AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Clinical Microbiology®

EPIDEMIOLOGY

High Prevalence of Toxigenic and Nontoxigenic *Clostridium difficile* Strains in Malaysia

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ABSTRACT Accumulating evidence shows a high prevalence of *Clostridium difficile* in Southeast Asia associated with a range of clinical presentations. However, severe infections are rarely reported. We investigated *C. difficile* infection (CDI) across four hospitals in Kuala Lumpur and Kota Bharu, Malaysia. Enzyme immunoassays for glutamate dehydrogenase (GDH) and toxin A or B were performed on diarrheal stool specimens collected from patients in 2015 and 2016. Specimens were also cultured and isolates of *C. difficile* characterized by PCR ribotyping and detection of toxin genes. In total, 437 specimens were collected and fecal toxin was detected in 3.0%. A further 16.2% of specimens were GDH positive and toxin negative. After culture, toxigenic strains were isolated from 10.3% and nontoxigenic strains from 12.4% of specimens. The most prevalent PCR ribotypes (RTs) were RT 017 (20.0%) and RT 043 (10.0%). The high prevalence of RT 017 and nontoxigenic strains in Malaysia and in neighboring Thailand and Indonesia suggests that they localize to the region of Southeast Asia, with an implication that they may mediate the burden of CDI in the region.

KEYWORDS *Clostridium difficile*, Malaysia, epidemiology, prevalence

Clostridium difficile, an antibiotic-resistant bacterium, is the most common cause of infectious diarrhea in hospitalized patients in the western world (1). Infection occurs following ingestion of highly resistant spores which persist in health care environments, evading many disinfection techniques. Risk factors for *C. difficile* infection (CDI) include advanced age, antibiotic use, and prolonged hospital stay (2). Notably, the incidence of CDI among younger people with no recent hospitalization is increasing worldwide (3, 4).

CDI ranges in severity from self-limiting diarrhea to life-threatening toxic megacolon and/or pseudomembranous colitis. CDI is mediated by toxins A (enterotoxin) and B (cytotoxin) and occasionally binary toxin (CDT). Toxigenic *C. difficile* strains generally produce both toxins A and B (A^+B^+); however, some are toxin A negative (A^-B^+) due to mutations in the *tcdA* gene (5). Diagnosis of CDI requires detection of toxin A and/or B in the stools of patients with confirmed diarrhea (6). Many laboratories rely on PCR detection of the gene encoding toxin B, *tcdB*, which confirms the presence of toxigenic *C. difficile* but cannot rule out colonization rather than disease (6).

Despite high incidence rates and extensive research in many developed countries,

Received 31 January 2018 **Returned for modification** 26 February 2018 **Accepted** 19 March 2018

Accepted manuscript posted online 21 March 2018

Citation Riley TV, Collins DA, Karunakaran R, Kahar MA, Adnan A, Hassan SA, Zainul NH, Rustam FRM, Wahab ZA, Ramli R, Lee YY, Hassan H. 2018. High prevalence of toxigenic and nontoxigenic *Clostridium difficile* strains in Malaysia. J Clin Microbiol 56:e00170-18. https://doi.org/10.1128/JCM.00170-18.

Editor Andrew B. Onderdonk, Brigham and Women's Hospital

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due to epidemics caused by strains of *C. difficile* with enhanced virulence such as the CDT-producing strain ribotype (RT) 027, the epidemiology of CDI in the developing countries of Southeast Asia is still understudied. The few reports available describe a lack of awareness among physicians (7, 8), a lack of appropriate testing, and a probable underdiagnosis of CDI in the region (9). Frequent unregulated and inappropriate antimicrobial use (10) suggests that the prevalence of CDI could be relatively high. RT 027 and other CDT-producing *C. difficile* strains are rarely reported from Asian countries, where the A^-B^+ strain RT 017 predominates, followed by RT 018 (A^+B^+), which is frequently reported from Korea and Japan (9). In Southeast Asia, reports indicate a high prevalence of both toxigenic and nontoxigenic *C. difficile* among hospital inpatients with diarrhea, in Thailand (9.2% toxigenic, 15.6% nontoxigenic strains) (11) and Indonesia (10.9% toxigenic, 10.6% nontoxigenic strains) (12). Surveillance of CDI in Asian countries is required to understand why awareness is so poor in the region and to determine the burden CDI places on Asian populations and health care systems.

