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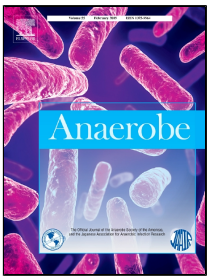

High prevalence and diversity of *tcdA*-negative and *tcdB*-positive, and non-toxigenic, *Clostridium difficile* in Thailand

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High prevalence and diversity of *tcdA*-negative and *tcdB*-positive, and non-toxigenic, *Clostridium difficile* in Thailand

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Abstract

Studies on the prevalence and diversity of *Clostridium difficile* in Thailand have been limited to those derived from a few tertiary hospitals in Central Thailand. In this study, 145 *C. difficile* isolates collected in 13 provinces in Thailand during 2006-2018 were characterized by ribotyping and detection of toxin genes. Minimum inhibitory concentrations of eight antimicrobial agents were determined also for all 100 *C. difficile* strains collected from 2006 until 2015. Of the 145 strains of *C. difficile*, 71 (49%) were non-toxigenic, 46 (32%) were toxin A-negative, toxin B-positive (A-B+) and 28 (19%) were A+B+. No binary toxin-positive strain was found. The most common ribotype (RT) was RT 017 (A-B+CDT-, 19%, 28/145). Besides RT 017, 20 novel non-toxigenic and A-B+ ribotyping profiles, which may be related to RT 017 by the similarity of ribotyping profile, were identified. All *C. difficile* strains remained susceptible to metronidazole and vancomycin, however, a slight increase in MIC for metronidazole was seen in both toxigenic and non-toxigenic strains (overall $MIC_{50/90}$ 0.25/0.25 mg/L during 2006 – 2010 compared to overall $MIC_{50/90}$ 1.0/2.0 mg/L during 2011 – 2015). There was a high rate of fluoroquinolone resistance among RT 017 strains (77%), but there was little resistance among non-toxigenic strains. These results suggest that RT 017 is endemic in Thailand, and that the misuse of fluoroquinolones may lead to outbreaks of RT 017 infection in this country. Further studies on non-toxigenic *C. difficile* are needed to understand whether they have a role in the pathogenesis of *C. difficile* infection in Asia.

Keywords: *Clostridium difficile*; Thailand; ribotype 017; epidemiology

Introduction

Clostridium difficile is an important cause of hospital-associated diarrhea with symptoms ranging from self-limiting watery diarrhea to life-threatening colitis (1, 2). *C. difficile* infection (CDI) occurs when the stability of intestinal microbiota is disturbed, often by exposure to antimicrobial agents, allowing *C. difficile* spores to germinate, multiply and eventually cause disease (1). CDI is mediated mainly by toxins and, to date, three toxins have been identified as the major virulence factors: toxin A (TcdA), toxin B (TcdB) and binary toxin (CDT) (3, 4).

C. difficile can be classified into different ribotypes (RTs) based on the banding patterns produced by PCR of the intergenic spacer region between the 16S and 23S rRNA genes (5). Previous studies in Bangkok, Thailand (6, 7), reported a high prevalence of *C. difficile* RT 017, a toxigenic strain of *C. difficile* with a deletion in the repeating region of the *tcdA* gene, resulting in a non-functional toxin A (A-B+CDT- *C. difficile*) (8). Another recent study found a high prevalence of A-B+CDT- *C. difficile* in two different regions of Thailand, but ribotyping was not performed (9).

C. difficile RT 017 has been associated with high rates of antimicrobial resistance, which may have facilitated its successful spread globally and promoted outbreaks in the past (10-14). In Thailand, RT 017 has also been associated with an accumulation of antimicrobial resistance in addition to reports of multidrug-resistant non-toxigenic *C. difficile* strains which may serve as a reservoir for accessory resistance genes (15).

Previously, knowledge of *C. difficile* in Thailand was limited to a few major hospitals in Bangkok. This study aimed to describe the strains of *C. difficile* found in other parts of the country. The National Institute of Health, Department of Medical Sciences, Ministry of

Public Health, Thailand (TH-NIH) is a reference laboratory that receives faecal specimens for *C. difficile* culture from medical centres, and secondary and tertiary hospitals, in various regions of Thailand. The TH-NIH has the largest collection of *C. difficile* strains in Thailand. Using this collection, which included some strains from a previous study that had not been ribotyped (9), we hoped to report on the diversity of *C. difficile* throughout Thailand.

Materials and Methods

Study sites and *C. difficile* isolates

C. difficile strains used in this study were cultured at the TH-NIH. When requested for *C. difficile* culture, a faecal specimen was collected and sent to the TH-NIH at 4 °C within 24 h. Upon arrival, specimens were treated with absolute ethanol at a 1:1 ratio for 1 h before inoculation onto Wilkins-Chalgren blood agar (CM 619B; Oxoid England). All agar plates were incubated anaerobically at 37 °C for 48 h and putative *C. difficile* colonies tested for phenotypic properties consistent with *C. difficile* (glucose and fructose fermentation, the presence of gelatinase and the positive result on esculin hydrolysis test). *C. difficile* ATCC 43255 (A+B+CDT-, RT 087) was used as a control strain. For long-term storage, all *C. difficile* strains were suspended in 2 mL of 15 % glycerol broth and kept at - 70 °C. A total of 145 *C. difficile* strains from clinical specimens processed from January 2006 to January 2018 were available in the TH-NIH collection. The strains were originally from the regions highlighted in Figure 1. For this study, *C. difficile* strains were inoculated on Wilkins-Chalgren blood agar and incubated anaerobically at 37 °C for 48 h before DNA extraction. DNA from all *C. difficile* strains was extracted using the NucleoSpin® tissue extraction kit (Macherey-Nagel, Germany). PCR for the *tpi* gene was initially done on all DNA samples to ensure the adequate quality of DNA using primers published by Lemee *et al.* (16). All DNA samples were

86 further sent to a reference laboratory in Western Australia for toxin gene detection and PCR
87 ribotyping.

