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SUSCEPTIBILITY

U.S.-Based National Surveillance for Fidaxomicin Susceptibility of *Clostridioides difficile*-Associated Diarrheal Isolates from 2013 to 2016

C. M. Thorpe,[a,b] L. A. McDermott,[a] M. K. Tran,[a] J. Chang,[a] S. G. Jenkins,[c] E. J. C. Goldstein,[d] R. Patel,[e] B. A. Forbes,[f] S. Johnson,[g,h] D. N. Gerding,[g,h] D. R. Snydman[a,b]

[a]Tufts Medical Center, Boston, Massachusetts, USA
[b]Tufts University School of Medicine, Boston, Massachusetts, USA
[c]Weill Cornell Medical College, New York, New York, USA
[d]R. M. Alden Research Laboratory, Santa Monica, California, USA
[e]Mayo Clinic, Rochester, Minnesota, USA
[f]Virginia Commonwealth University Medical Center, Richmond, Virginia, USA
[g]Hines VA Hospital, Hines, Illinois, USA
[h]Loyola University Chicago, Maywood, Illinois, USA

ABSTRACT In 2011, we initiated a sentinel surveillance network to assess changes in *Clostridioides* (formerly *Clostridium*) *difficile* antimicrobial susceptibility to fidaxomicin from 6 geographically dispersed medical centers in the United States. This report summarizes data from 2013 to 2016. *C. difficile* isolates or toxin-positive stools from patients were referred to a central laboratory. Antimicrobial susceptibility was determined by agar dilution. CLSI, EUCAST, or FDA breakpoints were used, where applicable. Toxin gene profiles were characterized by multiplex PCR on each isolate. A random sample of approximately 40% of isolates, stratified by institution and year, was typed by restriction endonuclease analysis (REA). Among 1,889 isolates from 2013 to 2016, the fidaxomicin MIC_{90} was 0.5 μg/ml; all isolates were inhibited at $\leq$1 μg/ml. There were decreases in metronidazole and vancomycin MICs over time. Clindamycin resistance remained unchanged (27.3%). An increase in imipenem resistance was observed. By 2015 to 2016, moxifloxacin resistance decreased in all centers. The proportion of BI isolates decreased from 25.5% in 2011 to 2012 to 12.8% in 2015 to 2016 ($P < 0.001$). The BI REA group correlated with moxifloxacin resistance (BI 84% resistant versus non-BI 12.5% resistant). Fidaxomicin MICs have not changed among *C. difficile* isolates of U.S. origin over 5 years post licensure. There has been an overall decrease in MICs for vancomycin, metronidazole, moxifloxacin, and rifampin and an increase in isolates resistant to imipenem. Moxifloxacin resistance remained high among the BI REA group, but the proportion of BI isolates has decreased. Continued geographic variations in REA groups and antimicrobial resistance persist.

KEYWORDS *Clostridioides difficile*, epidemiology, fidaxomicin, surveillance

Clostridioides (formerly *Clostridium*) *difficile* infection (CDI) continues to be a problem worldwide (1, 2), causing substantial morbidity and mortality. Approximately 450,000 cases of CDI occur annually in the United States, with approximately 29,300 deaths and 83,000 first recurrences (3). The elderly are particularly vulnerable. The U.S. 2012 national estimate for CDI incidence in nursing homes was approximately 100,000 cases, with approximately 20,000 recurrences and 8,700 deaths (4). The appearance of fluoroquinolone-resistant *C. difficile* strain NAP1/BI/027 isolates, which have been associated with epidemics of complicated CDI cases and increased mortality, has in-

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Address correspondence to D. R. Snydman, dsnydman@tuftsmedicalcenter.org.

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TABLE 1 Activities of fidaxomicin and comparative agents versus 1,889 isolates of *Clostridium difficile* from 2013 to 2016

Antibiotic	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Resistant	
				CLSI	EUCAST
Fidaxomicin	0.004–1	0.25	0.5	NA	NA
Rifaximin	≤0.004 to >4	0.03	0.06	NA	NA
Rifampin	≤0.004 to >4	≤0.004	0.008	NA	29.8
Tigecycline	≤0.06–1	0.12	0.25	0.0	1.0
Vancomycin	≤0.25–8	1	2	NA	6.5
Imipenem	≤0.12–16	4	8	6.2	NA
Moxifloxacin	≤0.5 to >16	2	16	23.2	23.2
Metronidazole	≤0.06–4	0.25	1	0.0	1.3
Clindamycin	≤0.5 to >16	4	16	27.6	NA
Chloramphenicol	≤0.5–16	4	8	0.0	NA

creased the urgency of this problem. The economic burden of CDI is also considerable. Two studies estimate an annual U.S. expenditure in the range of $4.8 billion to $5.4 billion (5, 6). A number of studies performed from Europe confirm a high cost burden as well (7). Thus, development and use of effective treatments for *C. difficile* infection that provide a durable cure are imperative. In addition, reduced susceptibilities to metronidazole and vancomycin have been noted, including in our previous surveillance efforts (8, 9). Ongoing national surveillance efforts are critical to allow for drug resistance monitoring in *C. difficile* to both metronidazole and vancomycin, as well as to newly licensed therapies that are entering into clinical practice.

