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Perspective

Population-Based Study of Ribotypes and Toxin-b Production in Clinical Isolates of Clostridium difficile in Victoria, Australia

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Abstract

Clostridium difficile infection (CDI) is an ongoing public health issue worldwide with increased incidence and severity of disease seen over the past two decades [1-3]. The recent implementation of a national surveillance model for *C. difficile* by the Australian Commission for Safety and Quality in Healthcare (ACSQH) has seen an increase in the number of CDI surveillance studies in Australia [2,4]. Presented here are the clinical, toxigenic, cytotoxic and isolate diversity findings from a CDI surveillance of the Barwon Health South West region of Victoria, Australia conducted between October 2011 and February 2012. The majority of cases were elderly (≥ 65 years) with 62% being female, 59% of cases were healthcare associated and 39% were community associated; and 91% of isolates were positive for toxin B. Hyper virulent ribotypes 027 and 078 were not detected. There were three cases of MDU-064 detected and six ribotypes that had not been previously identified by the Microbiological Diagnostic Unit Public Health Laboratory (MDU) in Victoria.

Keywords: *Clostridium difficile; Clinical epidemiology; Ribotypes; Toxin B*

Abbreviations: ACSQH: Australian Commission for Safety and Quality in Healthcare; BB: Brucella Broth; BHIA: Brain Heart Infusion Agar; CCA: Cell Cytotoxicity Assay; CDI: Clostridium difficile Infection; CDT: Clostridium difficile binary Toxin; cdtA: Binary toxin subunit A gene; cdtB: Binary toxin subunit B gene; gyrA: DNA gyrase subunit A gene; gyrB: DNA gyrase subunit B gene; HB: Horse Blood; HREC: Human Research Ethics Committee; MDU: Microbiological Diagnostic Unit Public Health Laboratory; NPV: Negative Predictive Value; PaLoc: Pathogenicity Locus; PCR: Polymerase Chain Reaction; PPV: Positive Predictive Value; tcdA: Toxin A gene; tcdB: Toxin B gene; TcdC: Anti-sigma factor for toxins A and B; tcdC: Anti-sigma factor gene; TcdE: Holin-like protein; TcdR: Alternate sigma factor for toxins A and B; tpi: Triose phosphate isomerase gene (housekeeping gene)

Introduction

Clostridium difficile infection (CDI) is a major problem in healthcare facilities worldwide. The incidence of CDI has been increasing over the past two decades, along with the severity of related diseases [1-3]. Although historically CDI is considered a healthcare associated disease, there have been an increasing number of cases seen in the community setting [1,2]. Traditional risk factors for CDI are antimicrobial therapy, prolonged hospitalization, residence in long-term care facilities and advanced age (≥ 65 years) [5]. There has been

an increase in association of community acquired CDI with groups previously considered to be at a low risk including people who are younger, have no exposure to antibiotics, and have fewer co-morbidities [1,2].

CDI typically occurs after antibiotic therapy reduces the host's intestinal microbiota, allowing C. difficile to colonize and overpopulate the gut [1,6]. Traditional therapies for CDI are the antibiotics metronidazole and vancomycin [6]. Both of these antibiotics have been associated with rates of recurrence as high as 25% due to further intestinal dysbiosis [7]. Symptoms of CDI range from mild self-limiting diarrhoea to more serious disease such as pseudo membranous colitis. Complications of severe CDI include toxic mega colon and bowel perforations, which are life threatening and require surgical intervention [6]. Host pathology of CDI is caused by toxin A and toxin B which are members of the large clostridial glycosylating toxin family [6]. These toxins are the main virulence factors for C. difficile however some strains also produce a binary toxin CDT, which may also play a role in virulence, although this role is less well established [6,7]. The toxin genes (tcdA and tcdB) are located on the pathogenicity locus (PaLoc) along with the genes for the alternate sigma factor TcdR, a positive regulator of toxin gene expression, the holin-like protein TcdE, which is thought to play a role in toxin protein export, and the anti-sigma factor TcdC, which negatively regulates toxin gene expression [6,8].

