

Development and Evaluation of a Novel, Semiautomated *Clostridium difficile* Typing Platform

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We describe a novel, semiautomated *Clostridium difficile* typing platform that is based on PCR-ribotyping in conjunction with a semiautomated molecular typing system. The platform is reproducible with minimal intra- or interassay variability. This method exhibited a discriminatory index of 0.954 and is therefore comparable to more arduous typing systems, such as pulsed-field gel electrophoresis.

Clostridium difficile, the etiological agent of *C. difficile* infection (CDI), is an important cause of both hospital- and community-acquired infectious diarrhea (1, 2). The emergence of hypervirulent *C. difficile* isolates and in particular the NAP1/BI/027 isolate has altered the epidemiology of *C. difficile* infections in many health care institutions, resulting in increased severity and duration of disease, with concomitant increases to the length and cost of hospitalization (1). Therefore, typing methods that can dis-

criminate NAP1/BI/027 and other emerging hypervirulent *C. difficile* isolates may be important for understanding the transmission dynamics of the organism. In addition, it has recently been demonstrated that the analytical sensitivity and specificity of *C. difficile* diagnostic assays may be dependent on the *C. difficile* strain type (3). In addition, recent reports suggest that the relapse rate following treatment with certain novel antianaerobic and *C. difficile*-specific antimicrobials could correlate with *C. difficile* strain type (4, 5); thus, an appreciation of the isolate type may play a role in patient management in the future. As such, *C. difficile* typing may have the potential to improve the management of CDI beyond clinical surveillance, especially in the hospital setting, and it may be important for clinical laboratorians and infection control specialists to have a baseline understanding of the different *C. difficile* isolates circulating in their institutions.

Pulsed-field gel electrophoresis (PFGE) is the principal reference method employed for *C. difficile* typing in North America (6). While PFGE affords acceptable discriminatory power (6), it does suffer from some important limitations, in particular the labor intensity, technical expertise, turnaround time, and necessity for control strains to be processed alongside isolates of epidemiological interest. In Europe, the predominant method for *C. difficile* typing is PCR-ribotyping, which involves the PCR amplification of the intergenic space region between the 16S and 23S rRNA genes (7, 8, 9). For many years, epidemiologic studies for *C. difficile* have relied on PFGE and PCR-ribotyping to determine strain relatedness; multiple-locus variable-number tandem-repeat analysis (MLVA) for *C. difficile* typing has also been described for recent studies (6, 10, 11, 12). Although this method does appear to be reproducible and discriminatory, it requires access to a

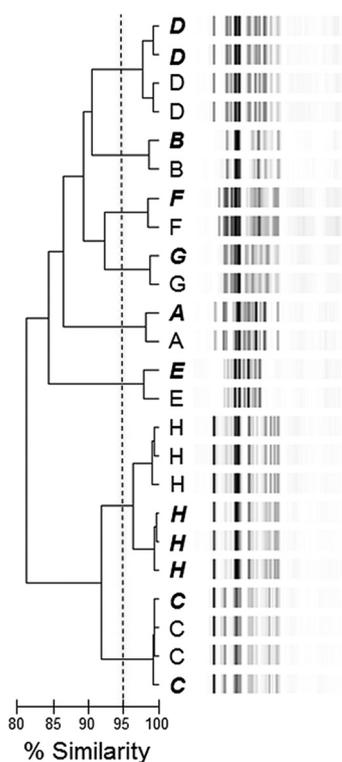


FIG 1 Intra- and interassay variability of the PCR-ribotyping/DiversiLab *C. difficile* typing platform. Dendrogram illustrating the gel electrophoresis profiles of 24 PCR-ribotyping reactions obtained for eight different *C. difficile* isolates (isolates A to H). Samples loaded onto the first Agilent chip are indicated by lightface capital letters, while PCR-ribotyping reactions loaded onto the second Agilent chip are indicated by letters in bold italics. The percent SI is indicated by the bar below the dendrogram; isolates with a SI of $\geq 95\%$ are considered very related or identical.

