JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2007, p. 1024–1028 Vol. 45, No. 3
0095-1137/07/$08.00+0 doi:10.1128/JCM.02023-06

Typing and Subtyping of *Clostridium difficile* Isolates by Using Multiple-Locus Variable-Number Tandem-Repeat Analysis▽

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Received 1 October 2006/Returned for modification 8 November 2006/Accepted 5 December 2006

Using the genomic sequence of *Clostridium difficile* strain 630, we developed multiple-locus variable-number tandem-repeat analysis (MLVA) with automated fragment analysis and multicolored capillary electrophoresis as a typing method for *C. difficile*. All reference strains, representing 31 serogroups, 25 toxinotypes, and 7 known subtypes of PCR ribotype 001, could be discriminated from each other. Application of MLVA to 28 isolates from 7 outbreaks due to the emerging hypervirulent PCR ribotype 027–pulsed-field gel electrophoresis type NAP1 resulted in recognition of 13 clusters. Additionally, 29 toxin A-negative, toxin B-positive isolates belonging to PCR ribotype 017 from eight different countries revealed eight country-specific clusters. MLVA is a highly discriminatory genotyping method and a new tool for subtyping of newly emerging variants of *C. difficile*.

To study the epidemiology of *Clostridium difficile*, a typing method with higher discriminatory power, typeability, and reproducibility than currently available methods is required. Multiple-locus variable-number tandem-repeat analysis (MLVA) is a new candidate technique that has already been applied successfully to a number of bacterial and fungal species (5, 10). Recently, MLVA using automated sequence detection and subsequent manual determination of the number of repeat loci has been developed for *C. difficile* (12). For faster and easier application of the MLVA to *C. difficile*, we developed an MLVA method using smaller short tandem repeats (2 to 9 bp) to facilitate automated fragment analysis with multicolored capillary electrophoresis instead of sequencing. Subsequently, we applied MLVA to seven subtypes of a common PCR ribotype, 001, and two other, emerging PCR ribotypes of *C. difficile*. Since 2004, a new toxin-hyperproducing *C. difficile* strain, characterized as PCR ribotype 027, toxinotype III, pulsed-field gel electrophoresis (PFGE) type NAP1, and restriction endonuclease analysis (REA) group BI, has been recognized in Canada, the United States, the United Kingdom, The Netherlands, Belgium, and France as an important cause of hospital outbreaks (3, 8, 9, 11, 13). Additionally, an increasing number of reports mention severe infections and outbreaks due to toxin A-negative, toxin B-positive isolates (1, 2, 7, 14). These toxin A-negative, toxin B-positive isolates belong to PCR ribotype 017, REA group CF, and toxinotype VIII and were first recognized as the cause of an outbreak in 1999 in Canada (1, 2).

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▽ Published ahead of print on 13 December 2006.

Bacterial strains. Isolates included in the analysis were 57 reference strains, all seven subtypes of PCR ribotype 001, 27 toxin A-negative, toxin B-positive isolates belonging to PCR ribotype 017 from eight different countries, and 29 isolates belonging to PCR ribotype 027 from The Netherlands (Table 1) and the United Kingdom. Of these 29 PCR ribotype 027 strains, 28 strains were outbreak related, from six different hospitals in The Netherlands and one in the United Kingdom, and 1 strain was a sporadic isolate from 2003 (8). The United Kingdom strain was obtained from Jon Brazier (Anaerobe Reference Laboratory, NPHS Microbiology Cardiff, Cardiff, United Kingdom). The outbreak strains of each hospital were randomly selected. DNA was isolated from colonies of *C. difficile* by QiaAmp DNA isolation columns (QIAGEN, Hilden, Germany) according to the manufacturer's recommendations. PCR ribotyping was performed as described previously (4), and the method of Rupnik et al. was used for toxinotyping (15).

