Molecular characterisation of, and antimicrobial resistance in, *Clostridioides difficile* from Thailand, 2017-2018

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Running title: *Clostridioides difficile* in Thailand

Abstract

Background: Antimicrobial resistance (AMR) plays an important role in the pathogenesis and spread of *Clostridioides difficile* infection (CDI). Many antimicrobials, such as fluoroquinolones, have been associated with outbreaks of CDI globally.

Objectives: This study aimed to characterise AMR among clinical *C. difficile* strains in Thailand, a country where the use of antimicrobials remains inadequately regulated.

Methods: Stool samples were screened for *tcdB* and positives were cultured. *C. difficile* isolates were characterised by toxin profiling and PCR ribotyping. Antimicrobial susceptibility testing was performed using an agar incorporation method, and whole-genome sequencing and AMR genotyping performed on a subset of strains.

Results: There were 321 *C. difficile* strains isolated from 326 stool samples. The most common toxigenic ribotype (RT) was RT 017 (18%), followed by RTs 014 (12%) and 020 (7%). There was a high resistance prevalence (≥ 10%) to clindamycin, erythromycin, moxifloxacin and rifaximin, and resistance prevalence was greatest among RT 017 strains. AMR genotyping revealed a strong correlation between resistance genotype and phenotype for moxifloxacin and rifampicin. The presence of *erm*-class genes was associated with high-level clindamycin and erythromycin resistance. Point substitutions on the penicillin-binding proteins (PBP1 and PBP3) were not sufficient to confer meropenem resistance, however, a Y721S substitution in PBP3 was associated with a slight increase in meropenem MIC. No resistance to metronidazole, vancomycin or fidaxomicin was observed.

Conclusion: There was a large proportion of *C. difficile* RT 017 in Thailand and a high AMR prevalence among these strains. The concordance between AMR phenotype and genotype was strong.

Introduction

Clostridioides (*Clostridium*) *difficile* is a major cause of antimicrobial-associated diarrhoea globally (1). *C. difficile* infection (CDI) is a toxin-mediated disease and, so far, there have been three different major toxins identified: toxin A (TcdA), toxin B (TcdB) and binary toxin (*C. difficile* transferase, CDT). The genes encoding TcdA and TcdB are located on a 19.6 kb pathogenicity locus (PaLoc) (2) and the genes for CDT are located in a different part of the chromosome, the CDT locus (3). In non-toxigenic *C. difficile* (NTCD), the PaLoc is replaced by a fixed 115 bp locus (2). The toxin genes (*tcdA*, *tcdB*, *cdtA* and *cdtB*) can be detected by PCR (4, 5), however, some *C. difficile* strains, such as *C. difficile* ribotype (RT) 017, have a deletion at the repeating region of the *tcdA* gene, resulting in a truncated and non-functional toxin A (6). Several methods have been developed to detect this deletion (7, 8).

C. difficile can be separated into different RTs by amplifying the intergenic spacer region between the 16S and 23S rRNA genes (9). This classification method has been used worldwide due to its simplicity and high discriminating power (10). Important *C. difficile* RTs include *C. difficile* RT 027, an A+B+CDT+ strain associated with outbreaks of severe CDI in North America and Europe in the early 2000s (11), *C. difficile* RT 078, another A+B+CDT+ strain associated with the zoonotic transmission (12), and *C. difficile* RT 017, a *tcdA*-negative (A-B+CDT-) strain associated with global outbreaks since 1995 (6).

Although resistance to antimicrobials used for the treatment of CDI is relatively rare (13), resistance to other commonly used antimicrobials plays an important role in the pathogenesis of CDI and the spread of *C. difficile*. While intrinsic resistance to cephalosporins was probably responsible for an increase in the rate of CDI worldwide in the 1980s (14), resistance to clindamycin, new generation fluoroquinolones, rifampicin and tetracycline has been associated with outbreaks of CDI (15). These antimicrobials are also associated with an increased risk of developing CDI in general (16). Strict regulation of antimicrobials can be a successful measure to control CDI. In the US, such regulation has resulted in a significant decrease in CDI cases and CDI-related deaths over the last decade (17). Fluoroquinolone regulation in Australia has resulted in a relatively low prevalence of fluoroquinolone-resistant organisms (18), including *C. difficile* (19).

