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PCR ribotyping and antimicrobial susceptibility testing of isolates of *Clostridium difficile* cultured from toxin-positive diarrheal stools of patients receiving medical care in Canadian hospitals: the Canadian *Clostridium difficile* Surveillance Study (CAN-DIFF) 2013–2015

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ABSTRACT

Clostridium difficile toxin-positive diarrheal stool specimens submitted to eight Canadian hospital laboratories from 2013 to 2015 were cultured. Polymerase chain reaction ribotyping of isolates was performed using an internationally standardized, high-resolution capillary gel-based electrophoresis protocol and antimicrobial susceptibility testing conducted by CLSI-defined agar dilution (M11-A8, 2012). Among the 1310 isolates of *C. difficile* cultured, 141 different ribotypes were identified; the most common ribotypes were 027 (24.5% of isolates), 014 (7.7%), 020 (6.6%), 106 (6.1%), and 002 (4.6%). Ribotype 027 was the commonest ribotype in all geographic regions of Canada and was more frequently isolated from patients aged ≥80 years (40.6%) than younger patients ($P<0.00001$). Ribotype 027 isolates were frequently moxifloxacin-resistant (92.2% of isolates) and multidrug-resistant (49.5%). Fidaxomicin demonstrated the greatest *in vitro* potency (lowest MIC_{90}, 0.5 μg/mL; lowest maximum MIC, 2 μg/mL) of eight antimicrobial agents tested and was the most active agent against each of the five commonest ribotypes (MIC_{90}, 0.25–1 μg/mL).

1. Introduction

Published reports indicate that up to 30% of patients receiving an antimicrobial agent in hospital will develop diarrhea (Bartlett and Gerding, 2008). *Clostridium difficile*, an anaerobic, spore-forming, gram-positive bacillus that causes gastrointestinal infection via toxin production, is the most frequently identified infectious cause of nosocomial diarrhea, accounting for 15%–30% of cases (Bartlett and Gerding, 2008). The severity, outcome, recurrence, and cost of *C. difficile* infections (CDIs) are influenced by patient age, immune status, length of hospitalization, treatment received, and other factors (Bartlett and Gerding, 2008; Freeman et al., 2010; Nanwa et al., 2016).

The management of patients with CDI includes withdrawal of the predisposing antimicrobial agent, if possible, and empiric therapy, most commonly with metronidazole or oral vancomycin. However, treatment of CDI with metronidazole is increasingly associated with recurrence of infection (20%–30% of treated patients) and may reflect growth suppression of patients' normal, protective microbiota and/or low concentrations of metronidazole in feces (Fekety et al., 1997; Freeman et al., 2010; Musher et al., 2005). CDI recurrence in patients treated with metronidazole has been reported to occur more frequently in association with ribotype 027 (also known as North American Pulsotype 1 [NAP1] or restriction endonuclease analysis [REA] type BI) than other ribotypes of *C. difficile* (Bourgault et al., 2006; Freeman et al., 2010; He et al., 2013; Johnson et al., 2014; Martin et al., 2008; Miller et al., 2010; Musher et al., 2005; Petrella et al., 2012). Reports of decreased susceptibility to metronidazole have resulted in revision of the ESCMID guidelines which now recommend treating patients with multiple recurrent (nonsevere) CDIs with either vancomycin or fidaxomicin (Debast et al., 2014).

Ribotype 027 produces binary toxin (*cdtB*), frequently hyperproduces spores as well as toxin A (*tcdA*) and toxin B (*tcdB*), possesses deletions in the regulatory gene *tcdC* and demonstrates resistance to fluoroquinolones (moxifloxacin) (Gerding, 2010; McDonald et al., 2005). The presence of binary toxin and *tcdC* deletions were initially suggested to underlie the enhanced virulence of ribotype 027 (McDonald et al., 2005); however, genomic studies later dispelled this

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hypothesis (Freeman et al., 2010; He et al., 2013; Martin et al., 2008; Murray et al., 2009) as these features are also found in other, less virulent ribotypes.

Clinical microbiology laboratories do not routinely culture toxin-positive stool specimens for *C. difficile* nor do they perform antimicrobial susceptibility testing because of its complexity, cost, and dubious clinical significance (given the high concentrations of antimicrobial agents achieved in the gastrointestinal tract following oral administration), and isolates are generally considered to be susceptible *in vitro* to the two main therapeutic agents, metronidazole and vancomycin (Goldstein et al., 2011). However, periodic assessment of toxin-positive *C. difficile* isolate ribotypes and antibiograms seems prudent and may inform healthcare providers of changes in the epidemiology of ribotypes and antimicrobial-resistant isolates and to help assess the effectiveness of infection control practices. The current study, the Canadian *Clostridium difficile* Surveillance Study (CAN-DIFF), was initiated in 2013 to provide an annual, ongoing prospective epidemiological assessment of changes in ribotype profiles and antimicrobial susceptibility of clinical isolates of *C. difficile* causing infection in patients across Canada. The current report summarizes data from the CAN-DIFF study for isolates of *C. difficile* from across Canada from 2013 to 2015, inclusive.

