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Detection of Mixed Populations of *Clostridium difficile* from Symptomatic Patients Using Capillary-Based Polymerase Chain Reaction Ribotyping

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Abstract

OBJECTIVE—To investigate the simultaneous occurrence of more than 1 *Clostridium difficile* ribotype in patients' stool samples at the time of diagnostic testing.

METHODS—Stool samples submitted for diagnostic testing for the presence of toxigenic *C. difficile* were obtained for 102 unique patients. A total of 95 single colonies of *C. difficile* per stool sample were isolated on selective media, subcultured alongside negative (uninoculated) controls, and polymerase chain reaction (PCR) ribotyped using capillary gel electrophoresis.

RESULTS—Capillary-based PCR ribotyping was successful for 9,335 *C. difficile* isolates, yielding a median of 93 characterized isolates per stool sample (range, 69–95). More than 1 *C. difficile* ribotype was present in 16 of 102 (16%) *C. difficile* infection (CDI) cases; 2 of the 16 mixtures were composed of at least 3 ribotypes, while the remaining 14 were composed of at least 2.

CONCLUSIONS—Deep sampling of patient stool samples coupled with capillary-based PCR ribotyping identified a high rate of mixed CDI cases compared with previous estimates. Studies seeking to quantify the clinical significance of particular *C. difficile* ribotypes should account for mixed cases of disease.

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Molecular epidemiologic studies of *Clostridium difficile* infection (CDI) are possible, in part, because of low-cost typing techniques that differentiate between genotypes. For *C. difficile*, restriction endonuclease analysis typing,¹ polymerase chain reaction (PCR) ribotyping,² and multilocus variable number tandem repeat analysis (MLVA)³ have been the most broadly applied. In practice, funds for molecular typing (eg, personnel and reagents) are almost always limited, so most studies consider only a single *C. difficile* isolate per patient stool sample. This practice conflicts with reports of mixed CDI cases (we refer to mixed CDI as the simultaneous occurrence of more than 1 genotype of *C. difficile* in a patient's gastrointestinal tract, where at least 1 of the genotypes encodes for a *C. difficile* toxin).^{4–7} Recently described high-throughput protocols for PCR ribotyping make it possible to screen thousands of isolates at relatively low cost.^{8,9} This technologic advance, however, has yet to gain widespread use and has not been applied to examine mixed CDI cases.

Previous studies of mixed CDI have either narrowly focused on community-acquired disease⁷ or considered only a few isolates (5 or fewer) per patient sample.^{4–6} Three important questions to be addressed are as follows: (1) What level of sampling is needed to adequately detect genotype mixtures? (2) How frequently can genotype mixtures be detected in patient stool samples? (3) At what ratio, if any, is a genotype mixture clinically important? In this study, we address the first 2 questions using high-throughput PCR ribotyping so that adequately powered studies can be designed to address the third question.

METHODS

Samples and Patient Information

The study population included hospitalized inpatients and ambulatory outpatients at the University of Michigan Hospital and Health System. Institutional review board approval was obtained for examination of stool samples and medical records. Stool samples were obtained from the Clinical Microbiology Laboratory between October 27, 2010, and May 21, 2012. All samples were submitted in Cary-Blair transport medium (Para-Pak C&S, Meridian Bioscience) and processed after diagnostic testing for the presence of toxigenic *C. difficile* (typically within 48 hours). A 3-step diagnostic algorithm was used to diagnose the presence of toxigenic *C. difficile* as follows: (1) enzyme immunoassays (EIAs) for the presence of glutamate dehydrogenase (GDH); (2) EIA for *C. difficile* toxins A and/or B as per C. Diff Quik Chek Complete cassettes (TechLab); and (3) PCR for the presence of the *tcdB* gene, which encodes for *C. difficile* toxin B (BD GeneOhm, BD Diagnostics). Small batches ($n = 2–7$) of samples per day were obtained and processed throughout the study (convenience sampling). Four additional samples were included that had been previously diagnosed as negative for the presence of toxigenic *C. difficile* (Figure 1). Given the diagnosis, we reasoned that *C. difficile* was at a low abundance in these 4 stool samples and that mixtures of more than 1 ribotype may be more probable when no single ribotype was dominant. These samples were originally identified during another study and had been stored at -80°C .

