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DNA Microarray-Based PCR Ribotyping of *Clostridium difficile*

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This study presents a DNA microarray-based assay for fast and simple PCR ribotyping of *Clostridium difficile* strains. Hybridization probes were designed to query the modularly structured intergenic spacer region (ISR), which is also the template for conventional and PCR ribotyping with subsequent capillary gel electrophoresis (seq-PCR) ribotyping. The probes were derived from sequences available in GenBank as well as from theoretical ISR module combinations. A database of reference hybridization patterns was set up from a collection of 142 well-characterized *C. difficile* isolates representing 48 seq-PCR ribotypes. The reference hybridization patterns calculated by the arithmetic mean were compared using a similarity matrix analysis. The 48 investigated seq-PCR ribotypes revealed 27 array profiles that were clearly distinguishable. The most frequent human-pathogenic ribotypes 001, 014/020, 027, and 078/126 were discriminated by the microarray. *C. difficile* strains related to 078/126 (033, 045/FLI01, 078, 126, 126/FLI01, 413, 413/FLI01, 598, 620, 652, and 660) and 014/020 (014, 020, and 449) showed similar hybridization patterns, confirming their genetic relatedness, which was previously reported. A panel of 50 *C. difficile* field isolates was tested by seq-PCR ribotyping and the DNA microarray-based assay in parallel. Taking into account that the current version of the microarray does not discriminate some closely related seq-PCR ribotypes, all isolates were typed correctly. Moreover, seq-PCR ribotypes without reference profiles available in the database (ribotype 009 and 5 new types) were correctly recognized as new ribotypes, confirming the performance and expansion potential of the microarray.

Clostridium difficile is the leading infectious agent of nosocomial diarrhea in humans and causes gastrointestinal infections also in various animal species (e.g., pigs, horses, and rodents) (1, 2). Over the last decade, increasing incidence and changes in the clinical presentation of human *C. difficile*-associated diarrhea have been reported worldwide (1). Newly emerging *C. difficile* genotypes (e.g., PCR ribotypes 027 and 078) are involved in these epidemiological changes, which have also been found in companion animals (i.e., calves and piglets), pets (i.e., cats and dogs), and foods (e.g., meat products and vegetables), indicating the possibility of zoonotic transmission (1, 3). Therefore, the genotyping of *C. difficile* isolates is important for epidemiological and clinical investigations. For genotyping, several molecular methods have been established so far: restriction endonuclease analysis (REA) (4, 5), pulsed-field gel electrophoresis (PFGE) (6, 7), toxinotyping (8), multilocus variable-number tandem repeat (VNTR) analysis (MLVA) (9, 10), multilocus sequence typing (MLST) (11), surface layer protein A typing (*slpA* typing) (12, 13), and PCR ribotyping (14, 15). PCR ribotyping is the standard typing method used in Europe and is widely used also in the United States and Canada (16). The target for this method is the intergenic spacer region (ISR) between the 16S and 23S rRNA genes (14, 15). The ISR is variable in length and is present up to 10 times in the *C. difficile* genome. Thus, PCR amplification results in a specific amplicon profile after separation in an agarose gel. However, agarose gel analysis needs a considerable effort in standardization, including a huge number of PCR ribotype reference strains, to correctly assign isolates for interlaboratory comparability (16). Recently, Indra et al. (17) developed a PCR ribotyping method with subsequent capillary gel electrophoresis (seq-PCR ribotyping) and Web database analysis. Compared to the conventional procedure, seq-PCR ribotyping is faster, has a higher resolution, and might be a tool for standardization (17). However, seq-PCR ribotyping causes high costs for laboratory infrastructure and requires special expertise to perform. The aim of this study was to develop a DNA microarray-based assay for efficient and reliable PCR ribotyping of *C. difficile* strains on a convenient laboratory platform.

MATERIALS AND METHODS

Bacterial strains, culture, DNA extraction, molecular characterization, and typing. A collection of 168 *C. difficile* reference, field, and type strains representing 48 seq-PCR ribotypes was used to validate the microarray-based assay (Table 1). Eighty-six field strains isolated from animal (calf, piglet, cat, and dog) and human (2 strains) sources were obtained from previous investigations (18–20). The University Hospital of Heidelberg (SZ) provided 41 strains isolated from diarrheic patients between 2006 and 2007. Fourteen isolates were kindly provided by the Robert Koch-Institute (H. Zaiss, Wernigerode, Germany). The Wellcome Trust Sanger Institute (T. Lawley, Cambridge, United Kingdom) kindly provided 12 strains, including *C. difficile* strains 630, R20291, CD196, M120, and CF5, for which complete genome sequences are available. Fourteen ribotype

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TABLE 1 *C. difficile* strains used for assay validation

Seq-PCR ribotypes	No. of strains	No. of strains from:						Presence of toxin gene:			
		Human	Bovine	Porcine	Canine	Feline	NA[a]	*tcdA*	*tcdB*	*cdtA*	*cdtB*
001	11	8					3	+	+	−	−
001/ecdc	2	2	0	0	0	0	0	+	+	−	−
002/2	6	3		3				+	+	−	−
003	3	2	0	0	0	0	1	+	+	−	−
003/FLI01	1		1					+	+	−	−
005	5	2	2				1	+	+	−	−
006	1						1	+	+	−	−
010	6	1	2		2		1	−	−	−	−
012	2	1					1	+	+	−	−
014/0	14	10	1	1	1	1		+	+	−	−
015	3	2					1	+	+	−	−
017	2	1					1	+	+	−	−
019	2	1					1	+	+	+	+
020	2				1		1	+	+	−	−
023	3	1	1				1	+	+	+	+
027	6	6						+	+	+	+
029	4		3				1	+	+	−	−
033	6		5				1	−	−	+	+
036	1						1	+	+	+	+
081/FLI01	1	1						+	+	+	+
039/2	2					1	1	−	−	−	−
045/FLI01	6		6					+	+	+	+
046	3	2					1	+	+	−	−
049	5			3			2	+	+	−	−
049/FLI01	1		1					+	+	−	−
049/FLI02	1			1				+	+	−	−
053/FLI01	1						1	+	+	−	−
054	2	1					1	+	+	−	−
054/FLI01	1	1						+	+	−	−
056	1						1	+	+	−	−
070	1	1						+	+	−	−
078	21	5	10	6				+	+	+	+
081	2						2	+	+	−	−
087	1						1	+	+	−	−
106	2	1					1	+	+	−	−
126	6	1	2	3				+	+	+	+
126/FLI01	1			1				+	+	+	+
413	6	1		5				+	+	+	+
413/FLI01	1			1				+	+	+	+
446	2		2					+	+	−	−
449	2	1				1		+	+	−	−
596	1		1					−	−	−	−
598	3		1	2				+	+	+	+
620	2		1	1				+	+	+	+
652	3		1		1	1		+	+	+	+
660	6		6					−	−	+	+
AI-15	2	2						+	+	−	−
AI-60	2	2						+	+	−	−
Total	168	59	46	27	5	4	27				