Increased incidence of CDI in North America and Europe has been largely attributed to the emergence of hyper virulent strains such as PCR ribotype 027 [1,2,4,5]. This ribotype was first isolated in Quebec, Canada in 2005 and has since caused outbreaks in North America, Europe, Asia and Central America [4]. Ribotype 027 is associated with increased toxin production, fluoroquinolone resistance, increased incidence and severity of disease, more frequent recurrences and higher mortality rates [2,4,8]. This ribotype has a deletion in tcdC at base pair 117 which results in an inactivated truncated protein. This mutation has been shown to increase production of toxin A and toxin B, increasing virulence in this ribotype [8]. In Australia the first case of ribotype 027 was reported in 2009 in a patient that had acquired it in North America, and the first locally acquired case was reported in Melbourne, Victoria in 2010 [2,4,9,10]. Other emergent strains include ribotypes 078, 017, 014, 020, 015, and 002. Ribotype 078 has the same genetic virulence as 027, also causing severe disease, but is more widely associated with community acquire CDI [8].

Until recently, surveillance of CDI in Australia was limited. The development of a national surveillance model for *C. difficile* by the Australian Commission for Safety and Quality in Healthcare (ACSQH) [2,4] has seen a dramatic increase in the number of surveillance studies in recent years. Overall, these studies have reported an increase in the number of cases of CDI being detected, with the majority of cases occurring in older people (≥ 65 years), people having exposure to antibiotics. Most cases are healthcare associated [1-5,11] with community associated CDI ranging from 30% to 40% of all cases [2]. The most common ribotypes reported were 014, 002 and 020 [5,8] with some reports of ribotype 027 and 078, a large proportion of which were associated with an outbreak at an aged care facility in October/November 2010 [4]. Continued surveillance is needed to gain a better understanding of the epidemiology in Australia and monitor the occurrence of strains with the potential to cause outbreaks.

The aim of this study was to monitor the occurrence of CDI in the Barwon Health South West region of Victoria, Australia by analyzing the diversity of strains being isolated from patients diagnosed with CDI. This study focused on strain and toxin gene diversity as well as the basic patient demographics.

Methods

Study design

This was a retrospective study of all *C. difficile* strains successfully isolated on selective chromogenic agar, cultured and stored during routine pathology investigations on stool samples sent to St John of God Pathology, Geelong, Victoria between October 2011 and February 2012 (n = 129). This pathology company services Barwon Health, some community clinics and other regional hospitals throughout Western Victoria.

Clinical epidemiology

All bacterial isolates (n = 129) were included in the initial laboratory studies for toxin genes, ribotype diversity and antimicrobial susceptibility (as detailed below). Patient data was collected after the exclusions of duplicates (n = 16). Children under 2 years of age (n = 2) were also excluded due to frequent asymptomatic carriage of *C. difficile* in this population [33]. The epidemiology of the age, gender and regional distribution of CDI was then assessed (n = 111). The data from a subset of patients that had been admitted to Barwon Health (n = 62) were examined for type of acquisition of CDI by the surveillance protocol outlined by ACSQH [4], using the time of first stool sample to define onset of CDI. Ethics approval for this project was gained from the Barwon Health Human Research Ethics Committee (HREC) and is HREC project number 12/49.

Routine bacterial culture and storage

C difficile isolates (n = 129) were received from St John of God Pathology (Geelong, Victoria, AUST) as frozen glycerol stocks and stored at -80°C. Isolates were cultured on Brain Heart Infusion Agar (BHIA) (Oxoid, SA, AUST) containing 10% Horse Blood (HB) (Oxoid) on 90 mm diameter sterile plates, and were incubated anaerobically for 48 hours at 37°C in 2.5L AnaeroGen® gas jars (Oxoid) using an AnaeroPak® (Oxoid) to create an anaerobic environment. Liquid cultures were inoculated with 4-6 bacterial colonies from solid agar cultures, into 6 ml of Brucella Broth (BB) (Bacto Laboratories, NSW, AUST) anaerobically for 48 hours at 37°C.

Detection of toxins

Multiplex Polymerase Chain Reaction (PCR) was used to detect the presence of tcdA and tcdB genes, as well as the housekeeping gene tpi to confirm *C. difficile*. This was performed on all isolates of *C. difficile*, using standard molecular biology techniques. The primers were obtained from Sigma Aldrich (NSW, AUST) and sequences were as previously described by Lemee., *et al*. [12] The Cell Cytotoxicity Assay (CCA) to detect the cytotoxic activity of toxin B was modified from Eastwood., *et al*. [13] Briefly, liquid cultures of each isolate were prepared (as above) and 4 ml of bacterial suspension was filtered using a Millex-GS 0.22 µm membrane filter (Millipore, VIC, AUST) to obtain a cell free supernatant. Aliquots (1 ml) were applied to Vero cell monolayers at 80% confluency, in triplicate, in a 12 well-plate (Nunclon, SA, AUST). Controls included sterile BB only, reference strains M7404 (toxin A+ B+, Ribotype 027) and VPI10463 (toxin A+ B+). Cell monolayers were incubated at 37°C in 5% CO_2 and photographed at 0, 48 and 72 hours. Cytotoxic effects observed in the test wells, such as a high proportion of rounded and floating cells, indicated the presence of toxin B protein in the isolate supernatants.

