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## MICROBIOLOGY

### Epidemiology of *Clostridium difficile* infection in a large teaching hospital in Singapore

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#### Summary

**Aims:** We undertook this study to define the incidence of toxigenic *Clostridium difficile* in our hospital and to characterise the isolates.

**Methods:** All unformed stool was tested for the presence of Toxin A (TcdA) and Toxin B (TcdB), and cultured for *C. difficile*. Culture filtrates were also tested for TcdA and TcdB. Detection of *tcdA* and *tcdB* genes was carried out for A<sup>-</sup>B<sup>+</sup> strains by polymerase chain reaction (PCR). The minimum inhibitory concentrations (MICs) of metronidazole, vancomycin and clindamycin for all isolates were tested using the Etest. PCR ribotyping was carried out on all isolates.

**Results:** The incidence of *Clostridium difficile* associated disease (CDAD) was 3.2 cases per 1000 admissions or discharges and 53.8 cases per 100 000 patient days. Most cases occurred in renal and haematology patients. CDAD was more common in patients aged over 50 years and of male gender. The Indian population was under-represented. Fourteen (11.8%) isolates were A<sup>-</sup>B<sup>+</sup>.

All strains were susceptible to metronidazole but one strain showed intermediate resistance to vancomycin. Only 12.8% of the isolates were susceptible to clindamycin. Thirty-five isolates had PCR ribotype A, of which 29 (83%) had a clindamycin MIC >256 mg/L. Thirty-three had PCR ribotype B, of which only one (3%) had a clindamycin MIC >256 mg/L. The 14 A<sup>-</sup>B<sup>+</sup> strains were all PCR ribotype C, and had a range of MICs for clindamycin from 2 to >256 mg/L.

**Conclusions:** The incidence of CDAD in our hospital is relatively low. Isolates remain susceptible to metronidazole and vancomycin.

**Key words:** PCR ribotyping, minimum inhibitory concentration, TcdA, TcdB, A<sup>-</sup>B<sup>+</sup>.

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#### INTRODUCTION

*Clostridium difficile* is an anaerobic pathogen which is an important cause of nosocomial diarrhoea. It produces two exotoxins which contribute to pathogenesis. Toxin A (TcdA) is a potent enterotoxin and thought to cause most of the clinical symptoms due to induced fluid secretion and mucosal damage.<sup>1</sup> Toxin B (TcdB) is approximately 1000

times more cytotoxic than toxin A. The effect of TcdB is thought to depend on initial tissue damage by TcdA, suggesting that both toxins work synergistically.<sup>2</sup>

While *Clostridium difficile* associated disease (CDAD) has been well described in North America and Europe, there are very little data on the prevalence of CDAD in south-east Asia.

Kumarasinghe *et al.* reported the prevalence of bacterial agents of diarrhoeal disease over a 50 month period from 1985 to 1989 in another large Singapore hospital.<sup>3</sup> In that study, *C. difficile* was isolated from 9.6% of cases tested. However, *C. difficile* was only sought when requested and toxin testing was not performed. All *C. difficile* isolates were sensitive to metronidazole.

We undertook this study to define the incidence of toxigenic *C. difficile* in our hospital and to characterise the isolates.

#### MATERIALS AND METHODS

##### Direct detection of TcdA and TcdB on samples

The Singapore General Hospital (SGH) is a 1600 bed tertiary referral hospital. All unformed stool from SGH inpatients sent to the Department of Pathology from 1 October 2002 to 28 February 2003 was tested for the presence of TcdA and TcdB using the Premier Toxin A and B enzyme immunoassay (EIA) kit (Meridian Diagnostics, USA) following the manufacturer's instructions. An overview of the specimen flow is given in Fig. 1.

##### Culture

The stool was further inoculated onto *C. difficile* Selective Agar and CDC anaerobe agar (Becton Dickinson, USA). Positive cultures were identified by microscopy, colony appearance, yellow-green fluorescence under long wave UV light, characteristic smell, negative reactions for lecithinase and lipase on egg yolk agar, and API20A (bioMérieux, France).

