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Sheila Waslawski
University of Michigan - Ann Arbor

Eugene S. Lo
University of Michigan - Ann Arbor

Sarah A. Ewing
University of Michigan - Ann Arbor

Vincent B. Young
University of Michigan - Ann Arbor

David M. Aronoff
University of Michigan - Ann Arbor

See next page for additional authors

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Clostridium difficile Ribotype Diversity at Six Health Care Institutions in the United States

Sheila Waslawski,[a] Eugene S. Lo,[a] Sarah A. Ewing,[a] Vincent B. Young,[a,b] David M. Aronoff,[a,b] Susan E. Sharp,[c] Susan M. Novak-Weekley,[d] Arthur E. Crist, Jr.,[e] W. Michael Dunne,[f,g] Joan Hoppe-Bauer,[g] Michelle Johnson,[g] Stephen M. Brecher,[h] Duane W. Newton,[i] Seth T. Walk[a,j]

Department of Internal Medicine, Division of Infectious Diseases, University of Michigan Hospital and Health Systems, Ann Arbor, Michigan, USA[a]; Department of Microbiology and Immunology, University of Michigan, Ann Arbor, Michigan, USA[b]; Kaiser Permanente-NW, Portland, Oregon, USA[c]; Southern California Permanente Medical Group Regional Reference Laboratories, North Hollywood, California, USA[d]; Department of Laboratory Services, Division of Clinical Microbiology, York Hospital, York, Pennsylvania, USA[e]; bioMérieux, Durham, North Carolina[f]; Department of Microbiology, Barnes-Jewish Hospital, St. Louis, Missouri, USA[g]; VA Boston Healthcare System, Boston, Massachusetts, USA[h]; Clinical Microbiology Laboratories, Department of Pathology, University of Michigan Hospital and Health Systems, Ann Arbor, Michigan, USA[i]; Department of Microbiology, Montana State University, Bozeman, Montana, USA[j]

Capillary-based PCR ribotyping was used to quantify the presence/absence and relative abundance of 98 *Clostridium difficile* ribotypes from clinical cases of disease at health care institutions in six states of the United States. Regionally important ribotypes were identified, and institutions in close proximity did not necessarily share more ribotype diversity than institutions that were farther apart.

National and international studies strongly suggest that *Clostridium difficile* populations are geographically distinct (1–5), meaning the composition of *C. difficile* genotypes varies from region to region. In the United States, there are currently few data concerning the composition, or structure, of the *C. difficile* population and whether health care institutions that are in close proximity are more similar with respect to ribotype diversity than those that are farther apart. The goals of this study were to quantify the abundance and diversity of *C. difficile* ribotypes from cases of *C. difficile* infection (CDI) at health care institutions in six states across the United States and to determine whether ribotype diversity was structured geographically.

Stool samples from CDI-positive patients were collected from diagnostic laboratories at six health care institutions (West Roxbury, MA; York, PA; Ann Arbor, MI; St. Louis, MO; Portland, OR; and North Hollywood, CA) between 15 February 2011 and 9 September 2011 and shipped in small batches (typically 5 to 10 samples) overnight on ice to the University of Michigan for processing (Ann Arbor, MI, samples were obtained directly from the Clinical Microbiology Laboratory at the University of Michigan Health System and were not shipped). A variety of CDI diagnostic methods were represented across the institutions (Table 1), and CDI cases were defined based on center-specific diagnostic results. Institutional review board (IRB) approval was obtained as appropriate by the participating institutions. Culture was attempted for all samples submitted by each center. On three occasions, samples from three centers were unable to be processed (i.e., plated) within 24 h of receiving due to the lack of available study personnel. These samples were not included in the study. An aliquot from each sample was cultured under anaerobic conditions on prereduced taurocholate cefoxitin cycloserine fructose agar (TCCFA) plates to select for individual *C. difficile* colonies (6). All TCCFA plates were prepared at the University of Michigan. All samples were frozen at −80°C and recultured a second time if the first attempt at culture was unsuccessful. A single colony was analyzed from each stool sample, and the toxigenicity of isolates was inferred based on results of PCR for a *C. difficile*-specific region of the 16S rRNA-encoding gene (7) and *tcdA* (toxin A) or *tcdB* (toxin B) (8). The absence of *tcdA* or *tcdB* was not confirmed for all isolates (i.e., *tcdA* can be variably present/absent and primers may or may not amplify all *tcdA*/*tcdB* alleles), but all toxigenic isolates were positive for at least one toxin gene. Potential nontoxigenic isolates identified as toxin gene negative by PCR were confirmed as *C. difficile* using specific 16S gene PCR and confirmed as nontoxigenic using a Vero cell assay (9). A range of isolates from each center (79% to 94%) were confirmed to be toxigenic *C. difficile* (Table 1), and the overall recovery of toxigenic isolates did not differ significantly across the six centers ($P = 0.074$). Although not significantly different, samples that tested positive by enzyme immunoassay (EIA) alone (Missouri) tended to have a higher culture-positive rate than the other centers that used nucleic acid amplification testing (NAAT) (Table 1). Whether EIA positivity is more predictive of *C. difficile* growth on TCCFA than NAAT positivity remains an interesting question to be addressed in further studies.