In previous studies, the epidemiology of CDI in Thailand has been characterised by a high prevalence of A-B+CDT- and NTCD, and an absence of A+B+CDT+ strains (20-22). *C. difficile* strains isolated in Thailand, especially *C. difficile* RT 017, were resistant to many antimicrobial groups, reflecting the use and misuse of these antimicrobials in the country (23). This study provides an update on the characterisation and antimicrobial susceptibility of *C. difficile* isolated from a tertiary hospital in Bangkok, Thailand.

Materials and Methods

Isolation and characterisation of *C. difficile*

This study was undertaken on 326 diarrhoeal stools samples collected from patients with a high index of suspicion of having CDI being treated at Siriraj Hospital, a large teaching hospital in Bangkok, Thailand, during 2017 – 2018. All stool samples were screened for the presence of the *tcdB* gene using the BD Max Cdiff assay (Becton Dickinson, US) as a part of routine investigations at Siriraj Hospital, and stools that were *tcdB* positive were then sent to a reference laboratory in Perth, Western Australia, for further investigation.

At the reference laboratory, stool samples were processed as previously described, including enrichment culture in cooked meat broth supplemented with gentamicin, cefoxitin and cycloserine (24). Characterisation of *C. difficile* was performed by PCR ribotyping and toxin gene profiling. PCR ribotyping was performed using the method described by Stubbs *et al* (9). The banding patterns were compared to a local database consisting of more than 80 internationally recognised RTs, including 15 reference RTs from the European Centre for Disease Prevention and Control. Patterns

82 that did not match strains in the database were given an internal nomenclature. Detection of *tcdA* and
83 *tcdB,* and the binary toxin genes (*cdtA* and *cdtB*), was performed using the methods described by Kato
84 *et al* (7) and Stubbs *et al* (5), respectively. All NTCD isolates in this study were confirmed as such by
85 the absence of the PaLoc using the method described by Braun *et al* (*lok* PCR) (2).

86 All stool samples were checked also for colonisation with multiple *C. difficile* strains. Briefly,
87 DNA extraction was performed on all enrichment broths. DNA was then screened with either *tcdB* (7)
88 or *lok* PCR (2)*,* based on the toxin profile of the first *C. difficile* strain isolated from the specimen (2,
89 7). For example, a specimen previously positive for toxigenic *C. difficile* (TCD) was screened with
90 *lok* PCR for NTCD and vice versa. All PCR-positive broths were re-cultured and the second *C. difficile*
91 strain characterised as described above.

92 Antimicrobial susceptibility testing

93 Antimicrobial susceptibility testing (AST) was performed using the agar incorporation method as
94 described by the Clinical and Laboratory Standards Institute (CLSI) against nine antimicrobials listed in
95 **Table 1** (25). *C. difficile* ATCC 700057, *Bacteroides fragilis* ATCC 25285, *Eubacterium lentum* ATCC
96 43055 and *B. thetaiotamicron* ATCC 29741 were included as control strains. Susceptibility results were
97 interpreted using the minimal inhibitory concentration (MIC) breakpoints listed in **Table 1** (25-29).
98 *C. difficile* strains that were resistant to at least three different antimicrobial classes were classified as
99 multidrug-resistant (MDR). Resistance to clindamycin and erythromycin was considered as resistance
100 to a single class (macrolide-lincosamide-streptogramin B; MLS_B).

101 **Table 1** – List of antimicrobials, test ranges and susceptibility breakpoints used in this study.