In Malaysia, the few studies of the epidemiology of CDI that have been published imply a high prevalence, comparable to that of neighboring Thailand and Indonesia, ranging from 6 to 14% (13, 14). More recently, in Kota Bharu, toxin A/B was detected among 9% of 76 hospital inpatients with diarrhea, while toxigenic and nontoxigenic *C. difficile* were isolated from 13 and 16% of those patients' stool samples. The same study described a low prevalence (2%) of *C. difficile* colonization in elderly community participants and identified RTs 043 and 017 as the most prevalent strains (14% each among 22 isolates) (8).

Given the paucity of data on *C. difficile* in Malaysia, particularly its molecular epidemiology, the aims of the present study were to describe the prevalence of CDI among inpatients of hospitals spread across Kuala Lumpur and Kota Bharu and to determine the RTs of *C. difficile* strains isolated.

MATERIALS AND METHODS

Study setting. The study was conducted in Kuala Lumpur, Sungai Buloh, Selangor, and in Kota Bharu, Kelantan, across four hospitals: Universiti Kebangsaan Malaysia Medical Centre (site 1), Kuala Lumpur, 1,040 beds; Hospital Sungai Buloh (site 2), Sungai Buloh, Selangor, 620 beds; University Malaya Medical Centre (site 3), Kuala Lumpur, 1,300 beds; and Hospital Universiti Sains Malaysia (site 4), Kota Bharu, 760 beds. Selangor has a large population (6.4 million) of mixed ethnic background, including Malay, Chinese, and Indian descent, while Kelantan has a smaller population (1.8 million) with a majority Malay population (dosm.gov.my). Ethical approval to conduct the study was received from each relevant institutional review board.

Sample collection and transport. Stool specimens from inpatients aged 18 to 80 years experiencing diarrhea (at least three episodes of loose or watery stool in 24 h), with a request for *C. difficile* testing or with a clinical history indicating antibiotic-associated diarrhea, were collected at each site over the following time periods: site 1, October 2015 to January 2016; site 2, December 2015 to July 2016; site 3, April to July 2016; and site 4, July 2015 to February 2016. Each specimen was tested with enzyme immunoassay (EIA) for glutamate dehydrogenase (GDH) and toxin A/B using C. diff Quik Chek Complete (TechLab, Blacksburg, VA). Specimens were then stored at −20°C for up to 18 weeks before being sent to a reference laboratory in Western Australia on transport swabs in Cary-Blair medium (Medical Wire and Equipment Co. Ltd., England) at 4°C for culture and molecular analysis.

Detection of *C. difficile*. Specimens were cultured directly on ChromID *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) and incubated at 35°C for 48 h in an A35 anaerobic chamber (Don Whitley Scientific, Ltd., Shipley, West Yorkshire, United Kingdom) in an atmosphere containing 80% nitrogen, 10% hydrogen, and 10% carbon dioxide at 75% relative humidity. Indirect culture was performed by enrichment of specimens in Robertson's cooked meat medium containing 5 mg/liter gentamicin, 250 mg/liter cycloserine, and 8 mg/liter cefoxitin (PathWest Laboratory Medicine Excel Media, Mount Claremont, Western Australia, Australia) with aerobic incubation at 37°C for 4 to 7 days, followed by alcohol shock and subculture on ChromID *C. difficile* agar. Putative *C. difficile* colonies were confirmed by characteristic odor, morphology, and chartreuse fluorescence on blood agar and by the L-proline aminopeptidase Diatabs (Rosco Diagnostica, Taastrup, Denmark) reaction.

Toxin gene detection and ribotyping. DNA was extracted from pure cultures on blood agar plates, and PCR ribotyping was performed using primers and conditions as described by O'Neill and coworkers (15). Toxin genes *tcdA*, *tcdB*, *cdtA*, and *cdtB* were detected by PCR as previously described (5, 16). PCR ribotyping products were analyzed on the QIAxcel capillary gel electrophoresis platform (Qiagen, Venlo, Limburg, The Netherlands). The resulting densitometric curve profiles were compared to profiles of a reference collection by cluster analysis using BioNumerics v.7.6 (Applied Maths, Saint-Martens-Latem, Belgium). RTs were assigned according to standard international typing numbers (17) or otherwise designated with internal nomenclature prefixed with "QX." The sensitivity, specificity, positive predictive,