[a] NA, information on source species was not available.

reference strains were obtained from the Leiden University Medical Centre (E. J. Kuijper, Leiden, The Netherlands), and *C. difficile* type strain DSM 1296 was purchased from the German Collection of Microorganism and Cell Cultures (DSMZ) (Braunschweig, Germany). Additionally, a panel of 50 *C. difficile* strains isolated from pet owners and their pets was used for assay verification.

Bacterial culture, genomic DNA extraction, toxin gene detection, and seq-PCR ribotyping were performed as previously described (20).

DNA sequence analysis and selection of hybridization probes. The ISR is formed by sequence modules that differ in their numbers and arrangements (21). The ISR begins with an ISRstart sequence, is terminated by an ISRend sequence, and contains up to five 9-bp direct repeats that separate 172-bp, 53-bp, and 33-bp sequence modules (Fig. 1). Furthermore, ISR modules occur in sequence variants, e.g., 14 variants of the 33-bp module were described (21).

For probe selection, two strategies were applied (Fig. 1). (i) Probe

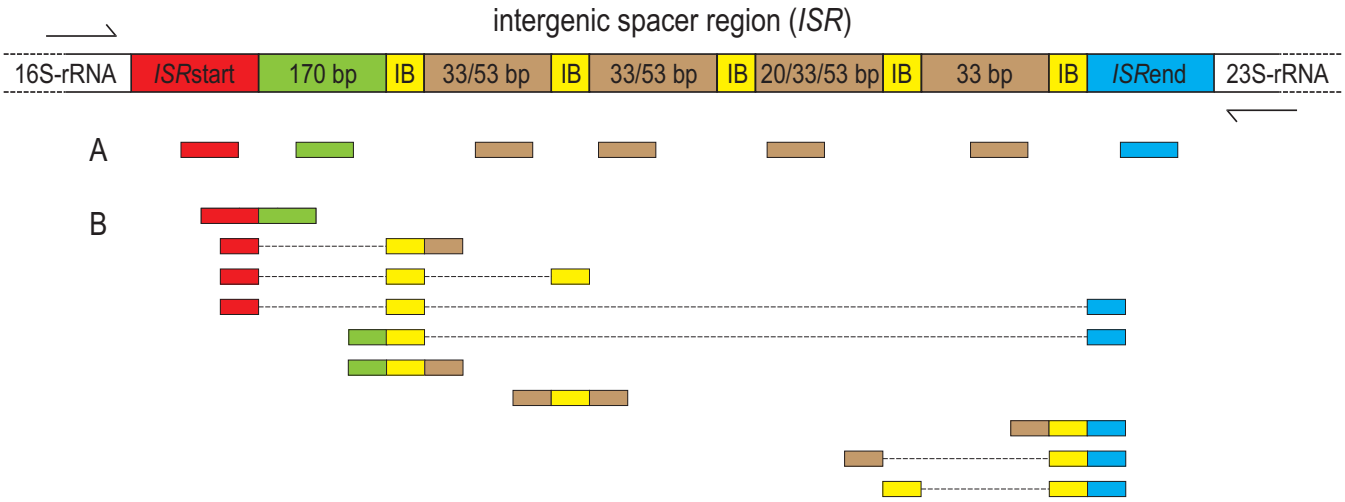

FIG 1 Probe selection for the DNA microarray using the intergenic spacer region (ISR) as molecular target. The ISR is formed by sequence modules that differ in their numbers and arrangements (21). The modules are separated by 9-bp direct repeats (IB). For probe selection, two strategies were applied: (A) probes binding within a single module and querying sequence variants of that module, and (B) probes for querying the synteny of the modules. The arrows display the PCR primers used for amplification of the ISR.

binding within a single ISR module and targeting sequence variants of that module was found. Therefore, a DNA sequence database was constructed that includes 182 complete ISR sequences available from GenBank (see Data Set S1 in the supplemental material) containing 1,249 ISR modules. Complete sequenced genomes and whole-genome shotgun (WGS) sequencing results were considered for this analysis. The alignment of the ISR modules revealed a total of 114 module sequence variants, from which 60 suitable probes were derived. (ii) In order to capture the synteny of the modules within the ISR, 84 probes were selected that overlap at least two ISR modules (Fig. 1). For example, hybridization probe 170bp_v1_IB_v1_53bp_v7 comprises the 3′ end of the 170-bp module (sequence variant 1), the direct repeat IB (sequence variant 1), and the 5′ end of the 53-bp module (sequence variant 7). For the selection of these probes, previously published ISR sequence arrangements and theoretically assembled arrangements of ISR modules and their sequence variants were analyzed (see Data Set S1 in the supplemental material, ISR structures sheet).

In order to yield similar binding efficiencies, comparable signal intensities, and to avoid pairwise hybridization, the following basic criteria were used for selecting hybridization probes: (i) a probe size of >22 bases, (ii) oligonucleotide melting temperatures in the range of 60 to 65.5°C, as calculated by the nearest-neighbor method of SantaLucia (22), and (iii) the absence of significant self-complementarity. In-house software developed by Alere Technologies (Jena, Germany) was used to check these properties. The alignments were performed in the BioEdit sequence alignment editor version 7.0.5.3.

A total of 144 distinct hybridization probes were finally designed. Their nucleotide sequences and the physical parameters of the 144 selected probes are provided in Data Set S2 in the supplemental material. All probes and additional staining and background controls were synthesized by Metabion International AG (Steinkirchen, Germany) and spotted in triplicate onto the surface-coated glass of the array, as previously described by Alere Technologies (Jena, Germany) (23). Finally, the microarrays were mounted into ArrayStrip reaction vials (Alere Technologies).

