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Antimicrobial Susceptibility Testing of *Clostridium difficile* Ribotypes Infecting Canadian Patients: The Canadian *Clostridium difficile* Surveillance Study (CAN-DIFF) 2013-2016

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Introduction

Clostridiodes difficile (formerly *Clostridium difficile*) is the most frequently identified infectious cause of nosocomial diarrhea. *C. difficile* infection (CDI) occurs primarily in patients previously receiving antimicrobial agents. Antimicrobial susceptibility testing of *C. difficile* is rarely performed in clinical laboratories because of its complexity, cost, and dubious clinical significance.

Management of patients with CDI includes withdrawal of the predisposing antimicrobial agent, if possible, and empiric therapy with either oral vancomycin or fidaxomicin recommended over metronidazole (1). Fidaxomicin was approved by the FDA to treat CDI in 2011. Fidaxomicin is an oral, narrow-spectrum macrocycle that inhibits the RNA polymerase of Gram-positive bacteria, especially *C. difficile*.

Treatment failure and CDI recurrence in patients treated with metronidazole occurs with considerable frequency (2-5). Vancomycin treatment of CDI may increase the risk for selection of vancomycin resistance in enterococci and staphylococci (6).

As the epidemiology and pathogenesis of *C. difficile* evolves, routine surveillance of clinical isolates to determine their ribotypes and *in vitro* susceptibility to both established and newer agents is warranted.

Materials and Methods

Bacterial Isolates Studied

1,747 isolates of *C. difficile* were cultured on *C. difficile* Moxalactam Norfloxacin (CDMN) Selective Supplement agar (Oxoid Canada, Nepean, ON, Canada) from toxin-positive stool specimens (following an ethanol shock step) by the clinical microbiology laboratory at the Winnipeg Health Sciences Centre. Each isolate's identity was confirmed by Gram stain, typical odor, latex agglutination (Microgen Bioproducts Ltd., Surrey, UK) or a positive L-proline aminopeptidase test, and chartreuse fluorescence under UV light (7). Chi-square testing was used to establish statistical significance (significance level, $P \leq 0.05$).

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was performed using the CLSI agar dilution reference method (8). Fidaxomicin and OP-1118 were supplied by Merck & Co., Inc.; the solvent for both of these compounds was DMSO and water was used as the diluent. *C. difficile* ATCC 700057 was used as the quality control strain for testing fidaxomicin; its reference MIC range for fidaxomicin is 0.06-0.25 µg/mL. *In vitro* antimicrobial susceptibility testing MIC interpretive criteria have not been established for fidaxomicin. CLSI breakpoints were used to interpret MICs for the other antimicrobial agents tested (9) except vancomycin. Vancomycin MICs were interpreted using the epidemiological cut-off value (ECOFF) established by EUCAST for vancomycin tested against *C. difficile* (vancomycin-wild-type [susceptible], ≤2 µg/mL; reduced susceptibility to vancomycin, >2 µg/mL) (10).

PCR Ribotyping

Isolates were ribotyped at the National Microbiology Laboratory, Public Health Agency of Canada, using an internationally-standardized, high-resolution capillary gel-based electrophoresis PCR-ribotyping protocol for *C. difficile* (11).

PCR for Toxin Genes

DNA extraction was performed using a commercial kit (InstaGene Matrix; Bio-Rad, Richmond, CA). The presence of the genes coding for toxin A (*tcdA*), toxin B (*tcdB*), negative regulator of toxin production (*tcdC*), binary toxin (*cdtB*), and triose phosphate isomerase (*tpi*) were determined for each cultured isolate using previously described PCR methods (12-14). PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized with ethidium bromide staining, and images captured using Alpha Imager software (Alpha Innotech Corp., San Leandro, CA). The presence of a deletion or mutations in the *tcdC* gene was investigated by PCR amplification of the *tcdC* gene by following the methods outlined by Spigaglia and Mastrantonio (15). PCR products were purified and sequenced. All PCR testing was performed at the National Microbiology Laboratory, Public Health Agency of Canada.

