Antimicrobial Susceptibility of Toxigenic *Clostridium difficile* strains at Rajavithi Hospital

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Background: *Clostridium difficile is an anaerobic gram-positive bacillus that is the leading cause of antibiotic-associated diarrhea (AAD) and pseudomembrane colitis. The symptoms of C. difficile infection (CDI) range from mild diarrhea to severe colitis and death. The incidence of CDI is on the increase, and it is a major cause of nosocomial and community-acquired infections. At the Microbiology Laboratory of Rajavithi Hospital, anaerobic culture has not been performed routinely; therefore, the antimicrobial susceptibility of these infections is not known.*

Objective: *To determine the antimicrobial susceptibility profiles of toxigenic C. difficile isolates at Rajavithi Hospital.*

Material and Method: *From August 2016 to April 2017, a total of 43 stool samples that were confirmed positive for C. difficile toxin by real-time polymerase chain reaction method were collected. Forty-three toxigenic C. difficile stool samples underwent anaerobic culture and identification using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS). All of these were tested to minimum inhibitory concentrations (MICs) of 4 antimicrobial agents; clindamycin (CD), metronidazole (MTZ), moxifloxacin (MXF) and vancomycin (VAN) using gradient diffusion test strips.*

Results: *All of the 43 toxigenic C. difficile isolates at Rajavithi Hospital were 100.0% susceptible to VAN and MTZ, while susceptibility rates to CD and MXF were 9.3% and 6.9%, respectively. High MIC values of CD (MICs ≥256 mcg/mL) and MXF (MICs ≥32 mcg/mL) were found for 55.8% and 81.4%, respectively. Co-resistance to CD and MXF was found in 74.4% of these isolates.*

Conclusion: *All toxigenic C. difficile isolates identified at Rajavithi Hospital were susceptible to VAN and MTZ but had high resistance rates to CD and MXF, and the antimicrobial susceptibility testing of other antibiotics should therefore be tested to ensure appropriate treatment. Further classification of these toxigenic isolates into PCR genotypes and ribotypes for the detection of hypervilurence strains should be studied because they have high resistance to MXF, fluoroquinolone and CD.*

Keywords: *Clostridium difficile, Toxin, MALDI-TOF MS, Antimicrobial susceptibility*

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Clostridium difficile is an anaerobic, gram-positive, spore-forming bacillus which is a major cause of antibiotic-associated pseudomembranous colitis[1,2], and it is also a significant nosocomial and community-acquired pathogen[1-3]. The clinical manifestations of *C. difficile* infection (CDI) range from being asymptomatic or having mild diarrhea to severe colitis and death[1,2]. The pathogenicity of CDI depends on toxin type such as Toxin A (enterotoxin) and/or Toxin B (cytotoxin), for example A^+B^+, A^-B^+. In addition, binary toxins, ribotypes 027[4], 078[5] (A^+B^+ genotypes) and 017[6] (A^-B^+ genotypes), are hypervilurent *C. difficile* strains which overproduce toxins and cause severe illness, resulting in increased mortality and more frequent outbreaks[7]. The distribution patterns of toxigenotype and ribotype *C. difficile* strains vary geographically: the most prevalent ribotypes in North America and Europe causing outbreaks are ribotypes 027 and 078, while ribotype 017 strains are widespread in Asia[6].

In Thailand, reports of CDI[8-13] and its antibiotic susceptibility to treatment[11] are few because the process of anaerobic culture is labor-intensive, complicated, time-consuming, and unreliable, especially in identification at the species level using conventional methods. The laboratories with anaerobic bacteria culture services are usually found only in the leading national university hospitals.

Anaerobic laboratory testing is not routinely provided in the Microbiology Laboratory at Rajavithi Hospital, and so only the test for *C. difficile* toxin detection in patients' stool samples by polymerase

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chain reaction (PCR) method has been normally performed. There is no antimicrobial test results history, as antimicrobial susceptibility testing (AST) of CDI in Rajavithi Hospital had not been performed. Fortunately, the lab now has an automated identification machine, the MALDI-TOF MS, that allows easy, convenient and reliable *C. difficile* identification[14-16]. The antimicrobial susceptibility profiles of toxigenic *C. difficile* at Rajavithi Hospital were studied in this research.

Material and Method

The protocol of this research was reviewed and approved by the ethics committee of Rajavithi Hospital (No. 009/2558).