Antimicrobial	Test range (mg/l)	Breakpoint (mg/l)			Reference
		S	I	R	
Clindamycin	0.008 – 256	≤ 2	4	≥ 8	(25)
Erythromycin	0.06 – 256	-	-	> 8	(26)
Moxifloxacin	0.06 – 64	≤ 2	4	≥ 8	(25)
Amoxicillin/clavulanate	0.015 – 16	≤ 4	8	≥ 16	(25)
Meropenem	0.12 – 16	≤ 4	8	≥ 16	(25)
Rifaximin	0.008 – 64	-	-	> 32	(27)
Metronidazole	0.008 – 4	≤ 2	-	> 2	(28)
Vancomycin	0.03 – 4	≤ 2	-	> 2	(28)
Fidaxomicin	0.002 – 2	-	-	> 1	(29)

102

103 Whole-genome sequencing, high-resolution typing and antimicrobial resistance characterisation

104 A subset of *C. difficile* strains (n = 37) was selected for whole-genome sequencing (WGS) to explore
105 possible antimicrobial resistance (AMR) genotypes. Genomic DNA was extracted, sequenced on an
106 Illumina HiSeq platform which generated 150 bp pair-end reads with a median coverage of 73X and
107 characterised by multi-locus sequence typing (MLST) as previously described (30). Clade assignment
108 of a newly defined sequence type (ST) was confirmed by comparing the average nucleotide identity
109 (ANI) with *C. difficile* strains 630 (clade 1, GenBank accession AM180355) and R20291 (clade 2,
110 GenBank accession FN545816) using FastANI (31). Known accessory AMR genes were identified by
111 interrogating the read files with SRST2 version 0.2.0 against ARGannot database version 3 (32, 33).
112 Draft annotated genomes were interrogated on Artemis version 17.0.1 to look for additional accessory
113 genes (34). The genomes were interrogated also for the presence of known point substitutions
114 associated with resistance to carbapenems (substitution in penicillin-binding proteins PBP1 and PBP3),
115 fluoroquinolones (substitution in GyrA and GyrB subunit of the gyrase enzyme) and rifaximin
116 (substitution in RpoB enzyme) (35, 36). The genotypes were then compared with phenotypic
117 susceptibility data.

118 Data availability

119 All sequence data generated in this study have been submitted to the European Nucleotide Archive
120 under BioProject PRJEB40974, sample accessions ERS5247348 – ERS5247384. Details of the genomes
121 are available in the **Supplementary Table S1**. The two newly characterised resistance determinants
122 were submitted to the Nomenclature Center for MLS_B Genes (37), and the sequences were submitted
123 to GenBank [accession numbers MW269959 (*erm*(52) gene) and MW269960 (*mef*(G) gene)]. The two
124 genomes containing the prototypes of the genes were submitted to Genbank under BioProject
125 PRJNA679085, accessions JADPMU000000000 (MAR225, carrying *erm*(52)) and JADPMT000000000
126 (MAR272, carrying *mef*(G)).

127 Statistical analysis

128 All statistical analyses were performed using online tools by Social Science Statistics available at
129 https://www.socscistatistics.com/. A p-value ≤ 0.05 was considered statistically significant.

130 Human research ethics approval

131 This study was approved by the Human Research Ethics Committee of The University of Western
132 Australia (reference file RA/4/20/4704) and the Siriraj Institutional Review Board (protocol number
133 061/2558 [EC1]).

134 **Results**

135 Characterisation of Thai *C. difficile*

136 A total of 296 *C. difficile* strains were initially isolated from the stool samples. Forty-four of the original
137 326 PCR positive stool samples were negative for TCD by culture; 30 contained no *C. difficile* while
138 from 14 only NTCD was cultured. The enrichment broths for these samples were re-screened for TCD
139 and were all negative by *tcdB* PCR. Another 25 strains were identified from the co-colonisation
140 screening process, yielding a total of 321 *C. difficile* strains. Of these, 221 (68.85%) were positive for
141 *tcdA* and *tcdB* (A+B+CDT-), 58 (18.07%) were positive for *tcdB* only and had a deletion in *tcdA* (A-
142 B+CDT-), three (0.93%) were positive for *tcdA*, *tcdB*, as well as *cdtA* and *cdtB* (A+B+CDT+) and 39 strains
143 (12.15%) were negative for all toxin genes (A-B-CDT-, NTCD).

