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

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Abstract

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. This study characterised AMR among clinical *C. difficile* strains in Thailand, where antimicrobial use remains inadequately regulated. 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 by agar incorporation, and whole-genome sequencing and AMR genotyping performed on a subset of strains. 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%). Resistance to clindamycin, erythromycin, moxifloxacin and rifaximin was common, especially among RT 017 strains. AMR genotyping revealed a strong correlation between resistance genotype and phenotype for moxifloxacin and rifaximin. The presence of *erm*-class genes was associated with high-level clindamycin and erythromycin resistance. Point substitutions in the penicillin-binding proteins were not sufficient to confer meropenem resistance, but a Y721S substitution in PBP3 was associated with a 4.37-fold increase in meropenem MIC. No resistance to metronidazole, vancomycin or fidaxomicin was observed.

Introduction

Clostridioides (*Clostridium*) *difficile* is a major cause of antimicrobial-associated diarrhoea.[1] *C. difficile* infection (CDI) is a toxin-mediated disease and there have been three different major toxins identified: toxin A (TcdA), toxin B (TcdB) and binary toxin (*C. difficile* transferase, CDT). The *tcdA* and *tcdB* genes are located on a 19.6 kb pathogenicity locus (PaLoc)[2] and the genes for CDT (*cdtA* and *cdtB*) are located on a different locus, the CDT locus.[3] In non-toxigenic *C. difficile* (NTCD), the PaLoc is replaced by a fixed 115 bp locus.[2] The toxin genes in toxigenic *C. difficile* (TCD) can be detected by PCR.[4,5] Some *C. difficile* strains have a deletion in the repeating region of the *tcdA* gene, resulting in a truncated and non-functional toxin A.[6]

C. difficile can be separated into different ribotypes (RTs) by amplifying the intergenic spacer region between the 16S and 23S rRNA genes.[7] This method has been used widely due to its simplicity and high discriminating power.[8] 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,[9] *C. difficile* RT 078, another A+B+CDT+ strain associated with the zoonotic transmission,[10] and *C. difficile* RT 017, a *tcdA*-negative (A-B+CDT-) strain associated with global outbreaks since 1995.[6]

Although resistance to the antimicrobials used for the treatment of CDI (metronidazole, vancomycin and fidaxomicin) is rare,[11] resistance to other antimicrobials plays an important role in the pathogenesis and spread of CDI. While intrinsic resistance to cephalosporins was probably responsible for an increase in the rate of CDI worldwide in the 1980s,[12] resistance to clindamycin, new generation fluoroquinolones, rifamycins and tetracyclines has been associated with CDI outbreaks.[13] These antimicrobials are also associated with an increased risk of developing CDI in general.[14] Strict regulation of antimicrobials is a successful measure to control CDI. In the US, such regulation has lead to a significant decrease in CDI cases and CDI-related deaths over the last decade.[15] Fluoroquinolone regulation in Australia has resulted in a relatively low prevalence of fluoroquinolone-resistant organisms,[16] including *C. difficile*.[17]

Several studies have reported an association between AMR genotypes and phenotypes for various antimicrobials. The most common clindamycin resistance determinant is the *erm*(B) gene, which methylates and protect 23S rRNA from the antimicrobial.[13] However, concordance between the presence of the *erm*(B) gene and the resistance phenotype is low.[18] A subsequent study suggested that *erm*(B) may only be associated with high-level clindamycin resistance and thus the mechanism underlying low-level clindamycin resistance remains unknown.[19] Carbapenem resistance is also poorly described. So far, only imipenem resistance has been characterised and is associated with point mutations on the penicillin-binding proteins PBP1 and PBP3.[20] On the contrary, fluoroquinolone and rifaximin resistance are well characterised and are associated with point substitutions in the quinolone resistance determining region (QRDR) on the DNA gyrase subunits (GyrA and GyrB) and RNA polymerase subunit B (RpoB), respectively.[21]

In previous studies, the epidemiology of CDI in Thailand has been characterised by a high prevalence of A-B+CDT- and an absence of A+B+CDT+ strains, as well as a high prevalence of NTCD, which may play a protective role against the development of CDI.[22-24] *C. difficile* strains isolated in Thailand, especially *C. difficile* RT 017, had a high prevalence of resistance to many antimicrobial groups, similar to other pathogenic bacteria in the country[25,26] reflecting, possibly, poor antimicrobial stewardship in the country.[27] 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 CDI at Siriraj Hospital, a large teaching hospital in Bangkok, Thailand, during 2017 – 2018. All stools were first positive for *tcdB* using the BD Max Cdiff assay (Becton Dickinson, US), as a part of routine investigations at Siriraj Hospital, and these were sent to a reference laboratory in Perth, Western Australia, for further investigation.