Bacterial collection

Between August 2016 and April 2017, all patient stools sent to Rajavithi's Microbiology section for detection of *C. difficile* toxin were tested by the BD MAX™ CDIFF assay (Becton Dickinson, Franklin Lakes, NJ). Forty-three stool samples with positive toxin results and non-duplicate patients were collected and the aliquots were stored at -70°C.

Bacterial isolates

All collected stool samples were cultured on to Chrom ID *C. difficile* agar (CDIF), a commercial chromogenic selective medium (bioMerieux, France), and incubated at 35°C under anaerobic condition (MGC AnaeroPacK™, Japan) for 48 hours. Colonies of *C. difficile* were selected on the basis of typical morphological features: dark flat, coarse and irregular structure.

Bacterial Identification

The typical colonies were confirmed to be *C. difficile* by MALDI-TOF MS (Biotyper 3.1; Bruker Daltonik, Germany). Briefly, a single colony was smeared on a steel MALDI target, spotted with 40.0% Formic acid in acetonitrile, overlaid with 1 microliter of a HCCA (alpha-4-cyano-4-hydroxycinnamic acid) matrix solution, which was then allowed to dry at room temperature, and the target was inserted into the MALDI-TOF MS to accurately detect and identify *C. difficile* (score of more than or equal 2.0). These colonies of *C. difficile* were transferred from CDIF and subcultured on sheep blood agar (SBA) plates in anaerobic condition (35°C) for 48 hours, and were then tested again for confirmation of *C. difficile* using MALDI-TOF MS.

Antimicrobial susceptibility testing

AST was performed, by picking 3-5 similar colonies from each isolate, diluted to a 0.5 McFarland standard suspension, and swabbed on Brucella agar plates, supplemented with haemin 5 mcg/mL, vitamin K_1 1 mcg/mL and sheep blood (5% v/v)[17].

All 43 clinical toxigenic *C. difficile* isolates were tested against the gradient minimum inhibitory concentrations (MICs) test strips (Liofilchem, Italy) of clindamycin (CD; 0.016 to 256 mcg/mL), metronidazole (MTZ; 0.016 to 256 mcg/mL), moxifloxacin (MXF; 0.002 to 32 mcg/mL), and vancomycin (VN; 0.016 to 256 mcg/mL). The MICs were determined under anaerobic incubation at 35°C after 48 hours. The interpretation of MIC results showed: CD (susceptible (S); ≤2 mcg/mL, intermediate (I), 4 mcg/mL, and resistant (R), ≥8 mcg/mL); MTZ (S; ≤8 mcg/mL, I; 16 mcg/mL, and R; ≥32 mcg/mL); and MXF (S; ≤2 mcg/mL, I; 4 mcg/mL, and R; ≥8 mcg/mL). Interpretation followed the Clinical Laboratory Standard Institute (CLSI) guidelines[17], except in the case of MIC of VAN (S; ≤2 mcg/mL, and R; >2 mcg/mL) which was interpreted by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines[18].

Clostridium difficile American Type Culture Collection (ATCC) 700057 was used as a control strain (Table 1).

Results

From August, 2016 through April, 2017, a total of 43 stool samples tested positive for *C. difficile* toxin by PCR, using the BD-Max CDIF. All *C. difficile* stool samples were cultured on CDIF agars in 35°C anaerobic condition for 48 hours, the typical colonies were selected and identified as *C. difficile* by MALDI-TOF MS, subcultured on SBA at 35°C anaerobic condition for 48 hours and then underwent a confirmatory test of *C. difficile* by MALDI-TOF MS again.

Each toxigenic *C. difficile* isolate was tested for all MICs strips of 4 antimicrobial agents; clindamycin (CD), metronidazole (MTZ), moxifloxacin (MXF) and vancomycin (VAN). Table 2 shows the results of antimicrobial susceptibility.

For clindamycin (CD), the rates of isolates' vulnerability were: susceptible (S), intermediate (I), and resistant (R) at 9.3% S (4/43), 14.0% I (6/43), and 76.7% R (33/43) respectively. The range of CD MICs were: 0.38 to >256 mcg/mL as follows: 0.38 mcg/mL (1 isolate); 0.75 mcg/mL (1 isolate); 1 mcg/mL (1 isolate); 2 mcg/mL (1 isolate); 3 mcg/mL (2 isolates); 4 mcg/mL (1 isolate); 6 mcg/mL (3 isolates); 8 mcg/mL (1 isolate); 12 mcg/mL