Because diagnosis of CDI is based on detection of the large clostridial toxins ToxA and ToxB by enzyme-linked immunosorbent assay (ELISA) and/or detection of *C. difficile*-specific DNA sequences, isolation of *C. difficile* from patient stool is not standard clinical microbiology laboratory practice. Strain isolation is performed in only a few reference laboratories. Lack of available isolates limits epidemiologic surveillance of strain characteristics and drug susceptibility testing. To meet this gap, we previously created a U.S. network to provide the scientific community (researchers, practitioners, clinical laboratories, and regulatory agencies) with accurate information on the changing drug susceptibility and strain epidemiology of *C. difficile* (9). We reported the drug susceptibility patterns of 925 isolates isolated from patients who developed CDI from 2011 to 2012, located in geographically diverse areas. Geographic variations in susceptibility, restriction endonuclease analysis (REA) group, and binary toxin gene presence were observed. Moxifloxacin and clindamycin resistance was higher among BI and binary toxin-positive isolates. This group also showed reduced susceptibility to metronidazole and vancomycin by EUCAST criteria. From 2011 to 2012, we observed uniform susceptibility to fidaxomicin.

Here, we report on our continued efforts in surveillance and susceptibility testing on 1,889 isolates obtained in a similar fashion over the 2013 to 2016 time period and compare these data in 2-year time intervals to the 2011 to 2012 baseline data.

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RESULTS

Susceptibility of the isolates. The results from the antimicrobial susceptibility testing of all 1,889 isolates are presented in Table 1 and expressed as MIC range, MIC_{50}, MIC_{90}, and percentage resistant based on CLSI and/or EUCAST breakpoints.

All isolates were inhibited at a fidaxomicin concentration of ≤1 μg/ml, and the MIC_{90} was 0.5 μg/ml. There has been no change in fidaxomicin resistance over time from 2011 through 2016.

The number of isolates of *C. difficile* inhibited at each drug dilution for all of the agents tested is shown in Table 2. Figure 1 shows overall trends in 2-year intervals for

TABLE 2 Distribution of MICs for antimicrobials tested against 2,814 isolates of *C. difficile* (2011 to 2016)

	No. of isolates at each MIC (μg/ml) value:												
Antimicrobial agent	**0.004**	**0.008**	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**
Fidaxomicin	23[a]	19	17	74	239	687	1,026	661	68	0			
Rifaximin	71	165	1,167	1,073	68	29	15	2	0	3	221[b]		
Rifampin	1,649	841	76	14	4	4	1	1	3	4	217[c]		
Tigecycline					129	1,934	721	26	4	0	0	0	0
Vancomycin							9	131	1,461	925	284	4	0
Imipenem						7	9	13	37	305	1,337	967	139
Moxifloxacin								47	559	1,430	29	95	654[d]
Metronidazole					10	57	1,052	828	423	385	59	0	0
Clindamycin								124	293	596	1,066	285	450[e]
Chloramphenicol								5	44	199	1,396	1,139	31

[a]Some MICs may be lower than lowest the dilution tested. Number shown is equal to or less than that dilution.
[b]Rifaximin: 215/221 MICs were >4.
[c]Rifampin: 215/221 MICs were >4.
[d]Moxifloxacin: 167/654 MICs were >16.
[e]Clindamycin: 186/450 MICs were >16.