***In silico* hybridization with eight fully sequenced *C. difficile* genomes.** For 8 fully sequenced *C. difficile* genomes available at GenBank (strains 630, CF5, M68, 2007855, BI1, CD196, R20291, and M120), a probe-matching matrix was calculated (Alere Software) to determine the number of mismatches for each probe (see Data Set S1 in the supplemental material).

The number of mismatches between a probe and the published chromosomal sequence was used to estimate the intensity of the hybridization signal based on the following relation: intensity value of 0.8 for a perfect match, 0.6 for 1 mismatch, 0.3 for 2 mismatches, 0.1 for 3 mismatches, and no signal for more mismatches. In the case of probes that show multiple matches, only the best matching result was considered for the calculation of theoretical signal intensities.

PCR ribotyping using biotinylated primers. PCR ribotyping was performed according to Bidet et al. (15) and Stubbs et al. (14). The primers Bidet_16S (5′-GTGCGGCTGGATCACCTCCT-3′), Stubbs_16S (5′-CTGGGGTGAAGTCGTAACAAGG-3′), Bidet_23S (5′-CCCTGCACCCTTAATAACTTGACC-3′), and Stubbs_23S (5′-GCGCCCTTTGTAGCTTGACC-3′) were synthesized and 5′ biontinylated (Eurofins MWG Synthesis GmbH, Ebersberg, Germany). For PCR, DreamTaq DNA polymerase (Fermentas, St. Leon-Rot, Germany) was used under the following conditions: initial denaturation at 95°C for 2 min, 30 cycles of 30 s at 95°C, 30 s at 52°C, 1 min at 72°C, and a final elongation of 5 min at 72°C carried out on a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). Initially, the amplified products were checked by electrophoresis on 1.5% agarose gels, which ran for 8 h at 80 V. Hybridization was done without purifying the PCR products.

Hybridization. Hybridization was conducted using the Hybridization kit (Alere Technologies, Germany [http://alere-technologies.com/en/products/lab-solutions/reagents.html]). The kit includes hybridization buffer C1, washing buffer C2, peroxidase-streptavidin conjugate C3, conjugation buffer C4, washing buffer C5, and peroxidase substrate D1.

The final protocol includes the following steps: initial washing with 150 μl of high-performance liquid chromatography (HPLC)-grade water per vial at 56°C and 550 rpm for 5 min and subsequent washing with 150 μl of hybridization buffer C1 at 56°C and 550 rpm for 5 min in a BioShake iQ thermomixer (Quantifoil Instruments, Jena, Germany). All liquids were carefully removed with a soft plastic pipette to avoid scratching the chip surface. In a separate tube, 1 μl of the biotinylated PCR product was diluted in 99 μl of hybridization buffer C1, heated at 95°C for 5 min, and subsequently cooled on ice for 1 min. This mixture (approximately 100 μl) was transferred into the ArrayStrip reaction vial, and hybridization was carried out at 56°C, with shaking at 550 rpm for 60 min. After hybridization, the liquids were removed, and each vial of the ArrayStrip was washed two times with 150 μl of washing buffer C2 at 56°C/ and 550 rpm. Peroxidase-streptavidin conjugate C3 was diluted 1:100 in conjugate buffer C4. One hundred microliters of this mixture was added to each reaction vial of the ArrayStrip and incubated for 15 min at 30°C and 550 rpm.

TABLE 2 Similarity matrix comparing MS/aMS values of predicted hybridization results for fully sequenced *C. difficile* genomes[a]

Strain	MS/aMS for ribotype/strain:							
	012/630	017/CF5	435/M68[b]	027/2007855	027/BI1	027/CD196	027/R20291	078/M120
630	0/0	37.2/132.8	28.2/64.6	28.7/73.3	32.5/87.4	34.8/97.3	33.1/90.7	33.1/103.6
CF5		0/0	16.0/51.3	33.7/90.3	35.7/97.4	38.0/105.1	35.7/96.7	34.3/92.9
M68			0/0	25.5/49.2	26.5/53.2	28.8/61.0	26.5/52.6	33.1/102.1
2007855				0/0	4.8/7.2	8.3/14.7	7.2/10.5	28.4/55.1
BI1					0/0	3.5/4.7	3.6/2.0	33.2/69.2
CD196						0/0	2.3/5.6	35.5/84.2
R20291							0/0	33.8/73.1
M120								0/0

[a] MS, MatchScore; aMS, aMatchScore.
[b] Assigned to PCR ribotype 017 in conventional PCR ribotyping.

After two washing steps with 150 μl of washing buffer C5 (30°C at 550 rpm for 2 min), a staining reaction was conducted by adding 100 μl of the peroxidase substrate D1 to each reaction vial of the ArrayStrip, followed by incubation at room temperature for 10 min without shaking. The staining intensities of the spots were measured using the ArrayMate device (Alere Technologies, Jena, Germany). The final protocol was achieved by empirically optimizing the hybridization conditions (48°C to 58°C) while comparing the PCR primers described by Bidet et al. (15) and Stubbs et al. (14). The strains with available sequenced genomes (630, R20291, M120, CF5, M68, and CD196) were used to compare the experimentally obtained hybridization results with the theoretically generated hybridization patterns.

Processing of hybridization data. Hybridization signals were processed using the IconoClust software, version 3.3, provided by Alere Technolgies (Jena, Germany). The intensities of the spots were normalized automatically by the software using the equation NI = 1 − (*M*/BG), where NI is the normalized intensity, *M* is the average intensity of the automatically recognized spot, and BG is the intensity of the local background. The NI values theoretically range from 0 (no signal) to 1 (maximum signal). The assignment of hybridization patterns to the experimentally obtained reference profile database was carried out using an adapted PatternMatch (Alere Technologies) algorithm. The final numerical output is given as the adapted MatchScore (aMS), which represents the overall quartic sum of the differences between corresponding signal intensities. Thus, the aMS value is a measure of the overall similarity/dissimilarity between two hybridization patterns. An ideal match of two patterns based on the same set of oligonucleotide probes will yield an aMS of 0, whereas values of >1.0 require critical analysis, because they may indicate a poor match. In the case of those scores of >1.0, additional manual scrutiny is necessary.