88 Toxin gene detection and PCR ribotyping

89 At the reference laboratory, all strains were tested for the presence of toxin A (*tcdA*),
90 toxin B (*tcdB*) and binary toxin (*cdtA* and *cdtB*) genes, and PCR ribotyped, as previously
91 described (5, 17-19). Ribotypes were identified by comparison of the band profiles with our
92 reference library, that consisted of a collection of >50 ribotypes that included 15 reference
93 strains from the European Centre for Disease Prevention and Control and the most
94 prevalent PCR ribotypes currently circulating in Australia (T. V. Riley *et al*., unpublished
95 data). Strains that did not match the reference strains but had been previously described in
96 our laboratory were given the internal nomenclature prefix"QX". Novel strains found in this
97 study were given the prefix "KI".

98 Antimicrobial susceptibility testing

99 Antimicrobial susceptibility testing (AST) results were retrieved from the TH-NIH
100 database. Minimal inhibitory concentrations (MICs) for eight antimicrobial agents
101 (vancomycin, metronidazole, clindamycin, moxifloxacin, levofloxacin, tetracycline,
102 erythromycin and chloramphenicol) were determined for all 100 *C. difficile* strains isolated
103 during 2006 – 2015 using the agar dilution method as recommended by the Clinical and
104 Laboratory Standard Institute (CLSI) (20). MIC breakpoints for metronidazole and
105 vancomycin were based on the European Committee on Antimicrobial Susceptibility Testing
106 (EUCAST) recommendations (21). MIC breakpoints for clindamycin, moxifloxacin,
107 tetracycline and chloramphenicol were based on CLSI recommendations (20) while the MIC
108 breakpoint for erythromycin was based on a previous publication (15). There were no

available MIC breakpoints for levofloxacin and so MIC_{50} and MIC_{90} values for this antimicrobial were used to compare the susceptibility of *C. diff*icile strains.

Results

High prevalence of non-toxigenic and A-B+CDT- *C. difficile* in Thailand

Table 1 summarises ribotypes and toxin gene profiles of *C. difficile* strains investigated in this study. Seventy-one strains (49%) were negative for all three toxin genes by PCR (A-B-CDT-). Among the 74 toxigenic strains, 46 (62%) were A-B+CDT- *C. difficile* while 28 (38%) were A+B+CDT- *C. difficile*. No binary toxin-positive *C. difficile* was found in this study. Table 2 shows the different distribution of common RTs in different regions of Thailand.

Forty different *C. difficile* RTs were found in this study (Table 1). Only five RTs matched reference strains from the ECDC, seven had been previously described in our laboratory reference database (nomenclature "QX") and 28 had not been previously described (nomenclature "KI"). The most common RT was *C. difficile* RT 017 (A-B+CDT-) [19%; 28/145], followed by the non-toxigenic RTs QX 578 [12%; 18/145], RT QX 500 [9%; 13/145] and RT QX 021 [8%; 11/145]. The most common A+B+CDT- *C. difficile* belonged to RT 014/020 [7%; 10/145].

Besides *C. difficile* RT 017, 13 new A-B+CDT- RTs were identified in this study. All A-B+CDT- *C. difficile* strains in this study were positive for the non-repeating region of *tcdA* gene but negative for the repeating region of *tcdA* gene (Figure 2).

Different *C. difficile* strains had very similar ribotyping pattern

Many novel RTs had ribotyping patterns similar to the RTs previously described. Among these, several *C. difficile* strains had similar patterns to RT QX 578 (A-B-CDT-) which are demonstrated in Figure 3. However, these strains of *C. difficile* had different toxin profiles (1 A+B+CDT- strain, 3 A-B+CDT- strains and 2 non-toxigenic strains). Besides these RTs, five RTs were similar to RT QX 108 and two RTs were similar to RT 017.

All *C. difficile* remained susceptible to metronidazole and vancomycin

Overall MIC and susceptibility data of 100 *C. difficile* strains from Thailand is shown in Table 3. All *C. difficile* were susceptible to metronidazole and vancomycin. Ninety-one *C. difficile* strains were resistant to clindamycin, while 31 were resistant to moxifloxacin, 28 of which also had high MIC levels (≥ 64 mg/L) of levofloxacin. Most strains remained susceptible to tetracycline and chloramphenicol (82% and 88%, respectively). MIC_{50} and MIC_{90} for eight antimicrobials against *C. difficile* by different toxin gene profiles are shown in Table 4. A-B+CDT- *C. difficile* had higher MIC_{50} values for clindamycin, moxifloxacin and levofloxacin compared to other *C. difficile* strains.

Fewer *C. difficile* RT 017 strains were susceptible to fluoroquinolones and tetracycline

Susceptibility profiles of common *C. difficile* RTs are shown in Table 5. A smaller proportion of *C. difficile* RT 017 strains was susceptible to moxifloxacin compared to other RTs (23% vs 72%; Chi-square $p < 0.001$). This RT also had higher MIC_{50} and MIC_{90} values for levofloxacin (128 mg/L and 128 mg/L, respectively) compared to other RTs (4 mg/L and 128 mg/L, respectively). *C. difficile* RT 017 also appeared to be less susceptible to tetracycline (55% vs 90%; Chi-square $p < 0.001$).

Discussion

The high prevalence of *C. difficile* RT 017 in this study is supported previous publications suggesting that RT 017 is endemic in South East Asia. *C. difficile* RT 017 has been reported as the most prevalent toxigenic RT of *C. difficile* in other South East Asian countries such as Indonesia and Malaysia (22-24) and is commonly found in China (25) and South Korea (26). In two previous studies in Thailand, RT 017 was also among the most prevalent toxigenic strains (6, 7). Besides the high prevalence of RT 017, we also found 13 novel toxigenic *C. difficile* RTs with a deletion in the repeating region of *tcdA* gene (A-B+CDT-). This characteristic has been described previously in *C. difficile* closely related to RT 017 (27-31), suggesting that these novel RTs may also be related to RT 017. A whole genome sequence (WGS) analysis will be needed to confirm the relatedness of new RT 017-like isolates to RT 017.