MLVA. Seven regions with short tandem repeats spread over the genome, designated markers MLVA *C. difficile* A6 ($A6_{Cd}$), $B7_{Cd}$, $C6_{Cd}$, $E7_{Cd}$, $F3_{Cd}$, $G8_{Cd}$, and $H9_{Cd}$, were identified using Tandem Repeat Finder, version 3.21, on the genome of *C. difficile* strain 630 (http://www.sanger.ac.uk/Projects/C_difficile/) (16). Four of these, MLVA $A6_{Cd}$, $B7_{Cd}$, $E7_{Cd}$, and $G8_{Cd}$, were identical to CDR4, CDR49, CDR48, and CDR9, respectively, in the assay described recently by Marsh et al. (12). Primers were designed based on the flanking sequences of the repeats using the Primer3 program (http://www.broad.mit.edu/cgi-bin/primer/primer3_www.cgi). Three separate duplex PCRs (MLVA $A6_{Cd}$-$H9_{Cd}$, $B7_{Cd}$-$F3_{Cd}$, and $C6_{Cd}$-$E7_{Cd}$) and one singleplex PCR (MLVA $G8_{Cd}$) were developed (Table 2). The repeats were amplified using a single PCR protocol. The amplification reactions were performed in a 50-μl final volume containing 25 μl of HotStar *Taq* master mix (QIAGEN, Hilden, Germany), 1 μM each primer, 3 mM magnesium chloride, and 5 μl of DNA. After an initial enzyme activation step

TABLE 1. Isolates included in this study

Characteristic	No. of isolates (total, 120)	Isolate identification	Outbreak or sporadic	Origin (reference)
Serogroups	31	A to I, K, X, A1 to A11, A13 to A17, S1 to S4	NA[a]	Gift from M. Delmée
Toxinotypes	25	I to XXII	NA	Gift from M. Rupnik
Strain 630	1	630	NA	Gift from P. Mastrantonio
Subtypes of PCR ribotype 001, by REP-PCR[b]	7	001-1 to 001-7	NA	Gift from J. Brazier
Toxin A-negative, toxin B-positive isolates, PCR ribotype 017	12	Arg28, -31, -32, -36 to -38, -77, -126, -127, -134, -143, -152	Outbreak	Argentina; gift from C. Legaria
	2	CD16, CD17	Outbreak	Amsterdam, The Netherlands (18)
	2	Can1, Can3	Outbreak	Canada (18)
	1	123825R	Endemic	Leiden, The Netherlands
	2	1110/98, 1745/00	Endemic	Poland (18)
	2	60, 99-3050	Endemic	France (18)
	2	CF2, CF4	Endemic	United States (18)
	2	R10205, R10430	Endemic	United Kingdom (18)
	2	GAI95601, GAI95602	Endemic	Japan (18)
Isolates belonging to PCR ribotype 027	5	AF1 to -5	Outbreak	Amersfoort, The Netherlands (8)
	7	AMC1 to -7	Outbreak	Amsterdam-1, The Netherlands (8)
	1	VUMC	Endemic	Amsterdam-2, The Netherlands (8)
	3	SV1 to -3	Outbreak	Amsterdam-3, The Netherlands (8)
	4	HL1 to -4	Outbreak	Haarlem, The Netherlands (8)
	5	HW1 to -5	Outbreak	Harderwijk, The Netherlands (8)
	3	UMC1 to -3	Outbreak	Utrecht, The Netherlands (8)
	1	UK027	Outbreak	United Kingdom (gift from J. Brazier)

[a] NA, not applicable.
[b] REP, repetitive extragenic palindromic.

of 15 min at 95°C, the protocol consisted of 35 cycles of 30 s at 94°C for denaturation, 30 s at 51°C for annealing, and 30 s at 72°C for elongation. A final elongation step was performed for 10 min at 72°C. The forward primer of each PCR was labeled at the 5′ end with either carboxyfluorescein (FAM), hexachlorofluorescein (HEX), 2′-chloro-7′-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC), or 2′-chloro-5′-fluoro-7′,8′-fused phenyl-1,4-dichloro-6-carboxyfluorescein (NED). PCR fragments were analyzed using multicolored capillary electrophoresis on an ABI3100 genetic analyzer, with a ROX500 marker as an internal marker for each sample. The size of each marker was determined by Genescan software (Applied Biosystems). Markers from a selected number of isolates were sequenced to verify accurate assignment of repeat numbers. All sequence results were equal to the results of fragment analysis by the ABI system and to the calculated repeat numbers. The repeat numbers were analyzed using BioNumerics (version 3.5) software (Applied Maths, Kortrijk, Belgium) and the unweighted-pair group method with arithmetic averages (UPGMA) with the multistate categorical similarity coefficient (MCSC). All markers were given equal weight, irrespective of the number of repeats. The percentages in the dendrogram reflect the percentage of homology between the specific markers. Thus, if two strains have an equal number of repeats in six of seven markers, they are 86% identical.

