, antibiotic misuse is prevalent [14]. Apart from encouraging the development of antibiotic resistance, such practices also increase the risk of CDI [13]. To assess the prevalence and molecular epidemiology of circulating *C. difficile* in Thailand, toxigenic culture was performed on 422 diarrhoeal stool specimens obtained from adult patients admitted at Siriraj Hospital in Bangkok.

By toxigenic culture, the prevalence of toxigenic *C. difficile* was 9.2%. Previous studies have reported the prevalence of CDI in Thailand to range between 4.8 and 52.2%, depending on the study protocol and population investigated [7]. Since 2000, two studies have investigated the prevalence of CDI among patients at Siriraj Hospital using a culture-based technique to isolate *C. difficile* and an in-house PCR to investigate the presence of toxin genes. In 2001, Wongwanich *et al.* [15] reported the prevalence of *C. difficile* among adult patients to be 25.0% (33/132) and that of toxigenic strains to be 13.6% (18/132). Although the prevalence of toxigenic *C. difficile* was slightly higher than that observed in the current study, it is still likely to be an underestimation at the time, as the authors only investigated the presence of *tcdA*. In 2003, a lower overall prevalence of *C. difficile* was reported among patients admitted at Siriraj Hospital (18.6%; 107/574). The prevalence of toxigenic *C. difficile* was 9.1% (52/574), all of which were positive for *tcdA* and *tcdB* [16]. The prevalence in the latter publication more closely resembled that seen in the current study and could suggest that the prevalence of CDI in Thailand has remained constant during the past decade.

As mentioned, the laboratory detection method used and the characteristics of the cohort under investigation affect the outcomes of any prevalence study. Compared to other

publications in the Asian region that involved direct stool culture and toxin gene detection by a PCR-based technique, the prevalence of toxigenic *C. difficile* in Thailand (9.2%) appeared to be lower than that reported in 2015 for China (19.2%, 80/416) [17] and similar to that reported in 2015 for India (10.9%, 121/1110) [18]. These figures are all higher than those reported in 2015 for Spain (6.0%, 108/1800) [19] and Australia (6.4% to 7.2%) [11,20]. Many studies conducted in Asia have investigated the prevalence of toxigenic *C. difficile* using direct detection of preformed toxins in stool. In 2014, a prevalence of toxigenic *C. difficile* similar to that of the current study was reported in Singapore (9.6%; 158/1642). That study used membrane-type EIA (C.DIFF Quik Chek Complete; TechLab, Blacksburg, VA, USA) to detect the presence of glutamate dehydrogenase and preformed toxins A and B, and the PCR-based Xpert *C. difficile* test (Cepheid, Sunnyvale, CA, USA) to detect the presence of *tcdB* [21]. On the basis of the existing data, the prevalence of CDI in Asia ranges between approximately 9.0 and 20.0%. The prevalence appeared to be lower among developed countries.

A high prevalence of A^-B^+ (RT 017) strains was noted in the current study (11.4%), in contrast to the report by Wongwanich *et al.* [16] for the same hospital in 2003. To detect the presence of *tcdA,* the authors used only the primer sequences which targets the nonrepeating region of *tcdA* (NK2 and NK3) described by Kato *et al.* [22]. Some strains of *C. difficile,* including RT 017, possess a deletion in the repeating region of *tcdA,* rendering the toxin nonfunctional. To detect the deletion, an additional primer pair (NK9 and NK11) is required, and failure to use these could explain the absence of A^-B^+ strains in their report [22].

Interestingly, a high prevalence of nontoxigenic *C. difficile* was observed in this study (15.6%). Previous studies from Thailand have reported the prevalence of nontoxigenic strains to be 9.6% (55/574) [16]. Studies conducted in China and India reported the prevalence of nontoxigenic isolates to be 7.7% (32/416) and 4.8% (53/1110), respectively [17,18]. There is some evidence suggesting an association between asymptomatic carriage of either toxigenic or nontoxigenic *C. difficile,* and a subsequent systemic anamnestic response of serum IgG antibody against toxin A leading to a reduced risk of developing CDI [23]. Given the high prevalence of nontoxigenic *C. difficile,* it is possible that such a mechanism plays a protective role against CDI in Asia.