TABLE 1 Results of EIA and culture analysis in inpatients with diarrhea at Kuala Lumpur and Kota Bharu

EIA result	Culture	Toxin profile	No. (%) of positive results at various sites[a]				
			Site 1 (n = 162)	Site 2 (n = 111)	Site 3 (n = 98)	Site 4 (n = 66)	Total (n = 437)
GDH$^+$/toxin$^+$	Positive	A$^+$B$^+$CDT$^-$	6 (3.7)	0	0	2 (3.0)	8 (1.8)
		A$^-$B$^+$CDT$^-$	4 (2.5)	0	0	1 (1.5)	5 (1.1)
		A$^-$B$^-$CDT$^-$	0	0	0	0	0
	Negative		0	0	0	0	0
GDH$^+$/toxin$^-$	Positive	A$^+$B$^+$CDT$^-$	5 (3.1)*	2 (1.8)	3 (3.1)	3 (4.5)**	13 (3.0)
		A$^-$B$^+$CDT$^-$	5 (3.1)*	4 (3.6)	0	0	9 (2.1)
		A$^-$B$^-$CDT$^-$	12 (7.4)	4 (3.6)	16 (16.3)	9 (13.6)**	41 (9.4)
	Negative		6 (3.7)	2 (1.8)	0	2 (3.0)	10 (2.3)
Negative	Positive	A$^+$B$^+$CDT$^-$	1 (0.6)	0	1 (1.0)	3 (4.5)	5 (1.1)
		A$^-$B$^+$CDT$^-$	1 (0.6)	4 (3.6)	0	1 (1.5)	6 (1.4)
		A$^-$B$^-$CDT$^-$	3 (1.9)	0	6 (6.1)	4 (6.1)	13 (3.0)
	Negative		120 (74.0)	95 (85.6)	72 (73.5)	42 (63.6)	329 (75.3)
Overall prevalence							
Toxigenic strains			21 (13.0)	10 (9.0)	4 (4.1)	10 (15.1)	45 (10.3)
Nontoxigenic strains			15 (9.3)	4 (3.6)	22 (22.4)	13 (19.7)	54 (12.4)

[a]Sites: 1, UKM Medical Centre, Kuala Lumpur, 1,040 beds; 2, Hospital Sungai Buloh, Sungai Buloh, Selangor, 620 beds; 3, University Malaya Medical Centre, Kuala Lumpur, 1,300 beds; 4, Hospital Universiti Sains Malaysia, Kota Bharu, 760 beds. *, one sample yielded one A$^+$B$^+$ and 1 A$^-$B$^+$ isolate; **, one sample yielded one A$^+$B$^+$ and one A$^-$B$^-$ isolate.

and negative predictive values of the Quik Chek Complete tests for the detection of GDH were calculated using direct culture as the reference standard.

RESULTS

A total of 437 nonrepeat samples were collected, from which 100 (22.9%) unique *C. difficile* strains were isolated. EIAs were GDH-positive/toxin-positive in 13 (3.0%) specimens, all of which yielded a toxigenic *C. difficile* strain by culture. The prevalence of toxin-positive specimens was higher in site 1 (6.9%) than site 4 (4.5%), whereas toxin-positive specimens were not detected in sites 2 and 3. Another 71 (16.2%) specimens were GDH positive/toxin negative; toxigenic strains were isolated from 21 (4.8% overall) of these samples, nontoxigenic strains were isolated from 41 (9.2%; one sample yielded one toxigenic and one nontoxigenic strain, another had two distinct toxigenic strains isolated), while 10 (2.3%) were culture negative. A further 11 toxigenic and 13 nontoxigenic isolates were cultured from GDH-negative specimens (Table 1), giving an overall prevalence of toxigenic strains of 45/437 (10.3%), and a prevalence of 54/437 (12.4%) for nontoxigenic strains. The prevalence of toxigenic and nontoxigenic strains varied between sites, from 4.1 to 15.1% (sites 3 and 4, respectively) for toxigenic strains and 3.6 to 22.4% (sites 2 and 3, respectively) for nontoxigenic strains (Table 1). Among the 100 isolates, nine resulted from enrichment culture only, from three GDH-positive/toxin-negative and six GDH-negative specimens. Five of these nine isolates were toxigenic, all from GDH-negative specimens.