The testing for TcdA and TcdB direct from stool ceased after 28 February 2003 because of the severe acute respiratory syndrome (SARS) outbreak in Singapore,<sup>4</sup> however, culture for *C. difficile* was continued until a total of 125 toxigenic isolates was reached.

##### Preparation of template DNA

Overnight growth on a CDC anaerobic plate was harvested. A 1 µL loopful was resuspended in 200 µL InstaGene matrix (Bio-Rad, USA). After boiling for 12 min, the suspension was centrifuged at 15 000 g for 10 min. The supernatant was removed and stored at -70°C.

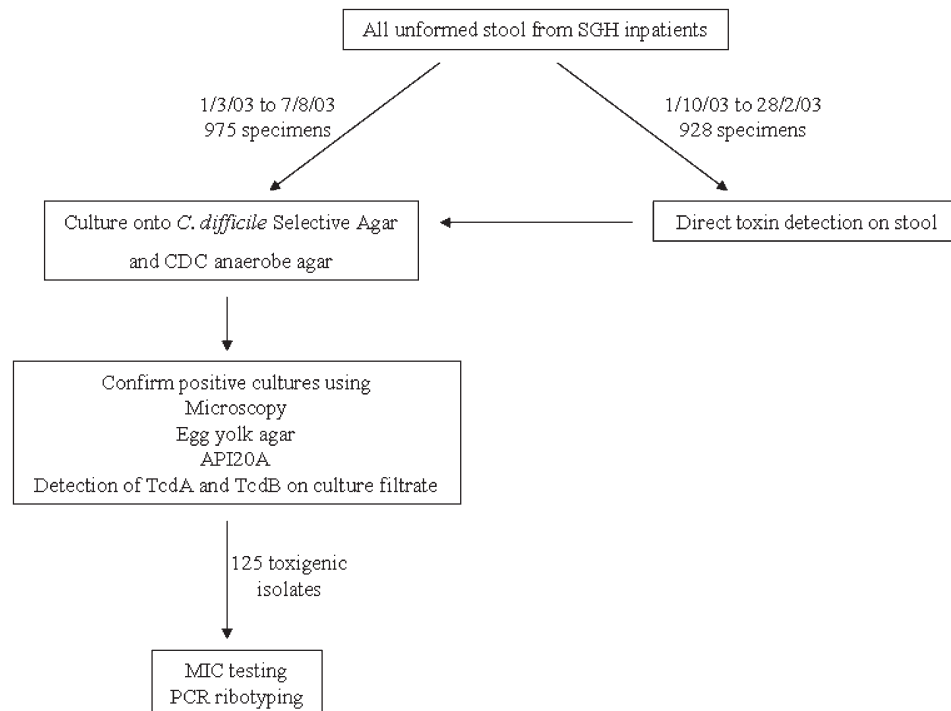


Fig. 1 Flow chart of specimen processing for this study. Direct toxin detection from stool was ceased after 28 February 2003 because of the SARS outbreak in Singapore. Culture for *C. difficile* was continued until a total of 125 toxigenic isolates was reached. PCR, polymerase chain reaction; MIC, minimum inhibitory concentration; SGH, Singapore General Hospital.

#### Preparation of culture filtrate

Strains were cultured in peptone-yeast extract-glucose broth at 35°C under anaerobic conditions for 24 h.<sup>5</sup> The culture was centrifuged at 2700 g for 15 min. The supernatant was removed and filtered through a 0.45 µm filter membrane into a Bijou bottle. Culture filtrate (0.2 mL) was then added to 1.8 mL of diluent.

#### Detection of TcdA and TcdB on culture filtrate

This was performed using the Premier Toxin A and B enzyme immunoassay (EIA) Kit (Meridian Diagnostics) following the manufacturer's instructions.

