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Modified Multiple-Locus Variable-Number Tandem-Repeat Analysis for Rapid Identification and Typing of *Clostridium difficile* during Institutional Outbreaks[▽]

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A modified multiple-locus variable-number tandem-repeat analysis (MMLVA) method was validated on *Clostridium difficile*-infected stool specimens from institutional outbreaks. The method allows simultaneous detection of toxin genes, deletions, and tandem repeats from cultured isolates or stool specimens. Results were used to aid institutional outbreak investigation by identifying clusters of NAP1/027.

Clostridium difficile infection (CDI) is a major cause of gastrointestinal disease in patients treated with antibiotics in hospitals (10, 15). Effective control of CDI outbreaks requires identification of true clusters of infected patients. The typing method must have sufficient discriminatory power to differentiate outbreak isolates from sporadic isolates, especially because the epidemic strain North American Pulsotype (NAP) 1/ribotype 027 is extremely common in North America and responsible for the vast majority of institutional outbreaks (13, 16). A previous comparison of genotyping methods revealed that multiple-locus variable-number tandem-repeat analysis (MLVA) was the most discriminatory and had the potential to identify true clusters of CDI (6). However, MLVA is technically challenging, requires considerable expertise beyond the medical laboratory technologist level, and relies on prior culture of the organism, resulting in significant delays in reporting during institutional outbreak investigation (5, 7, 12, 19). In our initial attempts to validate MLVA, we noted that there was no variability observed in the $F3_{Cd}$ and $H9_{Cd}$ loci for CDI outbreak isolates in our region, and, thus, these two loci added no

TABLE 1. Primers used in modified multiple-locus variable-number tandem-repeat analysis (MMLVA)[a]

Marker (PM)	Primer	Primer sequence (5′ to 3′)	Reference
$A6_{Cd}$ (1)	A6-F	FAM-TTAATTGAGGGAGAATGTTAAA	19
	A6-R	AAATACTTTTCCCACTTTCATAA	19
$B7_{Cd}$ (1)	B7-F	VIC-TTAATACTAAACTAACTCTAACCAGTAA	Modified from reference 19
	B7-NAP7/8-F	VIC-TTAATA**T**TAAACTAACTCTAACCAGTAA	Modified from reference 19
	B7-universal-R	TTATATTTTATGGG**Y**ATGTTAAA	Modified from reference 19
$C6_{Cd}$ (2)	C6-630, 91, and 96-F	VIC-GTTTAGAATCTACA**R**CATTATTTGA	Modified from reference 19
	C6-NAP7/8-F	**A**TTTAGAATCTA**T**A**C**TATTATTTGA	Modified from reference 19
	C6-R	ATTGGAATTGAATGTAACAAAA	19
	C6-NAP7/8-R	A**GC**GGAATTGAATGTAACAAAA	Modified from reference 19
$E7_{Cd}$ (2)	E7-F	FAM-TGGAGCTATGGAAATTGATAA	19
	E7-R	CAAATACATCTTGCATTAATTCTT	19
	E7-NAP7/8-R	CAAATACATCTTGCA**C**TA**G**TTCTT	Modified from reference 19
$G8_{Cd}$ (1)	G8-F	NED-TGTATGAAGCAAGCTTTTTATT	19
	G8-R	ACCAAAAATTTCTAACCCAAC	3
	G8-NAP7/8-R	ACCAAAA**T**TTTCTAACCCAAC	Modified from reference 3
tpi (3)	tpi-F	FAM-AAGAAGCTACTAAGGGTACAAA	9
	tpi-R	CATAATATTGGGTCTATTCCTAC	9
tcdA (3)	*tcdA*-F	FAM-AGATTCCTATATTTACATGACAATAT	9
	tcdA-R	GTATCAGGCATAAAGTAATATACTTT	9
tcdB (3)	*tcdB*-F	FAM-GGAAAAGAGAATGGTTTTATTAA	9
	tcdB-R	ATCTTTAGTTATAACTTTGACATCTTT	9
cdtB (3)	*cdtB*-F	FAM-CTTAATGCAAGTAAATACTGAG	18
	cdtB-R	AACGGATCTCTTGCTTCAGTC	18
tcdC deletion (2)	*tcdC*-F	FAM-AAGCTATTGAAGCTGAAAATC	1
	tcdC-R	GCTAATTGGTCATAAGTAATACC	1

[a] Modifications to published primers are indicated as underlined nucleotides. Three multiplex reactions were used simultaneously under identical cycling conditions. Primer mix (PM) combinations and forward (F) and reverse (R) primers are indicated. Fluorophores are named next to each relevant primer sequence.