Results

Table 1. PCR ribotype composition of the 1,747 isolates of toxin-positive *C. difficile* collected by the CAN-DIFF surveillance study from 2013 to 2016

PCR ribotype	Number of isolates (% of all isolates)
027	379 (21.7%)
106	131 (7.5%)
014	126 (7.2%)
020	116 (6.6%)
002	86 (4.9%)
056	63 (3.6%)
072	44 (2.5%)
078	43 (2.5%)
015	43 (2.5%)
057	40 (2.3%)
012	34 (1.9%)
076	32 (1.8%)
087	29 (1.7%)
054	25 (1.4%)
005	24 (1.4%)
176	23 (1.3%)
103	22 (1.3%)
153	21 (1.2%)
019	20 (1.1%)
Ribotypes with <20 isolates	446 (25.5%)
Total number of different ribotypes:	172

[a] West (British Columbia, Alberta, Manitoba; 3 laboratory sites/year), Central (Ontario; 2-3 laboratory sites/year), and East (Quebec, Nova Scotia; 3 laboratory sites/year).

Table 2. Annual prevalence of PCR ribotype 027 among toxin-positive *C. difficile* stratified by Canadian geographic region[a]

Year (number of isolates)	Canadian geographic region, number of isolates (% of total isolates from geographic region)		
	West	Central	East
2013 (411)	27 (17.4%)	25 (25.3%)	61 (38.9%)
2014 (410)	21 (13.5%)	30 (30.0%)	47 (30.5%)
2015 (485)	20 (12.8%)	21 (12.7%)	68 (41.7%)
2016 (441)	29 (17.1%)	12 (7.5%)	18 (16.2%)

Table 3. Prevalence of common PCR ribotypes among toxin-positive *C. difficile* stratified by patient age[a]

Age group (number of isolates)	Ribotype, number of isolates (% of total isolates from patient age group)				
	027	014	020	106	002
≤64 years (815)	110 (13.5%)	63 (7.7%)	57 (7.0%)	74 (9.1%)	39 (4.8%)
65-79 years (494)	100 (20.2%)	32 (6.5%)	36 (7.3%)	33 (6.7%)	26 (5.3%)
≥80 years (435)	167 (38.4%)	31 (7.1%)	23 (5.3%)	24 (5.5%)	21 (4.8%)

[a] Patient age was unknown for 3 of 1,747 (0.2%) isolates.

Figure 1. Prevalence of common PCR ribotypes among toxin-positive *C. difficile* stratified by Canadian geographic region[a]

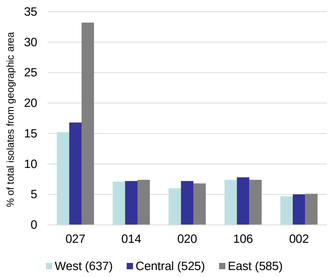

Table 4. CAN-DIFF 2013-2016 antimicrobial susceptibility testing results for 1,747 toxin-positive isolates of *C. difficile* stratified according to PCR ribotype