C. difficile RT 017 belongs to the multilocus sequence type (MLST) clade 4 of *C. difficile* (28). It is believed that *C. difficile* diverged into different clades millions of years ago and prior to the acquisition of the pathogenicity locus (PaLoc) region of the genome that contains the genes for toxins A and B. PaLoc acquisition has subsequently occurred at different times in different geographical areas of the world (28). Clade 4 consists mainly of non-toxigenic and A-B+CDT- *C. difficile* strains (27-31), similar to what was found in the present study and several previous studies of *C. difficile* in South East Asia (7, 22-24). These consistent findings of *C. difficile* RT 017 endemicity in South East Asia and the significant diversity of other clade 4 strains implies that the origin of RT 017 and clade 4 is in this region and not North America as reported by Cairns *et al.* (32). In order to confirm that these

C. difficile strains are related to RT 017 and belong in the same clade, MLST will be required following WGS.

PCR ribotyping has been used globally in prevalence studies to determine strain relatedness due to its simplicity (33). It is also used in Europe for CDI surveillance purposes (34), however, its limitations should be noted (35, 36). This study reports several novel *C. difficile* RTs with similar ribotyping patterns, as demonstrated in Figure 3. Some banding patterns were almost indistinguishable from one another highlighting the limits in discriminatory power of this method. Also, similar banding patterns do not guarantee strain relatedness as the banding pattern relies mainly on differences in PCR amplicon size, not the actual nucleotide sequence. The use of toxin gene profiles can help further classify these strains, however, other methods, such as MLST and/or whole genome sequencing (WGS), are needed to confirm strain relatedness.

Though the number of strains from some parts of Thailand was low, some differences in the distribution of *C. difficile* can be seen (Table 2). In the Northern region, there was a higher prevalence of A+B+CDT- *C. difficile*, primarily RT 014/020, and lower prevalence of non-toxigenic strains. In the Western region, there was a lower prevalence of A-B+CDT- *C. difficile* and no RT 017 was found. Notably, the prevalence of *C. difficile* in a large tertiary hospital in Central Thailand in previous studies was different from the prevalence found in this study. The previous studies reported a high prevalence of RT 014/020 (6, 7), similar to that found in Northern Thailand in this study. Given the marked increase in the prevalence of *C. difficile* RT 014/020 between two study periods (25% during 2006 – 2008 to 44% in 2015), it is possible that there was an outbreak of RT 014/020 infection during the previous studies (6, 7). Strains from Northern Thailand in the present

196 study also came from a single tertiary hospital and the high prevalence of RT 014/020 could
197 also be due to an outbreak. Also, Chiang Rai is one of the top tourist destinations in
198 Thailand, attracting significant numbers of tourists from Europe, and this may have
199 impacted on the appearance of *C. difficile* RT 014/020 in the community
200 (https://www.mots.go.th/mots_en57/more_news.php?cid=332&filename=index).

201 Though the high prevalence of non-toxigenic *C. difficile* was consistent with previous
202 studies, the RTs and antimicrobial susceptibility patterns were different (15). The most
203 common non-toxigenic *C. difficile* strains in a previous study belonged to RTs 010 (11%;
204 12/105), 039 (9%; 9/105) and 009 (6%; 6/105). Only one RT 010 and no RTs 039 and 009
205 were identified in this study, and the three most common non-toxigenic RTs had rarely been
206 isolated in the previous studies. Only one RT QX 578 had been isolated in the Southern part
207 of Thailand in 2014 (Collins DA, unpublished data), RT QX 021 was previously found in 5% of
208 *C. difficile* in Malaysia (22) and no RT QX 500 had been isolated in South East Asia before. It
209 appears that the population of non-toxigenic *C. difficile* in South East Asia is more diverse
210 than previously thought. Given that non-toxigenic *C. difficile* may have a protective role
211 against the development of CDI which could result in a lower prevalence and severity of
212 disease in the region (37-39), further studies should be conducted on these non-toxigenic
213 strains.

214 The non-toxigenic *C. difficile* isolated in this study also had low rates of antimicrobial
215 resistance in contrast to previous reports (15). This could be due to the difference in the
216 time when *C. difficile* strains were isolated. The AST data in this study was from *C. difficile*
217 strains isolated from 2006 – 2015, while a previous study reported AST data on *C. difficile*
218 isolated during 2015 (15). This difference in time-frames may have contributed to the

219 apparent differences in susceptibility pattern. However, with the exception of
220 metronidazole, the strains of *C. difficile* isolated from 2011 – 2015 in this study were not less
221 susceptible than strains from 2006 – 2010 as shown by a comparison of MIC_{50} and MIC_{90}
222 data in Table 6. Another possible explanation is the difference in the study sites. *C. difficile*
223 strains in this study came from different levels of healthcare facilities where patients may
224 have been exposed to fewer antimicrobial agents. The previous study (15) was conducted in
225 a large tertiary hospital in Bangkok where there is a higher rate of antimicrobial use.

226 While all *C. difficile* in this study remained susceptible to metronidazole and
227 vancomycin, similar to the earlier study (15), there did appear to be some creep in
228 metronidazole MICs similar to that described by Baines *et al.* (40). How this increase in
229 metronidazole MIC may affect the outcome of CDI treatment in Thailand is unknown.
230 However, despite updates in the international guideline for the treatment of CDI (41),
231 metronidazole and vancomycin remain the treatment of choice in Thailand. The most recent
232 clinical study of CDI in Thailand was conducted in 2008 and most patients responded well to
233 either metronidazole or vancomycin with low mortality and recurrence (37). It should also
234 be noted that the metronidazole MICs remained low in the previous study, while there was
235 a slight increase in vancomycin MICs, suggesting that different treatment regimens may
236 have been used in different hospitals (15). MICs of fidaxomicin were not determined as the
237 drug is not available in Thailand, however, since fidaxomicin has not been used previously, it
238 is likely that *C. difficile* in Thailand remain susceptible to this agent. This was shown in the
239 previous study which reported low MIC (0.004 – 0.25 mg/L) of fidaxomicin in *C. difficile*
240 strains from Bangkok, Thailand in 2015 (15).