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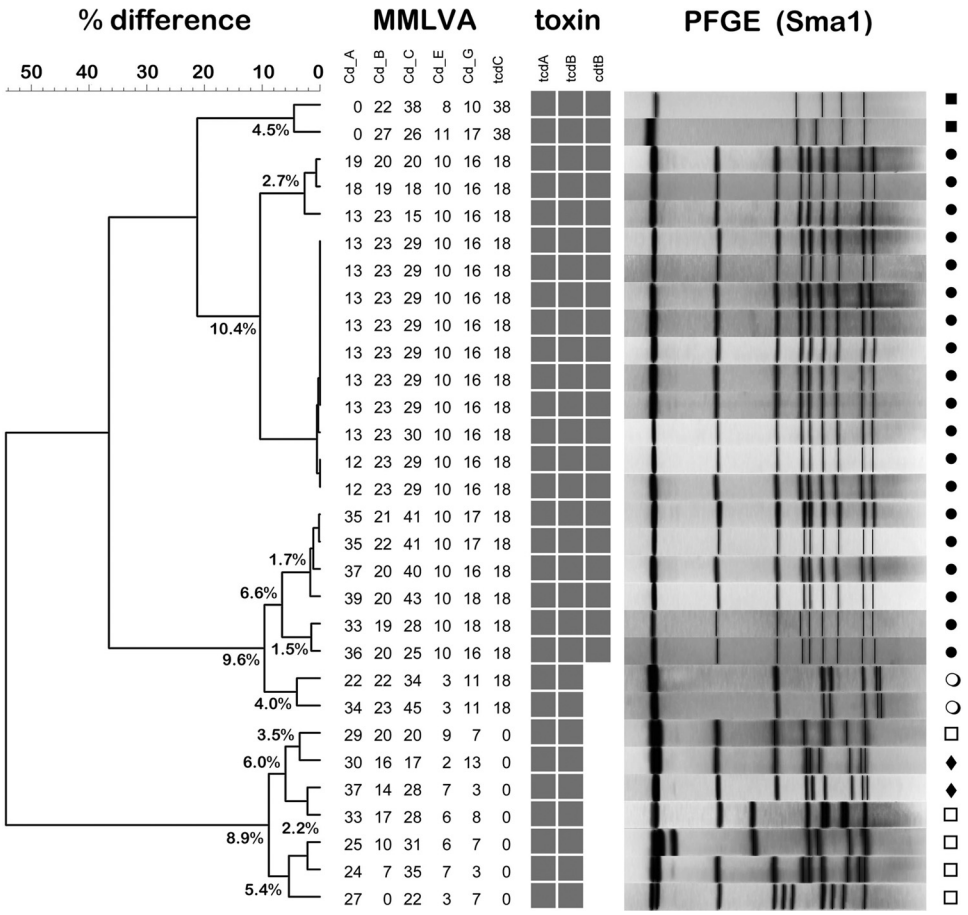

FIG. 1. Dendrogram depicting MMLVA types compared to pulsotypes ($n = 30$) for an institutional outbreak. Clusters were defined as <5% difference based on the Manhattan distance measure. Within the NAP1 pulsotype (closed circles), four clusters are identified. A pair of NAP7 (closed squares) isolates formed a cluster, whereas a pair of NAP11 (closed diamonds) isolates did not form a cluster. Other unclassified pulsotypes (open circles, open squares) are noted. A matrix array format is used to identify the presence or absence of toxin genes (the *tpi* control gene is used as an amplification control and is not shown). Note the unusual pair of CDI cases (open circles) which has the *tcdC* deletion but not the binary toxin. PFGE (SmaI digest) results correlated closely with those of MMLVA.

value in discriminating among the isolates (our unpublished observations). Additionally, MLVA does not indicate whether the isolate is the epidemic strain NAP1/027, which contains the *tcdC* deletion and elaborates binary toxin (*cdtB*) in addition to toxins A (*tcdA*) and B (*tcdB*), all constituents of the pathogenicity locus (PaLoc) of toxigenic strains. Finally, MLVA does not identify NAP7, a hypervirulent emerging strain in North America, due to DNA sequence variation (14). Thus, MLVA still requires complementary pulsed-field gel electrophoresis (PFGE) or ribotyping in order to confirm that it is NAP1/027. In this study, we have developed a modified MLVA (MMLVA) method which enables simultaneous detection of CDI and identification of hypervirulent epidemic strains, as well as providing typing information which enables rapid identification of clusters of outbreak isolates in institutions.