antimicrobial resistance according to either EUCAST or CLSI breakpoints, where applicable. Although there is no CLSI resistance breakpoint for vancomycin, the recently published CLSI epidemiological cutoff value (ECV) for non-wild-type *C. difficile* is ≥4 μg/ml, which is consistent with the EUCAST epidemiological cutoff (ECOFF) value. In this study, the MIC_{50} and MIC_{90} were 1 and 2 μg/ml, respectively, in both 2013 to 2014 and 2015 to 2016 (Table 1); whereas, in the 2011 to 2012 baseline period, the MIC_{50} and MIC_{90} were 2 and 4 μg/ml. Using these epidemiological cutoff values, 17.9% of isolates in the baseline period had decreased susceptibility to vancomycin, but this proportion diminished over time to a low of 5.5% in 2015 to 2016 (Fig. 1). In 2011 to 2012, 3.6% of isolates demonstrated decreased susceptibility to metronidazole by EUCAST ECOFF values; this proportion also diminished over time to a low of 0.6% in 2015 to 2016. There were no isolates resistant to metronidazole by the CLSI breakpoint. Overall, 33.5% of isolates were moxifloxacin resistant in 2011 to 2012; this decreased over time to 25.7% in 2013 to 2014 and 20.7% in 2015 to 2016 ($P < 0.001$). Tigecycline resistance remained low by the EUCAST breakpoint; no isolate was considered resistant to tigecycline by the FDA breakpoint. Isolates showed increasing susceptibility to rifampin over time, with 65.3% of isolates resistant by EUCAST breakpoints in 2011 to 2012, decreasing to 39.7% of isolates resistant in 2013 to 2014 and 19.7% of isolates resistant in 2015 to 2016. Clindamycin resistance remained relatively stable. In 2011 to 2012, 23.8% of isolates were resistant to clindamycin; this rose only slightly over time to 27.9% in 2013 to 2014 and 27.3% in 2015 to 2016 ($P = 0.0894$). Imipenem resistance

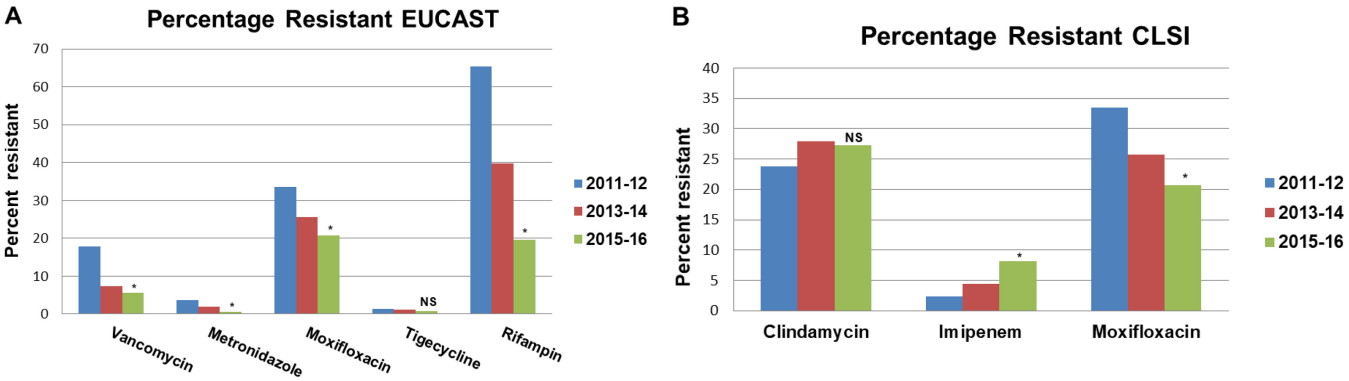

FIG 1 Antibacterial-resistant *C. difficile* strains by 2-year intervals. (A) Proportion of strains resistant when EUCAST criteria is applied. (B) Proportion of strains resistant when CLSI criteria is applied. Of note, no strain was considered metronidazole resistant by CLSI criteria, and no strain was considered tigecycline resistant by FDA criteria. *, A statistically significant increase or decrease over time between the 2011 to 2012 and 2015 to 2016 time intervals; NS, no significant change.

TABLE 3 Toxin gene profiles by 2-year intervals, 2011 to 2016

Toxin gene profile	Base pair deletion in *tcdC*	Percent of isolates with the given profile during[a]:		
		2011 to 2012	2013 to 2014	2015 to 2016
tcdA positive, *tcdB* positive	0	59.0	67.4	69.6
tcdA positive, tcdB positive, *cdtA* positive, and *cdtB* positive	18	28.1	19.0	17.6
tcdA positive, *tcdB* positive	18	5.8	5.3	4.5
tcdA positive, *tcdB* positive, *cdtA* positive, and *cdtB* positive	39	2.3	3.4	2.8
tcdA positive, *tcdB* positive, *cdtA* positive, and *cdtB* positive	0	2.1	1.8	2.1
tcdA positive, *tcdB* positive, *cdtA* positive, and *cdtB* positive	54	0.2	0	0
tcdA negative, *tcdB* positive, *cdtA* positive, and *cdtB* positive	0	0	0	0.1
Nontoxigenic		2.5	3.1	3.3

[a]In 2011–2012, 914 (98.8%) isolates were profiled; in 2013–2014, 945 (99.7%) isolates were profiled; and in 2015–2016, 941 (100%) isolates were profiled.

increased and reached 8.1% in 2015 to 2016 compared to 2.3% in 2011 to 2012 and 4.4% in 2013 to 2014 ($P < 0.001$).