Similarity matrix and unweighted-pair group method with arithmetic mean (UPGMA) analyses were performed with the BioNumerics software version 7.1.

RESULTS

Adjustment of the PatternMatch algorithm and *in silico* probe matching. In order to put a disproportionally large mathematical weight on probes showing strong deviations between corresponding signal intensities, matching scores were calculated using a quartic equation (adapted MatchScore):

$$\mathrm{aMS}\ (X,\ Y) = 10 \times \sum_{i=1}^{144} \left(x_i - y_i\right)^4$$

where X and Y are hybridization patterns, and x_i and y_i are the corresponding probe signals.

A similarity matrix that compares the conventional MatchScore (MS) and adapted MatchScore (aMS) values was created showing the predicted hybridization results for 8 fully sequenced *C. difficile* genomes (Table 2). The comparison of strains that were assigned to the same ribotype (027) resulted in an MS between 2.3 and 8.3, while the aMS were between 2.0 and 14.7. Strains of different ribotypes showed MS between 16 and 38, while the aMS calculation yielded values between 49.2 and 132.8.

Predicted versus empirically obtained hybridization patterns. A comparison of the predicted and real hybridization patterns was performed for *C. difficile* strains 630, R20291, CD196, and M120. Most probes showed more than one possible match per genome. The lowest mismatch value was used to calculate the theoretical signal intensities.

Experimentally obtained signal intensities of probes that match virtual signals in a range of ±0.15 were considered congruent (see Data Set S3 in the supplemental material). The percentages of congruent probes for the real and virtual hybridization results in relation to all probes were 38% for strain 630, 31% for R20291, 26% for CD196, and 32% for M120. For 11 probes (8%), the results of virtual and real hybridization were in concordance for all tested strains, while 56 probes (40%) showed no agreement for any strain.

Comparison of hybridization patterns generated using primers described by Stubbs et al. (14) and Bidet et al. (15). Eight strains of PCR ribotype 078 were array typed using PCR products obtained by the primers of Stubbs et al. (14) and Bidet et al. (15). In comparison to Bidet et al. (15), the summed signal intensities at a hybridization temperature of 56°C were 23 to 41% (mean, 33%) higher when using PCR products generated by Stubbs et al. (14). There was no significant difference in the proportion of the signal intensities between both methods. The final protocol used the primer set of Stubbs et al. (14).

Effects of PCR template amounts on seq-PCR ribotyping and PCR product amounts on hybridization. Genomic DNA of *C. difficile* DSM 1296 (ribotype 001) was diluted in 8 steps (1, 10, and 100 pg, and 1, 5, 10, 25, and 50 ng) to obtain PCR template amounts of 1 pg to 50 ng per reaction. Seq-PCR ribotyping was performed using the primers of Stubbs et al. (14). The PCR failed only when using the 1-pg (approximately 200 *C. difficile* genomes) template. Template amounts of 10 pg up to 50 ng always resulted in ribotype 001-specific seq-PCR ribotype profiles. Differences in the proportion of peak intensities were not recorded. However, increasing PCR product concentrations led to increasing peak intensities (e.g., height of peak, 232; 10-pg template/reaction [rct] revealed 258 fluorescent units [FU], while 50 ng/rct yielded 4,364 FU).

The effects of the PCR product amounts on the array hybridization were not recorded for 0.5 to 3 μl of PCR mix.

Reproducibility of ribotype-specific hybridization patterns. In order to assess the reproducibility of hybridization patterns, 4 *C. difficile* strains (Liv024, 10S0106, R20291, and M120) of seq-PCR ribotypes 001/ecdc, 014/0, 027, and 078 were array typed based on 4 independent ribotyping PCRs, whereby each PCR contained 4 separate reactions per strain (e.g., PCR1 with 4 *C. difficile* strains × 4 reactions). Thus, 16 array hybridizations were performed per strain under identical hybridization conditions. Within the ribotype sets, the hybridization patterns were compared by similarity matrix analyses. The matrices showed average aMS values of 0.1 for strains Liv024, 10S0106, and M120 and 0.04 for strain R20291. The maximum aMS were 0.04, 0.1, 0.53, and 0.09 for strains Liv024, 10S0106, R20291, and M120, respectively (see Data Set S4 in the supplemental material). The maximum aMS obtained for ribotype 027 was caused by a hybridization reaction with comparatively strong signal intensities (PCR3, reaction 3; see Data Set S4 in the supplemental material).

In order to examine whether the seq-PCR ribotypes isolated from different sources and/or geographical origin reveal type-specific hybridization patterns, 24 strains of seq-PCR ribotypes 001, 014/0, and 078 (8 different isolates per ribotype) were hybridized based on two independent PCRs. Ribotype-related similarity matrix analyses showed maximum aMS of 0.51, 0.38, and 0.13 for the strains of ribotypes 001, 014/0, and 078, respectively (see Data Set S5 in the supplemental material). The calculated average aMS were 0.09 for strains of ribotypes 001 and 014/0, whereas for strains of ribotype 078, an average aMS of 0.03 was recorded.

Database of experimentally obtained reference hybridization patterns. A total of 142 *C. difficile* strains were used to obtain reference hybridization patterns for 48 seq-PCR ribotypes. At least 6 hybridizations were performed per ribotype (except ribotype 049, with 5 hybridization reactions). The results were used to calculate 3 reference hybridization patterns for each ribotype based either on the arithmetic mean, the median, or on the arithmetic mean of the minimum and maximum signal intensities that were recorded per probe. Subsequently, all hybridization patterns were matched against their 3 corresponding reference profiles to evaluate the best method for calculating reference hybridization patterns. The average deviation of the individual hybridization results to corresponding reference profiles were aMS of 0.066 ± 0.104 for the arithmetic mean of the minimum and maximum signals intensities, 0.075 ± 0.188 for the arithmetic mean of all hybridizations, and 0.109 ± 0.302 for the median of all hybridizations. Therefore, the minimum/maximum (min/max) method was chosen to calculate the database reference profiles, since it revealed the lowest aMS. However, 99% of all recorded aMS obtained in this experiment were ≤1, regardless of the method of calculation used.