CDI specimens associated with institutional outbreaks (defined as ≥6 cases per ward per month) were received by our laboratory for typing by PFGE between December 2008 and December 2009. A case was defined as the presence of toxin A/B by enzyme immunoassay (EIA) or molecular test in a patient with clinical diarrhea. Outbreak-associated specimens ($n = 155$) were subjected to culture using *C. difficile* moxalactam-norfloxacin (CDMN) agar as described previously (2, 16). For PFGE, extraction of DNA from colonies grown on CDMN agar was performed using a commercial extraction method (InstaGene Matrix, Bio-Rad Laboratories, Hercules, CA). For MMLVA, DNA was extracted from colonies with the InstaGene Matrix (Bio-Rad, Hercules, CA). A single colony was resuspended in 100 μl of InstaGene, heated at 100°C for 10 min, vortexed for 10 s, and centrifuged at 10,000 rpm for 2 min. The supernatant was used for PCR. For MMLVA performed directly from stool, DNA was extracted using the QIAamp stool minikit (Qiagen, Valencia, CA) adapted to the QIAcube platform. In order to amplify five tandem repeat loci (denoted $A6_{Cd}$, $B7_{Cd}$, $C6_{Cd}$, $E7_{Cd}$, and $G8_{Cd}$ according to the method of van den Berg [19]), the *tcdC* deletion, toxin genes (*tcdA*, *tcdB*, *cdtB*), and the housekeeping gene *tpi*, three multiplex PCRs were conducted under identical cycling conditions. *C. difficile* genome sequences from GenBank (630: taxonomy ID [TID], 272563; M120: TID, 699035; NAP07: TID, 525258; NAP08: TID, 525259; CD196: TID, 645462; R20291: TID, 645463) were used to modify primer sequences to accommo-

date a broader set of strain types (Table 1). Each primer stock solution was prepared at a concentration of 100 μM and mixed with other primers to make the primer mixes (PM) indicated in Table 1. Each PM (1 μl) was added to 7 μl of the Type-it Microsatellite master mix (Qiagen, Valencia, CA) for a total reaction volume of 8 μl. The cycling conditions were as follows: hot start at 90°C for 5 min, then 36 cycles of 30 s at 90°C, 60 s at 50°C, and 30 s at 72°C, with a final extension step of 30 min at 60°C. Amplicons were diluted 1:20 with distilled water, and 1 μl was transferred to 11 μl of formamide mixed with LIZ500 DNA ladder (0.1 μl per well), heated for 3 min at 95°C, and snap-cooled to 4°C. The Applied Biosystems (Carlsbad, CA) 3130xl genetic analyzer was used to separate the fragments, and GeneScan software was used to identify their fragment length. BioNumerics (Applied Maths, Austin, TX) software was used to perform cluster analysis and generate dendrograms of MMLVA types compared to results of PFGE. Cluster analysis was performed using the Manhattan distance measure (numerical coefficient which sums differences in repeat units and deletions) and the Ward clustering algorithm to generate the dendrogram (6). To facilitate the interpretation, a cluster was defined as having <5% difference based on summed tandem repeat differences (STRD) at all five loci.

MMLVA types were compared with traditional PFGE for all outbreak-associated CDI. A representative dendrogram from a group of hospital outbreak specimens ($n = 30$) is depicted in Fig. 1. Confirmation of the presence of toxin genes is demonstrated using a matrix array format. By including the 18-bp NAP1 *tcdC* deletion in the MMLVA analysis, the NAP1/027 strain responsible for all outbreaks in our region is immediately separated from non-NAP1/027 strains. This includes NAP1/027 variants with single-nucleotide polymorphisms (SNPs) upstream of the deletion (11). Good correlation between MMLVA and PFGE was observed, with MMLVA being able to further segregate four clusters within 19 NAP1/027 isolates and two NAP7 isolates forming a cluster, as well as four other non-NAP1 pairs. A pair of NAP11 isolates was also noted but did not form a cluster by MMLVA, highlighting the discriminatory power of MMLVA for non-NAP1 isolates as well. The absence of tandem repeats at locus $A6_{Cd}$ and the 38-bp deletion at *tcdC* is typical of the NAP7 strain. A numerical measure (Manhattan) rather than categorical coefficient was used in our cluster analysis because categorical metrics tend to inflate variability when subtle changes occur at multiple loci. A more liberal cutoff value of <5% difference by Manhattan coefficient was used because the previously reported cutoff value of 2 STRD to define a "clone" is likely too stringent in the context of an outbreak (4). For example, we and others have observed that a single stool specimen with a polyclonal infection can contain greater variation than 2 STRD (17). As shown in this representative outbreak, variability in tandem repeats can be observed for clusters at loci $A6_{Cd}$, $B7_{Cd}$, and $C6_{Cd}$, but little or no variability is seen at loci $E7_{Cd}$ and $G8_{Cd}$ within outbreak clusters. The fact that not all loci demonstrate equal variability hampers facile interpretation of true clusters and reinforces the fact that sound epidemiological investigation is required to confirm or refute the laboratory typing. Similar results were obtained whether using cultured isolates or those directly from stool, with the exception that certain stool specimens may produce more than one MMLVA