C. difficile control strain 630 revealed identical results in five different experiments using both separate cultures and DNA extractions. The stability of the repeat numbers of the different markers was tested in duplicate after isolates belonging to PCR ribotypes 014 and 027 were subcultured a total of 10 and 30 times. The repeats from the isolate belonging to type 014 were stable in all experiments. An expansion of 1 repeat unit in marker $A6_{Cd}$ was observed in one duplicate sample of the type 027 isolate after 10 subcultures, which subsequently returned to the original number of repeats after 30 subcultures. For marker $C6_{Cd}$, a reduction of 1 repeat unit could be detected after 30 subcultures for this isolate. Based on the stability tests, we concluded that a difference of 1 repeat unit between strains should not be interpreted as indicative of separate types or subtypes. This conclusion is in complete concordance with the

TABLE 2. Characteristics of markers and primers

Marker	Repeat motif	Location[a]	Forward primer sequence (label–5′→3′)	Reverse primer sequence (5′→3′)
$A6_{Cd}$	AAGAGC	755721	FAM-TTAATTGAGGGAGAATGTTAAA	AAATACTTTTCCCACTTTCATAA
$B7_{Cd}$	ATCTTCT	3688632	FAM-CTTAATACTAAACTAACTCTAACCAGTAA	TTATATTTTATGGGCATGTTAAA
$C6_{Cd}$	TATTGC	3239736	HEX-GTTTAGAATCTACAGCATTATTTGA	ATTGGAATTGAATGTAACAAAA
$E7_{Cd}$	ATAGATT	167124	FAM-TGGAGCTATGGAAATTGATAA	CAAATACATCTTGCATTAATTCTT
$F3_{Cd}$	TTA	1954915	HEX-TTTTTGAAACTGAACCAACATA	ACAAAAGACTGTGCAAATATACTAA
$G8_{Cd}$	TAAAAGAG	664660	NED-TGTATGAAGCAAGCTTTTTATT	AATCCAGCAATCTAATAATCCA
$H9_{Cd}$	TCTTCTTCC	4116072	VIC-GTTTTGAGGAAACAAACCTATC	GATGAGGAAATAGAAGAGTTCAA

[a] Location on the genomic sequence of strain 630 (36).