Determination of toxin gene profiles. The majority of isolates from 2013 to 2016 were able to be assayed for a toxin gene profile (Table 3). Compared to the baseline period of 2011 to 2012, a similar proportion of isolates were nontoxigenic (~3% each year), with the vast majority testing positive by PCR for both *tcdA* and *tcdB* (one isolate did not test positive for *tcdA* with the primers used). The percentage of toxigenic isolates testing positive for binary toxin genes (*cdtA* positive and *cdtB* positive) in the baseline 2011 to 2012 period was 33.6% (299/891). This decreased in the ensuing years of surveillance, with only 25.0% in 2013 to 2014 and 23.3% in 2015 to 2016 testing positive by PCR for binary toxin genes. This difference was primarily in isolates with the toxin profile *tcdA*, *tcdB*, *cdtA* and *cdtB*, *tcdC* 18-bp deletion. This toxin profile is the most common one associated with the NAP1/BI/027 strain and continued to correlate with BI over time (see Fig. S1A through C in the supplemental material).

REA strain typing. A random sample subset, stratified by center, was chosen for REA typing in each surveillance period (approximately 40% in each year). The distribution of all isolates by REA group by 2-year intervals is shown in Table 4. There was a significant decrease in BI isolates over time ($P < 0.001$). BI comprised 25.5% of isolates that were REA typed in 2011 to 2012 but had decreased by one-quarter in 2013 to 2014 (16.9%) and then by one-half (12.8%) in 2015 to 2016. When all centers were analyzed, the proportion of nonspecific REA groups increased over time ($P < 0.001$), while other REA groups stayed relatively constant. Figure S1 shows relationships between REA group and toxin profile by 2-year intervals, which remained relatively consistent.

We noted a significant difference between the BI REA group and others with respect to antimicrobial resistance (Table 5). Differences in MIC_{50} and MIC_{90} were noted between BI and non-BI strains for rifaximin, rifampin, vancomycin, moxifloxacin, metronidazole, and clindamycin. The MIC_{50} for imipenem for BI isolates was 8 μg/ml versus 4 μg/ml for non-BI isolates, but the MIC_{90} was the same. No difference was noted for tigecycline. Moreover, for vancomycin, 29.3% of EUCAST ECOFF values were above the

TABLE 4 Percentage of isolates with REA type by 2-year interval

REA group	Percent of isolates in group during:		
	2011 to 2012 (n = 322)	2013 to 2014 (n = 356)	2015 to 2016 (n = 360)
A group	4.0	3.9	4.2
BI group	25.5	16.9	12.8
BK group	2.2	2.2	2.8
CF group	2.5	2.0	1.7
DH group	10.2	12.6	13.3
G group	4.3	7.3	5.3
J group	3.7	2.2	1.7
K group	1.9	1.7	1.1
W group	0.9	0.6	0.0
Y group	15.8	15.7	15.6
Nonspecific	28.9	34.8	41.7

TABLE 5 Antimicrobial susceptibility of the most prevalent REA groups 2011 to 2016