Antimicrobial agent	Ribotype[a]	MIC (µg/mL)				MIC interpretation		
		Range	Mode	MIC_{50}	MIC_{90}	% S	% I	% R
Amoxicillin-clavulanate	027	≤0.25-2	2	2	2	100	0	0
	014	0.5-2	1	1	2	100	0	0
	020	0.5-2	1	1	2	100	0	0
	106	0.5-2	1	1	2	100	0	0
	002	1-4	1	1	2	100	0	0
	All non-027 ribotypes	≤0.25-8	1	1	2	100	0	0
Ceftriaxone	027	8->128	64	64	64	1.3	23.5	75.2
	014	8-128	32	32	64	7.9	69.9	22.2
	020	16-128	32	32	64	7.8	79.3	12.9
	106	16->128	32	32	64	5.3	62.6	32.1
	002	16->128	32	32	32	5.8	86.1	8.1
	All non-027 ribotypes	8->128	32	32	64	8.9	71.8	19.3
Clindamycin	027	1->64	8	8	>64	4	27.9	68.1
	014	2->64	8	8	8	5.6	37.3	57.1
	020	≤0.12->64	8	8	8	8.6	21.6	69.8
	106	1->64	8	8	8	3.1	23.6	73.3
	002	1-8	8	8	8	2.3	33.7	64
	All non-027 ribotypes	≤0.12->64	8	8	>64	5.6	32	62.4
Fidaxomicin	027	0.12-1	0.5	0.5	0.5	NA	NA	NA
	014	0.06-1	0.25	0.25	0.5	NA	NA	NA
	020	0.06-0.5	0.25	0.25	0.25	NA	NA	NA
	106	0.03-2	0.5	0.5	0.5	NA	NA	NA
	002	0.06-0.5	0.12	0.25	0.5	NA	NA	NA
	All non-027 ribotypes	≤0.015->8	0.25	0.25	0.5	NA	NA	NA
Metronidazole	027	0.25-4	2	2	4	100	0	0
	014	0.25-4	0.5	0.5	1	100	0	0
	020	0.25-4	0.5	0.5	1	100	0	0
	106	0.25-2	0.5	0.5	1	100	0	0
	002	0.25-2	0.5	0.5	1	100	0	0
	All non-027 ribotypes	0.12-4	0.5	0.5	1	100	0	0
Moxifloxacin	027	1->32	>32	32	>32	10.3	0	89.7
	014	1->32	2	2	16	84.1	1.6	14.3
	020	1-16	2	2	2	92.2	1.8	6
	106	1-32	2	2	2	92.4	1.5	6.1
	002	0.5-32	1	1	2	90.7	1.2	8.1
	All non-027 ribotypes	0.5->32	1	2	4	88.9	1.1	10
OP-1118	027	1-32	16	16	16	NA	NA	NA
	014	0.5-16	4	4	8	NA	NA	NA
	020	1-16	4	4	8	NA	NA	NA
	106	0.5-16	8	8	16	NA	NA	NA
	002	1-16	4	4	8	NA	NA	NA
	All non-027 ribotypes	0.12-64	4	4	8	NA	NA	NA
Vancomycin	027	≤0.25-4	1	1	2	97.6[b]	NA	NA
	014	0.5-2	1	1	2	100	NA	NA
	020	0.5-2	1	1	2	100	NA	NA
	106	0.5-4	1	1	2	99.2	NA	NA
	002	0.5-4	1	1	2	98.8	NA	NA
	All non-027 ribotypes	0.5-4	1	1	2	99	NA	NA

[a] There were 379 isolates with ribotype 027 and 1368 non-ribotype 027 isolates. Non-ribotype 027 isolates with >85 isolates are shown individually and are also included in the "All non-027 ribotypes" group.
[b] MICs were interpreted using the EUCAST epidemiological cut-off value (ECOFF) (vancomycin-wild-type [susceptible], ≤2 µg/mL; reduced susceptibility to vancomycin, >2 µg/mL)

Table 5. Prevalence of common PCR ribotypes among toxin-positive *C. difficile* collected by the CAN-DIFF surveillance study

Year (number of isolates)	Ribotype, number of isolates (% of total isolates from study year)				
	027	014	020	106	002
2013 (411)	113 (27.5%)	33 (8.0%)	17 (4.1%)	24 (5.8%)	16 (3.9%)
2014 (410)	98 (23.9%)	37 (9.0%)	32 (7.8%)	20 (4.9%)	19 (4.6%)
2015 (485)	109 (22.5%)	31 (6.4%)	38 (7.8%)	36 (7.4%)	25 (5.2%)
2016 (441)	59 (13.4%)	25 (5.7%)	29 (6.6%)	51 (11.6%)	26 (5.9%)

Table 6. CAN-DIFF 2013-2016 surveillance study antimicrobial susceptibility testing results for 1,747 toxin-positive isolates of *C. difficile*