At the reference laboratory, stools were processed as previously described.[28] Briefly, a portion of each stool sample was directly inoculated on ChromID *C. difficile* agar (bioMérieux, Marcy l'Etoile, France) and incubated anaerobically for 48 hours before the putative *C. difficile* colonies were identified. The remainder of each sample underwent enrichment culture in supplemented brain heart infusion broth, followed by ethanol shock to increase the sensitivity of the culture process. *C. difficile* isolates were characterised by PCR ribotyping, performed as described by Stubbs *et al,* with a QIAxcel Advanced System capillary gel electrophoresis platform (QIAGEN, Venlo, The Netherlands).[7] The banding patterns were compared to a local database consisting of 80 internationally recognised RTs, including 15 reference RTs from the European Centre for Disease Prevention and Control. This method can differentiate similar RTs, such as RTs 014 and 020 (**Supplementary Figure S1**). Patterns that did not match strains in the database were given an internal nomenclature. Detection of *tcdA* and *tcdB,* and the binary toxin genes, was performed as described by Kato *et al*[29] and Stubbs *et al*,[5] respectively. All NTCD isolates in this study were confirmed as such by PCR as described by Braun *et al* (*lok* PCR).[2]

All stool samples were tested also for colonisation with multiple *C. difficile* strains. Briefly, DNA extraction was performed on all enrichment broths. DNA was then screened with either *tcdB*[29] or *lok*[2] PCR based on the toxin profile of the first *C. difficile* strain isolated from the specimen. For example, a specimen previously positive for toxigenic TCD was screened with *lok* PCR for NTCD and *vice versa*. All PCR-positive broths were re-cultured and up to 30 putative *C. difficile* colonies per broth were selected and characterised by toxin gene profiling. An isolate with a different toxin profile from the first strain was treated as the second strain from the same sample and underwent further characterisation by PCR ribotyping.

<u>Antimicrobial susceptibility testing</u>

Antimicrobial susceptibility testing (AST) was performed by agar incorporation, as described by the Clinical and Laboratory Standards Institute (CLSI), against the eight antimicrobials listed in **Supplementary Table S1.**[30] *C. difficile* ATCC 700057, *Bacteroides fragilis* ATCC 25285, *Eubacterium*

lentum ATCC 43055 and *B. thetaiotaomicron* ATCC 29741 were included as controls. Susceptibility results were interpreted using the minimal inhibitory concentration (MIC) breakpoints listed in **Supplementary Table S1.**[30-34] *C. difficile* strains resistant to at least three antimicrobial classes were classified as multidrug-resistant (MDR). Resistance to clindamycin and erythromycin was considered as resistance to a single class (macrolide-lincosamide-streptogramin B; MLS_B).

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

A subset of 37 *C. difficile* strains was selected for whole-genome sequencing (WGS). Genomic DNA was extracted, sequenced on an Illumina HiSeq platform which generated 150 bp pair-end reads with a median coverage of 73X and characterised by multi-locus sequence typing (MLST) as previously described.[35] Clade assignment of a new sequence type (ST) was confirmed by comparing the average nucleotide identity (ANI) with *C. difficile* strains 630 (clade 1, accession AM180355) and R20291 (clade 2, accession FN545816) using FastANI.[36] Accessory AMR genes were identified by interrogating the read files with SRST2 version 0.2.0 against ARGannot database version 3.[37,38] Draft annotated genomes were interrogated on Artemis version 17.0.1, and additional accessory genes identified.[39] Known point substitutions associated with resistance to carbapenems (substitution in penicillin-binding proteins PBP1 and PBP3), fluoroquinolones (substitution in the GyrA and GyrB subunits of the gyrase enzyme) and rifaximin (substitution in the RpoB enzyme)[20,21] were also identified using SRST2 as previously described.[19]