Isolate diversity by PCR ribotyping

A subset of isolates (n = 68) was sent to the Microbiological Diagnostic Unit Public Health Laboratory (MDU) for ribotyping, and analysis of toxigenic profile. The tcdA and tcdB genes were detected as detailed above [12]. The tcdC gene was amplified using primers described in Spigaglia and Mastrantonio and sequenced using big dye terminator chemistry (Applied Biosystems) on a 3130xl genetic analyzer (Applied Biosystems). Isolate sequences were aligned to the reference sequence VPI10463 (Genbank, NCBI) to determine if any mutations were present. PCR ribotyping was based on the Stubbs, Brazier, O'Neill and Duerden method [14]. Isolates with ribotype patterns that did not match MDU's international reference collection but did match MDU's local collection were designated with an MDU ribotype number, while previously unidentified ribotypes were allocated a new MDU ribotype number.

Results

Clinical epidemiology

Demographic data (age and gender) for the entire isolate cohort was divided into age ranges as shown in Figure 1 below. There were more females diagnosed with CDI (62%) than males (38%), and the majority of cases occurred in elderly persons (> 65). The ages of female cases range from 16 to 96 years old (median 69 years), while the male patients ranged from 16 to 93 years old (median 73 years).

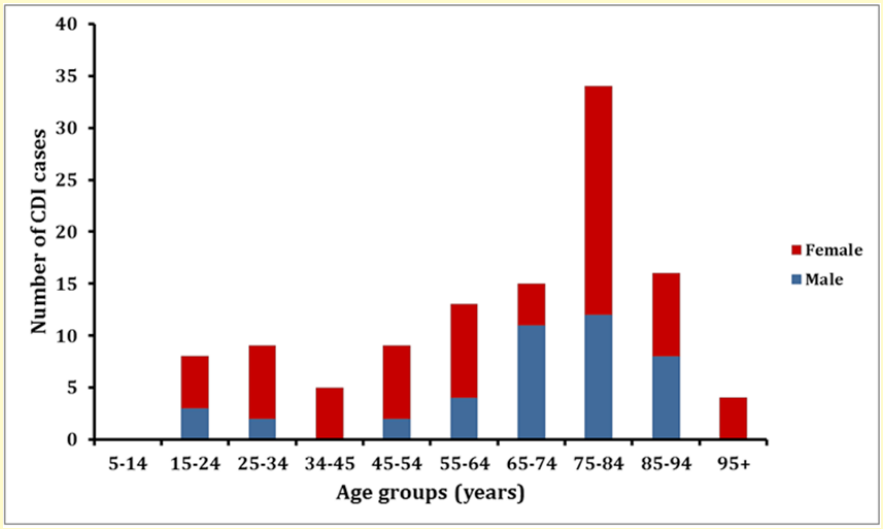

Figure 1: *Age and gender distribution of CDI cases (n = 111). Frequency histograms showing the distribution of CDI cases across age groups. Each age group is divided into male (blue) and female (green) cases.*

