

Comparison of Seven Techniques for Typing International Epidemic Strains of *Clostridium difficile*: Restriction Endonuclease Analysis, Pulsed-Field Gel Electrophoresis, PCR-Ribotyping, Multilocus Sequence Typing, Multilocus Variable-Number Tandem-Repeat Analysis, Amplified Fragment Length Polymorphism, and Surface Layer Protein A Gene Sequence Typing[∇]

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Using 42 isolates contributed by laboratories in Canada, The Netherlands, the United Kingdom, and the United States, we compared the results of analyses done with seven *Clostridium difficile* typing techniques: multilocus variable-number tandem-repeat analysis (MLVA), amplified fragment length polymorphism (AFLP), surface layer protein A gene sequence typing (*slpAST*), PCR-ribotyping, restriction endonuclease analysis (REA), multilocus sequence typing (MLST), and pulsed-field gel electrophoresis (PFGE). We assessed the discriminating ability and typeability of each technique as well as the agreement among techniques in grouping isolates by allele profile A (AP-A) through AP-F, which are defined by toxinotype, the presence of the binary toxin gene, and deletion in the *tcdC* gene. We found that all isolates were typeable by all techniques and that discrimination index scores for the techniques tested ranged from 0.964 to 0.631 in the following order: MLVA, REA, PFGE, *slpAST*, PCR-ribotyping, MLST, and AFLP. All the techniques were able to distinguish the current epidemic strain of *C. difficile* (BI/027/NAP1) from other strains. All of the techniques showed multiple types for AP-A (toxinotype 0, binary toxin negative, and no *tcdC* gene deletion). REA, *slpAST*, MLST, and PCR-ribotyping all included AP-B (toxinotype III, binary toxin positive, and an 18-bp deletion in *tcdC*) in a single group that excluded other APs. PFGE, AFLP, and MLVA grouped two, one, and two different non-AP-B isolates, respectively, with their AP-B isolates. All techniques appear to be capable of detecting outbreak strains, but only REA and MLVA showed sufficient discrimination to distinguish strains from different outbreaks.

Since Bartlett et al. (2) implicated *Clostridium difficile* as an agent of antimicrobial-associated colitis nearly 30 years ago, *C. difficile* strain typing methods have been explored as aids in epidemiologic investigations. Early typing techniques reflected the technology of the time and were based on phenotypic characteristics such as antibiotic resistance patterns, soluble protein patterns (11, 34), bacteriophage and bacteriocin patterns (29), slide agglutination schemes (8), and Western immunoblotting (13). In the 1980s, typing methods began to evolve from phenotype-based methods toward genotype-based methods such as restriction endonuclease analysis (REA) of

the total bacterial genome (13, 17), pulsed-field gel electrophoresis (PFGE), and arbitrarily primed PCR (AP-PCR) (9, 15, 26). Brazier et al. compared the results of different typing methods using international isolates, but their study included results from only one genotypic typing method and was conducted before the emergence of the current international epidemic strain of *C. difficile* (5).

Currently, a strain known as type BI by REA, North American (NA) pulsed-field type 1 (NAP1) by PFGE, and 027 by PCR-ribotyping (BI/NAP1/027) is the single most important epidemic strain causing *C. difficile*-associated disease (CDAD) in North America and Europe (16, 21, 24). The emergence of this hypervirulent strain has increased interest in *C. difficile* typing and stimulated the application of newer genotype-based methods such as PCR-ribotyping, amplified fragment length polymorphism (AFLP), multilocus sequence typing (MLST),

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TABLE 1. *C. difficile* typing techniques evaluated

Laboratory	Technique used	Target
Leiden University Medical Center, Leiden, The Netherlands	MLVA	DNA repeat units
VU University Medical Center, Amsterdam, The Netherlands	AFLP	PstI and MseI restriction sites
National Institute of Infectious Diseases, Tokyo, Japan	<i>slpAST</i>	Surface layer protein A gene
Anaerobe Reference Unit, PHL, Cardiff, United Kingdom	PCR ribotyping	16S-23S spacer region
Loyola University Medical Center and Hines VA Hospital	REA	HindIII restriction sites
Duke University	MLST	Housekeeping loci
Duke University	PCR-ribotyping	16S-23S spacer region
CDC	PFGE	SmaI restriction sites

multilocus variable-number tandem-repeat analysis (MLVA), and surface layer protein A gene sequence typing (*slpAST*). In view of the emergence of BI/NAP1/027 and the proliferation of typing techniques, we conducted an international multilaboratory comparison of seven typing methods to determine their utility in epidemiologic investigations of *C. difficile* outbreaks.