Data availability

All sequence data were submitted to the European Nucleotide Archive under BioProject PRJEB40974, accessions ERS5247348 – ERS5247384 (**Supplementary Tables S2 and S3**). Two newly characterised resistance determinants were submitted to the Nomenclature Center for MLS_B Genes,[40] and the sequences were submitted to GenBank [accessions MW269959 (*erm*(52) gene) and MW269960 (*mef*(H) gene)]. Genomes containing the prototypes of these genes were submitted to Genbank under

BioProject PRJNA679085, accessions JADPMU000000000 (MAR225, carrying *erm*(52)) and JADPMT000000000 (MAR272, carrying *mef*(H)).

Statistical analysis

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

Results

Characterisation of Thai *C. difficile*

A total of 296 *C. difficile* strains were initially isolated from the stools and another 25 strains were identified from the co-colonisation screening process, yielding a total of 321 *C. difficile* strains. Of these, 221 (68.85%) were positive for *tcdA* and *tcdB* (A+B+CDT-), 58 (18.07%) were positive for *tcdB* only and had a deletion in *tcdA* (A-B+CDT-), three (0.93%) were positive for all toxin genes (A+B+CDT+) and 39 strains (12.15%) were negative for all toxin genes (A-B-CDT-, NTCD). A list of samples with multiple *C. difficile* strains is provided in the **Supplementary Table S4**.

The 321 *C. difficile* strains belonged to 63 RTs, 19 of which were internationally recognised. The remaining RTs were given internal nomenclature (prefix "QX-" or "KI-"). The prevalence of the common RTs is summarised in **Table 1**. The most common TCD strain was *C. difficile* RT 017 (A-B+CDT-), followed by RTs 014 and 020 (both A+B+CDT-). The most common NTCD was *C. difficile* RT 010.

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

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

Antimicrobial susceptibility of Thai *C. difficile*

AST results are shown in **Table 2** and the MIC distribution of selected six antimicrobial classes is displayed in **Figure 1**. Based on the MIC value, clindamycin-resistant *C. difficile* strains could be divided into two groups: those with MIC ≥ 32 mg/l (n = 97) and those with MIC < 32 mg/l (n = 166). There was a strong correlation between high-level clindamycin resistance and erythromycin resistance: 95 strains (97.94%) that had clindamycin MIC ≥ 32 mg/l were also resistant to erythromycin while only 16 strains (9.64%) in the other group were resistant to erythromycin (Cohen's kappa = 0.857).

When classified by toxin gene profiles, resistance to clindamycin, erythromycin, moxifloxacin and rifaximin were more prevalent among A-B+CDT- *C. difficile*, all belonging to RT 017, than A+B+CDT- and NTCD (**Figure 1**). Twenty-nine (9.03%) *C. difficile* strains were MDR, 26 (8.10%) of which were *C. difficile* RT 017. The remaining strains were NTCD (n=2) and A+B+CDT- *C. difficile* (n=1). All MDR strains were resistant to MLS_B (both clindamycin and erythromycin), moxifloxacin and rifaximin. One MDR strain was also resistant to meropenem (RT 017, MIC = 16 mg/l).

AMR genotypes in Thai *C. difficile*

A summary of MIC values and AMR genotypes of 37 sequenced *C. difficile* strains is available in **Supplementary Table S3**. Thirty-one *C. difficile* strains had high-level resistance to clindamycin: 23 strains carried *erm*(B), five carried *erm*(G) and three carried a gene encoding an rRNA adenine N(6)-methyltransferase protein. This gene was given the name *erm*(52). Of the 23 *erm*(B)-positive strains, 19 carried the gene on transposon Tn*6194* (82.61%), while the other four (17.39%) carried the gene on Tn*6189*. No *erm*-class genes were identified among strains with low-level clindamycin resistance. The concordance between the presence of *erm*-class genes and high-level clindamycin resistance was 100%. A gene encoding a macrolide efflux protein was identified in two strains with high-level erythromycin resistance (MIC > 256 mg/l) and only low-level clindamycin resistance, and given the name *mef*(H). No significant genotypic resistance determinants were identified in strains with low-level clindamycin resistance.