Diarrhea was noted in patient medical records for CDI-positive patients (see 3-step diagnostic below), with the following rare exceptions: ileus, colonoscopy-confirmed colonic inflammation, and when recurrent CDI was directly stated. Samples were collected before

CDI treatment, and only a single sample per patient was considered. Patient records were used to categorize CDI cases on the basis of age, gender, and surveillance definitions¹⁰ (hospital onset, healthcare facility associated; community onset, healthcare facility associated; community acquired; indeterminate; and unknown). Additionally, patients with a history of inflammatory bowel disease and 60-day post-CDI treatment outcome of recurrence (recurrence of CDI symptoms within 60 days after antibiotic treatment for CDI) or death were identified.

Isolate Processing

Stool samples were serially diluted in anaerobic, sterile, phosphate buffered saline. Aliquots from each dilution were plated onto prereduced, taurocholate cefoxitin cycloserine fructose agar (TCCFA), as per Sorg and Dineen.¹¹ Plates were incubated (37°C) anaerobically overnight and evaluated for the following: round, flat, opaque colonies characteristic of *C. difficile*; adequate growth and resolution of colonies; and minimal growth of contaminating organisms that can grow on TCCFA. On rare occasions, where an insufficient number of colonies were observed (fewer than 10 per plate), the colonies were not adequately resolved, or colonies appeared to be something other than *C. difficile*, the stool sample was not used.

All surfaces were precleaned with sporicidal (bleach) wipes. Colonies were selected from plates using sterile pipette tips (a new, sterile tip was used to pick each colony) and cultured in 96-well deep-well plates containing brain heart infusion broth with cysteine, as per Sorg and Dineen.¹¹ A single well in each plate containing sterile broth was not inoculated and served as a negative control. Sterile, breathable lids (Breathable Sealing Film, Thermo Fisher Scientific) were placed onto plates, which were subsequently incubated between 24 and 48 hours at 37°C. Aliquots of turbid cultures were diluted 10-fold with sterile, PCR-grade water (UltraPure Distilled Water, Invitrogen) in a new, sterile 96-well plate, taking care to preserve the original plate's orientation. This dilution served as a template for PCR ribotyping. Anaerobic glycerol was added to the remaining turbid culture, sealed with aluminum lids (Thermowell Sealing Tape, Corning), and frozen at -80°C. This archived plate was used to confirm results.

Taxonomic identity of each ribotype was confirmed using PCR assays for a *C. difficile*-specific 16S rRNA encoding gene locus,¹² and toxigenicity was inferred on the basis of results of a multiplex PCR assay for toxin genes.¹³

PCR Ribotyping

Diluted cultures were heated in a thermocycler for 18 minutes at 95°C to release DNA from vegetative cells. After heating, plates were frozen at -20°C before ribotyping. The details of the PCR ribotyping protocol were recently published,⁹ including reaction conditions, data analysis, and reproducibility. Briefly, a PCR reaction was used to generate fluorescently labeled DNA fragments of varying length. Fragment length depended on genotype-specific differences in the length of noncoding nucleotides between the 16S and 23S encoding genes. Fragments were analyzed using an ABI3730xl (Life Technologies), and chromatogram peaks were normalized and quantified using computer programs available from S.T.W.

Statistical Analysis

General statistical tests are referred to as they appear in the text. Bonferroni correction for family-wise error rate (P value/total number of tests) and the alternative P value correction by Benjamini and Yekutieli¹⁴ were considered.