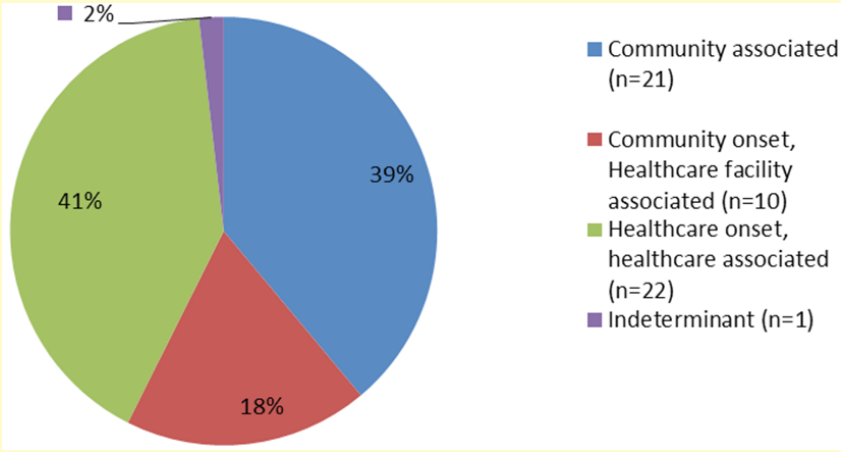

Figure 2: *C. difficile infection (CDI) acquisition for Geelong Hospital subset (n = 54). A total of 62 samples were taken in the study period for these patients, this data excludes duplicates from patients (n = 8) who had recurrent CDI during their hospital stay.*

The subset of patients that were admitted to Barwon Health during the study had detailed records of previous or subsequent hospitalization and/or CDI events and this enabled the stratification of community acquired versus the healthcare associated CDI, shown in Figure 2 below. The overall percentage of community associated CDI was 39%. The percentage of community onset, healthcare associated CDI was 18% and the percentage of healthcare onset, healthcare associated CDI was 41%, making the total percentage of healthcare associated CDI 59%. The acquisition of one case (2%) was unable to be determined. The rate of recurrence of CDI within this cohort was 12.9% (8 cases).

Ribotype	Occurrence	tcdA/B gene	tcdC sequence	Binary Toxin
002	9	+/+	Wild type	-/-
014	8	+/+	Wild type	-/-
056	5	+/+	Wild type	-/-
MDU-013	4	+/+	18bp deletion	-/-
MDU-023	4	+/+	Wild type	-/-
MDU-022	3	+/+	Wild type	+/+
MDU-051	3	+/+	Wild type	-/-
MDU-064	3	+/+	1 bp deletion at 117	+/+
005	2	+/+	Wild type	-/-
020	2	+/+	Wild type	-/-
054	2	+/+	Wild type	-/-
MDU-009	2	+/+	Wild type	-/-
MDU-019	2	+/+	Wild type	-/-
MDU-020	2	+/+	Wild type	-/-
MDU-075	2	+/+	Wild type	-/-
010	1	-/-	Not detected	-/-
103	1	+/+	Wild type	-/-
003	1	+/+	Wild type	-/-
MDU-049	1	+/+	Wild type	-/-
MDU-077	1	+/+	Wild type	-/-
MDU-109	1	+/+	Wild type	-/-
MDU-110	1	+/+	Wild type	-/-
MDU-115	1	+/+	Wild type	-/-
MDU-167	1	-/+	Wild type	-/-
MDU-174 (new)	1	+/+	Wild type	-/-
MDU-175 (new)	1	+/+	Wild type	-/-
MDU-176 (new)	1	+/+	Wild type	-/-
MDU-177 (new)	1	+/+	Wild type	-/-
MDU-178 (new)	1	+/+	18bp deletion	-/-
MDU-179 (new)	1	+/+	Wild type	-/-

Table 1: *List of ribotypes detected in MDU subset (n = 68) and their toxigenic profile.*

Toxigenicity, Cytotoxicity and Diversity

Toxigenicity of isolates was determined by multiplex PCR. Amplification of the housekeeping gene tpi confirmed that all isolates were *C. difficile*. Of the 129 samples, 99 (76.7%) were shown to be positive for toxin genes, tcdA and tcdB. Seven isolates (5.4%) had just tcdB and 11 (8.5%) were negative for both toxin genes. Surprisingly, 12 isolates (9.9%) were positive for tcdA but negative for tcdB. The multiplex PCR results were confirmed by CCA, which showed the tcdB negative result for these 12 isolates to be false negatives. Cytotoxic activity was assessed by CCA, which tests the cytotoxic activity of toxin B against Vero cells. Cytotoxic activity was observed for 117 isolates (90.7%) including some isolates that were negative for toxin B by multiplex PCR. A comparison of the multiplex PCR to CCA showed that the multiplex PCR had a sensitivity of 87%, specificity of 67%, a positive predictive value (PPV) of 96% and a negative predictive value (NPV) of 33%. These results indicate that the multiplex PCR method described here is likely to result in a large proportion of false negatives for the detection of tcdB.