The rates of resistance to clindamycin, erythromycin and moxifloxacin in this study were similar to the previous study in Thailand (15). Most fluoroquinolone-resistant strains belonged to *C. difficile* RT 017, which is also consistent with earlier reports in several countries (12, 13, 15, 42). For many years, antimicrobial use in Thailand has been loosely regulated (43), and the misuse of antimicrobials, especially fluoroquinolones in diarrhoeal patients, is common (44). Fluoroquinolone resistance provides RT 017 with a survival advantage and this was a factor that led to an outbreak of CDI caused by RT 017 in Ireland (45, 46). This could also happen in Thailand given that no regulation in antimicrobial usage has been implemented (47).

A limitation of this study is that all *C. difficile* strains were from an existing collection. The TH-NIH is a reference laboratory which does not have any records of patients' information or other diagnostic tests which may have been done in the referring hospital. Thus, it was impossible to tell whether the strains were from patients with CDI or asymptomatic carriers. However, the aim of this study was to describe the diversity of *C. difficile* strains in Thailand, not the prevalence of CDI in the country nor the characteristics of patients. Since the TH-NIH had the largest collection of *C. difficile* from various locations, it was suitable for this study.

Conclusion

There was a high prevalence of non-toxigenic *C. difficile* and *C. difficile* RT 017 (A-B+CDT-) in Thailand, similar to many countries in South East Asia, suggesting that these *C. difficile* strains are endemic to the region. There were also high rates of antimicrobial resistance among Thai toxigenic *C. difficile* strains, especially fluoroquinolone resistance in

263 RT 017. Given the common misuse of fluoroquinolone in Thailand, there is a significant risk
264 that an outbreak of CDI caused by RT 017 will occur in this country.

265

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269 Thailand.

270

271 **Conflict of Interest**

272 TVR received grants from Cepheid, Merck, Sanofi and Otsuka during the conduct of
273 the study. Other authors declare no conflicts of interest relevant to this article.

References

1. Leffler DA, Lamont JT. *Clostridium difficile* Infection. N Engl J Med. 2015;373(3):287-8.
2. Barbut F, Petit JC. Epidemiology of *Clostridium difficile*-associated infections. Clin Microbiol Infect. 2001;7(8):405-10.
3. Chumbler NM, Rutherford SA, Zhang Z, Farrow MA, Lisher JP, Farquhar E, et al. Crystal structure of *Clostridium difficile* toxin A. Nat Microbiol. 2016;1:15002.
4. Bauer MP, Notermans DW, van Benthem BH, Brazier JS, Wilcox MH, Rupnik M, et al. *Clostridium difficile* infection in Europe: a hospital-based survey. Lancet. 2011;377(9759):63-73.
5. Stubbs SL, Brazier JS, O'Neill GL, Duerden BI. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. J Clin Microbiol. 1999;37(2):461-3.
6. Ngamskulrungroj P, Sanmee S, Putsathit P, Piewngam P, Elliott B, Riley TV, et al. Molecular epidemiology of *Clostridium difficile* infection in a large teaching hospital in Thailand. PLoS One. 2015;10(5):e0127026.
7. Putsathit P, Maneerattanaporn M, Piewngam P, Kiratisin P, Riley TV. Prevalence and molecular epidemiology of *Clostridium difficile* infection in Thailand. New Microbes New Infect. 2017;15:27-32.
8. Drudy D, Fanning S, Kyne L. Toxin A-negative, toxin B-positive *Clostridium difficile*. Int J Infect Dis. 2007;11(1):5-10.
9. Wangroongsarb P, Kamthalang T, Jittaprasatsin C, Cheunban N, Sriwanthana B, Sangkitporn S. Antimicrobial susceptibility and toxin production of *Clostridium difficile* isolated from diarrheal patients during 2012-2015. J Assoc Med Sci. 2017;50(2):187-96.
10. Huang H, Weintraub A, Fang H, Nord CE. Antimicrobial resistance in *Clostridium difficile*. Int J Antimicrob Agents. 2009;34(6):516-22.
11. Chow VCY, Kwong TNY, So EWM, Ho YII, Wong SH, Lai RWM, et al. Surveillance of antibiotic resistance among common *Clostridium difficile* ribotypes in Hong Kong. Sci Rep. 2017;7(1):17218.

12. Gao Q, Wu S, Huang H, Ni Y, Chen Y, Hu Y, et al. Toxin profiles, PCR ribotypes and resistance patterns of *Clostridium difficile*: a multicentre study in China, 2012-2013. Int J Antimicrob Agents. 2016;48(6):736-9.

13. Lee JH, Lee Y, Lee K, Riley TV, Kim H. The changes of PCR ribotype and antimicrobial resistance of *Clostridium difficile* in a tertiary care hospital over 10 years. J Med Microbiol. 2014;63(Pt 6):819-23.

14. Barbut F, Mastrantonio P, Delmee M, Brazier J, Kuijper E, Poxton I, et al. Prospective study of *Clostridium difficile* infections in Europe with phenotypic and genotypic characterisation of the isolates. Clin Microbiol Infect. 2007;13(11):1048-57.

15. Putsathit P, Maneerattanaporn M, Piewngam P, Knight DR, Kiratisin P, Riley TV. Antimicrobial susceptibility of *Clostridium difficile* isolated in Thailand. Antimicrob Resist Infect Control. 2017;6:58.

16. Lemee L, Dhalluin A, Testelin S, Mattrat M-A, Maillard K, Lemeland J-F, 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(12):5710-4.

17. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, et al. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J Clin Microbiol. 1998;36(8):2178-82.

18. Kato N, Ou CY, Kato H, Bartley SL, Brown VK, Dowell VR, Jr., et al. Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction. J Clin Microbiol. 1991;29(1):33-7.

19. Stubbs S, Rupnik M, Gibert M, Brazier J, Duerden B, Popoff M. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol Lett. 2000;186(2):307-12.

20. Clinical and Laboratory Standards Institute (CLSI). Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria, Approved Standard M11-A8. 8th ed. Wayne, PA: Clinical and Laboratory Standards Institute; 2012.

325 21. European Committee on Antimicrobial Susceptibility Testing. Clinical breakpoint tables,
326 version 8.1 London, United Kingdom [updated 16 May 2018. Available from:
327 http://www.eucast.org/clinical_breakpoints/.
328 22. Riley TV, Collins DA, Karunakaran R, Kahar MA, Adnan A, Hassan SA, et al. High prevalence of
329 toxigenic and non-toxigenic *Clostridium difficile* in Malaysia. J Clin Microbiol. 2018;56(6):e00170-18.
330 23. Zainul NH, Ma ZF, Besari A, Siti Asma H, Rahman RA, Collins DA, et al. Prevalence of
331 *Clostridium difficile* infection and colonization in a tertiary hospital and elderly community of North-
332 Eastern Peninsular Malaysia. Epidemiol Infect. 2017;145(14):3012-9.
333 24. Collins DA, Gasem MH, Habibie TH, Arinton IG, Hendriyanto P, Hartana AP, et al. Prevalence
334 and molecular epidemiology of *Clostridium difficile* infection in Indonesia. New Microbes New Infect.
335 2017;18:34-7.
336 25. Huang H, Weintraub A, Fang H, Wu S, Zhang Y, Nord CE. Antimicrobial susceptibility and
337 heteroresistance in Chinese *Clostridium difficile* strains. Anaerobe. 2010;16(6):633-5.
338 26. Kim J, Kim Y, Pai H. Clinical characteristics and treatment outcomes of *Clostridium difficile*
339 infections by PCR ribotype 017 and 018 strains. PLoS One. 2016;11(12):e0168849.
340 27. Chen YB, Gu SL, Wei ZQ, Shen P, Kong HS, Yang Q, et al. Molecular epidemiology of
341 *Clostridium difficile* in a tertiary hospital of China. J Med Microbiol. 2014;63(Pt 4):562-9.
342 28. Dingle KE, Elliott B, Robinson E, Griffiths D, Eyre DW, Stoesser N, et al. Evolutionary history of
343 the *Clostridium difficile* pathogenicity locus. Genome Biol Evol. 2014;6(1):36-52.
344 29. Qin J, Dai Y, Ma X, Wang Y, Gao Q, Lu H, et al. Nosocomial transmission of *Clostridium*
345 *difficile* genotype ST81 in a general teaching hospital in China traced by whole genome sequencing.
346 Sci Rep. 2017;7(1):9627.
347 30. Wang X, Cai L, Yu R, Huang W, Zong Z. ICU-Onset *Clostridium difficile* infection in a university
348 hospital in China: a prospective cohort study. PLoS One. 2014;9(11):e111735.

31. Cheng J-W, Liu C, Kudinha T, Xiao M, Yu S-Y, Yang C-X, et al. Use of matrix-assisted laser desorption ionization-time of flight mass spectrometry to identify MLST clade 4 *Clostridium difficile* isolates. Diagn Microbiol Infect Dis. 2018;92(1):19-24.

32. Cairns MD, Preston MD, Hall CL, Gerding DN, Hawkey PM, Kato H, et al. Comparative genome analysis and global phylogeny of the toxin variant *Clostridium difficile* PCR ribotype 017 reveals the evolution of two independent sublineages. J Clin Microbiol. 2017;55(3):865-76.

33. Elliott B, Androga GO, Knight DR, Riley TV. *Clostridium difficile* infection: Evolution, phylogeny and molecular epidemiology. Infect Genet Evol. 2017;49:1-11.

34. Krutova M, Kinross P, Barbut F, Hajdu A, Wilcox MH, Kuijper EJ, et al. How to: Surveillance of *Clostridium difficile* infections. Clin Microbiol Infect. 2018;24(5):469-75.

35. Tenover FC, Akerlund T, Gerding DN, Goering RV, Bostrom T, Jonsson AM, et al. Comparison of strain typing results for *Clostridium difficile* isolates from North America. J Clin Microbiol. 2011;49(5):1831-7.

36. Cohen SH, Tang YJ, Silva J, Jr. Molecular typing methods for the epidemiological identification of *Clostridium difficile* strains. Expert Rev Mol Diagn. 2001;1(1):61-70.

37. 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.

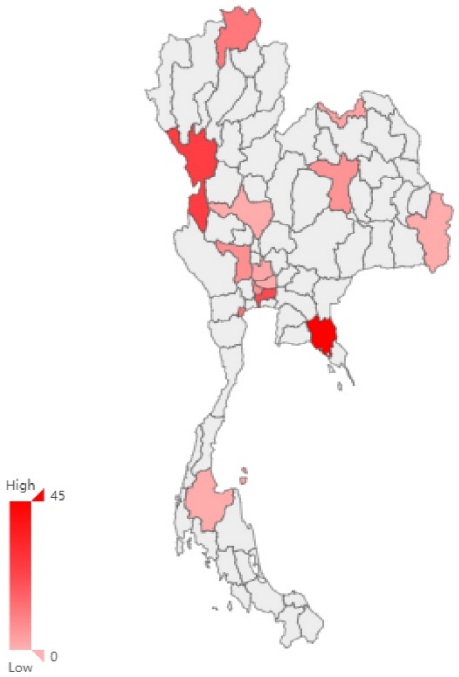
38. Oliveira Junior CA, Silveira Silva RO, Goncalves Cruz DS, Pires IH, Carvalho Guedes RM, Faria Lobato FC. The non-toxigenic strain of *Clostridioides difficile* Z31 can prevent infection by *C. difficile* in experimental model piglets. Anaerobe. 2018.