The probability of mixed infection was calculated under the assumptions that (1) all ribotypes had an equal ability to cause a mixed infection and (2) the probability of causing a mixed infection was independent for each ribotype. In other words, if p is the frequency of ribotype A and q is the frequency of ribotype B, then $p \times q$ is the probability of a (pairwise) mixture containing ribotypes A and B. An online calculator (http://www.stattools.net/SSizRareInc_Pgm.php) for power and prevalence of rare events (λ) was used to examine the rate of mixed cases, as described by Machin et al.¹⁵

Power analysis was conducted with G*Power 3 (ver. 3.1.3; F. Faul, Universität Kiel): test family = χ^2 test, statistical test = goodness-of-fit tests: contingency tables, type of power analysis = post hoc: compute achieved power – given α , sample size, and effect size. A 2×2 design was used, where rows represented hypothetical ribotypes x and y and columns represented expected and observed proportions. Expected proportions were set at 0.99 and 0.01 (H_0), and observed proportions were set at 0.96 and 0.04 (H_1). This analysis was intended to identify power to detect a mixed infection based on sample size and the frequency of genotypes in a mixed case and not to determine adequate power for testing between different patient demographics.

RESULTS

Only stool samples from distinct patients were considered. Diagnostic results (Figure 1) were used to identify samples that were likely to contain toxigenic isolates. Eight samples failed to yield a sufficient number of *C. difficile* colonies for testing and were excluded. In total, 98 samples either were GDH/*C. difficile* toxin positive by EIA testing ($n = 34$; 35%) or were GDH positive by EIA/PCR positive for the *tcdB* gene ($n = 64$; 65%) and yielded sufficient colonies for analysis. We also included 4 samples where the presence of GDH ($n = 2$) or *C. difficile* toxin ($n = 2$) was not detected by EIA but where a *C. difficile* isolate had been previously isolated (see “Methods”).

Analysis of 95 single *C. difficile* colonies from each of the 102 patient stool samples resulted in a total of 9,335 ribotyped isolates and a median of 93 typed isolates per sample (range, 69–95). This median represents the success rate of our ribotyping protocol across all samples or a failure rate of approximately 1% (range, 0%–27%). Ribotyping results indicated that 16 of the 102 samples (16%) contained mixtures, and Table 1 lists the identity and abundance ratios of the ribotypes involved. For example, a mixed CDI case containing ribotypes UM9 and UM24 was observed at a ratio of 65 : 25, or where UM24 comprised 26.9% of the 2-part mixture. All mixtures were confirmed by repeat analysis of representative isolates. Three mixtures were composed of a single toxigenic ribotype and/or a single nontoxigenic ribotype. The remaining 13 mixtures were composed of at least 2 toxigenic ribotypes. Of these 13 toxigenic mixtures, the less common members in 8 mixtures represented at least 5% of the isolates examined. The less common members in the

remaining 5 mixtures were observed only once (~1%). Power analysis suggested that adequate sampling was achieved to differentiate mixtures at this level (for 1% vs 5%, power greater than 0.9). Two mixtures were composed of at least 3 different ribotypes, and no mixtures were observed among the 4 patients that originally tested negative for the presence of toxigenic *C. difficile*.

No combination of ribotypes was observed more than once (ie, each mixture was different), and the ribotypes in mixed cases were not observed in other samples that were processed on the same day, suggesting that cross-contamination was minimal. Ribotype 001 was observed in 3 of 16 mixed CDI cases (19%) but was not observed among nonmixed CDI cases. This association was significant ($P = .003$) and remained so after a Benjamini and Yekutieli correction but not a Bonferroni P value correction. Ribotype frequency of the most abundant ribotypes, 027 and 014-020, among nonmixed cases was 0.186 and 0.174, respectively (Figure 2); thus, the probability of observing a 027 : 014-020 mixture was 0.032 (3.2%). One mixed case of 027 : 014-020 was observed (Table 1), indicating that the observed rate of mixed CDI (~1%) was similar to the expected (~3%) for the most abundant ribotypes.

The median age of patients with and without mixed infections was not significantly different (Mann-Whitney test, $P = .6355$), and mixtures were not associated with gender ($\chi^2 = 1.78$, $df = 1$, $P = .182$). Patient information beyond age and gender was unavailable for 15 nonmixed CDI cases. In the remaining 87 cases, no significant associations were found between mixed cases and CDI surveillance groups ($\chi^2 = 4.63$, $df = 4$, $P = .327$), a history of inflammatory bowel disease ($\chi^2 = 3.79$, $df = 2$, $P = .151$), or whether the case represented a recurrent episode ($\chi^2 = 2.80$, $df = 2$, $P = .247$).