2. Materials and methods

2.1. Bacterial isolates

From January 2013 to December 2015, eight Canadian hospital clinical microbiology laboratories submitted aliquots (1–4 mL) of diarrheal stool specimens identified in their respective laboratories as positive for the presence of *C. difficile* toxin to the study's coordinating laboratory at the Winnipeg Health Sciences Centre (Winnipeg, Manitoba, Canada). Five diarrheal stool aliquots were requested per month from each participating laboratory, resulting in a maximum of 60 stool aliquots per year per laboratory. Each diarrheal stool aliquot was from a unique patient. Basic patient demographic information was submitted by the participating laboratory with each stool aliquot (i.e., patient geographic location, gender, age). Aliquots were frozen at −20°C by the participating laboratory and shipped quarterly to the coordinating laboratory where they were cultured on *Clostridium difficile* Moxalactam Norfloxacin (CDMN) Selective Supplement agar (Oxoid Canada, Nepean, Ontario, Canada) following a 1-h ethanol shock step at room temperature using equal volumes of 95% ethanol and diarrheal stool. The identity of each isolate of *C. difficile* was confirmed phenotypically by gram stain, typical odor, latex agglutination (Microgen Bioproducts Ltd., Surrey, UK) or a positive L-proline aminopeptidase activity test, and chartreuse fluorescence under UV light (CLSI, 2008). Isolates were stocked at the coordinating laboratory in skim milk at −70°C. In total, 1310 isolates of *C. difficile* were cultured and available for antimicrobial susceptibility testing and molecular characterization.

2.2. Polymerase chain reaction for C. difficile toxin genes

DNA extraction was performed using a commercial kit (InstaGene Matrix; Bio-Rad, Richmond, CA). The presence of genes coding for *C. difficile* toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdtB*) was determined for each isolate using previously described polymerase chain reaction (PCR) methods (Kato et al., 1998; Lemee et al., 2004; Stubbs et al., 2000). PCR products were separated by electrophoresis on a 1.5% agarose gel, visualized with ethidium bromide staining, and identified by size using bands generated by control strains.

2.3. PCR ribotyping

Isolates were ribotyped using an internationally standardized, high-resolution capillary gel-based electrophoresis PCR ribotyping protocol for *C. difficile* (Fawley et al., 2015). PCR products were analyzed on an Applied Biosystems 3130xl genetic analyzer using a 16-capillary 36-cm array with POP-7 separation matrix (Life Technologies, Grand Island, NY). The genetic analyzer was calibrated for the G5 dye set. GeneScan 1200 LIZ standard was used as internal sizing reference (Life Technologies). Samples contained 1 μL of amplified DNA, 0.5 μL of 1200 LIZ standard, and 8.5 μL of Hi-Di formamide (Life Technologies, Carlsbad, CA). Samples were injected at 5 kV for 5 s and resolved using a separation voltage of 6.5 kV for 103 min. Major peaks in fluorescent signal were imported into BioNumerics v.5.1 software (Applied Maths, Austin, TX) for analysis. Fragments were initially sized using GeneMapper v.4.0 software (Life Technologies) before being imported into BioNumerics. All signals with a height <10% of the highest peak in the individual profile were excluded (as these were considered background rather than evidence of a major DNA fragment).

2.4. Antimicrobial susceptibility testing

The *in vitro* activities of eight antianaerobic agents, including fidaxomicin and its active metabolite OP-1118, were determined using CLSI-defined agar dilution antimicrobial susceptibility testing (CLSI, 2015). Fidaxomicin and OP-1118 were provided by Merck & Co., Inc. (Kenilworth, NJ); the other antimicrobial agents were obtained from commercial sources. All antimicrobial agents other than OP-1118 were dissolved and diluted following CLSI guidelines (CLSI, 2017); OP-1118 was dissolved in DMSO and diluted in water (as recommended for fidaxomicin). *C. difficile* ATCC 700057 was used as the quality control strain; its MIC reference range for fidaxomicin is 0.06–0.25 μg/mL (CLSI, 2017). MIC interpretive criteria have not been established for fidaxomicin or OP-1118; CLSI breakpoints (CLSI, 2017) were used to interpret MICs for the other antimicrobial agents tested with the exception of vancomycin for which the EUCAST epidemiological cutoff value (ECOFF) for vancomycin tested against *C. difficile* was used (i.e., vancomycin wild-type, MIC ≤2 μg/mL; reduced susceptibility to vancomycin, MIC >2 μg/mL) (EUCAST, 2017).

2.5. Statistical analysis

Chi-square testing was used to establish statistical significance (significance level, $P<0.05$) between variables.

3. Results

There was tremendous ribotype diversity (141 different ribotypes) among the isolates of *C. difficile* cultured from diarrheal stool specimens of patients receiving medical care at hospitals in Canada from 2013 to 2015 (Table 1). Ribotype 027 was the most frequently identified ribotype, accounting for 24.5% of isolates followed by ribotypes 014 (7.7%), 020 (6.6%), 106 (6.1%), and 002 (4.6%). Ribotypes with <15 isolates accounted for 25.9% of the 1310 isolates.

Ribotype 027 was found more commonly among isolates of toxin-positive *C. difficile* in eastern Canada (37.1% of isolates in Quebec and Nova Scotia) than among isolates from central (21.0% of isolates in Ontario) and western (14.5% of isolates in British Columbia, Alberta, Manitoba) Canada ($P<0.00001$) (Table 2). The prevalence of ribotypes 014, 020, 106, and 002 was constant across the three regions of Canada ($P>0.05$). The overall prevalence of ribotype 027 was similar across the 3 years of the study ($P=0.208$); however, when analyzed by geographic region, the prevalence of ribotype 027 decreased from 2013 to 2015 in central Canada ($P=0.001$) but remained unchanged over time in western ($P>0.05$) and eastern Canada ($P>0.05$) (Table 3). From 2013 to 2015, the prevalence of ribotype 014 significantly decreased in eastern Canada ($P=0.018$) but remained unchanged in western and central Canada (data not shown), while the prevalence of ribotype 020 significantly increased in western ($P=0.041$) and central ($P=0.010$) Canada but remained unchanged in eastern Canada (data

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