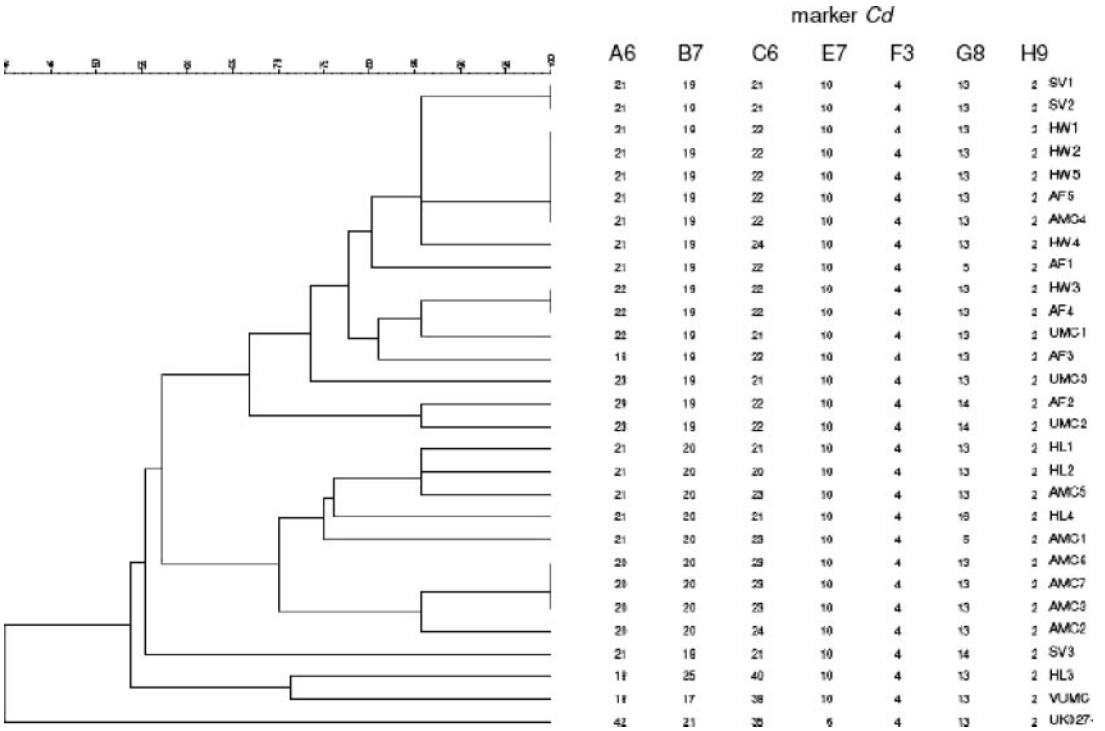

FIG. 1. Dendrogram based on profiles of seven markers for all PCR ribotype 027 isolates ($n = 29$) tested in this study. Numbers of repeats for the specified markers in each strain are given on the right.

study of the stability of *C. difficile* MLVA loci by Marsh et al. (12). They found three pairs of serial isolates from individual patients with a single-locus variation of only 1 tandem repeat and one pair of isolates with a double-locus variation of 1 tandem repeat each. Therefore, they concluded that isolates with a summed tandem repeat difference of ≤ 2 are genetically related. MLVA discriminated between isolates belonging to all 31 serogroups, the 7 subtypes of PCR ribotype 001, and all 25 toxinotypes, except for toxinotypes XII, XIII, and XIV. An isolate belonging to serogroup A15 was completely identical (100%) to toxinotype V, as has been observed previously (15). Toxinotypes XII, XIII, and XIV were clustered into one MLVA type with 100% similarity, indicating that toxinotyping is a method that merely reflects the status of the toxin genes (15). With one marker difference, toxinotype XIb was comparable to the 100% cluster of toxinotypes XII to XIV. The similarity of isolate 630 to serogroup C (the closest match) was only 43% (3 of the 7 markers), although markers $B7_{Cd}$ and $E7_{Cd}$ differed by only 1 repeat. PCR ribotype 001 isolates were quite stable in markers $E7_{Cd}$ (5 to 7 repeats), $F3_{Cd}$ (5 repeats), $G8_{Cd}$ (6 to 8 repeats), and $H9_{Cd}$ (2 repeats). By using these characteristics, type 001 isolates can be discriminated from most serogroups and toxinotypes. Consequently, MLVA is able to replace PCR ribotyping and PFGE for identification and recognition of subtypes of PCR ribotype 001. Until recently, strains belonging to PCR ribotype 001 were the most common in the United Kingdom, and the importance of the ability to subtype these strains is high (6, 17). A recent Health Protection Agency report (available at http://www.hpa.org.uk/) indicated that PCR ribotypes 106 and 027 are the most common in the United Kingdom, followed by type 001 (still approximately 25%) (6a).

Among the isolates belonging to PCR ribotype 027 ($n = 29$), 100% similarity (Fig. 1) was detected for isolate AF4 with HW3, for isolate SV1 with SV2, for isolate AF5 with AMC4, HW1, HW2, and HW5, and for isolate AMC3 with AMC6 and AMC7. With 86% similarity, 14 clusters were detected among the 29 isolates. Hospital-specific clusters were seen for SV, HW, HL, and AMC (Fig. 1). The sporadic endemic isolate recovered in 2003 was only 53% similar to the outbreak isolates and 71% similar to isolate HL3. The United Kingdom isolate was only 40% identical to all Dutch outbreak isolates. For all type 027 isolates, markers $E7_{Cd}$, $F3_{Cd}$, and $H9_{Cd}$ were completely identical, except for the United Kingdom isolate, which had 6 repeats for marker $E7_{Cd}$ (Fig. 1).

Toxin A-negative, toxin B-positive isolates (including the two reference strains belonging to serogroup F and toxinotype VIII) ($n = 29$), belonging to PCR ribotype 017 and toxinotype VIII, could be divided into eight clusters at a similarity of 86% (6 markers identical) (Table 3, clusters G to N). Six clusters with 100% homology were recognized (Table 3, clusters A to F). All isolates with 100% similarity were country specific (clusters A to F), as were clusters H and I. Toxin A-negative, toxin B-positive isolates could be differentiated from all other types by using the combination of markers $A6_{Cd}$ (2 repeats), $F3_{Cd}$ (5 repeats), $G8_{Cd}$ (fragment size, >400 bp), and $H9_{Cd}$ (2 repeats). For marker $G8_{Cd}$, all PCR ribotype 017 isolates showed the previously described larger fragment size, exceeding the 400 bp detectable by our system. MLVA discriminated toxin A-negative, toxin B-positive isolates better than amplified fragment length polymorphism (18).