Sufficient patient information to assess CDI outcome (60 days post-CDI treatment) was available for 73 patients (57 nonmixed and 16 mixed). However, recurrence rates among patients with nonmixed (18 of 57; 32%) and mixed (7 of 16; 44%) infection were not significantly different ($\chi^2 = 0.822$, $df = 1$, $P = .365$). Three deaths occurred among patients with nonmixed infection within 60 days after treatment of their CDI, and no deaths were observed among patients with mixed infection.

DISCUSSION

The presence of more than 1 toxigenic *C. difficile* genotype during infection of the human gastrointestinal tract may be an important clinical and microbiologic phenomenon. Such events hold the potential to differentially influence host immune responses, infection dynamics, pathogen evolution, horizontal gene flow, and patient therapy compared with clonal infections.¹⁶ Mixtures of toxigenic and nontoxigenic *C. difficile* from 6 symptomatic CDI patients were first observed by Borriello and Honour.¹⁷ Sharp and Poxton¹⁸ similarly found mixtures in stool from 2 of 3 CDI patients after characterizing only 8 colonies per sample (the toxigenicity of these isolates was not reported). More recently, van den Berg et al⁶ and Hell et al⁵ characterized 5 or fewer *C. difficile* colonies per stool sample and found that 2 of 23 (8.7%) and 1 of 11 (9.1%) patients had mixtures of more than 1 PCR ribotype. Wroblewski et al⁷ used real-time PCR to detect 3 mixed populations in 23 patients (13%), although these patients were selected specifically to represent community-acquired

infection. Eyre et al⁴ conducted multilocus sequence typing on isolates from CDI patients, for whom at least 2 stool samples were collected on the same day. Although not directly comparable with studies that consider mixed populations from the same stool sample, this study reported mixed populations in 3 out of 109 (2.9%) patients. Collectively, these data were generated using different patient populations, sampling designs, *C. difficile* culture conditions, and genotyping techniques. Although it is clear that mixed CDI occurs, methodological differences between studies mask the true prevalence of this phenomenon.

The consideration of a small number of colonies (5 or fewer) per stool sample may lead to an underestimation or underappreciation of mixed CDI. In support of this hypothesis, the rate of mixed-population CDI in our study (16%) is higher than most previous studies and may be due to our increased sampling depth. That being said, our estimate was based on a convenience sample, so caution should be taken when generalizing results to other patient populations. However, it is likely that mixtures identified in our study would not be detected by considering fewer than 10 isolates per stool sample.

The sensitivity of our approach (~95 colonies per sample) was not without limits, and mixtures where less abundant members were less than 3% may have been missed. Similarly, it is possible that some of these rare events would not be observed if the samples were reanalyzed. On the other hand, other techniques—such as pulsed-field gel electrophoresis, MLVA, or whole genome sequencing—are more discriminant than ribotyping (eg, we could not distinguish between 2 different 027 isolates), so the observed rate of mixed CDI (16%) is conservative. It is therefore important for future studies to replicate our results and to determine the prevalence of mixed CDI from serially collected CDI-positive stool samples.

Our study was not designed to identify clinical differences between patients with mixed and nonmixed CDI. However, we feel that future epidemiologic studies should incorporate some level of mixed CDI detection into study designs. For example, suppose a study wanted to estimate mortality attributable to specific *C. difficile* genotypes and wanted to exclude mixed cases where the less abundant ribotype was more than 5%. The same sample size calculations used in drug/treatment trials to detect rare events¹⁵ can be used to determine how many individual *C. difficile* colonies should be examined. For the scenario above, if more than 1 ribotype is observed among 33 colonies from a stool sample, then there is reasonable power (0.8) to exclude the sample.

Three of our results support the hypothesis that mixed CDI occurs at random. First, the probability observing a mixture composed of the 2 most abundant ribotypes was low (3%). Second, no combination of ribotypes was observed more than once. Third, none of the patient epidemiologic factors were associated with mixed cases. We did find a significant association between ribotype 001 and mixed CDI, but more data are needed to confirm this result. Our study was not powered to detect differences between mixed and nonmixed patient factors, and there are few data with which to assess the differential roles of *C. difficile* genotypes during infection of the same host.