144 The 321 *C. difficile* strains belonged to 63 different RTs, 19 of which were internationally
145 recognised. The remaining RTs were given internal nomenclature beginning with either "QX" or "KI".
146 The prevalence of the most common RTs is summarised in **Table 2**. The most common TCD strain was
147 *C. difficile* RT 017 (A-B+CDT-), followed by RTs 014 and 020 (both A+B+CDT-). The most common NTCD
148 was *C. difficile* RT 010.

149 Characterisation of a novel binary toxin-positive *C. difficile* isolate

150 One of the *C. difficile* strains isolated in this study was positive for all three toxin genes (A+B+CDT+)
151 and had a unique ribotyping pattern compared to the local reference library. According to the MLST
152 scheme, this isolate was characterised as the novel ST 692 within evolutionary clade 1. However,
153 pairwise ANI analysis showed that this strain was more closely related to *C. difficile* R20291 (clade 2,
154 ANI = 99.17%) than *C. difficile* 630 (clade 1, ANI = 98.89%).

155 Antimicrobial susceptibility of Thai *C. difficile*

156 Overall antimicrobial susceptibility results are shown in **Table 3** and the MIC distribution of selected
157 six antimicrobial classes is displayed in **Figure 1**. Based on the MIC value, clindamycin-resistant
158 *C. difficile* strains could be divided into two groups: those with MIC ≥ 32 mg/l (n = 97) and those with
159 MIC < 32 mg/l (n = 166). There was a strong correlation between high-level clindamycin resistance and
160 erythromycin resistance: 95 strains (97.94%) that had clindamycin MIC ≥ 32 mg/l were also resistant
161 to erythromycin while only 16 strains (9.64%) in the other group were resistant to erythromycin

162 (Cohen's kappa = 0.857). There was also a clear separation in MIC value between strains with and
163 without rifaximin resistance. The separation was less clear for moxifloxacin and was not observed in
164 amoxicillin/clavulanate and meropenem.

165 **Table 2** – Ribotypes of 321 *C. difficile* strains from Thailand, by toxin profile.

Toxin Profile	Ribotype	Number	% of toxigenic strains	% of all
A+B+CDT-	**Total**	**221**	**78%**	**69%**
	014	40	14%	12%
	020	24	9%	7%
	046	18	6%	6%
	QX517	17	6%	5%
	297	13	5%	4%
	QX026	11	4%	3%
	043	10	4%	3%
	Others	88	31%	27%
A-B+CDT-	**Total**	**58**	**21%**	**18%**
	017	58	21%	18%
A+B+CDT+	**Total**	**3**	**1%**	**1%**
	078	1	<1%	<1%
	QX273	1	<1%	<1%
	KI008	1	<1%	<1%
A-B-CDT-	**Total**	**39**	-	**12%**
	010	7	-	2%
	QX002	5	-	2%
	009	4	-	1%
	QX011	4	-	1%
	Others	19	-	6%

166 Note: RTs designated with "QX" and "KI" were RTs that did not match the internationally recognised
167 RTs in the database and were given internal nomenclature.