Application of MLVA to *C. difficile* isolates was easy to

TABLE 3. MLVA results and cluster information for toxin A-negative, toxin B-positive isolates belonging to PCR ribotype 017 isolates[a] tested in this study

Isolate	Origin	No. of repeats detected for the following marker:							Cluster(s)[b]
		$A6_{Cd}$	$B7_{Cd}$	$C6_{Cd}$	$E7_{Cd}$	$F3_{Cd}$	$G8_{Cd}$	$H9_{Cd}$	
Arg32	Argentina	2	7	18	7	5	>400	2	G
Arg38	Argentina	2	7	17	7	5	>400	2	G
CF2	United States	2	7	31	7	5	>400	2	G
Can3	Canada	2	9	20	7	5	>400	2	H
1110/98	Poland	2	10	23	7	5	>400	2	I
1745/00	Poland	2	10	15	7	5	>400	2	I
R10205	United Kingdom	2	8	26	8	5	>400	2	A, J
R10430	United Kingdom	2	8	26	8	5	>400	2	A, J
Can1	Canada	2	8	22	8	5	>400	2	J
CF4	United States	2	8	30	8	5	>400	2	J
F	Reference strain	2	13	22	8	5	>400	2	K
CD16	Amsterdam	2	10	30	8	5	>400	2	B, L
CD17	Amsterdam	2	10	30	8	5	>400	2	B, L
123825R	Leiden	2	10	32	8	5	>400	2	L
GAI95601	Japan	2	10	43	8	5	>400	2	L
GAI95602	Japan	2	10	40	8	5	>400	2	L
Arg77	Argentina	2	7	20	8	5	>400	2	C, M
Arg134	Argentina	2	7	20	8	5	>400	2	C, M
Arg152	Argentina	2	7	19	8	5	>400	2	D, M
Arg37	Argentina	2	7	19	8	5	>400	2	D, M
Arg127	Argentina	2	7	19	8	5	>400	2	D, M
Arg31	Argentina	2	7	18	8	5	>400	2	E, M
Arg126	Argentina	2	7	18	8	5	>400	2	E, M
Arg28	Argentina	2	7	21	8	5	>400	2	F, M
Arg36	Argentina	2	7	21	8	5	>400	2	F, M
Arg143	Argentina	2	7	22	8	5	>400	2	M
60 (fr)	France	2	7	28	8	5	>400	2	M
99-3050	France	2	7	24	8	5	>400	2	M
VIII	Reference strain	2	9	21	8	5	>400	2	N

[a] $n = 29$.
[b] Clusters at 100% similarity: A to F. Clusters at 86% similarity: G to N.

perform and consisted of four separate PCR mixes and a single PCR protocol. Although MLVA has yet to show its value in longer-term epidemiology or phylogeny studies, MLVA can be widely applied in outbreak situations. Therefore, MLVA is an important new tool for study of the epidemiology of toxin A-negative, toxin B-positive PCR ribotype 017 isolates and PCR ribotype 027–PFGE NAP1–REA BI isolates, which are newly emerging worldwide. MLVA is a highly discriminatory genotyping method for *C. difficile* and is able to discriminate between isolates with identical PCR ribotypes belonging to types 001, 017, and 027. MLVA also clearly differentiated these PCR ribotypes from other PCR ribotypes included in this study. Future studies should be performed on all currently available PCR ribotypes to explore this in more detail.

This work was supported by a grant from the Foundation Microbiology Leiden, The Netherlands.

We thank, in alphabetical order, Michelle Alfa (University of Manitoba, Winnipeg, Canada), Frederic Barbut (Centre Hospitalo-Universitaire Saint-Antoine, Paris, France), Jon Brazier (University Hospital of Wales, United Kingdom), Stu Johnson (Northwestern University, Chicago, IL), Haru Kato (Gifu University School of Medicine, Gifu, Japan), Cristina Legaria (Hospital Tornú, Buenos Aires, Argentina), Paola Mastrantonio (Instituto Superiore di Sanita, Rome, Italy), Hanna Pituch (Medical University of Warsaw, Warsaw, Poland), and Maja Rupnik (University of Maribor, Maribor, Slovenia) for kindly providing the isolates used in this study.

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