#### TcdA assay on culture filtrate

This was carried out using the Premier Toxin A enzyme immunoassay (EIA) Kit (Meridian Diagnostics) following the manufacturer's instructions.

#### TcdB assay on culture filtrate

The *C. difficile* Tox-B cytotoxicity assay (Techlab, USA) was used to confirm the production of Toxin B. Phosphate buffered saline and antitoxin were added to pairs of wells in a microtitre plate containing a monolayer of HeLa cells. Fifty µL of the culture filtrate and appropriate controls were added to each pair of wells in duplicate. The microtitre plate was incubated in 5% CO<sub>2</sub> at 35°C for 24 h. An inverted microscope was used to observe for rounding of cells. If necessary the plate was incubated for a further 24 h.

#### PCR detection of TcdA (*tcdA*) and Tcd (*tcdB*) genes

This was done for A<sup>-</sup>B<sup>+</sup> strains using the method described by Kato *et al.*<sup>6</sup>

#### Minimum inhibitory concentration (MIC)

The MICs of metronidazole, vancomycin and clindamycin for all isolates were tested using the Etest (AB Biodisk, Sweden) on Brucella agar with 5% sheep blood, haemin and vitamin K (Becton Dickinson). We tested the

MIC of clindamycin, even though this is inappropriate therapy for CDAD, because resistance to this antimicrobial is a surrogate strain marker. The data were entered and analysed in WHONET 5.<sup>7</sup>

#### PCR ribotyping

PCR ribotyping was carried out on all isolates using the method described by Stubbs *et al.*<sup>8</sup>

## RESULTS

Between October 2002 and February 2003, 928 stool samples were received. There were 50 stools positive for TcdA and TcdB by EIA (5.4%). On second look culture, there were an additional 38 stools which grew *C. difficile* whose culture filtrates were TcdA and TcdB positive by EIA (overall 9.5%). We were unable to isolate *C. difficile* from 20 stools which were toxin positive by EIA on direct testing.

Combining the numbers of toxigenic strains and culture negative/direct toxin positive specimens, the incidence of CDAD was 3.2 cases per 1000 admissions or discharges and 53.8 cases per 100 000 patient days. The breakdown of cases by specialty is shown in Table 1. The breakdown of cases by age group is shown in Table 2. The breakdown of cases by ethnic origin and sex are shown in Tables 3 and 4, respectively.

Fourteen (11.8%) isolates were negative for TcdA but positive for TcdB. All A<sup>-</sup>B<sup>+</sup> strains had a smaller PCR product (700 bp) for *tcdA*, in keeping with the phenotype.<sup>6</sup>

All isolates were susceptible to metronidazole, one strain showed intermediate resistance to vancomycin, and only 12.8% of the strains were susceptible to clindamycin. Sixty-three percent of isolates were fully resistant to clindamycin

TABLE 1 Occurrence of CDAD by hospital specialty

Speciality	Cases	Admissions	Cases per 1000 admissions
Renal	21	1688	12.4
Internal medicine	13	4006	3.2
Haematology	11	665	16.5
Gastroenterology	9	1417	6.4
Oncology	5	1554	3.2
Cardiothoracic surgery	5	595	8.4
Respiratory	5	2081	2.4
Orthopaedics	3	2603	1.2
Cardiology	3	3631	0.8
General surgery	3	3734	0.8
Other medical	6	2575	Not done
Other surgical	4	2468	Not done

TABLE 2 Number of cases of CDAD by age group

Age (years)	Cases	Cases per 1000 patients
20–29	4	1.37
30–39	6	1.87
40–49	7	1.79
50–59	15	3.22
60–69	21	4.42
70–79	21	4.99
80+	11	4.88

Data for patients aged <20 years not shown as the number of children admitted to our hospital is small.