The database reference profiles that were finally obtained were compared by generating an aMS similarity matrix and the corresponding unweighted-pair group method with arithmetic mean (UPGMA) tree (Fig. 2). An aMS of ≥1 was defined as indicating hybridization patterns that can be discriminated by the microarray. Based on this definition, 27 distinct ribotypes or ribotype groups were differentiated. A comparison of the molecular typing and characterization results discussed in this study is shown in Fig. 3. The potential of the array for the discrimination of >350 currently known ribotypes cannot be predicted theoretically.

Microarray-based PCR ribotyping of field isolates. *C. difficile* strains previously isolated from dogs, cats, and their owners were characterized using seq-PCR ribotyping and the microarray-based assay in parallel. Seq-PCR ribotyping of 50 isolates yielded 17 ribotypes, including 5 new ribotypes (009/FLI01, 010/FLI01, 003/FLI02, 441/FLI01, and 001/5/FLI01; Table 3). Using the microarray-based assay, 49 strains (one hybridization reaction failed) were assigned to 13 ribotypes or ribotype groups (Table 3). The strains of seq-PCR ribotypes 014/0, 014/5, 020, and 449 showed similar hybridization patterns (014-020-449 group). These 4 strains therefore were not differentiated by the array, which explains the discrepancy with the results obtained by seq-PCR ribotyping.

From 40 field strains that were correctly assigned to ribotype reference profiles, 39 (97.5%) displayed an aMS of <0.75. The remaining strain showed an aMS of 1.37 for the best match to ribotype 003/FLI01. Nevertheless, the isolate was assigned to the ribotype 003-070 group, since it showed a consistent hybridization pattern with two other 003-070 group strains (*C. difficile* 13S0325.DP.A, aMS = 0.16, and *C. difficile* 13S0748.DP.A, aMS = 0.31). Seq-PCR ribotypes that were not present in the array database (9 strains, from 5 new ribotypes and ribotype 009) yielded aMS scores between 1.2 and 5.7, correctly indicating new ribotypes (Table 3).

DISCUSSION

The classification of *C. difficile* strains in PCR ribotypes is important for epidemiological investigations. This study describes a newly developed DNA microarray-based assay for PCR ribotyping of *C. difficile*.

The probe design of the array was carried out using previously published sequence data on the intergenic spacer regions (ISR) of *C. difficile* genomes (21). Accordingly, the microarray-based assay was constructed on the same molecular target sequences as the PCR ribotyping to provide the best conditions for consistent typing results.

PCR ribotyping detects the lengths of ISR sequences, which in turn are determined by ISR module types and their arrangements (21). In order to consider these two parameters, the microarray operates by recognizing ISR sequence module variants and detecting the synteny of those module variants. The ISR as a target for probe design avoids the need for additional probes in case of newly occurring ribotypes, since any *C. difficile* strain will display a hybridization pattern. New ribotypes can be implemented simply into the assay by depositing a reference hybridization pattern in the database. In contrast to previously published arrays that create genotype-unique yes/no signals, the array generates hybridization patterns of various signal intensities (23–25). Each hybridization signal of a probe contains a measurement error that affects the analysis. The conventional PatternMatch algorithm implies the summation of all differences in the signal intensities of two hybridization profiles (Alere Technologies, Jena, Germany). Thereby, the absolute value of the difference between corresponding probes has a direct proportional effect on the summed MatchScore. As a consequence, the sum of measurement errors due to multiple signal intensities results in high MatchScores that may hide significant deviations in single probes. To avoid this limitation, the MatchScore algorithm was replaced by a quartic equation (adapted MatchScore) in order to set a disproportionally large mathematical weight on probes that show strong deviations be-