Antibiotic	REA group	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Resistant	
					CLSI	EUCAST
Fidaxomicin	BI [188]	≤0.004–1	0.25	0.5	NA	NA
	All non-BI types [850][a]	≤0.004–1	0.25	0.5	NA	NA
	A [42]	≤0.004–1	0.25	0.5	NA	NA
	DH [126]	0.03–1	0.25	0.5	NA	NA
	G [59]	0.008–1	0.25	0.5	NA	NA
	Y [163]	≤0.004–0.5	0.25	0.5	NA	NA
Rifaximin	BI	≤0.004 to >4	0.03	>4	NA	NA
	All non-BI types	≤0.004 to >4	0.015	0.03	NA	NA
	A	≤0.004 to >4	0.03	0.06	NA	NA
	DH	0.008 to >4	0.03	0.03	NA	NA
	G	≤0.004 to >4	0.03	0.03	NA	NA
	Y	≤0.004 to >4	0.015	0.03	NA	NA
Rifampin	BI	≤0.004 to >4	0.008	>4	NA	74.5
	All non-BI types	≤0.004 to >4	≤ 0.004	0.008	NA	35.3
	A	≤0.004 to >4	≤ 0.004	0.008	NA	33.3
	DH	≤0.004 to >4	≤ 0.004	0.008	NA	49.2
	G	≤0.004–4	≤ 0.004	0.008	NA	25.4
	Y	≤0.004 to >4	≤ 0.004	0.008	NA	35.0
Tigecycline	BI	≤0.06–0.5	0.12	0.25	0.0	1.6
	All non-BI types	≤0.06–1	0.12	0.25	0.0	1.8
	A	≤0.06–0.25	0.12	0.25	0.0	0.0
	DH	≤0.06–0.5	0.12	0.25	0.0	1.6
	G	≤0.06–0.25	0.12	0.12	0.0	0.0
	Y	≤0.06–1	0.12	0.25	0.0	1.8
Vancomycin	BI	≤0.25–8	2	4	NA	29.3
	All non-BI types	≤0.25–4	1	2	NA	7.5
	A	0.5–4	1	2	NA	7.1
	DH	0.5–4	1	4	NA	11.9
	G	0.5–4	1	2	NA	6.8
	Y	0.5–4	1	2	NA	4.3
Imipenem	BI	≤0.12–16	8	8	7.4	NA
	All non-BI types	≤0.12–16	4	8	3.9	NA
	A	≤0.12–16	4	8	2.4	NA
	DH	1–16	4	8	5.6	NA
	G	1–8	4	8	0.0	NA
	Y	≤0.12–16	4	8	3.7	NA
Moxifloxacin	BI	≤0.5 to >16	16	>16	84.0	84.0
	All non-BI types	≤0.5 to >16	2	8	12.5	12.5
	A	≤0.5–16	2	2	4.8	4.8
	DH	≤0.5–16	2	2	5.6	5.6
	G	≤0.5 to >16	2	16	18.6	18.6
	Y	≤0.5 to >16	2	4	8.0	8.0
Metronidazole	BI	0.12 to 4	2	2	0.0	7.4
	All non-BI types	≤0.06–4	0.5	1	0.0	0.4
	A	≤0.06–2	0.5	1	0.0	0.0
	DH	≤0.06–2	0.5	1	0.0	0.0
	G	0.12–4	0.25	2	0.0	1.7
	Y	≤0.06–2	0.5	2	0.0	0.0
Clindamycin	BI	≤0.5 to >16	4	>16	48.4	NA
	All non-BI types	≤0.5 to >16	4	16	23.5	NA
	A	≤0.5 to >16	4	8	16.7	NA
	DH	≤0.5 to >16	4	16	20.6	NA
	G	≤0.5–16	4	8	15.3	NA
	Y	≤0.5 to >16	4	8	17.2	NA

(Continued on next page)

TABLE 5 (Continued)

Antibiotic	REA group	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)	% Resistant	
					CLSI	EUCAST
Chloramphenicol	BI	1–16	4	8	0.0	NA
	All non-BI types	≤0.5 to 16	4	8	0.0	NA
	A	1–8	4	8	0.0	NA
	DH	1–8	4	8	0.0	NA
	G	1–8	4	8	0.0	NA
	Y	≤0.5–8	4	8	0.0	NA

[a]All non-BI types includes the groups listed separately plus an additional 384 isolates from REA groups with <30 isolates.

cutoff for BI strains and only 7.5% were above the cutoff for non-BI strains. Metronidazole demonstrated a similar difference in EUCAST ECOFF values with 7.4% above the cutoff for BI strains and 0.4% above the cutoff for non-BI strains. Moxifloxacin demonstrated 84% resistance using CLSI breakpoints for the BI group compared to 12.5% for the non-BI group. Finally, the percentage of clindamycin resistance was much higher in BI (48.4%) than in non-BI (23.5%) strains.

Center-specific differences. Center-specific differences in isolate antimicrobial susceptibility patterns are shown in Fig. S2 in the supplemental material. Increasing vancomycin and metronidazole susceptibility was seen at almost all centers by the 2015 to 2016 interval (Fig. S2A and B). Decreased moxifloxacin resistance was observed at all centers by the 2015 to 2016 interval (Fig. S2C). The proportion of imipenem-resistant isolates was variable among institutions and over time (Fig. S2D). In 3 of the 5 institutions, approximately 11 to 15% of isolates were imipenem resistant by 2015 to 2016, a substantial increase over the time interval (Hines, New York Presbyterian, and Tufts). In contrast, one center (Virginia Commonwealth University Medical Center [VCU]) had a dramatic decrease in the proportion of imipenem-resistant isolates; however, the small number of samples contributed in 2012 from this center compared to the subsequent years may have skewed these data (38 isolates versus 155 and 126 isolates in subsequent intervals, respectively). Two of five centers had relatively minimal changes, maintaining resistance levels of ~5% or lower (Mayo Clinic and R. M. Alden). Resistance to clindamycin (Fig. S2E) was variable across centers/time. At NYP, there was a spike in the 2013 to 2014 interval that returned to baseline in 2015 to 2016. At Mayo Clinic, there was a substantial increase in clindamycin resistance (7.9% in the baseline interval to 30.1% in 2015 to 2016). A decrease in proportion of rifampin-resistant isolates was observed across all institutions (Fig. S2F).