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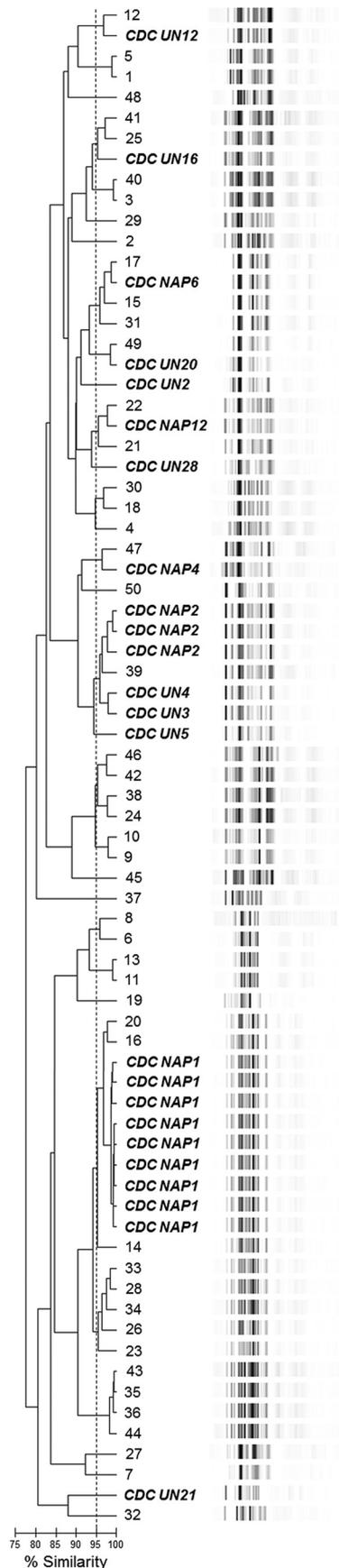
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genetic analyzer/DNA sequencer, which is cost prohibitive to many laboratories.

The objective of this study was to develop and validate a semi-automated PCR-ribotyping platform that would further reduce labor intensity, allow analysis of isolates in real time, provide objective downstream data analysis, maintain discriminatory power, and be a feasible option for routine benchtop typing of *C. difficile* isolates in a clinical setting. As such, we evaluated the performance of PCR-ribotyping in conjunction with the DiversiLab system (bioMérieux, Durham, NC) and the 2100 Bioanalyzer instrument (Agilent; Santa Clara, CA, USA) for fragment separation and PCR-ribotype band pattern analysis. A significant advantage of this platform is that all resultant banding patterns are banked electronically so that they may be recalled and compared retrospectively.

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Initially, we evaluated the intra- and interassay variability of the PCR-ribotyping/DiversiLab platform. Therefore, in a blinded experiment, prior to PCR-ribotype amplification, genomic DNA isolated from eight *C. difficile* isolates (labeled A to H) previously characterized as having unique PFGE types (6) was distributed into 24 tubes such that each tube contained DNA from only one isolate (*C. difficile* DNA was isolated using the BiOStic bacteremia DNA kit [MO BIO; Carlsbad, CA]). To perform the PCR-ribotyping reaction, a 25- μ l PCR mixture that included 100 ng of *C. difficile* DNA, a Ready-To-Go randomly amplified polymorphic DNA (RAPD) analysis bead (GE Healthcare Life Sciences; Piscataway, NJ), and primers complementary to the 3' end of the 16S rRNA gene (5'-CTGGGGTGAAGTCGTAACAAGG-3') and the 5' end of the 23S ribosomal gene (5'-GCGCCCTTTGTAGCTTG ACC-3') at a final concentration of 0.5 μ M was assembled (13). The amplification conditions were 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The PCR products were loaded into two different DiversiLab DNA chips (bioMérieux; Durham, NC) according to the manufacturer's specifications and resolved using the 2100 Bioanalyzer instrument. The resultant banding patterns were analyzed using the DiversiLab Bacterial Barcodes software program, which uses curve-based Pearson coefficients for pairwise similarity scores (14). The data were organized into a dendrogram, and a similarity index (SI) for each pair was calculated (Fig. 1). By applying a SI of $\geq 95\%$, the PCR-ribotype band patterns were separated into eight clusters, with each cluster corresponding to an isolate. Upon examination of the resultant dendrograms, it is clear that independent PCR-ribotype patterns obtained for each *C. difficile* isolate cluster together when loaded onto either the same or different DNA chips and that this is both discriminatory and reproducible. Taken together, these data support our assessment of limited intra- and interassay variability.

To assess the effect of culture duration and subculture fre-

FIG 2 Evaluation of the performance of the PCR-ribotyping/DiversiLab *C. difficile* typing platform at the local level. The dendrogram illustrates the electrophoresis profiles of a collection of 74 *C. difficile* isolates obtained from both the CDC and our institution. Isolates obtained from the CDC are indicated by their PFGE pulsotype and are in bold italics, while patient isolates are indicated numerically. The collection was differentiated into 17 clusters with 15 unrelated strains, yielding a discrimination index of 0.954. The percent SI is indicated by the bar below the dendrogram; $\geq 95\%$ are very related or identical.