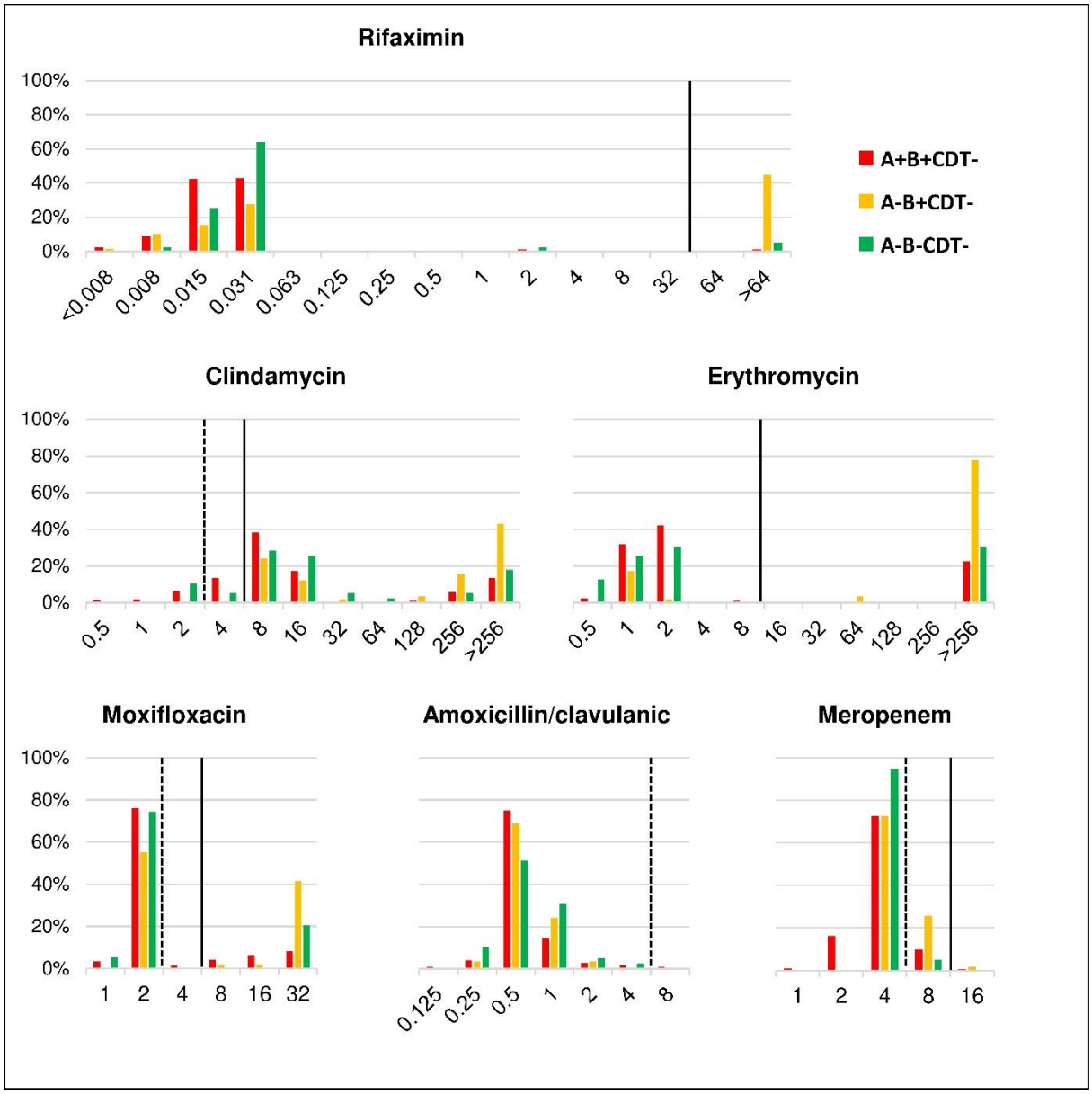
168

169 **Table 3** – Antimicrobial susceptibility of 321 *C. difficile* strains from Thailand.

Antimicrobial	MIC range (mg/l)	MIC_{50} (mg/l)	MIC_{90} (mg/l)	Susceptibility (%)		
				S	I	R
Clindamycin	0.5 – >256	8	>256	26 (8%)	32 (10%)	263 (82%)
Erythromycin	0.5 – >256	2	>256	-	-	109 (34%)
Moxifloxacin	0.5 – >64	2	32	242 (75%)	3 (1%)	76 (24%)
Amoxicillin/clavulanate	0.25 – 8	0.5	1	320 (>99%)	1 (<1%)	0
Meropenem	1 – 16	4	8	280 (87%)	39 (12%)	2 (1%)
Rifaximin	≤0.008 – >64	0.03	2	-	-	31 (10%)
Metronidazole	0.06 – 1	0.25	0.25	321 (100%)	-	0
Vancomycin	0.5 – 2	1	2	321 (100%)	-	0
Fidaxomicin	0.03 – 0.25	0.125	0.25	-	-	0

170

171 When classified by toxin gene profiles, resistance to clindamycin, erythromycin, moxifloxacin
172 and rifaximin were more prevalent among A-B+CDT- *C. difficile*, all of which belonged to RT 017, than
173 A+B+CDT- and NTCD (**Figure 1**). A total of 29 (9.03%) *C. difficile* isolates were MDR, 26 (8.10%) of which
174 were *C. difficile* RT 017. The remaining isolates were NTCD (n=2) and A+B+CDT- *C. difficile* (n=1). All
175 MDR isolates were resistant to the MLS_B group (both clindamycin and erythromycin), moxifloxacin and
176 rifaximin. One MDR isolate was also resistant to meropenem (A-B+CDI-, RT 017, MIC = 16 mg/l).