There were significant differences between centers in proportions of REA types isolated (see Fig. S3 in the supplemental material). For 5/6 of the centers, the proportion of BI isolates decreased in 2015 to 2016 compared to that in 2011 to 2012, but the percentages were variable (Fig. S3B). Different REA types filled the niche evacuated by BI at the different centers. However, there was variability in the proportions of non-BI REA types at each center in a given time interval, and a consistent finding at all centers was an increase in nonspecific REA types in 2015 to 2016 compared to that in 2011 to 2012 (Fig. S3C).

DISCUSSION

This report provides a summary of U.S. national surveillance of fidaxomicin activity against isolates of *C. difficile* obtained from 6 medical centers from 2013 to 2016 and compares these data to our baseline results from 2011 to 2012. No fidaxomicin resistance was noted among the 1,889 isolates tested from 2013 to 2016 (distributed equally with approximately 450 isolates obtained yearly). There were no differences in fidaxomicin MIC_{50} and MIC_{90} values over the three time intervals. Studies from other countries have shown similar results; over 2,500 isolates obtained from patients between 2011 and 2014 across 22 European countries remained highly susceptible to fidaxomicin (10). This was also true in surveillance studies undertaken in Canada from 2013 to 2015 (11) and Australia from 2013 to 2015 (12).

Only a handful of isolates with decreased susceptibility to fidaxomicin have been reported as follows: one isolate in Florida, isolated in 2016 from a patient with CDI with an MIC of 16 μg/ml (13), and 4 isolates in Mexico from patients with recurrent CDI with MICs of 2 μg/ml (14). Interestingly, fidaxomicin is not in clinical use in Mexico. The clinical significance of reduced fidaxomicin susceptibility is not clear. Like vancomycin, fidaxomicin reaches very high levels in stool (15).

The proportion of isolates that were vancomycin resistant by EUCAST criteria decreased significantly ($P < 0.001$) over the course of our surveillance, in contrast to the studies cited above which demonstrated low rates that varied little over time. In this study, the MIC_{90} for vancomycin was 2 μg/ml in both 2013 to 2014 and 2015 to 2016. In our 2011 to 2012 study, the MIC_{90} was 4 μg/ml, which contrasted with historical values of 1 μg/ml (9).

In this study, we also observed a decrease in metronidazole resistance over time ($P < 0.001$), with both the MIC_{50} and MIC_{90} values decreasing from 1 μg/ml and 2 μg/ml in 2011 to 2012 to 0.25 μg/ml and 1 μg/ml in 2015 to 2016. We observed the same decrease in isolates that were resistant by EUCAST (overall 2.1% from 2011 to 2016), with a rate of 0.6% in 2015 to 2016. Conversely, others have noted increases in resistance over time (8). The annual rate of metronidazole-resistant organisms reported in the European ClosER study (2011 to 2014) (10) ranged from 0.1% to 0.5%. In a 2013 to 2014 study from Australia, no metronidazole-resistant organisms were reported. In the CAN-Diff study (2013 to 2015) (11), CLSI breakpoints were reported rather than EUCAST ECOFFs; 58/1,310 isolates were above the EUCAST ECOFF. A relationship between ribotype 027 (REA group BI) and higher metronidazole MICs was observed in both the ClosER and CAN-Diff studies as well as in our study. Unlike fidaxomicin and vancomycin, which reach very high levels in the gastrointestinal tract (8), metronidazole gastrointestinal tract concentrations can be low, so increased metronidazole MICs may be clinically relevant. Furthermore, strain heteroresistance may result in clinical failures. In the recently updated Infectious Diseases Society of America (IDSA)/Society for Healthcare Epidemiology of America (SHEA) guidelines, metronidazole is no longer recommended as a first-line therapy for uncomplicated CDI (16).