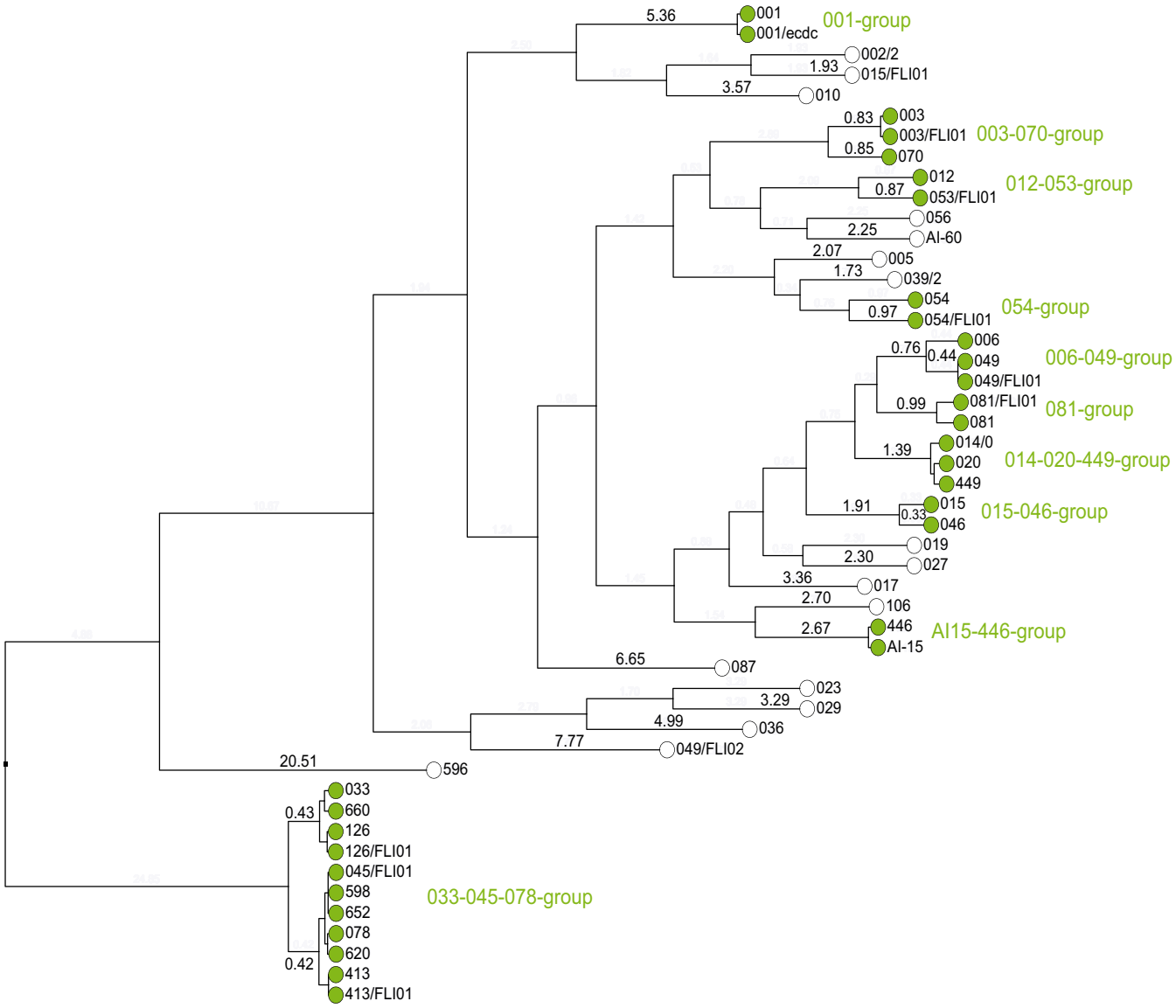

FIG 2 Comparison of seq-PCR ribotypes using the developed DNA microarray. The UPGMA tree was constructed based on a similarity matrix analysis of the seq-PCR ribotype reference profiles. Per seq-PCR ribotype, ≥6 hybridization patterns were generated experimentally (except ribotype 049, with 5 hybridization patterns). On that basis, the reference profiles were determined by calculating the average of the minimum and maximum hybridization intensities for each probe of the DNA microarray. The reference profiles were compared on the basis of the adapted match score algorithm (aMS) in order to create a similarity (square) matrix with zero diagonal. A logarithmic scaling was used for branch length scaling. The adapted match scores were displayed on the branches, whereby only values of >0.3 were considered. Adapted match scores of >1 indicate ribotype hybridization patterns that were discriminated by the microarray. Groups of seq-PCR ribotypes with closely related hybridization patterns (aMS of <1) are highlighted in green. The analysis was performed with BioNumerics software version 7.1.

tween the corresponding signal intensities. Doubling the signal difference of two corresponding probes will thus result in a 16-times-higher summand. Consequently, signal differences in the range of 0 to 0.15 are practically negligible, and signal differences of >0.57 in one probe only result in an aMS of >1. An aMS of >1 was set as the cutoff that indicates different array type patterns. The value was defined due to the maximum aMS obtained in the experiments concerning the reproducibility of array patterns of identical ribotypes (all aMS were <0.53).

For a comparison of the predicted and experimental hybridization results, 4 fully sequenced *C. difficile* strains were analyzed that showed a poor correlation. This finding might be explained by the redundant occurrence of the ISR and its module variants in the genome of *C. difficile*. Most probes have multiple binding sites in the genome that differ in their number of probe mismatches. For example, probe 33bp_v10 showed ISR binding sites in the fully sequenced genome of strain 630 that displayed 0 to 3 mismatches (see Data Set S2 in the supplemental material). However, for a calculation of the theoretical signal intensities, only the best matching results (in the example, 0 mismatches) were considered. It could be assumed that in the hybridization experiment, sequence variants of different probe binding affinities compete for