Table 1. Minimal Inhibitory Concentrations (MICs) Breakpoints for *Clostridium difficile* isolates and Quality Control Ranges for *Clostridium difficile* ATCC 700057

AntimicrobialAgent	Interpretive Categories and MIC Breakpoints (mcg/mL)			MIC QC Range (mcg/mL) *Clostridium difficile* ATCC 700057
	Susceptible (S)	Intermediate (I)	Resistant (R)	
Clindamycin (CD)	≤2	4	≥8	2 to 8
Metronidazole (MTZ)	≤8	16	≥32	0.125 to 0.5
Moxifloxacin (MXF)	≤2	4	≥8	1 to 4
Vancomycin (VAN)*	≤2	-	>2	0.5 to 4

MIC breakpoints applied for CD, MTZ and MXF are those recommended by CLSI
*For VAN, breakpoints are those recommended by EUCAST

Table 2. Antimicrobial susceptibility of 43 clinical toxigenic *Clostridium difficile* isolates isolated at Rajavithi Hospital, 2016 to 2017

Antimicrobial agent	Interpretation			Range of MIC (mcg/mL)	
	%S	%I	%R		(No. of isolates)
Clindamycin (CD)	9.3 (4/43)	14.0 (6/43)	76.7 (33/43)	0.38 to >256	0.38 (1), 0.75 (1), 1 (1), 2 (1), 3 (2), 4 (1), 6 (3), 8 (1), 12 (1), 16 (2), 64 (1), 128 (2), 192 (2) and >256 (24)
Metronidazole (MTZ)	100 (43/43)	0 (0/43)	0 (0/43)	0.023 to 0.38	0.023(1), 0.032 (4), 0.047 (5), 0.064 (5), 0.094 (7), 0.125 (6), 0.19 (7), 0.25 (7) and 0.38 (1)
Moxifloxacin (MXF)	6.9 (3/43)	4.7 (2/43)	88.4 (38/43)	1.0 to >32	1 (2), 1.5 (1), 3 (1), 4 (1), 6 (1), 24 (2), 32 (2) and >32 (33)
Vancomycin (VAN)	100 (43/43)	0 (0/43)	0 (0/43)	0.064 to 1.5	0.064 (1), 0.094 (2), 0.19 (4), 0.125 (2), 0.25 (10), 0.38 (15), 0.5 (6), 0.75 (1), 1 (1) and 1.5 (1)

(1 isolate); 16 mcg/mL (2 isolates); 64 mcg/mL (1 isolate); 128 mcg/mL (2 isolates); 192 mcg/mL (2 isolates); and >256 mcg/mL (24 isolates). All MICs of CD that showed ≥8 mcg/mL were interpreted as resistant (R). Twenty-four isolates (55.8%) had high-level resistance to CD (MICs ≥256 mcg/mL).

With regard to metronidazole (MTZ), all the isolates (43/43) were susceptible (100%S) with MTZ MICs ≤0.38 mcg/mL, and there was a narrow range of distribution of MIC values at 0.023 to 0.38 mcg/ml.

For moxifloxacin (MXF), most of the toxigenic *C. difficile* isolates (88.4%, 38/43) were resistant. MICs of MXF >8 mcg/mL were interpreted as resistant, as follows: 6 mcg/mL (1 isolate); 24 mcg/mL (2 isolates); 32 mcg/mL (2 isolates); and >32 mcg/mL (33 isolates). The rates of vulnerability were susceptible (S) 6.9% S (3/43) and intermediate (I) 4.7% I (2/43). Thirty-five isolates (81.4%) also had high-level resistance to MXF (MICs ≥32 mcg/mL).

For vancomycin (VAN), all the isolates (43/43) were susceptible (100% S) and had a narrow range of distribution of MIC values of 0.064 to 1.5 mcg/mL. Most MIC ranges of VAN were distributed within 0.25 mcg/mL (10 isolates), 0.38 mcg/mL (15 isolates) and 0.5 mcg/mL (6 isolates).

Overall, co-resistance to CD and MXF was found in 74.4% (32/43) of clinical toxigenic *C. difficile* isolates and MIC values of both CD and MXF showed high-level resistance.

Only 3 isolates (3/43) were susceptible to CD, but resistant to MXF (data not shown). Only one isolate was susceptible to all four antimicrobial agents (data not shown). Overall, no isolate was resistant to all four antimicrobial agents.