177

178 **Figure 1 – MIC distribution for six antimicrobials against 321 strains of C. difficile in Thailand.**
179 *C. difficile* strains were classified according to their toxin gene profiles: A+B+CDT-, red; A-B+CDT-,
180 yellow; A-B-CDT-, green. The number of A+B+CDT+ C. difficile strains was low (n = 3) and these were
181 excluded. Breakpoints for intermediate resistance (I) and resistance (R) are shown by broken and
182 solid lines, respectively.

183 Genotypic resistance determinants in Thai *C. difficile*

184 A summary of MIC values and genotypic resistance determinants of 37 sequenced *C. difficile* strains is
185 available in **Supplementary Table S2**. Of these strains, 31 had high-level resistance to clindamycin: 23
186 strains carried an *erm*(B) gene, five carried an *erm*(G) gene and three carried a gene encoding an rRNA
187 adenine N(6)-methyltransferase family protein, the closest match to which in the CARD database was
188 the Erm(42) protein [39% identity, E-value = 1.19E-53] (38). This newly characterised gene was given
189 the name *erm*(52). Of the 23 *erm*(B)-positive strains, 19 carried the *erm*(B) gene on transposon Tn*6194*
190 (82.61%), while the other four (17.39%) carried the gene on Tn*6189*. No *erm*-class genes were
191 identified among strains with low-level clindamycin resistance. The concordance between the
192 presence of *erm*-class genes and high-level clindamycin resistance was 100%. A gene encoding a
193 macrolide efflux protein was identified in two strains with high-level erythromycin resistance (MIC
194 > 256 mg/l), and given the name *mef*(G). No significant genotypic resistance determinants were
195 identified in strains with low-level clindamycin resistance.

196 A T82I substitution in GyrA was found in 23 strains and a D426V substitution in GyrB found in
197 one strain with a moxifloxacin MIC of 16 – 32 mg/l. There were no known point substitutions in the
198 remaining 14 strains, 13 of which were moxifloxacin-susceptible; one had low-level moxifloxacin
199 resistance (MIC 8 mg/l) [97.37% concordance]. There were H502N and R505K substitutions in RpoB in
200 all 23 rifaximin-resistant strains and none of the susceptible strains [100% concordance].

201 Twelve strains had an A555T substitution in penicillin-binding protein 1 (PBP1) and another
202 seven had a Y721S substitution in PBP3. A multiple linear regression analysis was performed to assess
203 the association between the presence of these substitutions and the MIC values for meropenem. The
204 Y721S substitution in PBP3 was associated with an increase in meropenem MIC (adjusted $R^2 = 0.516$,
205 $t = 5.521$, $p < 0.0001$), while the A555T substitution in PBP1 was not associated with the change in
206 meropenem MIC ($t = -1.127$, $p = 0.268$).

207 **Discussion**

208 This study provides an update on the molecular epidemiology and antimicrobial susceptibility of
209 *C. difficile* strains circulating in Thailand. It also explores the genomic basis of important AMR in these
210 strains. As the focus was on patients suspected of having CDI and not on the prevalence of *C. difficile*
211 in the general population, the stool samples included in this study were first screened for the *tcdB*
212 gene by PCR before culture. Thus the overall prevalence of TCD was higher than previous studies in
213 Thailand (20-22), however, the common strains found were similar. The majority of A+B+CDT- strains
214 belonged to *C. difficile* RT 014/020 group, all A-B+CDT- strains belonged to *C. difficile* RT 017 and most
215 NTCD belonged to *C. difficile* RTs 009, 010 and 039. Three binary toxin-positive isolates were found in
216 this study, one of which was *C. difficile* RT 078. The epidemic *C. difficile* RT 027 remained absent in
217 Thailand despite its successful spread in other regions (39).