TABLE 3 Number of cases of CDAD by ethnic origin

Ethnic origin	Cases (%)	No. patients	Cases per 1000 patients	95% CI (per 1000 patients)
Chinese	66 (75)	20208	3.3	2.5–4.1
Malay	15 (17)	3357	4.5	2.5–7.4
Indian	3 (3.5)	2439	1.2	0.3–3.6
Others	4 (4.5)	1426	Not done	Not done

CI, confidence interval.

TABLE 4 Distribution of CDAD cases by sex

Sex	Cases (%)	No. patients (%)
Females	34 (39)	14 095 (51)
Males	54 (61)	13 335 (49)

and 24% showed intermediate resistance. The MIC data are summarised in Table 5.

A selection of the most representative PCR ribotypes is shown in Fig. 2. Thirty-five isolates had PCR ribotype A of which 29 (83%) had a clindamycin MIC >256 mg/L. Thirty-three had PCR ribotype B, of which only one (3%) had a clindamycin MIC >256 mg/L. The 14 A<sup>-</sup>B<sup>+</sup> isolates were all PCR ribotype C and had a range of MICs to clindamycin from 2 to >256 mg/L. There were seven PCR ribotypes representing 2–3 isolates each. The remaining isolates had unique PCR ribotypes.

TABLE 5 Summary of MIC data for clindamycin, metronidazole and vancomycin

Antimicrobial	MIC (mg/L)		
	Range	50%	90%
Clindamycin	1–>512	8.0	>512
Metronidazole	0.125–2.0	0.5	1
Vancomycin	0.5–8.0	1.0	1

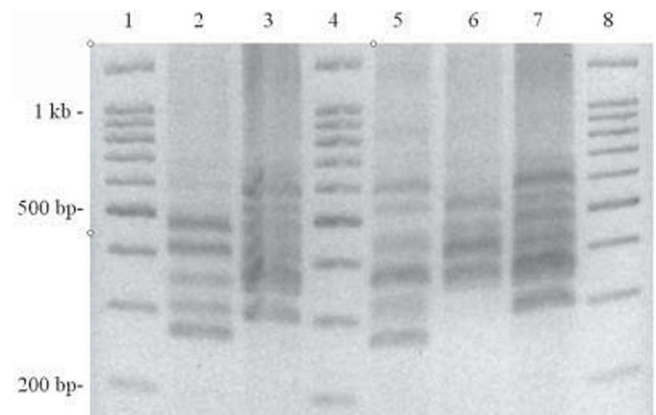


Fig. 2 Examples of three most common PCR ribotypes. Lane 1, 100 bp marker; Lane 2, PCR ribotype C; Lane 3, PCR ribotype A; Lane 4, 100 bp marker; Lane 5, PCR ribotype B; Lane 6, isolated PCR ribotype; Lane 7, PCR ribotype A; Lane 8, 100 bp marker.

## DISCUSSION

The general impression in Singapore is that *C. difficile* does not cause the same degree of morbidity and mortality locally compared with hospital populations in Western countries. Perceived low rates of severe disease and death may have led to an underestimate of the importance of CDAD.

In a recent outbreak in Quebec, hospitals were experiencing 22 cases of CDAD per 1000 admissions, although this decreased to 12.4 per 1000 admissions after infection control measures were implemented.<sup>9</sup> In the absence of an outbreak, the incidence may be very low. For example in a Paris hospital, the annual incidence varied from 0.7–1.2 per 1000 admissions.<sup>10</sup>

In the United States, from 1989 to 1992, the reported incidence in four hospitals varied from about 20 per 1000 admission/discharges during an outbreak, to seven per 1000 discharges in the absence of an outbreak. These epidemics were largely caused by clindamycin-resistant strains.<sup>11</sup> US hospital discharges for which CDAD was listed as any diagnosis doubled from 82 000 or 31 per 100 000 population in 1996 to 178 000 or 61 per 100 000 in 2003. This overall rate was several-fold higher in persons >65 years of age (228 cases per 100 000) than in the age group with the next highest rate, 45–64 years (40 cases per 100 000).<sup>12</sup>

Our hospital data are similar to those of Sweden, where the incidence of CDAD in a country-wide survey in 1995 varied from 1.0–3.3 cases per 1000 admissions.<sup>13</sup> However, the incidence in terms of bed-days was different (27 per 100 000 bed days in Sweden compared with 53.8 per 100 000 bed days in Singapore).