39. Gerding DN, Sambol SP, Johnson S. Non-toxigenic *Clostridioides* (Formerly *Clostridium*) *difficile* for Prevention of *C. difficile* Infection: From Bench to Bedside Back to Bench and Back to Bedside. Front Microbiol. 2018;9:1700.

373 40. Baines SD, O'Connor R, Freeman J, Fawley WN, Harmanus C, Mastrantonio P, et al.
374 Emergence of reduced susceptibility to metronidazole in *Clostridium difficile*. J Antimicrob
375 Chemother. 2008;62(5):1046-52.
376 41. McDonald LC, Gerding DN, Johnson S, Bakken JS, Carroll KC, Coffin SE, et al. Clinical Practice
377 Guidelines for *Clostridium difficile* Infection in Adults and Children: 2017 Update by the Infectious
378 Diseases Society of America (IDSA) and Society for Healthcare Epidemiology of America (SHEA). Clin
379 Infect Dis. 2018;66(7):987-94.
380 42. Kullin B, Wojno J, Abratt V, Reid SJ. Toxin A-negative toxin B-positive ribotype 017
381 *Clostridium difficile* is the dominant strain type in patients with diarrhoea attending tuberculosis
382 hospitals in Cape Town, South Africa. Eur J Clin Microbiol Infect Dis. 2017;36(1):163-75.
383 43. Aswapokee N, Vaithayapichet S, Heller RF. Pattern of antibiotic use in medical wards of a
384 university hospital, Bangkok, Thailand. Rev Infect Dis. 1990;12(1):136-41.
385 44. Supcharassaeng S, Suankratay C. Antibiotic prescription for adults with acute diarrhea at
386 King Chulalongkorn Memorial Hospital, Thailand. J Med Assoc Thai. 2011;94(5):545-50.
387 45. Drudy D, Harnedy N, Fanning S, Hannan M, Kyne L. Emergence and control of
388 fluoroquinolone-resistant, toxin A-negative, toxin B-positive *Clostridium difficile*. Infect Control Hosp
389 Epidemiol. 2007;28(8):932-40.
390 46. Drudy D, Quinn T, O'Mahony R, Kyne L, O'Gaora P, Fanning S. High-level resistance to
391 moxifloxacin and gatifloxacin associated with a novel mutation in *gyrB* in toxin-A-negative, toxin-B-
392 positive *Clostridium difficile*. J Antimicrob Chemother. 2006;58(6):1264-7.
393 47. Putsathit P, Kiratisin P, Ngamwongsatit P, Riley TV. *Clostridium difficile* infection in Thailand.
394 Int J Antimicrob Agents. 2015;45(1):1-7.

395

396 **Figures and Tables**



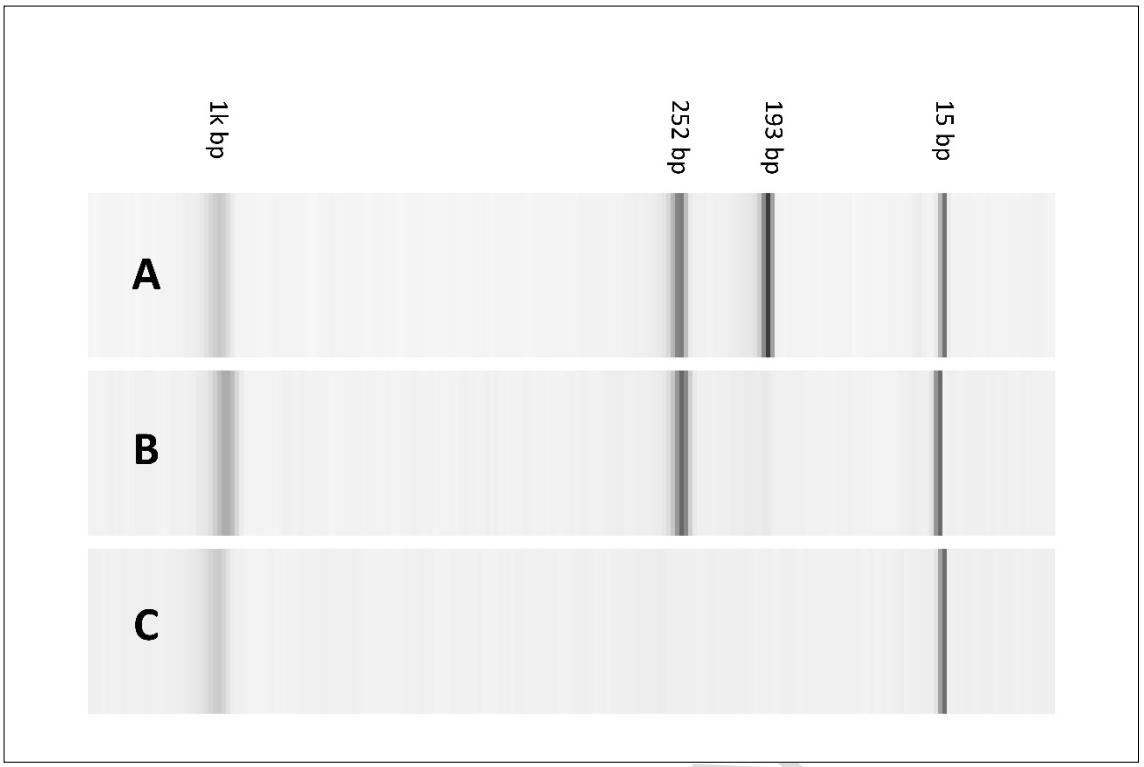
Province	Number of strains
Central Thailand	**52**
Bangkok	22
Samut Songkhram	13
Suphanburi	7
Nonthaburi	6
Ayutthaya	2
Nakhon Sawan	1
Pathum Thani	1
Northern Thailand	**12**
Chiang Rai	12
North-eastern Thailand	**9**
Khon Kaen	6
Nong Khai	3
Eastern Thailand	**45**
Chanthaburi	45
Western Thailand	**25**
Tak	25
Southern Thailand	**1**
Surat Thani	1
Total	**145**