Antimicrobial agent	MIC (µg/mL)				MIC interpretation		
	Range	Mode	MIC_{50}	MIC_{90}	% S	% I	% R
Amoxicillin-clavulanate	≤0.25-8	1	1	2	99.8	0.2	0
Ceftriaxone	8->128	32	32	64	7.3	61.3	31.4
Clindamycin	≤0.12->64	8	8	>64	5.3	31.1	63.6
Fidaxomicin	≤0.015-8	0.25	0.25	0.5	NA[a]	NA	NA
Metronidazole	0.12-4	0.5	0.5	2	100	0	0
Moxifloxacin	0.5->32	1	2	32	71.8	0.9	27.3
OP-1118	0.12-64	4	4	16	NA	NA	NA
Vancomycin	≤0.25-4	1	1	2	98.7[b]	NA	NA

[a] NA, CLSI MIC interpretative breakpoints not available; [b] MICs were interpreted using the EUCAST epidemiological cut-off value (ECOFF) for vancomycin

Table 7. CAN-DIFF 2013-2016 surveillance study distribution of MICs for antimicrobial agents tested against 1,747 toxin-positive isolates of *C. difficile*

Antimicrobial agent	Number of isolates for which the antimicrobial agent MIC (µg/mL) was:												
	0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	≥64
Amoxicillin-clavulanate					5[a]	73	979	674	13	3			
Ceftriaxone										3	124	1071	549[b]
Clindamycin				2[a]			17	72	544	878	15	2	216[c]
Fidaxomicin	3[a]	28	74	426	634	534	46	1		1[d]			
Metronidazole				6	203	796	349	326	67				
Moxifloxacin						3	670	582	15	20	185	272[e]	
OP-1118				1	5	33	97	283	554	464	308	1	1[f]
Vancomycin					1[a]	192	1184	347	23				

[a] Isolate count shown for lowest the dilution tested; some MICs may be lower than the lowest dilution tested; [b] 79/549 isolate MICs for ceftriaxone were >64 µg/mL; [c] 206/216 isolate MICs for clindamycin were >64 µg/mL; [d] One isolate MIC for fidaxomicin was >8 µg/mL; [e] 175/272 isolate MICs for moxifloxacin were >32 µg/mL; [f] One isolate MIC for OP-1118 was 64 µg/mL.

Conclusions

- Ribotype 027 was the most common ribotype identified among isolates of toxin-positive *C. difficile* infecting Canadian patients from 2013 to 2016. Ribotype 027 isolates were frequently resistant to moxifloxacin (fluoroquinolones) and MDR. However, the prevalence of ribotype 027 is decreasing in both central and eastern Canada.
- Ribotype 027 accounted for 13.5, 20.2, and 38.4% of isolates from patients aged ≤64, 65-79, and ≥80 years, respectively ($P<0.00001$).
- 89.7% of ribotype 027 isolates were resistant to moxifloxacin compared with only 10.0% of non-ribotype 027 isolates.
- Ribotype 027 was found more commonly among isolates of toxin-positive *C. difficile* in eastern Canada (33.2%) than among isolates from central (16.8%) and western (15.2%) Canada ($P<0.00001$).
- The prevalence of ribotype 106 increased significantly in western Canada ($P=0.002$) and the prevalence of both ribotype 106 ($P=0.039$) and ribotype 002 ($P=0.045$) significantly increased in central Canada from 2013 to 2016.
- 49.9% of ribotype 027 isolates were multidrug-resistant (MDR defined as resistant to ceftriaxone, clindamycin, and moxifloxacin) compared to 3.9% for non-027 ribotypes.
- All isolates of toxin-positive *C. difficile* tested were susceptible to metronidazole and had vancomycin MICs of ≤0.25-4 µg/mL (98.7% of vancomycin MICs were ≤2 µg/mL).
- Fidaxomicin demonstrated greater *in vitro* potency than metronidazole, vancomycin and all other antimicrobial agents tested based upon their MIC_{90} values and MIC distributions.
- Fidaxomicin demonstrated potent *in vitro* activity (MIC_{90}, 0.5 µg/mL) against all genotypes of toxin-positive *C. difficile* including ribotype 027 (NAP1/BI).
- Metronidazole had an MIC_{50} and MIC_{90} two doubling-dilutions higher for ribotype 027 isolates than for non-ribotype 027 isolates; vancomycin and fidaxomicin MIC_{50}s and MIC_{90}s were identical for both ribotype 027 and non-ribotype 027 isolates.

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