Among the specimens investigated, 12.3% were stools submitted for non–*C. difficile*–related tests. The prevalence of toxigenic *C. difficile* among this population was 7.7%. Although these may represent missed cases of CDI, it is possible that *C. difficile* testing was not requested because of a lack of clinical suspicion. This could not be confirmed, as no clinical data were collected. Furthermore, some recent studies suggest that asymptomatic carriage of toxigenic *C. difficile* occurs more commonly than previously thought, in 7 to 15% of healthy adults [24]. Further investigation is required to better interpret the significance of such missed cases.

Using toxigenic culture as the reference standard, the sensitivity, specificity, PPV and NPV of the BD MAX Cdiff assay were 68.6, 95.1, 63.2 and 96.1%, respectively. The values appeared inferior to the previously reported sensitivity, specificity, PPV and NPV figures for BD MAX (94.0–97.7, 97.9–99.7, 87.5–97.7 and 98.9–99.7%, respectively) [11]. It is possible that patients may have been exposed to antibiotics before the stool collection. In such scenarios, BD MAX may have detected the presence of *tcdB* released by the dead *C. difficile,* leading to a relatively higher number of false-positive results. One possible explanation of false-negative findings might be an alteration in the *tcdB* sequence, rendering it unrecognizable by the BD MAX Cdiff assay. However, this was not the case, as isolates from all false-negative samples signalled positive upon retesting on the BD MAX assay. False-negative results may occur as a result of the low bacterial load in the stool specimens.

The five most prevalent RTs were 014/020 group (16.2%), 010 (11.4%), 017 (11.4%), 039 (8.6%) and 009 (5.7%). An earlier study investigating the molecular epidemiology of *C. difficile* collected between 2006 and 2008 from inpatients at Siriraj Hospital also reported RTs 017 (41.5%) and 014/020 group (20.7%) among the top RTs [9]. Additionally, RT 014/020 group was reported to be highly prevalent among humans in Australia [20], Korea [25], Europe [19,26] and North America [27]. In contrast, RT 046, which was previously reported as the third most common RT at Siriraj Hospital (6/53), was not found [9]. The second most common RT, 017, is highly prevalent in Asian countries, including China, Japan, Korea and Taiwan [28]. RT 017 has also caused significant epidemics in the Netherlands and Ireland [29].

Reports on nontoxigenic *C. difficile* in Asia are scarce. Hawkey *et al.* [30] identified two isolates of RT 009 from 21 culture-positive specimens collected from patients in China. The lack of reporting has likely stemmed from the fact that most molecular studies conducted in Asia focussed on the characterization of the toxigenic strains [28]. As such, the prevalence and molecular characteristics of nontoxigenic strains such as RTs 009, 010 and 039 observed in this study may have largely gone undocumented.

In addition to the top five RTs, 33 other RTs were observed, the majority of which were novel and did not match the PCR ribotyping patterns available in the reference library. Such a great heterogeneity could suggest a previously undefined and widely distributed reservoir of infection in Thailand. Furthermore, the absence of binary toxin producers, the lack of hypervirulent RTs 027 and 078 and the high prevalence of

nontoxigenic strains imply that the mix of *C. difficile* strains circulating in Thailand may be different from that in other parts of the world. Epidemiologic studies involving humans, companion and production animals, and the environment would shed light in possible sources of infection in both Thai healthcare facilities and communities.

CDI is common in Thailand, and continuing surveillance and research are required to monitor its changing epidemiology. Misuse of antibiotics remains a major driver of CDI in Asia, and greater efforts must be made to encourage antibiotic stewardship by healthcare practitioners and the public.

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Conflict of Interest

None declared.

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