TABLE 2. Summary of the performance characteristics and costs of commercially available molecular detection and typing methods for *C. difficile* compared to those of MMLVA

Assay	Manufacturer	Function	Test method	Batch size	Instrumentation for:		Interpretation	Concern(s)	Advantage(s)	Avg time	Cost[a]
					DNA extraction	Detection					
BD GeneOhm *C. difficile*	Becton Dickinson	Detects *C. difficile* toxin B gene	PCR	8	Heat lysis	SmartCycler	Cepheid SmartCycler software	Cost, throughput	Good specificity	2-3 h	$25-$30
ProGastro CD	GenProbe	Detects *C. difficile* toxin B gene	PCR	8	Automated extraction MagNa Pure	SmartCycler	Cepheid SmartCycler software	Cost, throughput	Good specificity	2-3 h	$25-$30
Illumigene *C. difficile*	Meridian Bioscience	Detects PaLoc gene segment; codes for both toxin A and B genes	Loop-mediated amplification technology (LAMP)	10	Illumigene sample prepn apparatus, heat lysis	Illumipro-10	Illumipro-10 ratio of initial to final signal (turbidity)	Newer methodology	Minimal equipment required; rapid	40 min	$25-$30
Xpert *C. difficile*	Cepheid	Detects *C. difficile* toxin B gene	PCR	10 cartridges, 120 cartridges	GeneXpert System	GeneXpert System	Cepheid SmartCycler software	Cost, throughput	Good specificity	2 h	$35-$40
MMLVA	This study—in-house test	Detects *C. difficile*, toxin A, toxin B, binary toxin, *tcdC* gene plus VNTRs[b]	PCR sequencing and fragment analysis	1 to 48	QIAcube (Qiagen)/ InstaGene Matrix	3130xl genetic analyzer (Life Technologies)	GeneMapper software	High setup cost, technical expertise	Highly discriminatory detection and typing	5 h	$5-$10
GenoType *CDiff*	Hain Lifescience	Detects *C. difficile*, toxin A, toxin B, binary toxin, *tcdC* gene, resistance to moxifloxacin	DNA strip technology	12	GenoType *CDiff*	GenoType *CDiff*	Visual assessment	Typing lacks discriminatory power	Detection and typing performed simultaneously	5 h (stool direct)	$30-$35

[a] Costs are in Canadian dollars and meant only to be approximate for reagents alone.
[b] VNTRs, variable-number tandem repeats.

type, but this does not interfere with the cluster analysis when using a <5% cutoff value (our unpublished observations). Ten stool specimens can be run concurrently when using a liquid handler for DNA extraction and capillary electrophoresis to separate fragments. CDI identification and typing results are obtained within 5 h and reported back to the institution (Table 2).

Due to an ongoing epidemic of NAP1/027 resulting in CDI institutional outbreaks in our region, we have been conducting PFGE to support outbreak investigation and infection control (16). However, PFGE lacks discriminatory power to distinguish outbreak clusters from sporadic NAP1/027 and requires laborious culture (8). More discriminatory methods such as MLVA are highly specialized, result in amplification of uninformative loci (from the point of view of outbreak investigation), and do not identify the critical NAP1/027 strains *per se*. MMLVA couples the discriminatory power of tandem-repeat loci with the capacity to identify toxin genes and deletions as well as determine whether an isolate is related to the NAP1/027 epidemic strain in a single run. Identification of MMLVA clusters early in the course of an outbreak enables infection prevention and control workers to pinpoint possible breakdowns in strict barrier precautions, reinforcement of hand hygiene practices with soap and water, enhanced environmental cleaning, and equipment disinfection on the affected units that may have led to the increase in case load. The method is not restricted to NAP1 clusters, as the tandem-repeat units will also identify clusters of other pulsotypes. Importantly, the method works from cultured isolates or from stool, eliminating the need for culture, which can result in delays for infection prevention and control measures. We believe this method provides rapid identification of current outbreak clusters to aid investigation which may curb the spread of the NAP1/027 epidemic strain as well as other emerging strains.

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