Overall, the most common toxigenic strain was RT 017 (A$^-$B$^+$, n = 20, 20.0% of isolates), followed by RT 043 (A$^+$B$^+$, n = 10, 10.0%; described as QX 001 in some previous publications [11, 18]), RT 053, QX 026, and RT 014/020 (all A$^+$B$^+$, n = 4, 4.0%). QX 002 was the most common nontoxigenic strain (n = 8, 8.0%), followed by QX 021 (n = 5, 5.0%) and RTs 009, 010, and 039 (all n = 4, 4.0%). The remaining strains represented 32 different RTs. No CDT$^+$ strains were identified. Among the 15 toxin-positive specimens, RT 017 was most common (n = 5), followed by QX 026 (n = 4) and then RTs 043 and 053 (each n = 3, Table 2). RT distributions varied somewhat across sites; RT 017 was the most common type at sites 1 (27.5% of isolates) and 2 (57.1%), QX 021 and RT 010 were the most common types at site 3 (11.5% each), while RT 043 was most common in site 4 (21.7%). RT 017 was not isolated at site 3.

The sensitivity, specificity, positive predictive value, and negative predictive value of GDH for detection of *C. difficile* by Quik Chek Complete were 75.5% (95% confidence

TABLE 2 Molecular types of Malaysian *C. difficile* isolates, collected in Kuala Lumpur, Sungai Buloh, and Kota Bharu, between July 2015 and August 2016

Toxin gene profile and specific ribotype	No. (%) of isolates
$A^-B^+CDT^-$	
RT 017	20 (20.0)
$A^+B^+CDT^-$	
RT 043	10 (10.0)
RT 053	3 (3.0)
QX 026	3 (3.0)
RT 014/020	3 (3.0)
RT 001	1 (1.0)
QX 005	1 (1.0)
QX 068	1 (1.0)
QX 079	1 (1.0)
QX 103	1 (1.0)
Other	1 (1.0)
$A^-B^-CDT^-$	
QX 002	8 (8.0)
QX 021	5 (5.0)
RT 009	4 (4.0)
RT 010	4 (4.0)
RT 039	4 (4.0)
QX 011	2 (2.0)
QX 083	2 (2.0)
QX 327	2 (2.0)
QX 631	2 (2.0)
QX 633	2 (2.0)
QX 077	1 (1.0)
QX 138	1 (1.0)
QX 140	1 (1.0)
QX 238	1 (1.0)
QX 362	1 (1.0)
QX 380	1 (1.0)
QX 541	1 (1.0)
QX 553	1 (1.0)
QX 562	1 (1.0)
QX 602	1 (1.0)
QX 632	1 (1.0)
Others	9 (9.0)

interval [CI] = 66 to 84%), 97.1% (95% CI = 95 to 99%), 88.1% (95% CI = 79 to 94%), and 93.2% (95% CI = 90 to 96%), respectively.

DISCUSSION

The significance of *C. difficile* and CDI in Asia is still poorly understood (9). The prevalence of toxins A and B (3.0%) was lower than in previous studies in Malaysia, where the reported prevalence was 6 to 14% (8, 13, 14), and in Indonesia, where the reported prevalence was 5.6% (12). In Singapore, prevalence of toxins A and B was lower at 2.7% among 973 inpatients tested in 2013 (19). However, the prevalence of both toxigenic (10.3%) and nontoxigenic (12.4%) *C. difficile* identified in Malaysia was high and comparable to the neighboring Southeast Asian countries Thailand (9.2% toxigenic, 15.6% nontoxigenic) (11), Indonesia (10.9% toxigenic, 10.6% nontoxigenic) (12), and Singapore (8.9% toxigenic) (19), and higher than the reported prevalence rates in Australia (6.4 to 7.2%) (20, 21) and in Europe (6.0% in Spain in 2015) (22). However, the inclusion criteria for patients varied over these studies, which could account for the differences in the reported prevalences.