To determine diversity of the isolates PCR ribotyping was performed on a subset of strains by MDU (n = 68). The ribotypes for this subset are shown in table 1 below. Toxigenicity, tcdC mutations and fluoroquinolone resistance were also assessed by MDU for this subset. The most prevalent ribotype was ribotype 002, followed by 014 and 056. Only one isolate of this subset (ribotype 010) lacked both the PaLoc and binary toxin, and was therefore non-toxigenic. There was one isolate (ribotype MDU-167) that was positive for tcdB, but negative for tcdA. Six *C. difficile* isolates had ribotypes that had not previously been identified at MDU (MDU-174 to MDU-179) and are now included in the MDU reference laboratory collection for Victoria. Three of the ribotypes (ribotype 024, MDU-178 and MDU-064) had mutations in tcdC. Ribotypes 024 (4 isolates) and MDU-178 (1 isolate) contain an 18bp deletion in tcdC, while MDU-064 (3 isolates) had a single base pair deletion at position 117. Ribotype MDU-064 is also positive for the binary toxin genes (cdtA and cdtB). There were three other isolates that were positive for the binary toxin genes, all belonging to ribotype MDU-022. The mutations in gyrA and gyrB are associated with fluoroquinolone resistance [15] were not detected in any isolate. Susceptibility to moxifloxacin was not tested in bacterial culture. No isolates from the hyper virulent ribotypes 027 or 078 were identified in this subset of isolates.

Discussion

This population based study aimed to provide data on CDI in the Barwon Health South West region of Victoria. It is limited by an incomplete data set, due to commercial partnerships between health care providers and other pathology companies in this region and small group sizes of stratified data. Overall the patient demographics were consistent with most other Australian and international studies, with the majority of patients being elderly (≥ 65 years) and female [1,4,5]. There was one study in Western Australia where there were more male patients than female (51.4% male) [5]. The number of community and hospital associated cases is also consistent with the other Australian studies. The rate of recurrence was lower than the ranges typically reported [7] but should still be monitored.

The high level of toxin producing strains in this cohort was not surprising, since all isolates were from confirmed cases of CDI. What was surprising was the detection by multiplex PCR of isolates that were positive for tcdA but negative for tcdB, as there were no previous records of this toxigenic profile reported. Further analysis by CCA indicated that these strains did in fact produce toxin B, and the PCR results were simply a false negative for tcdB. Six isolates (three MDU-064 and three MDU-022) were positive for the binary toxin genes (cdtA and cdtB). The binary toxin is an actin specific ADP-ribosylating toxin [16]. The exact role of the binary toxin in *C. difficile* virulence is not yet well established [5,8].

There were 30 individual ribotypes identified in the 68 isolates sent to MDU. The most prevalent ribotypes were 014, 002 and 056. These ribotypes are among the most frequently occurring ribotypes in Australia [3,8]. Ribotypes 014 and 002 are also frequently seen in North America and Europe [17,18] No isolates of the hyper virulent ribotype 027 was identified in this study. Other recent studies in Australia have reported only low numbers of ribotype 027, and there have been very few outbreaks [1,2,4]. It is clear that ribotype 027

is still not endemic in Australia, and this may be due to Australia's geographic isolation which may slow the spread of new strains to this country. Australia also has conservative policies on fluoroquinolone use in both humans and animals, and this may also offer protection against fluoroquinolone resistant strains such as ribotype 027 [2]. There were three MDU-064 isolates detected in the MDU cohort (n = 68), which is certainly a point of interest. This ribotype was first detected in Victoria in September 2010 and was then associated with a cluster of cases in April 2011 with approximately 50 isolates detected in various laboratories in Victoria. MDU-064 has a single base pair mutation at position 117 of tcdC, the same mutation that has been attributed to increased toxin production and virulence in ribotype 027 [8]. Another ribotype with this mutation is ribotype 244, which is very similar to MDU-064 and has been associated with severe CDI in New Zealand [19] and more recently in Australia [20]. The detection of previously unidentified ribotypes as well as ribotypes associated with clusters of CDI cases highlights the need for ongoing surveillance of CDI in Australia, not just to maintain current knowledge of the epidemiology of this infection, but also to allow early detection of potentially hyper virulent strains.

Conclusions

Clostridium difficile is an ongoing public health threat. The epidemiological findings reported here for the Barwon Health South West region of Victoria are consistent with those reported both in Australia and internationally. We strongly recommend ongoing surveillance of this infection to monitor the occurrence of strains capable of causing severe disease and outbreaks, as well as monitoring recurrence rates. Surveillance should cover both healthcare facilities and community settings, and should aim to identify potential reservoirs in both settings.

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