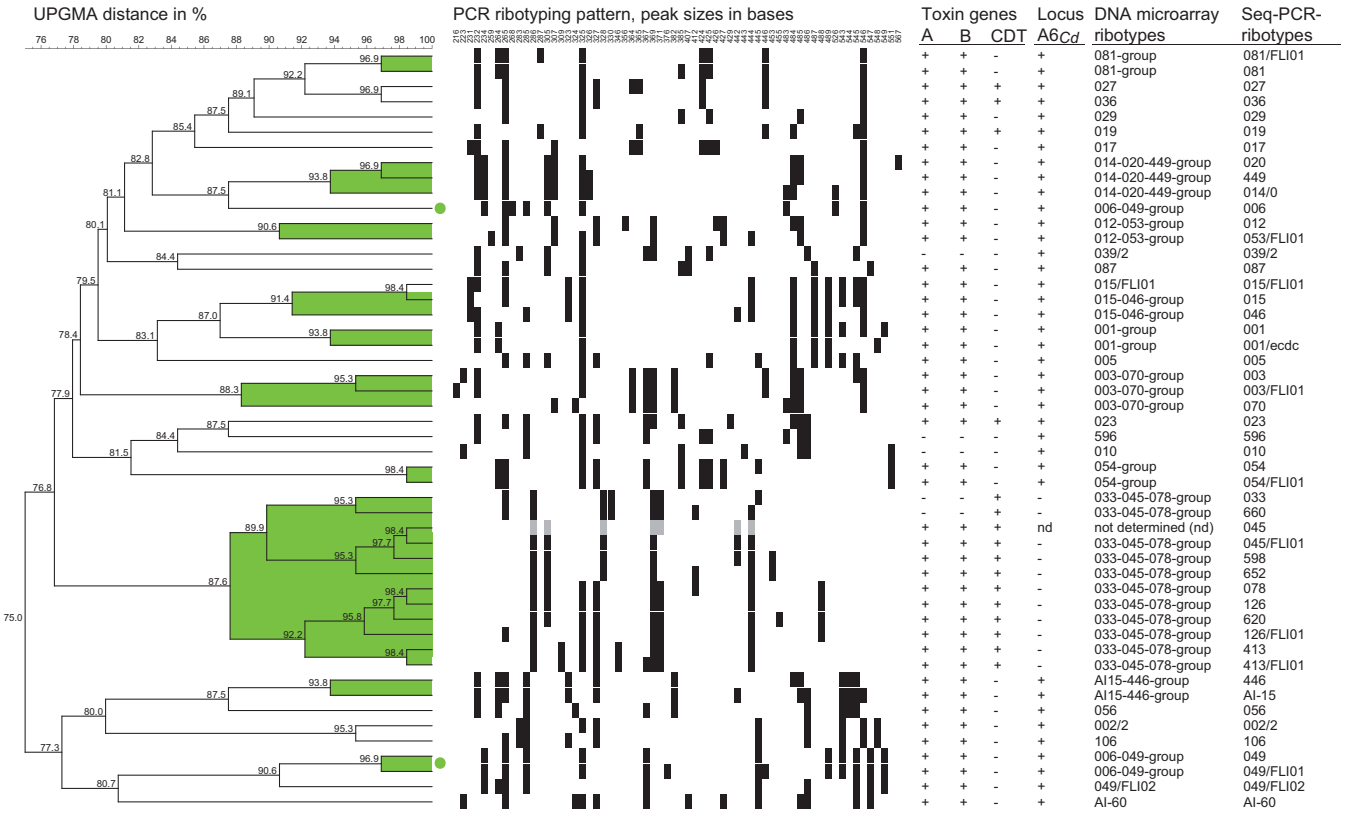

FIG 3 Seq-PCR ribotyping, DNA microarray PCR ribotyping, and molecular characterization of *C. difficile* isolated from human and animal sources. The branches highlighted in green show clusters of seq-PCR ribotypes that were assigned to microarray ribotype groups using the microarray-based assay we developed. The branches of the microarray ribotype 006-049 group are labeled with green circles. UPGMA distance analysis is based on the PCR ribotyping peak profile obtained by capillary gel electrophoresis; the fluorescence intensities of the peaks were not considered. In the case of incomplete matches, PCR ribotypes were designated with the best matching PCR ribotype and the suffix/FLI. PCR ribotype 045 was used for comparison only; the pattern is therefore displayed in gray. Multiple-locus VNTR analysis locus A6*Cd* is absent only in strains of the microarray ribotype 033-045-078 group. nd, not determined.

free binding sites on the array, which may cause deviations from the theoretically best possible hybridization signals.

It was also observed that the results of seq-PCR ribotyping can differ from the predicted ribotyping results that were compiled from the genome sequences. For example, *C. difficile* strain CD196 contains an ISR with a size of 361 bases, which is not detected by seq-PCR ribotyping. Furthermore, the full genomes of *C. difficile* strains CD196, R20291, 2007855, and BI1, which belong to ribotype 027, differ from each other in at least one ISR length, indicating partially incorrect DNA sequencing results or insufficient PCR ribotype assignment. It is therefore difficult to compare the predicted and experimentally obtained results. Furthermore, Alere Technologies often observed (our unpublished data) that a distinct region of a PCR product did not hybridize on a microarray, while the neighboring region in the same amplicon yielded good hybridization results. This effect is possibly caused by the structural chemistry of the oligonucleotides. Braun et al. (24) explained the observed discrepancies between predicted and experimentally obtained hybridizations with amplicon-related secondary structures, which decrease the affinity to the probes.

The current version of the DNA microarray permits the discrimination of the most prevalent human-pathogenic ribotypes (014/020, 001, 078/128, and 027) found in European hospitals (26). However, seq-PCR ribotypes displaying closely related typing profiles were often not distinguished by the array, particularly those of the 033-045-078 and 014-020-449 groups, respectively (Fig. 3). The seq-PCR ribotypes of the 033-45-078 group have been shown to be highly related. Knetsch et al. (27) found that the PCR ribotypes 078, 126, 033, and 045 are highly related in MLST, as they displayed the same sequence type, ST-11, and contained a deletion (Δ39 bp) in the *tcdC* gene. Recently published data on the sequence similarities of conserved genes (e.g., *gyrA*, *tcdB*, and RNA polymerases) in *C. difficile* PCR ribotypes showed that strains of ribotypes 033, 078, and 126 were very similar, forming a distinct ribotype group or cluster (19, 20, 28). Accordingly, the results of the array typing confirm the relatedness of ribotypes assigned to the 033-045-078 group. Ribotypes 014 and 020 or 078 and 126 are difficult to discriminate in conventional PCR ribotyping and show the equivalent typing performance of the array (27, 29). However, these ribotypes can be clearly distinguished by seq-PCR ribotyping, which is therefore better suited for epidemiological investigations. Ribotype 176, which is closely related to 027 and is currently emerging in eastern Europe, was not available for this study (30). It cannot be estimated whether these two types can be differentiated by the array. The seq-PCR ribotype 006 differed significantly in its ribotyping profile from that of ribotype 049 and 049/FLI01 (Fig. 2 and 3) but showed a similar hybridization pattern. It is evident that the discriminative ISR for these seq-PCR ribotypes are not covered by the current assay layout. With this exception, the array-based PCR ribotyping showed a good corre-

TABLE 3 Field study with a blind panel of 50 isolates comparing the *C. difficile* ribotyping array and seq-PCR ribotyping