These high prevalence rates of toxigenic and nontoxigenic strains suggest there is a high rate of *C. difficile* colonization in general in Southeast Asia, given that presence of a toxigenic strain does not necessarily confirm the presence of CDI, and the prevalence of toxin was considerably lower than the prevalence of a toxigenic strain in the present study (3.0% versus 10.3%). However, it is interesting that the only Southeast

Asian study of *C. difficile* colonization in community members, performed previously in Kota Bharu, detected a low prevalence of colonization of 2% among 138 elderly community participants, with isolation of nontoxigenic strains only. There may be a local issue of increased contamination of hospitals with *C. difficile* spores which would contribute to the high rates of *C. difficile* colonization seen here and elsewhere in Southeast Asia. Our results show wide variation in prevalence of toxigenic versus nontoxigenic strains across the sites in the study (Table 1), which could be due to differences in cleaning and disinfection practices, in local animal or environmental reservoirs of *C. difficile*, in characteristics of patient populations, in antimicrobial stewardship practices, or a combination of these factors. Information on comorbidities and recent medications was not collected for study participants, so we were unable to explore these ideas further.

The molecular epidemiological data reported here broaden our overview of the strains of *C. difficile* circulating around greater Southeast Asia. The strains of toxigenic *C. difficile* circulating in Malaysia were similar to those in Indonesia, where RT 017 comprised 24.3% of isolates, and RTs 053 (4.1%), 014/020 (2.7%), and 043 (2.7%) were also among the eight most common RTs. In the Indonesian study, the most prevalent nontoxigenic strains differed from the present study, with QX 002, QX 021, RT 009, RT 010, and RT 039 not reported (12). In Thailand, RT 017 and RT 014/020 were the most prevalent toxigenic strains (11, 18), and the nontoxigenic strains circulating in Thailand were more similar to those in the present study, with RT 010, RT 009, RT 039, and QX 002 being the most common (11). In Singapore, only toxigenic strains have been described in detail, showing a greater predominance of RTs 053 and 012, with the most common strains also including RTs 014/020, 043, and 017 (19, 23).

Many nontoxigenic strains of *C. difficile*, together with RT 017, are placed in clade 4 of the five main phylogenetic clades of *C. difficile* (24). Given the high prevalence of both nontoxigenic and RT 017 strains in this study, and others in Thailand (11) and Indonesia (12), it is likely that clade 4 of *C. difficile* has evolved in the Asian region in contrast to a recent report suggesting that RT 017 *C. difficile* evolved in North America (25). However, little is known about the prevalence of nontoxigenic strains in other regions of the world, with a publication bias toward toxigenic strains because they cause infection. While more research is required to determine whether the high prevalence of nontoxigenic *C. difficile* is unique to Asia, clinical studies may shed some light on a possible protective role of nontoxigenic strains in Southeast Asia. *C. difficile* RT 017 is, however, a strain of international importance, having caused significant outbreaks in North America and Europe previously (26, 27), with enhanced virulence and clindamycin and fluoroquinolone resistance. It is interesting that outbreaks of severe CDI are not reported from Asia more frequently given the high prevalence of RT 017 and frequent overuse of antibiotics. Again, this may be due to a protective role of nontoxigenic *C. difficile* in the region.

Therapeutic administration of nontoxigenic *C. difficile* in patients receiving treatment for CDI reduces their risk of recurrent infection (28), showing that colonization with nontoxigenic strains can prevent infection with toxigenic strains. Anecdotal reports and a recent multicountry study in Asia (29) indicate that CDI rarely has severe outcomes in Southeast Asia and generally presents as self-limiting diarrhea and that recurrence is rare. It is plausible that the high prevalence of nontoxigenic strains in the region could contribute to low recurrence rates and apparently milder outcomes of CDI. The self-limiting nature of CDI in the region would also explain why there is poor awareness of CDI among local physicians. A study in the Philippines demonstrated that CDI was frequently misdiagnosed as amoebic colitis, given that the signs and symptoms of infection are similar and that both can be treated successfully with metronidazole (30).

The present study has enhanced our understanding of the molecular epidemiology of *C. difficile* in Southeast Asia. However, data on the clinical characteristics of CDI in the region are still scarce. Further studies on the molecular and clinical epidemiology of CDI

in Southeast Asia are needed to determine any role nontoxigenic strains may play in reducing the burden of CDI in the region.

ACKNOWLEDGMENTS

Some consumables and logistical support were kindly supplied by Alere Pty Ltd. We especially thank Yunnie Tan for assistance with this project.

T.V.R. has received grants from Merck and Otsuka outside the present work.

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