quency on the reproducibility of the PCR-ribotype band patterns, a *C. difficile* isolate (ATCC 9689) was incubated anaerobically at 35°C on cycloserine-cefoxitin-fructose agar (CCFA) plates for 2, 5 or 14 days or consecutively passaged onto CCFA plates one, three or five times. Subsequently, the cells were collected, their DNA was extracted, and PCR-ribotyping was performed. The PCR-ribotype band patterns were analyzed using the DiversiLab Bacterial Barcodes software program, and dendrograms to illustrate strain relatedness were generated. From inspection of the PCR product electrophoresis profiles and the associated dendrograms, it was found that PCR-ribotype patterns are not altered significantly in response to either culture duration (SI > 98% across all conditions) or subculture frequency (SI > 99% across all conditions), indicating that the semiautomated typing platform can tolerate considerable variation in culture conditions; this has been demonstrated previously for other typing methods (15, 16).

In an effort to evaluate the performance of the semiautomated, PCR-ribotyping platform at typing *C. difficile* isolates at the local level, a collection of 50 *C. difficile* isolates cultured from the stools of patients with a positive toxin A/B assay in our institution and, as a calibrant, 24 *C. difficile* isolates whose PFGE pulsotypes had been previously determined (6) was analyzed (Fig. 2). To ensure a highly diverse calibrant collection, *C. difficile* isolates with the NAP1, NAP2, NAP4, NAP6, and NAP12 PFGE pulsotypes and isolates that did not map to previously characterized PFGE pulsotypes (UN for “unnamed” types) were included. The 74 isolates were separated into 17 clusters, with 15 unrelated isolates yielding a discrimination index (*D*) (17) of 0.954 with 95% confidence intervals for *D* of 0.931 to 0.977 (18), which is in very close agreement with findings of previous studies evaluating the discriminatory power of different *C. difficile* typing methods (6, 9). However, the discriminatory index of some of these prior studies could be somewhat skewed, since NAP1 strains are typically in abundance in contemporary epidemiological studies.

A requirement of molecular typing methods is an ability to group related isolates and differentiate unrelated isolates. Our method can do both, grouping the PCR-ribotyping patterns obtained for isolates with identical PFGE pulsotypes and differentiating the PCR-ribotyping patterns attained for isolates with different PFGE pulsotypes. In an effort to further validate the discriminatory power of the semiautomated, PCR-ribotyping platform, a collection of patient isolates whose PCR-ribotyping patterns clustered with those obtained for isolates with known PFGE pulsotypes (i.e., calibrant isolates) was analyzed using PFGE. In all cases, the patient isolate yielded a PFGE pulsotype very similar, if not identical, to that of the calibrant isolate it had clustered with after PCR-ribotyping analysis (Table 1).

The novel, semiautomated *C. difficile* typing platform described here has some advantages over PFGE and other more intensive molecular typing methodologies; in particular, it is less labor-intensive. Data analysis is automated, easy to perform, and can be done within a matter of minutes, while ensuring a high discriminatory power. In addition, the ability to bank isolate PCR-ribotype patterns for retrospective and prospective analysis is an advancement in *C. difficile* typing and could facilitate the rapid, streamlined examination of epidemiological trends.

At present, the primary utility of *C. difficile* typing is to assist epidemiological studies; however, it is possible that real-time *C. difficile* typing will become increasingly relevant in routine clinical management to identify the predominant *C. difficile* isolates in an

TABLE 1 Agreement between PFGE and the semiautomated *C. difficile* PCR-ribotyping platform

Patient isolate	Pulsotype inferred from PCR-ribotyping	Pulsotype determined by PFGE
15	NAP6	NAP6
16	NAP1	NAP1
17	NAP6	NAP6
20	NAP1	NAP1
23	NAP1	NAP1
28	NAP1	NAP1 related
35	NAP1	NAP1
39	NAP2	NAP2
47	NAP4	NAP4

institution prior to administration of expensive *C. difficile*-specific antimicrobials and to monitor diagnostic assay performance. After *C. difficile* is recovered in culture, the hands-on time per isolate to complete this typing method is approximately 1 h. After the initial investment of acquiring the hardware and software for this method, the cost of analyzing a single isolate is approximately \$50. However, up to 12 samples can be analyzed in a single chip or run, and if 12 isolates are run in a batch, the cost per isolate is reduced to approximately \$35. With the ability to bank banding patterns and compare isolates in real-time to previously typed isolates, it will be relatively easy for institutions to create their own *C. difficile* strain typing database.

Therefore, we believe that the rapid, facile, and discriminatory novel, semiautomated PCR-ribotyping platform described herein will be favorably useful to epidemiologists, clinicians, and laboratory medicine professionals involved in managing CDIs.

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The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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