Even though the less common ribotype(s) in 5 mixed cases were observed a single time, their relative distribution throughout the patient's gastrointestinal tract may have been

heterogeneous. *C. difficile* colonization of the gastrointestinal tract is thought to be mediated, in part, by adhesins and colonization factors that facilitate attachment to the intestinal wall.¹⁹ Therefore, the transintestinal distribution of cells (from proximal to distal colon) may be ribotype specific. If so, each genotype's contribution to virulence will be site specific in the gastrointestinal tract. Alternatively, attachment may not be necessary for *C. difficile* pathogenesis. If this is the case, the ratio of genotypes in stool may indeed reflect the relative contribution of each member to virulence throughout the gastrointestinal tract. It will be important to address these hypotheses in future studies.

Although 16% of cases were mixed, only 13% were composed of more than 1 toxigenic isolate. Therefore, the actual estimate of mixed cases depends on the epidemiologic question being addressed. For example, 16% represents the rate at which any 2 genotypes come into contact in a patient, whereas 13% represents the rate at which 2 pathogens come into contact in a patient. It is difficult to know what role nontoxigenic members play during mixed infection, but it has been reported that they compete with pathogenic cells for colonic nutrients and resources and ultimately decrease virulence under certain circumstances.²⁰ Molecular epidemiologic investigations that seek to understand virulence attributable to a particular genotype should take into account the possibility that a significant proportion of CDI cases are actually mixed. However, other epidemiologic investigations that are interested in the relative influence of genotypes⁹ should be less affected by this phenomenon because our data suggest that mixtures are random events that occur at similar frequencies across CDI patients.

In conclusion, the rate of mixed CDI reported here (16%) is higher than previously reported but may be conservative. Similarly collected data are needed to provide confidence in this estimate. A high rate of mixed CDI increases the risk that epidemiologic links between disease episodes will be missed or misinterpreted. It is therefore imperative to identify cases of mixed CDI when differentiating between sporadic versus outbreak cases or when considering multiple cases from the same patient (disease recurrence). On the basis of our results, studies that do not or cannot identify mixed populations may be at risk of incorrectly linking *C. difficile* genotypes with epidemiologic factors.

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FIGURE 1.

Clostridium difficile diagnostic outcomes and mixed infection results. *C. difficile* was isolated on selective media from all stool samples included in this study. Results for the presence of glutamate dehydrogenase (GDH) enzyme immunoassay (EIA), *C. difficile* toxin EIA, and real-time polymerase chain reaction (PCR) for the *tcdB* gene are shown, along with the number and percentage of mixed cases.

FIGURE 2.

Frequency of ribotypes from nonmixed cases and expected ribotype mixtures.

TABLE 1

Mixed *Clostridium difficile* Infection Observed among 102 Cases

Type of mixed infection	Ratio	Less common ribotype(s), %
Less common ribotype more than 5% of mixture ($n=8$; 7.8%)		
UM9 : UM24	68 : 25	26.9
001 : UM27	71 : 23	24.5
UM22 : 001	74 : 20	21.3
UM70 : UM66	75 : 20	21.1
014-020 : 027	74 : 15	16.9
UM21 : 078-126	84 : 7	7.7
UM19 : UM70	85 : 6	6.6
UM18 : 001	87 : 6	6.5
Less common ribotype observed a single time ($n=5$; 4.9%)		
014-020 : UM9 : UM61	84 : 1 : 1	1.2
002 : UM2	89 : 1	1.1
UM13 : 014-020	92 : 1	1.1
002 : unique : UM12 ^a	92 : 1 : 1	1.1
002 : unique	94 : 1	1.1
Toxigenic/nontoxigenic mixtures ($n=3$; 2.9%)		
UM12 ^a : 014-020	80 : 15	15.8
UM10 ^a : 014-020	93 : 2	2.1

NOTE. Ratio refers to the number of one ribotype to the number of another ribotype. Numbers are listed in the same order as ribotype identifications in the first column.

^aNontoxigenic isolate. Unless noted, isolates were toxigenic.