Fluorescent PCR ribotyping was conducted using capillary gel electrophoresis as previously reported (10), and a total of 98 different ribotypes were identified. Approximately half ($n = 50$) of the ribotypes were observed at least twice, and the remaining half ($n = 48$) were observed a single time (i.e., a single isolate). The number of toxigenic ribotypes at a particular site correlated significantly with the number of isolates examined (Pearson correlation; $P = 0.006$; $R^2 = 0.87$), indicating that additional ribotypes would have been identified with deeper sampling. We had initially hoped that our sampling design would provide an overall estimate of the number of toxigenic *C. difficile* ribotypes in U.S. circulation. However, the strong ($R^2 = 0.87$), near-linear relationship between

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Address correspondence to Seth T. Walk, seth.walk@montana.edu.

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TABLE 1 Stool samples and *C. difficile* isolates obtained from confirmed CDI cases at six health care institutions in the United States

Institution (by state)	CDI diagnostic[a]	No. of samples obtained	No. of culture-positive samples (%)	No. of nontoxigenic isolates (%)	No. of toxigenic isolates (%)
MA	GeneXpert	125	103 (82)	0 (0)	103 (82)
PA	GeneOhm	120	106 (88)	3 (3)	103 (86)
MI	GDH/TOX EIA + GeneOhm	289	261 (90)	8 (3)	253 (88)
MO	C. difficile TOX A/B II	48	45 (94)	0 (0)	45 (94)
OR	GDH/TOX EIA + GeneXpert	120	98 (82)	3 (3)	95 (79)
CA	GeneXpert	150	121 (81)	0 (0)	121 (81)
Total		852	734 (86)	14 (2)	720 (85)

[a] CDI diagnostic test performed at each institution prior to shipping of samples: GeneXpert (Cephied, Sunnyvale, CA), GeneOhm (Becton, Dickinson, and Company, Franklin Lakes, NJ), GDH/TOX EIA + (C. Diff Quik Chek Complete; TechLab, Blacksburg, VA), and C. difficile TOX (C. difficile TOX A/B II; TechLab, Blacksburg, VA).

the number of ribotypes observed and the number of isolates examined indicated that we identified only the most abundant ribotypes at each institution and that the actual number of toxigenic *C. difficile* ribotypes is much greater than 98. Other collections have identified, or at least named, over 270 different ribotypes (11). However, there are few data with which to compare the total number of ribotypes within or between countries because there is currently no internationally recognized *C. difficile* ribotype reference collection. For example, a collection of 80 *C. difficile* ribotypes was recently published (11), but it is not clear whether non-European representative isolates were included in this collection. In this study, we identified 10 ribotypes for which we had reference cultures. The identity of the remaining 88 ribotypes and international data comparison rely on the future establishment of a representative *C. difficile* ribotype collection.