397

398 **Figure 1 – Source of *C. difficile* strains used in this study.** Strains from Bangkok came from 7

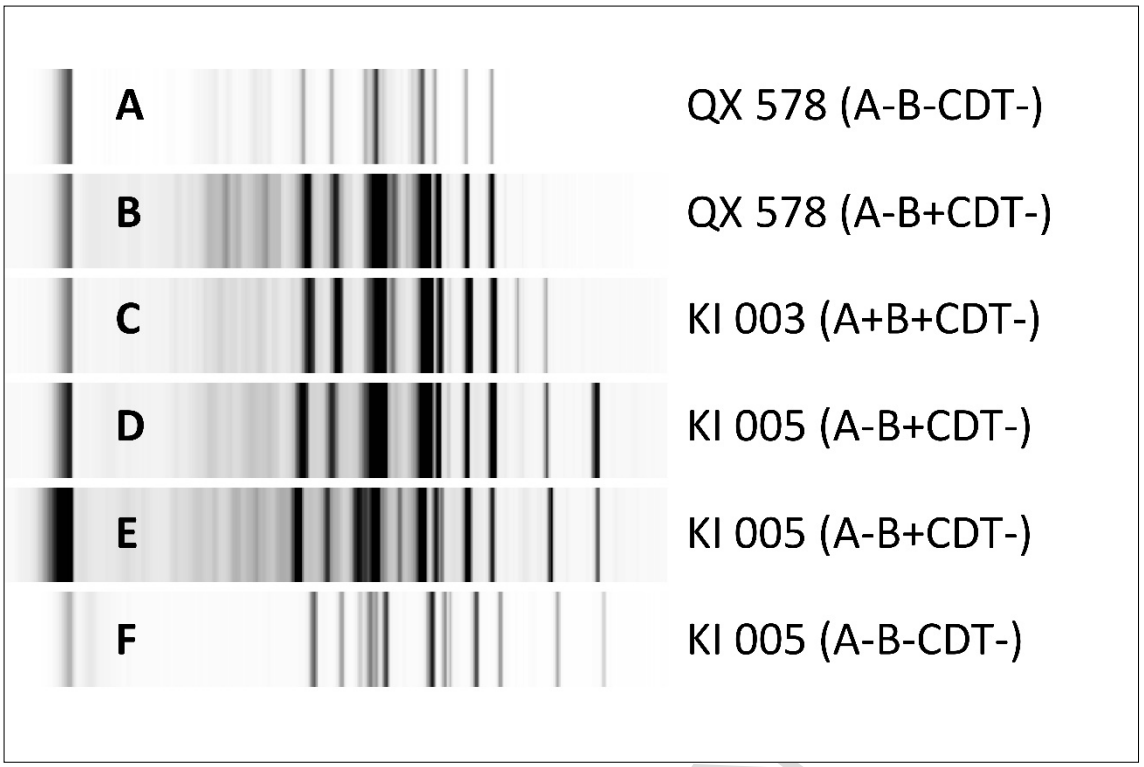
399 different healthcare facilities (number of strains: 11, 4, 2, 2, 1, 1, 1). Strains from other

400 provinces came from a single healthcare facility in each province.



401

402 **Figure 2 – PCR product of *tcdA* gene in different *C. difficile* strains.** (A) Both the non-
403 repeating region (demonstrated by a 252 bp band) and the repeating region (demonstrated
404 by 193 bp band) of *tcdA* were present in every A+B+CDT- *C. difficile* strains. (B) Only the
405 non-repeating region of *tcdA* was present in A-B+CDT- *C. difficile* strains, indicating a
406 deletion in the repeating region of *tcdA* gene. (C) Non-toxigenic *C. difficile* strains do not
407 have the *tcdA* gene.



408

409 **Figure 3 – *C. difficile* strains with similar ribotyping patterns.** (A) *C. difficile* RT QX 578 (A-B-
410 CDT-) has eight distinct bands with the mirror-image arrangement. (B) An A-B+CDT- *C.*
411 *difficile* strain was classified as RT QX 578. (C) One A+B+CDT- *C. difficile* strain had a similar
412 banding pattern to RT QX 578 (RT KI 003). (D) and (E) Two A-B+CDT- *C. difficile* strains and
413 (F) one non-toxigenic *C. difficile* strain had similar ribotyping pattern and were classified into
414 the same ribotype (RT KI 005).

415

416 Table 1 – Ribotypes and toxin gene profiles of *C. difficile* found in this study.

Ribotypes and toxin gene profiles	**Number of strains**	**Proportion %**
A+B+CDT-	**28**	**19**
014/020	10	7
QX 070	4	3
001, KI 001	2 (each)	1
046, KI 002, KI 003, KI 004, KI 007, KI 009, KI 010, KI 011, KI 012, KI 013	1 (each)	1
A-B+CDT-	**46**	**32**
017	28	19
KI 017, KI 005	3 (each)	2
KI 015, KI 020	2 (each)	1
KI 014, KI 016, KI 018, KI 019, KI 021, KI 022, QX 021*,QX 578*	1 (each)	1
A-B-CDT- (non-toxigenic)	**71**	**49**
QX 578	19	13
QX 500	13	9
QX 021	11	8
KI 026	7	5
KI 025	5	3
KI 005**, KI 023, KI 027, KI 028, QX 238	2 (each)	1
010, KI 024, KI 029, KI 030, QX 011, QX 511	1 (each)	1

417

418 Note: * One strain each of ribotypes QX 021 and QX 578 was A-B+CDT- *C. difficile*.

419 ** Three strains of ribotype KI 005 were A-B+CDT- while two were non-toxigenic.