Twenty-five sequenced strains were resistant to moxifloxacin (MIC 8 – 32 mg/l). Of these, the T82I substitution in GyrA and the D426V substitution in GyrB were found in 23 strains and one strain, respectively. No known point substitutions were found in one strain with low-level moxifloxacin resistance (MIC 8 mg/l), as well as all moxifloxacin-susceptible strains [97.37% concordance]. There were H502N and R505K substitutions in RpoB in all 23 rifaximin-resistant strains and none of the susceptible strains [100% concordance].

Twelve strains had an A555T substitution in PBP1 and another seven had a Y721S substitution in PBP3. A multiple linear regression analysis suggested that the Y721S substitution in PBP3 was associated with a 4.37 fold increase in meropenem MIC (95% confidence interval: 2.78 – 5.96, adjusted $R^2 = 0.516$, $t = 5.521$, $p < 0.0001$), while the A555T substitution in PBP1 was not associated with the change in meropenem MIC ($t = -1.127$, $p = 0.268$).

Discussion

This study provides an update on the molecular epidemiology and antimicrobial susceptibility of *C. difficile* strains circulating in Thailand. It also explores the genomic basis of important AMR in these strains. The overall epidemiology of *C. difficile* was similar to the previous studies.[22-24] The majority of A+B+CDT- strains belonged to *C. difficile* RTs 014 and 020, all A-B+CDT- strains belonged to *C. difficile* RT 017 and most NTCD belonged to *C. difficile* RTs 009, 010 and 039. Three binary toxin-positive strains were found in this study, one of which was *C. difficile* RT 078. The epidemic *C. difficile* RT 027 remained absent in Thailand despite its successful spread in some other regions.[41]

Why *C. difficile* RT 027 has failed to spread and to establish in Thailand remains unknown. One possible reason is that the successful spread of this RT was mainly due to its resistance to fluoroquinolones which provided a selective advantage over other less resistant RTs.[42] Although there is high consumption of fluoroquinolones, such as levofloxacin, in the country,[43] Thailand already harbours *C. difficile* RT 017, another epidemic RT many of which are resistant to fluoroquinolones, as

well as other antimicrobials.[13] Thus, it may have been difficult for *C. difficile* RT 027 to compete with this local RT compared to other regions.

Though *C. difficile* RT 027 was not identified, a possible relative of this hypervirulent strain, ST 692, was isolated. The MLST result was unusual, as it was classified into clade 1 despite carrying a complete CDT locus, a common feature in *C. difficile* clades 2 and 5 but rare in clade 1.[44] Thus, an ANI analysis was performed In a previous study, *C. difficile* strains from the same clade generally shared > 99% ANI.[44] Thus the ANI results suggested that this newly characterised strain was a member of clade 2 rather than clade 1, as expected from the toxin gene profile. The average ANI between clades 1 and 2 in a previous study was around 98%, which further supports the results.[44] Clades 1 and 2 *C. difficile* are closely related and share a large proportion of housekeeping gene alleles used in the MLST scheme. As a result, it may be difficult to properly discriminate these two clades by MLST. The use of ANI analysis, which involves the whole genome rather than a specific set of housekeeping genes, can help in the correct classification of some borderline strains as shown in a previous study.[44] According to the ANI analysis, it is more likely that *C. difficile* ST 692 belongs to clade 2 and is related to *C. difficile* RT 027.

A discordance between culture results and the result of a conventional real-time *tcdB* PCR was observed in 44 stool samples. The false-positive rate of the real-time PCR method (13.50%) was comparable to the previous report comparing *tcdB* PCR with a similar culture method but without the colonisation screening step,[28] suggesting that the additional screening step does not increase the yield of the culture method, although it may help identify stool samples with multiple *C. difficile* strains. This false-positive rate also highlights the importance of patient clinical data or additional tests to improve the accuracy of CDI diagnosis. In the latest guidelines for the treatment and diagnosis of CDI, *tcdB* PCR in combination with another diagnostic test is recommended, commonly a toxin antigen detection kit, for a proper diagnosis of CDI and the use of stand-alone *tcdB* PCR should be interpreted with caution.[45]