In another Swedish study in 1999–2000 from Örebro county, the incidence was similar and varied from 0.4–6.5 per 1000 admissions. The high incidence wards were geriatrics (36 cases per 1000 admissions), infectious disease (19 cases per 1000 admissions) and nephrology (13 cases per 1000 admissions).<sup>14</sup>

A later study from a Swedish teaching hospital in 2001 had a slightly higher incidence of 7 cases per 1000 admissions. Like our study, there was a higher incidence in nephrology (37.1 cases per 1000 admissions) and haematology (30.2 cases per 1000 admissions) wards. This is a likely reflection of antimicrobial and chemotherapeutic drug use in these specialties. In contrast to our study, the three most common PCR ribotypes comprised only 30% of hospital associated cases (versus 66% in Singapore) indicating that this was due to endogenous strains rather than hospital spread.<sup>15</sup>

It is quite difficult to draw comparisons about the incidence of CDAD, as papers from different countries are often comparing different measures and hospital populations. However, it appears that the incidence in Singapore is at the lower end of the spectrum, although the affected age groups and ward populations at risk are similar. Most CDAD in our study occurred in males and there was up to four-fold difference in incidence between patients representing the three major ethnic groups in Singapore. However, the 95% confidence intervals for the latter were wide and overlapping and our study was not designed to exclude confounding factors like underlying illnesses and principal diagnosis in the different subpopulations.

Most laboratories detect CDAD based on toxin assays. The performance of a toxin assay on *C. difficile* isolates after a negative direct toxin assay on stool ('second-look') has been shown to detect an extra 15% of toxin-producing strains which would have gone undetected otherwise.<sup>16</sup> In our study, direct toxin detection on stools would only have detected 56.8% of toxigenic isolates. This is almost identical to the result (56.7%) obtained by Delmée *et al.*<sup>17</sup> Like Delmée, we also used HeLa cells which are considered slightly less sensitive than Vero cells, which may partly explain the low sensitivity.

The reasons for culture not being performed are linked to cost and technical difficulties. However, Delmée *et al.* estimate that the total cost including labour, agar and reagents for toxin detection on colonies does not exceed 10 euros (~AU\$16).<sup>17</sup> In our laboratory, culture was not a problem because we have a large dedicated anaerobic section and a virology section to provide cell cultures. However, these resources may not be readily available in all laboratories. A disadvantage is the much longer turn-around time of culture (at least 3 days) compared with direct toxin assays (several minutes). The trade off between sensitivity and timeliness of result will need to be evaluated in each laboratory as sometimes the speed of the toxin assay is negated by the batching of specimens. More studies may need to be performed comparing the culture filtrate ('second-look') toxin assay versus repeating a direct toxin assay on a repeat specimen. It should be noted that culture is the only way to type strains and test their antimicrobial susceptibility.

Clinical isolates from patients with CDAD usually produce both TcdA and Tcd (A<sup>+</sup>B<sup>+</sup>) but an increasing number of infections are due to A<sup>-</sup>B<sup>+</sup> strains.<sup>18,19</sup>

*Clostridium difficile* A<sup>-</sup>B<sup>+</sup> strains of apparent clonal origin are widely distributed in North America and Europe. In a study of 39 A<sup>-</sup>B<sup>+</sup> isolates from Canada, the United States, Poland, United Kingdom, France, Japan and the Netherlands, 37 had the same PCR ribotype (017/20), and belonged to serogroup F.<sup>20</sup> The majority (85%) of isolates showed resistance to clindamycin.