218 Why *C. difficile* RT 027 has failed to spread and to establish in Thailand, and Southeast Asia in
219 general, remains unknown. One possible reason is that the successful spread of this RT was mainly
220 due to its resistance to fluoroquinolones which provided a selective advantage over other less
221 resistant RTs (40). Although there is high consumption of fluoroquinolones, such as levofloxacin, in
222 the country (41), Thailand already harbours *C. difficile* RT 017, another epidemic RT many of which are
223 resistant to fluoroquinolones, as well as other antimicrobials (15). Thus, it may have been difficult for
224 *C. difficile* RT 027 to compete with this local RT compared to other regions.

225 Though *C. difficile* RT 027 was not identified in this study, a possible relative of this
226 hypervirulent strain, ST 692, was isolated. The MLST for this strain was unusual, as it was classified
227 into clade 1 despite containing binary toxin genes in a complete CDT locus. The presence of binary
228 toxin genes is a feature common in *C. difficile* clades 2 and 5, but rare in clade 1 (42). Thus, an ANI
229 analysis was performed, which indicated that this newly characterised strain was more related to clade

230 2 than clade 1 *C. difficile*, as expected from the toxin gene profile. Clades 1 and 2 *C. difficile* are closely
231 related and share a large proportion of housekeeping gene alleles used in the MLST scheme. As a
232 result, it may be difficult to properly discriminate these two clades by this method. The use of ANI
233 analysis, which involves the whole genome rather than a specific set of housekeeping genes, can help
234 in the correct classification of some borderline strains as shown in a previous study (42). According to
235 the ANI analysis, it is more likely that this newly described *C. difficile* ST 692 belongs to clade 2 and is
236 related to *C. difficile* RT 027.

237 A discordance between culture results and the result of a conventional real-time *tcdB* PCR was
238 observed in 44 stool samples. The false-positive rate of the real-time PCR method (13.50%) was
239 comparable to the previous report comparing *tcdB* PCR with a similar enrichment culture method but
240 without the colonisation screening step (24). This suggests that the additional screening step does not
241 significantly increase the yield of the culture method, although it may help identify stool samples with
242 multiple *C. difficile* strains. This false-positive rate also highlights the importance of patient clinical
243 data or additional tests to improve the accuracy of CDI diagnosis. In the latest guidelines for the
244 treatment and diagnosis of CDI, *tcdB* PCR is no longer recommended as a stand-alone test unless the
245 patient has symptoms suggestive of CDI (43).

246 AMR in *C. difficile* affects both the pathogenesis and treatment of CDI. To cause the disease,
247 *C. difficile* must tolerate the presence of antimicrobials in the intestinal lumen while the microbiota
248 perishes (15). Many successful *C. difficile* lineages have indeed been characterised with increased
249 resistance to at least one major drug group (15). In this study, *C. difficile* RT 017, which was the most
250 common RT, had greater resistance to MLS_B (both clindamycin and erythromycin), moxifloxacin and
251 rifaximin than other RTs. It was also the most common MDR *C. difficile* strain in this study. *C. difficile*
252 RT 017 has been reported also to be the most prevalent RT with significant resistance to many
253 antimicrobials in other parts of Thailand (22). This particular RT has been associated with resistance
254 to at least six antimicrobial groups (15), which may have accounted for its successful global spread (6).
255 As regulation of antimicrobial use has reduced the impact of *C. difficile* in many countries (17, 19), a
256 similar approach should be effective for the control CDI in Thailand.

257 All *erm*(B)-positive *C. difficile* strains carried the gene on two well-characterised *erm*(B)-
258 positive transposons: Tn*6189* and Tn*6194*, the latter being found also in *C. difficile* M68, a *C. difficile*
259 RT 017 strain widely used as a reference in genomic studies (15). Tn*6194*, the most prevalent
260 transposon in this study, is capable of inter-species transfer, most notably between *C. difficile* and
261 *Enterococcus faecalis* (44). This emphasises another aspect of AMR in *C. difficile*; its possible role as a
262 reservoir of AMR genes for other pathogenic bacteria residing in the colon.