420 Table 2 – Distribution of common *C. difficile* ribotypes in Thailand.

Ribotypes	Distribution (%)						
	Overall	**North**	**Northeast**	**West**	**Centre**	**East**	**South**
A+B+CDT-	**28 (19)**	**7 (58)**	**2 (20)**	**6 (24)**	**5 (10)**	**8 (18)**	**0 (0)**
014/020	10 (7)	5 (41)	1 (10)	-	2 (4)	2 (5)	-
Others	18 (12)	2 (17)	1 (10)	6 (24)	3 (6)	6 (13)	-
A-B+CDT-	**46 (32)**	**3 (25)**	**6 (60)**	**3 (12)**	**20 (38)**	**14 (31)**	**0 (0)**
017	28 (19)	3 (25)	1 (10)	-	16 (31)	8 (18)	-
Others	18 (12)	-	5 (50)	3 (12)	4 (7)	6 (13)	-
A-B-CDT-	**71 (49)**	**2 (17)**	**2 (20)**	**16 (64)**	**27 (52)**	**23 (51)**	**1 (100)**
QX 578	19 (13)	-	-	6 (24)	7 (13)	5 (11)	1 (100)
QX 500	13 (9)	-	1 (10)	2 (8)	5 (10)	5 (11)	-
QX 021	11 (8)	-	-	4 (16)	5 (10)	2 (5)	-
Others	28 (19)	2 (17)	1 (10)	4 (16)	10 (29)	11 (24)	-
Total	**145**	**12**	**10**	**25**	**52**	**45**	**1**

421

422 Table 3 – Antimicrobial susceptibility for 100 *C. difficile* strains isolated in Thailand from

423 2006 until 2015.

Antimicrobial	**MIC Range** mg/l	**MIC_{50}** mg/l	**MIC_{90}** mg/l	**MIC breakpoint** mg/l			**Susceptibility (%)**		
				S	**I**	**R**	**S**	**I**	**R**
Vancomycin †	0.25 – 1	0.5	0.5	≤ 2	-	> 2	100	-	0
Metronidazole †	≤0.125 – 2	0.25	2	≤ 2	-	> 2	100	-	0
Clindamycin ‡	0.5 – > 256	32	>256	≤ 2	4	≥ 8	4	5	91
Moxifloxacin ‡	1 – 32	2	32	≤ 2	4	≥ 8	61	8	31
Levofloxacin	1 – >128	4	128	-	-	-	-	-	-
Tetracycline ‡	≤0.125 – 8	2	8	≤ 4	8	> 16	82	18	0
Erythromycin §	≤0.5 – >128	2	>128	-	-	> 8	-	-	35
Chloramphenicol ‡	≤0.5 – 64	4	16	≤ 8	16	> 32	88	4	8

424

425 Note: † MIC breakpoints based on EUCAST recommendation (21)

426 ‡ MIC breakpoints based on CLSI recommendation (20)

427 § MIC breakpoint based on a previous publication (15)

428

429 Table 4 – Summary of MIC data for eight antimicrobials against Thai *C. difficile* strains by

430 their toxin gene profiles

Antimicrobials	A+B+CDT- (n = 14)		A-B+CDT- (n = 31)		A-B-CDT- (n = 55)	
	MIC_{50} mg/l	**MIC_{90}** mg/l	**MIC_{50}** mg/l	**MIC_{90}** mg/l	**MIC_{50}** mg/l	**MIC_{90}** mg/l
Vancomycin	0.5	1	0.5	0.5	0.5	0.5
Metronidazole	0.5	2	0.25	1	1	2
Clindamycin	32	> 256	256	> 256	32	> 256
Moxifloxacin	2	32	16	32	2	16
Levofloxacin	4	> 128	128	128	4	128
Tetracycline	1	8	4	8	1	4
Erythromycin	4	> 128	16	> 128	2	> 128
Chloramphenicol	8	8	4	16	4	32

431

Table 5 – Antimicrobial susceptibility rate of common *C. difficile* ribotypes in Thailand.

Ribotype (n)	**Antimicrobial**			
	Clindamycin	**Moxifloxacin**	**Tetracycline**	**Chloramphenicol**
017 (22)	14%	23%	55%	100%
Other toxigenic ribotypes (23)	0%	65%	78%	89%
QX 578 (18)	0%	79%	95%	63%
QX 500 (12)	0%	67%	83%	100%
QX 021 (11)	9%	73%	100%	82%
Other non-toxigenic ribotypes (13)	0%	77%	100%	92%

434 Table 6 – Comparison of $MIC_{50/90}$ between *C. difficile* strains from 2006 – 10 and 2011 – 15.

Antimicrobials	Strains from 2006 – 2010				Strains from 2011 – 2015			
	Toxigenic (n = 22)		Non-toxigenic (n = 22)		Toxigenic (n=23)		Non-toxigenic (n = 33)	
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}
Vancomycin	0.5	1	0.5	0.5	0.5	0.5	0.5	0.5
Metronidazole	≤0.125	0.25	0.25	0.25	0.5	2	1	2
Clindamycin	128	> 256	64	> 256	128	> 256	32	> 256
Moxifloxacin	16	32	2	32	4	32	2	32
Levofloxacin	128	128	128	128	8	128	4	128
Tetracycline	4	8	4	8	4	8	0.125	4
Erythromycin	4	> 128	2	> 128	8	> 128	2	256
Chloramphenicol	4	32	4	32	4	8	4	8

435

Highlights

- Around half of *Clostridium difficile* strains from Thailand were non-toxigenic.
- The most common strain was *tcdA*-negative, *tcdB*-positive ribotype 017.
- 20 new *C. difficile* ribotypes were found.
- There was a slight creep in metronidazole MIC over time.