In another international study of A<sup>-</sup>B<sup>+</sup> strains from the United Kingdom, Belgium and the United States, 21 of 23 strains had a 1.8 kb truncation of the *tcdA* gene, characteristic of toxinotype VIII strains. Twenty of these had PCR ribotype 017.<sup>21</sup>

The prevalence of A<sup>-</sup>B<sup>+</sup> strains reported was initially low. Lysterly found two strains (0.2%) in a collection of 102 toxigenic *C. difficile* isolates from a multicentre trial in the United States.<sup>22</sup> In the United Kingdom, 3% of strains sent to a reference centre for typing were A<sup>-</sup>B<sup>+</sup>.<sup>23</sup> In France, A<sup>-</sup>B<sup>+</sup> strains comprised 2.7% of toxigenic *C. difficile* isolates. Most had a 1.7 kb deletion and the same PCR ribotype.<sup>24</sup>

However, recently a paper from Poland reported 14% of toxigenic *C. difficile* strains isolated were A<sup>-</sup>B<sup>+</sup>. Of these, 41% were clonal and indistinguishable from a Japanese control strain by random amplification of polymorphic DNA (RAPD) and PCR ribotyping. These all had a deletion in *tcdA* and had high level resistance to clindamycin.<sup>25</sup>

Against this backdrop, the prevalence of A<sup>-</sup>B<sup>+</sup> isolates in our study is relatively high. Even though our A<sup>-</sup>B<sup>+</sup> isolates all had the same PCR ribotype, their clindamycin MICs were variable, ranging from 2 to >256 µg/L.

Routine antimicrobial susceptibility testing of *C. difficile* is not carried out because resistance is very rare and most laboratories do not culture the organism. However there is an increasing number of reports of resistance.

In a study from France over two time periods (1991 and 1997), there was decreased susceptibility to metronidazole for six strains (8–32 mg/L).<sup>26</sup> The minimal inhibitory concentration of 50% of the isolates (MIC<sub>50</sub>) for metronidazole remained static at 0.25 mg/L, whereas the minimal inhibitory concentration of 90% of the isolates (MIC<sub>90</sub>) decreased from 2 mg/L to 0.5 mg/L. The vancomycin MIC<sub>50</sub> and MIC<sub>90</sub> remained static at 1 mg/L and 2 mg/L, respectively. They recommended that periodic studies be conducted to detect the emergence of resistant strains.

In a study in Sweden, 238 *C. difficile* isolates collected between the years 2000 and 2001 were all susceptible to metronidazole and vancomycin, whereas 31.1% were intermediate and 12.6% were fully resistant to clindamycin. Unlike our study, no particular ribotype was associated with clindamycin resistance.<sup>27</sup> However in another part of Sweden, 45% of the isolates were resistant to clindamycin and belonged to the same fingerprinting group, suggesting clonal spread.<sup>28</sup> Clones of clindamycin-resistant *C. difficile* have also been associated with large outbreaks in the United States.<sup>11</sup>

Recently, Pelaez reported from Spain an overall rate of resistance to metronidazole of 6.3%. Although full resistance to vancomycin was not observed, the overall rate of intermediate resistance was 3.1%.<sup>29</sup>

Both metronidazole, and vancomycin retained good activity against our isolates, although one isolate showed reduced susceptibility to vancomycin. This is a little

surprising as the standard therapy for CDAD in our hospital is metronidazole and oral vancomycin is hardly used. Compared with other studies, a high proportion (87%) of our isolates were resistant to clindamycin.

In conclusion, the incidence of CDAD in our hospital is relatively low. It must be emphasised that this is likely to be an underestimate as patients were only diagnosed if a stool specimen was submitted to the laboratory. Isolates remain susceptible to the recommended antimicrobials metronidazole and vancomycin. If an EIA is to be used for diagnosis of CDAD in our population, it should be able to detect TcdB, as the prevalence of A<sup>-</sup>B<sup>+</sup> isolates is relatively high. Continued surveillance should be carried out to pre-empt the introduction of potentially more virulent strains of *C. difficile*.

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