We observed an increasing proportion of isolates resistant to imipenem, with 8.1% of isolates found to be resistant in the 2015 to 2016 interval compared to 2.3% at baseline, with differences between centers. This increase in resistance is contrary to European surveillance of isolates obtained from July 2011 to July 2014, where rates decreased from 7.2% to 2.2% over time (10). However, a recent single-center study from Spain reported that 20% of 70 isolates isolated from 2015 to 2016 were imipenem resistant (17). In this study, clindamycin resistance remained high (27.6%), but moxifloxacin resistance decreased over time from 33.5% in 2011 to 2012 to 20.7% in 2015 to 2016.

As has been observed in other studies, the proportion of isolates with BI/NAP1/027 decreased over time. This decrease was seen at five out of six centers contributing isolates. Dingle et al. has noted the profound effect of restricted fluoroquinolone usage on the proportion of BI/027 strains in the UK (18).

There are a number of limitations in this study that must be stated. These include a lack of information on antimicrobial exposure prior to the onset of *C. difficile* infection, information on the specific treatments used for *C. difficile* disease, clinical outcome data for treatment of *C. difficile*, and estimates of fidaxomicin usage for treatment of *C. difficile* in the medical centers that contributed the isolates to the survey. These important limitations should be explored for future surveillance and analysis of relationships of exposure to *C. difficile* isolates.

In summary, we report the results of ongoing surveillance efforts for *C. difficile* epidemiology and antimicrobial susceptibility, showing changing trends in the prevalence of REA groups, including a decrease in the epidemic BI group and changing susceptibility to a number of different agents. Importantly, 5 years after licensure, isolates remain highly susceptible to fidaxomicin.

TABLE 6 Isolates referred by medical center

Medical center	No. of isolates referred		
	2011 to 2012[a]	2013 to 2014	2015 to 2016
Hines VA Hospital	137	137	125
Mayo Clinic	139	151	136
NY Presbyterian Hospital	179	155	192
R. M. Alden Research Laboratory	163	194	206
Tufts Medical Center	208	156	156
Virginia Medical College	38[b]	155	126
Total isolates	925	948	941

[a]Duke University Medical Center provided 61 isolates in 2011.
[b]2012 isolates only.

MATERIALS AND METHODS

Medical centers. From 2013 to 2016, a total of 1,889 *C. difficile* isolates were referred by 6 medical centers for processing to the Special Studies Laboratory (SSL) at Tufts Medical Center. The medical centers were Hines VA Hospital, Hines, IL; Mayo Clinic, Rochester, MN; New York Presbyterian Hospital/Weill Cornell Medical Center, New York, NY; Tufts Medical Center, Boston, MA; RM Alden Research Laboratory, Culver City, CA; and Virginia Commonwealth University Medical Center (VCU), Richmond, VA. Duke supplied isolates for 2011 only.

Clinical samples. Isolates of *C. difficile* from toxin-positive stool samples were obtained from 6 different locations around the United States, from institutions that had excellent anaerobic bacteriology laboratories and investigators willing to collaborate (Table 6). Each institution that performed isolation of *C. difficile* was invited to send at least 75 isolates collected throughout the year. These were forwarded in chopped meat broth (Anaerobe Systems, Morgan Hill, CA) to the SSL at Tufts Medical Center for antimicrobial sensitivity testing and toxin gene profiling. Alternatively, some centers sent approximately 100 frozen stool samples from toxin-positive patients; these were processed for isolation of *C. difficile* at the SSL as outlined below.

Processing of samples. After arrival of stool samples, *C. difficile* was isolated by plating on selective *C. difficile* medium (cycloserine-cefoxitin-fructose agar with taurocholate) and observing for characteristic colonial morphology (19, 20). This was followed by using the rapid identifying method API20A (bioMérieux Inc., Durham, NC) on the isolate. If identification by this methodology was not conclusive, methods outlined in the Wadsworth Anaerobic Bacteriology Laboratory Manual were followed (20). Confirmation of purity and identification of isolates as *C. difficile* submitted by participating centers was accomplished by following the same procedure. All isolates were maintained in chopped meat broth until testing, after which a cell paste swabbed from fresh plates was suspended directly into skim milk medium and frozen at −80°C for future reference (21).

Antimicrobial susceptibility testing. MICs of the isolates were determined using the agar dilution method per Clinical and Laboratory Standards Institute (CLSI) recommendations, M11-A8 (21, 22), for the following antimicrobial agents: fidaxomicin, vancomycin, metronidazole, rifaximin, rifampin, tigecycline, imipenem, moxifloxacin, clindamycin, and chloramphenicol. Fidaxomicin (obtained from Optimer, Cubist, and/or Merck depending on time of isolate testing) was solubilized in dimethyl sulfoxide (DMSO) and further diluted with distilled water to a final concentration in the agar of <0.5% DMSO. Rifampin (Sigma-Aldrich, St. Louis, MO) was dissolved in methanol and further diluted with distilled water. Rifaximin (Salix Pharmaceuticals, Inc., Morrisville, NC) was dissolved in methanol and then diluted in 0.1 M phosphate buffer (pH 7.4) plus 0.45% sodium dodecyl sulfate. All other antibiotics, provided by their respective manufacturers, were dissolved in distilled water.