A median of 7.6% (range, 0.0% to 11.0%) of isolates per institution were specific to a single location, indicating that a significant percentage of CDI-causing isolates were regionally versus nationally important. Contingency table analysis with a Bonferroni correction (*P* value/total number of tests) indicated that the abundance of ribotype 027 was statistically different ($P < 0.0005$) across the six institutions (Table 2). Ribotype 027 was more abundant than any other ribotype at four out of six sites, but 014-020 was the most abundant at the Oregon site and nearly equal to 027 at the Michigan site. To visualize how similar institutions were with respect to the overall composition of *C. difficile* ribotypes, we used an ecological diversity metric (Morisita-Horn) to cluster sites based on similarity (Fig. 1). This type of analysis considers both the presence/absence and the relative abundance of all ribotypes (not just a select few). Branch lengths in the resulting dendrogram are then proportional to *C. difficile* population similarity (i.e., the more similar that sites are in ribotype composition, the shorter the branches connecting them). Therefore, if the distance between institutions has a strong influence on ribotype diversity, we would expect institutions in close proximity to cluster together in the dendrogram. Two main clusters (A and B) were observed, but they were not easily explained by geographic proximity (e.g., Oregon is closer to California than Michigan). Alternatively, abundance data (Table 2) indicated that the two clusters differed noticeably in ribotype 027 and 014-020 abundance. For example, a median of 32% of isolates (range, 25% to 51%) were ribotype 027 at sites in Pennsylvania, Missouri, California, and Massachusetts (cluster A), as opposed to only 11% in Oregon and 19% in Michigan (cluster B).

TABLE 2 Ribotype abundance at six U.S. health care institutions

Ribotype	No. (%) of isolates for ribotypes from each center (by state)						Total no. of isolates for ribotypes (mean %, 95% CI)[a]
	PA	MO	CA	OR	MA	MI	
027	43 (42)	23 (51)	29 (24)	10 (11)	28 (27)	48 (19)	181 (29, 13–45)
014-020	15 (15)	6 (13)	5 (4)	14 (15)	7 (7)	39 (15)	86 (12, 7–17)
UM11	7 (7)	2 (4)	11 (9)	4 (4)	4 (4)	8 (3)	36 (5, 3–8)
053-163	4 (4)	1 (2)	4 (3)	6 (6)	8 (8)	13 (5)	36 (5, 2–7)
002	2 (2)	2 (4)	9 (7)	5 (5)	6 (6)	9 (4)	33 (5, 3–7)
001	1 (1)	1 (2)	7 (6)	3 (3)	2 (2)	12 (5)	26 (3, 1–5)
078-126	1 (1)	1 (2)	2 (2)	4 (4)	3 (3)	11 (4)	22 (3, 1–4)
017	1 (1)	1 (2)	4 (3)	3 (3)	1 (1)	10 (4)	20 (2, 1–4)
UM67-69	5 (5)	2 (4)	1 (1)	1 (1)	5 (5)	4 (2)	18 (3, 1–5)
UM5	4 (4)	1 (2)	2 (2)	3 (3)	1 (1)	7 (3)	18 (3, 1–4)
UM33-35	5 (5)	0 (0)	0 (0)	2 (2)	3 (3)	7 (3)	17 (2, 0–4)
UM8	3 (3)	0 (0)	2 (2)	2 (2)	1 (1)	9 (4)	17 (2, 1–4)
UM9	0 (0)	0 (0)	1 (1)	4 (4)	0 (0)	6 (2)	11 (1, 0–3)
UM16	3 (3)	0 (0)	1 (1)	1 (1)	0 (0)	5 (2)	10 (1, 0–2)
UM24	0 (0)	0 (0)	2 (2)	2 (2)	2 (2)	4 (2)	10 (1, 0–2)
Other	9 (9)	5 (11)	41 (34)	31 (33)	32 (31)	61 (24)	179 (24, 12–35)
Total	103 (14)	45 (6)	121 (17)	95 (13)	103 (14)	253 (35)	720

[a] CI, confidence interval.

At this level of sampling, it appears that U.S. health care institutions can be differentiated by the high versus moderate abundance of ribotype 027. Data from Wilcox et al. found that 027 abundance decreased significantly in England from 2007 to 2010 (5) but remained the most abundant ribotype in the country (overall, ~30%). The Wilcox et al. study included 11,294 isolates, compared to 720 isolates in our study, so there is a large discrepancy between the two with respect to overall sample size. However, it is not directly apparent from the Wilcox et al. paper how many isolates were included from each institution and over what period of time, so a direct per-center comparison of sample size is difficult to assess. Temporal data are needed to determine whether U.S. trends mirror those of England and other countries, but our results confirm that ribotype 027 is currently as abundant as or even less abundant than other toxigenic ribotypes at some U.S. institutions.