AMR in *C. difficile* mainly impacts the pathogenesis of CDI. To cause the disease, *C. difficile* must tolerate the presence of antimicrobials in the intestinal lumen while the microbiota perishes.[13] Many successful *C. difficile* lineages have been characterised with increased resistance to at least one major drug group.[13] In this study, *C. difficile* RT 017, the most prevalent RT, had greater resistance to MLS_B (both clindamycin and erythromycin), moxifloxacin and rifaximin than other RTs. It was also the most common MDR *C. difficile* strain. *C. difficile* RT 017 has been reported also to be the most prevalent RT with significant resistance to many antimicrobials in other parts of Thailand.[24] This particular RT has been associated with resistance to at least six antimicrobial groups,[13] which may account for its successful global spread.[6] As regulation of antimicrobial use has reduced the impact of *C. difficile* in many countries,[15,17] a similar approach should be effective in Thailand.

All *erm*(B)-positive *C. difficile* strains carried the gene on two well-characterised *erm*(B)-positive transposons: Tn*6189* and Tn*6194*, the latter being found also in *C. difficile* M68, a *C. difficile* RT 017 strain widely used as a reference in genomic studies.[13] Tn*6194*, the most prevalent transposon in this study, is capable of inter-species transfer, most notably between *C. difficile* and *Enterococcus faecalis*.[46] This emphasises another aspect of AMR in *C. difficile*; its possible role as a reservoir of AMR genes for other pathogenic bacteria residing in the colon.

Previously, the low concordance between the presence of the *erm*(B) gene and an MLS_B resistance phenotype was reported,[18] likely due to the presence of multiple resistance mechanisms. However, another study suggested that the *erm*(B) gene may be associated only with high-level MLS_B resistance.[47] We also observed separation between *C. difficile* strains with high-level and low-level clindamycin and erythromycin resistance (**Figure 1**). Upon genomic analysis, there was a strong correlation between the presence of an *erm*-class gene (*erm*(B), *erm*(G) and *erm*(52) genes) and high-level clindamycin resistance, which is usually accompanied by high-level erythromycin resistance, supporting the earlier study.[47] Resistance determinants were not identified among strains with low-level clindamycin resistance, however, this underestimation is likely irrelevant, as the median

clindamycin MIC in this population (8 mg/l) remained lower than the clindamycin level in stools (approximately 240 mg/g of stool).[48] Besides MLS_B, a separation between strains resistant and susceptible to rifaximin and fluoroquinolones was observed (**Figure 1**). The concordance between resistant phenotype and known genotype was high, similar to a previous study.[18]

Compared to the study at the same hospital in 2015, there was no difference in overall resistance prevalence,[31] however, there was a slight increase in meropenem MICs and the emergence of carbapenem resistance. Carbapenem resistance in *C. difficile* is poorly characterised, possibly due to its rare occurrence. A previous study reported an association between point substitutions in PBP1 and PBP3 and high-level imipenem resistance, though these substitutions do not confer meropenem resistance.[20] We confirmed that neither the substitution on PBP1 nor PBP3 was associated with meropenem resistance. However, linear regression analysis suggested that the Y721S substitution in PBP3 may have contributed to a 4.3 folds increase in meropenem MIC. Thus, this substitution could be a part of a multistep meropenem resistance mechanism. Indeed, two *C. difficile* strain in this study had meropenem MICs of 16 mg/l (resistance breakpoint ≥ 16 mg/l), one of which was confirmed to have the Y721S substitution in PBP3.

C. difficile remained susceptible to metronidazole, vancomycin and fidaxomicin, similar to the other parts of the world.[49] Thus, these antimicrobials should remain effective treatments for CDI. There was a slight increase in vancomycin MIC reaching the clinical breakpoint, consistent with a previous study,[31] however, this should have little impact on the treatment of CDI as the stool vancomycin concentration remains far greater than the MIC (>2,000 mg/l vs 2 mg/l).[50] The increase in vancomycin MIC in this study is in contrast to other hospitals in Thailand and this could reflect usage of vancomycin at the study site.[24] Overuse of vancomycin can lead to the emergence of vancomycin-resistant *Enterococcus* spp., which can have a devastating effect on patients.[51,52] Therefore, vancomycin usage should be carefully monitored.

Conclusion

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

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Ethical conduct of research statement

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

Authors' disclosure statement

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