Discussion

The emergence of CDI is a worldwide problem, especially the increasing incidence of hypervirulent strains[1-6]. In one study, the prevalence of toxigenic *C. difficile* isolates in Thailand was approximately 10%[12,13]; however; as yet there is no database of antimicrobial susceptibility results. The present study is the first report of clinical toxigenic *C. difficile* isolates at Rajavithi Hospital, where anaerobic culture had not been a routine service.

In our study, no resistance of clinical toxigenic *C. difficile* isolates to VAN and MTZ were reported, and this is in agreement with other studies[19-23]. VAN and MTZ are therefore recommended for treatment of CDI[24], and although Vancomycin is more effective, it is also more expensive[24]. Some studies have reported reduced susceptibility to VAN[13,25] and MTZ[25]: the greater the use of VAN, the greater the risk of selection of Vancomycin-resistant enterocci (VRE)[24], which is still a concern at Rajavithi Hospital[26].

With regard to resistance to CD and MXF, most of the toxigenic *C. difficile* isolates at Rajavithi Hospital had high resistance rates to both (76.7%R and 88.4% R, respectively) and there were high level MIC values: CD (≥256 mcg/mL) and MXF (≥32 mcg/mL) (55.8% and 81.4%, respectively).

In contrast, Keessen EC, et al[23] found that ribotype 078 was susceptible to CD and MXF in 96% and 84% of cases, respectively, while Knight DR, et al[22] found that the most common virulent ribotypes, 014 and 002, were highly susceptible (96.6% S) to MXF. A study by Lidan C, et al[19] found that A^-B^+ *C. difficile* isolates were highly resistant to CD with MICs ≥256 mcg/mL while they were highly susceptible to MXF (77.8%); however, 22.2% showed high-level resistance with MXF MICs >32 mcg/mL.

Similarly, the results from a study by Lachowicz D, et al[20] showed that 27.7% of ribotype 027 and 176 isolates had high-level CD resistance, and with MICs >256 mcg/mL, 83.1% of these were resistant to MXF.

In another study by Lee JH, et al[21], most of the ribotypes 001 and 017 had high resistance to CD and MXF at 81% and 78.4%, respectively.

The limitation of this study was unclassified toxin genotypes (A^+B^+ or A^-B^+) and ribotypes (027 or 078 or 017) because the genotypes and ribotypes of toxigenic *C. difficile* vary geographically[6] and multidrug resistance has been associated with hypervirulent strains[20].

In future, culturing and AST of toxigenic *C. difficile* isolates from patients' stools should be performed routinely at Rajavithi Hospital because accuracy of treatment helps to limit the spread of virulent strains. The isolates in this study showed high resistance to CD and MXF, and repeated studies are required to classify them into toxin genotype (A^+B^+ or A^-B^+) and hypervilurent ribotype (027 or 078 or 017) strains.

Conclusion

This study is the first report of the antimicrobial susceptibility profile of clinical toxigenic *C. difficile* infection (CDI) at Rajavithi Hospital. All of the clinical isolates were susceptible to MTZ and VAN, but it was worrying that high resistance rates to CD and MXF were found. AST should be tested for the individual isolates to ensure accuracy of treatment, control of infection, and the spread of these organisms. These CDI organisms should be classified into toxin PCR genotypes and ribotypes for epidemiology and CDI database.

What is already known on this topic?

Antimicrobial susceptibility testing was performed of 4 agents; CD, MTZ, MXF, and VAN on 43 clinical toxigenic *C. difficile* isolates from patients' stools (CDI) at Rajavithi Hospital. The database of antimicrobial susceptibility showed that all CDI isolates were 100% susceptible to VAN and MTZ. CDI isolates at Rajavithi Hospital were highly resistant to CD and MXF with high-level MICs. To ensure accuracy of treatment, individual antibiotic agents should be tested.

What this study adds?

All clinical toxigenic *C. difficile* isolates at Rajavithi Hospital will in future be studied for classification into PCR genotypes (A^+B^+ or A^-B^+) and ribotypes (ribotype 027 or 078 or 017) for detection of hypervirulent strains, and testing of new antibiotics for antimicrobial susceptibility. This study was of AST of stool samples, and it should be followed by studies of other samples such as blood. Protein peaks of MALDI-TOF MS for all of the clinical toxigenic *C. difficile* isolates at Rajavithi Hospital will in future be compared with the results of PCR genotypes (A^+B^+ or A^-B^+) and ribotypes (ribotype 027 or 078 or 017) for detection of hypervirulent strains using MALDI-TOF MS.

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Potential conflict of interest

None.

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