A tracking study of *C. difficile* disease at health care institutions in selected counties of 10 U.S. states was initiated in 2009 by the Centers for Disease Control and Prevention (http://www.cdc.gov/hai/eip/clostridium-difficile.html). Results from this study presented at the Society for Healthcare Epidemiology of America

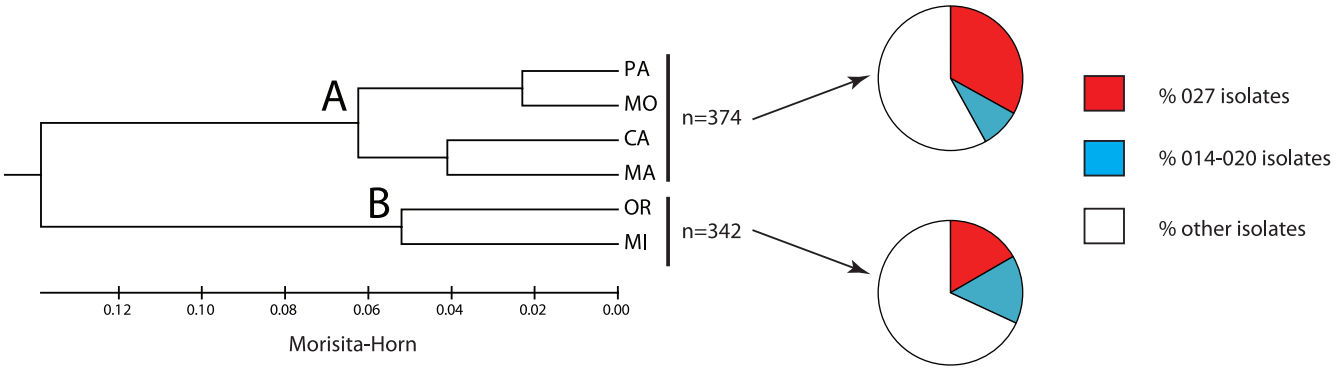

FIG 1 Ribotype-based *C. difficile* population diversity. An unweighted-pair group method using average linkages (UPGMA) dendrogram displays the similarity between six U.S. health care institutions based on the presence/absence and relative abundance of all ribotypes observed in this study (Morisita-Horn index). Pie charts display the percentages of two main ribotypes (027 and 014-020) and all remaining ("other") ribotypes for clusters A and B.

(SHEA) conference in 2011 indicated that ribotype 027 isolates (also known as North American pulsed-field gel electrophoresis type NAP1) comprised 28% of the *C. difficile* population (322 isolates from hospitalized patients across five centers in 2010) (12). Our finding of a mean 027 abundance of 29% across six centers supports this general finding. An important detail that our data show, however, is that 027 abundance varies widely across institutions (13% to 45%).

In light of recent data from Tenover et al. (13), heterogeneity in *C. difficile* ribotype composition may potentially influence CDI diagnostic results. Recently published guidelines recommend either the sole use of high-sensitivity nucleic acid amplification testing (NAAT) or a two- or three-step algorithm composed of EIA (for glutamate dehydrogenase [GDH] and/or *C. difficile* toxin) and NAAT (14). Since EIA is currently cheaper than NAAT, EIA may remain useful as a prescreening tool in clinical laboratories. Thus, additional studies should be conducted to estimate the effect of ribotype diversity on false-positive/false-negative rates across institutions.

A limitation of our study design was the use of deidentified patient samples. We therefore were unable to account for duplicate samples or recurrent and nonnosocomial cases. Another important limitation was that only one *C. difficile* colony per sample was considered. It is possible that cultivable, toxigenic isolates would have been recovered from samples that yielded nontoxigenic isolates, although the overall number of nontoxigenic isolates was low and not likely to have significantly influenced our overall results. It is also possible that more than one toxigenic *C. difficile* ribotype was present in the same sample. Such cases are not necessarily rare and may comprise up to 13% of samples in some situations (15). Future studies that incorporate deeper sampling are needed to understand the rate of mixed CDI among health care institutions.

In summary, *C. difficile* ribotype data from six health care institutions across the United States suggest that there are many (>98) different ribotypes in circulation and that there is significant heterogeneity in ribotype composition across these regions. The abundance of ribotypes 027 and 014-020, but not geographic proximity, correlated with compositional variation between *C. difficile* populations. Finally, our results illustrate the potential importance of regionally versus nationally circulating isolates (~7.6%) as well as the presence of a large amount of undersampled *C. difficile* genetic diversity. More-representative data of *C. difficile* populations, including these rarer members, are needed to better assess the impact of ribotype on sensitivity/specificity of currently used diagnostic algorithms and clinical treatment of CDI.

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