Proband ID	Strain	Source	Ribotype from seq-PCR ribotyping	Results of DNA microarray-based ribotyping			
				Best matched ribotype	aMS	Alternative ribotypes aMS <1[a]	Assigned ribotype/ ribotype group
0248T1	12S0490.DP.A	Canine	014/0	014/0	0.17	020, 449	014-020-449 group
0231T1	12S0599.EC.A	Canine	014/0	014/0	0.09	020, 449	014-020-449 group
0269T1	12S0600.EC.A	Canine	010	010	0.31	No	010
0382T1	12S0735.EC.A	Canine	027	027	0.13	No	027
0382T1	12S0735.EC.B	Canine	027	027	0.26	036	027
0199M1	13S0046.DP.A	Human	009/FLI01	029	3.27	No	New ribotype
0456M1	13S0127.EC.A	Human	014/0	020	0.07	014/0, 449	014-020-449 group
0435M1	13S0131.EC.A	Human	078	620	0.06	033, 045/FLI01, 078, 126, 126/FLI01, 413, 413/FLI01, 598, 652, 660	033-045-078 group
0652T1	13S0239.EC.A	Canine	014/0	014/0	0.01	020, 449	014-020-449 group
0673T1	13S0240.EC.A	Feline	010	010	0.74	No	010
0672T1	13S0272.EC.A	Canine	078	Hybrid; failed			
0748M2	13S0325.DP.A	Human	003	003/FLI01	0.43	003	003-070 group
0748M2	13S0325.EC.A	Human	078	126	0.07	033, 045/FLI01, 078, 126/FLI01, 413, 413/FLI01, 598, 620, 652, 660	033-045-078 group
0730T1	13S0326.DP.A	Canine	009	029	1.68	13S0378.DP.A	New ribotype
0203M1	13S0330.EC.A	Human	078	126	0.06	033, 045/FLI01, 078, 126/FLI01, 413, 413/FLI01, 598, 620, 652, 660	033-045-078 group
0212M1	13S0045.EC.A	Human	010	010	0.36	No	010
0416M1	13S0375.DP.A	Human	070	070	0.47	No	003-070 group
0382T1	13S0376.DP.A	Canine	001/5/FLI01	446	1.6	13S0594.EC.A, 13S0596.EC.A	New ribotype
0773T2	13S0378.DP.A	Canine	009	029	1.67	13S0326.DP.A	New ribotype
0783T4	13S0490.EC.A	Feline	449	014/0	0.03	020, 449	014-020-449 group
0762T1	13S0491.DP.A	Canine	014/0	014/0	0.03	020, 449	014-020-449 group
0798M1	13S0492.DP.A	Human	003/FLI02	017	1.21	13S0325.DP.A	New ribotype
0721M2	13S0493.DP.A	Human	020	020	0.03	014/0, 449	014-020-449 group
0674M1	13S0494.EC.A	Human	010/FLI01	049/FLI02	5.7	No	New ribotype
0810M1	13S0525.EC.A	Human	087	087	0.01	No	087
0831T1	13S0566.DP.A	Canine	014/0	449	0.16	014/0, 020	014-020-449 group
0838T1E	13S0569.DP.A	Canine	010	010	0.02	No	010
0715M1	13S0570.DP.A	Human	014/0	020	0.02	014/0, 449	014-020-449 group
0715M1	13S0570.EC.A	Human	014/0	020	0.03	014/0, 449	014-020-449 group
0838T1	13S0571.DP.A	Canine	010	010	0.04	No	010
0838T1	13S0571.EC.A	Canine	010	010	0.05	No	010
0837T3	13S0592.DP.A	Canine	014/0	020	0.04	014/0, 449	014-020-449 group
0718M1	13S0593.DP.A	Human	014/0	014/0	0.14	020, 449	014-020-449 group
0829T5	13S0594.EC.A	Feline	001/5/FLI01	446	1.94	13S0376.DP.A, 13S0596.EC.A	New ribotype
0765T2	13S0596.EC.A	Feline	001/5/FLI01	AI-15	1.7	13S0376.DP.A, 13S0594.EC.A	New ribotype
0824T1	13S0608.DP.A	Canine	039/2	039/2	0.27	No	039/2
0858M1	13S0612.EC.A	Human	441/FLI01	033	6.7	No	New ribotype
0770T2	13S0613.EC.A	Feline	014/0	020	0.04	014/0, 449	014-020-449 group
0770T4	13S0614.EC.A	Feline	014/0	020	0.16	014/0, 449	014-020-449 group
0818M2	13S0639.EC.A	Human	014/0	020	0.03	014/0, 449	014-020-449 group
0824T1	13S0641.EC.A	Canine	039/2	039/2	0.17	No	039/2
0895T1	13S0675.EC.A	Canine	010	010	0.17	No	010
0934T1	13S0747.DP.A	Canine	039/2	039/2	0.23	No	039/2
0934T1	13S0747.EC.A	Canine	010	010	0.03	No	010
0926M3	13S0748.DP.A	Human	003	003	0.09	003/FLI01	003-070 group
0926M3	13S0748.EC.A	Human	003	003/FLI01	1.37	13S0325.DP.A, 13S0748.DP.A	003-070 group[b]
0920T1	13S0779.EC.A	Human	039/2	039/2	0.05	No	039/2
0919T2	13S0782.EC.A	Feline	014/0	020	0.02	014/0, 449	014-020-449 group
0919T3	13S0783.EC.A	Feline	014/0	020	0.05	014/0, 449	014-020-449 group
0947M2	13S0784.DP.A	Human	014/5	449	0.11	006, 014/0, 449	014-020-449 group

[a] Tested strains generated a ribotyping pattern that is shared by multiple ribotypes. In case of putative new ribotypes (aMS > 1), the best matching strains of the field panel showing aMS of <1 were listed.

[b] Assigned to 003-070 group based on consistent hybridization pattern with strains 13S0325.DP.A (aMS = 0.16) and 13S0748.DP.A (aMS = 0.31).

lation with seq-PCR ribotyping, which was also approved in the field study.

Taking into account that the DNA microarray cannot discriminate some closely related seq-PCR ribotypes (in this experiment, strains of the 003, 014-020, and 033-045-078 groups), all *C. difficile* field isolates were typed correctly. The seq-PCR ribotypes that were not part of the microarray database (009, and 5 new types) were correctly recognized as putative new ribotypes. These ribotypes showed unique hybridization patterns, demonstrating the capability of the assay for database expansion. The field study also implies the suitability of an aMS value of 1 as the appropriate limit for the distinction of known and unknown ribotypes, since 97.5% of the strains of known ribotypes displayed an aMS of <0.75, while 89% of the unknown ribotypes showed an aMS of ≥ 1.6.

Future versions of the ribotyping array will have to include probes in order to raise typing capability. In order to avoid a possible interaction of the sense and antisense amplicons, an approach using single-stranded PCR should be applied. Single-stranded DNA prevents competition between the probe and antisense strand and increases the probability of the single-stranded amplicon binding to the probe (24). In this study, biotin-labeled primers were used. Braun et al. (24) assumed that the cross-hybridization of highly concentrated biotin-labeled primers may cause false-positive signals more often. An approach should be conducted using biotin-labeled dUTP for internal labeling, which may prevent false-positive signals due to unused primer binding on empty probes (24). Furthermore, the DNA microarray technology offers the opportunity to simultaneously query virulence and resistance information, which should be considered for future versions of the array. Compared to seq-PCR ribotyping, such an assay would retrieve all clinically relevant information about *C. difficile* isolates from one reaction within a couple of hours. We assume that the typing resolution of array typing will not attain the performance of seq-PCR ribotyping. Therefore, seq-PCR ribotyping will remain the method of choice for epidemiological purposes, while the array typing will be applied mainly to clinical diagnostics. Finally, validation is needed to prove the system suitability for clinical diagnostics and to demonstrate the performance capacity of the array.

This study presents the first DNA microarray-based assay for rapid PCR ribotyping of *C. difficile*. Compared to conventional and seq-PCR ribotyping, the need for special laboratory infrastructure and expertise is low. The most prevalent human-pathogenic ribotypes 001, 014/020, 078/126, and 027 can reliably be discriminated. The typing results are in good concordance with those of common PCR ribotyping. However, seq-PCR ribotypes that are closely related are hard to discriminate using the current layout.

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