263 Previously, it has been reported that concordance between the presence of the *erm*(B) gene
264 and an MLS_B resistance phenotype was low (45), likely due to the presence of multiple resistance
265 mechanisms. In a previous study, however, the *erm*(B) gene was found in all *C. difficile* isolates with
266 high-level resistance to both clindamycin and erythromycin, hinting that the gene may be associated
267 with high-level MLS_B resistance (46). We also observed separation between *C. difficile* strains with
268 high-level and low-level clindamycin and erythromycin resistance (**Figure 1**). Upon genomic analysis
269 of a subset of strains, there was a strong correlation between the presence of an *erm*-class gene
270 (*erm*(B), *erm*(G) and *erm*(52) genes) with high-level clindamycin resistance, which is usually
271 accompanied by high-level erythromycin resistance, supporting the previous study (46). A macrolide
272 efflux gene *mef*(G) was identified also in strains with high-level erythromycin resistance and not in
273 erythromycin-susceptible strains, suggesting an association between the presence of this efflux
274 protein and phenotypic erythromycin resistance, although the function of this gene was not
275 characterised. Resistance determinants were not identified among strains with low-level clindamycin
276 resistance, however, this underestimation is likely irrelevant, as the median clindamycin MIC in this
277 population (8 mg/l) remained significantly lower than the clindamycin level in stools (approximately
278 240 mg/g of stool) (47). Besides MLS_B, a separation between strains resistant and susceptible to

279 rifaximin and fluoroquinolones was observed (**Figure 1**). The concordance between resistant
280 phenotype and known genotype was also high, similar to a previous study (45).

281 Compared to the study at the same hospital in 2015, there was no difference in overall
282 resistance prevalence (26), however, there was a slight increase in meropenem MICs and the
283 emergence of carbapenem resistance. In a previous study, high-level imipenem resistance was
284 associated with point substitutions in PBP1 and PBP3 (36). It appears that these substitutions only
285 confer high-level resistance to imipenem and not to meropenem, although the linear regression
286 analysis suggests that the Y721S substitution in PBP3 may have contributed to a slight increase in
287 meropenem MIC in this study.

288 *C. difficile* remained susceptible to metronidazole, vancomycin and fidaxomicin, similar to the
289 other parts of the world (48). Thus, these antimicrobials should remain effective treatments for CDI.
290 There was a slight increase in vancomycin MIC reaching the clinical breakpoint, consistent with a
291 previous study (26), however, this should have little impact on the treatment of CDI given that the
292 faecal vancomycin concentration remains far greater than the MIC (>2,000 mg/l vs 2 mg/l) (49). The
293 increase in vancomycin MIC in this study is in contrast to other hospitals in Thailand and this could
294 reflect usage of vancomycin at the study site (22). Overuse of vancomycin can lead to the emergence
295 of vancomycin-resistant *Enterococcus* spp., which can have a devastating effect on patients (50, 51).
296 Therefore, vancomycin usage should be carefully monitored.

297 **Conclusion**

298 A-B+CDT- *C. difficile* and NTCD remained prevalent in Thailand. Few binary toxin-positive strains
299 (A+B+CDT+) were identified; one belonging to a known epidemic lineage and another a novel strain
300 related to *C. difficile* RT 027. The most common RT in this study was *C. difficile* RT 017 (A-B+CDT-), a
301 large proportion of which was resistant to MLS_B, moxifloxacin and rifaximin. Many strains were also
302 MDR. Such resistance may have played a role in the success of *C. difficile* RT 017 in Thailand. There
303 was a strong concordance between the presence of *erm*-class genes and high-level clindamycin
304 resistance, as well as significant concordance between point substitutions in gyrase subunits and RpoB
305 with fluoroquinolone and rifaximin resistance, respectively. Resistance to antimicrobials suitable for
306 the treatment of CDI was not detected.

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Transparency declaration

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