Antibiotic-containing plates were prepared on the day of the test. The medium was brucella agar (Becton, Dickinson, Sparksville, MD), supplemented with 5 μg of hemin/ml, 1 μg vitamin K_1/ml, and 5% (vol/vol) laked sheep blood. Serial 2-fold dilutions of the antibiotics were added to the molten agar, poured into 100-mm Petri dishes and allowed to solidify and dry. Non-antibiotic-containing control plates were prepared in the same manner. The isolates were grown to log phase in brain heart infusion supplemented (yeast and hemin) broth and the turbidity adjusted to that of a 0.5 McFarland standard. For *C. difficile* ATCC 700057, the density was 1×10^7 to 4×10^7 CFU/ml. The inocula were deposited on the agar surface using a Steer replicator, resulting in a final concentration of 10^4 CFU/spot. The plates were incubated at 35 to 37°C in an anaerobic chamber with an atmosphere of 85% N_2, 5% CO_2, and 10% H_2. MICs were read after 48 h of incubation. The following ATCC reference organisms were used as controls in all tests: *C. difficile* ATCC 700057, *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741, and *Staphylococcus aureus* ATCC 29213. The rates of resistance of the antimicrobial agents were determined using currently accepted breakpoint recommendations by the CLSI and/or by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (22, 23). Resistance breakpoints for tigecycline were those recommended by the FDA (24). The antibiotics tested, range of concentrations, and breakpoints are listed in Table 7. Optimer, the sponsor at the time, choose the antibiotics to be tested.

Toxin determination. Toxin gene profiles for *C. difficile* were performed using PCR methodology as described by Persson et al. (25, 26). Isolates were tested in duplicate, with nonconcordant tests repeated. The following controls were included with each test: (i) a nontoxigenic strain of *C. difficile* VPI 11186

TABLE 7 Antimicrobial agents

Antimicrobial agent	Range of test (μg/ml)	Breakpoint for resistance	
		CLSI	EUCAST
Fidaxomicin	0.004–4	NA[a]	NA
Rifaximin	0.004–4	NA	NA
Rifampin	0.004–4	NA	>0.004
Vancomycin	0.25–64	≥4[b]	>2
Metronidazole	0.06–16	≥32	>2
Moxifloxacin	0.5–16	≥8	>4
Clindamycin	0.5–16	≥8	NA
Imipenem	0.12–8	≥16	NA
Tigecycline[c]	0.06–64	≥16	>0.25
Chloramphenicol	0.5–16	≥32	NA

[a]NA, resistance breakpoint not established when study was performed.
[b]CLSI epidemiological cutoff value for vancomycin.
[c]FDA resistance breakpoint used for tigecycline.

(ATCC 700057) with a PCR profile of *tcdA* negative, *tcdB* negative, *cdtA* negative, and *cdtB* negative, in which the entire PaLoc is absent (27) and in which no functional *cdt* locus is present; (ii) VPI 10463 with a PCR profile of *tcdA* positive, *tcdB* positive, *cdtA* negative, and *cdtB* negative with an intact *tcdC* region (28, 29); and (iii) R20291 with a PCR profile of *tcdA* positive, *tcdB* positive, *cdtA* positive, and *cdtB* positive with 18-bp deletion in the *tcdC* hypervariable region (30). Loss of PaLoc was confirmed in nontoxigenic isolates by performing a PCR using Lok1/Lok3 primers as previously described (31).

Restriction endonuclease analysis typing. A randomly selected sample of isolates, stratified by center (~30 isolates from each center per year), were referred to the Microbiology Reference Laboratory at the Hines Veterans Administration Hospital (Hines, IL) for restriction endonuclease analysis (REA) strain typing. Following purification, total cellular DNA was cut with HindIII restriction enzyme, and fragments were separated by electrophoresis on a 0.7% agarose gel as previously described (32, 33). HindIII restriction patterns with a 90% similarity index were included in the same REA group (letter designation).

Data storage and analysis. Results from the various tests were sent to the SSL for analysis. All data were stored and analyzed using Microsoft Excel spreadsheets. Fisher's exact test was used to determine equality of proportions using a *P* value of 0.05 (GraphPad 7.01).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00391-19.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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