MATERIALS AND METHODS

Isolates. The pool of *C. difficile* isolates for testing consisted of 42 isolates submitted by four countries: 10 from Canada, 10 from the United Kingdom, 10 from The Netherlands, and 12 from the United States. Each country was instructed to submit five epidemic strains (including three of a single, identical subtype from different geographic locales and two additional strain subtypes, if available; otherwise, these were also to be from different locales) and five non-epidemic strains (three of the most common strains and two of the next most common strains). Two laboratories did not examine all 42 isolates because some isolates were nonviable upon receipt.

All isolates were further characterized by toxinotyping (27), PCR for the presence of the *cdtB* binary toxin gene using primers described previously by Stubbs et al. (32), and PCR for a *tdcC* DNA deletion using primers *tdc1* (GACCTCATCACCATCTTC) and *tdc2* (TGGTTCAAATGAAAGACG AC), prepared by the CDC core facility. The *tdcC* PCR was performed on a 50- μ l sample containing 5 μ l Ampliqaq Gold 10 \times buffer (Applied Biosystems, Foster City, CA), 1.5 mM MgCl₂, 25 pmol each primer, and 2.5 U Ampliqaq Gold PCR polymerase. Amplification was performed on an Applied Biosystems model 9700 thermocycler with 1 cycle at 95°C for 5 min; 35 cycles at 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s; and 1 cycle at 72°C for 2 min. PCR products were detected by gel electrophoresis in 2% agarose-1 \times Tris-borate-EDTA (TBE) buffer, with an applied voltage of 200 V for 4 h. Gels were stained with ethidium bromide and photographed.

Typing techniques. All seven of the typing methods that we assessed were DNA based. Two methods (REA and PFGE) used enzyme restriction of total genomic DNA, two methods (MLVA and PCR-ribotyping) relied on PCR products alone, two methods (MLST and *slpAST*) relied on sequencing of selected gene segments, and one method (AFLP) used a combination of enzyme restriction and PCR.

The seven typing methods (AFLP, *slpAST*, PCR ribotyping, MLST, MLVA, REA, and PFGE) were evaluated in six laboratories, and PCR ribotyping was evaluated twice because the U.S. and United Kingdom laboratories used different primer sets (Table 1). MLVA was performed as described previously by van den Berg et al. (33). AFLP was performed as described previously by Mohammadi et al. (22): isolates clustering with $\geq 86\%$ similarity were considered to be the same AFLP type.

United Kingdom PCR-ribotyping was performed as described previously by Stubbs et al. (31): isolates were considered to be of a new PCR ribotype if their pattern was at least one band different from previously named patterns. U.S. PCR-ribotyping was performed according to the method described previously by Bidet et al. (3), with a slight modification, in that the thermocycling protocol denaturation, annealing, and extension times were 30 s instead of 1 min, and the PCR products were run on 3% SeaKem LE agarose gels in 1 \times TBE at 85 V for 4 h. Again, isolates were considered to be of a new PCR ribotype if their patterns were at least one band different from previously named patterns.

MLST was performed according to a method described previously by Lemee et al. (18). REA was done by HindIII digestion of total genomic DNA as described previously by Clabots et al. (6). *slpAST* was done by sequencing a PCR product from the variable region of the surface layer protein A gene (*slpA*) and comparing the amino acid sequences deduced from the DNA sequences: a new

type was established if the deduced amino acid sequences differed from existing types by more than 20 amino acid residues. Subtypes consisted of groups differing by ≤ 20 amino acid residues, as described previously by Kato et al. (14).

For PFGE, broth cultures of the isolates were harvested at 7 h, and agar plugs were prepared. Plugs were incubated overnight at 37°C with lysozyme (5 mg/ml) and RNase (0.4 mg/ml) and treated with proteinase K (1 mg/ml). DNA plugs were digested with SmaI for 4 h at room temperature. DNA was separated on 1% SeaKem Gold agar in 0.5% TBE buffer for 18 h with the use of a 5- to 40-s switch time. Thiourea (200 μ M) was added to the running buffer and gel for any DNA samples showing degradation. PFGE patterns were analyzed with BioNumerics 4.01 (Applied Maths, Austin, TX) software. To show the similarity between PFGE patterns, we created dendrograms by the unweighted-pair group method with arithmetic mean using Dice coefficients with position tolerance and optimization of 1.10%. We considered clusters with $\geq 80\%$ similarity to be distinct NAP types and clusters with $\geq 95\%$ similarity within a type to be distinct NAP subtypes.

Discrimination index. To compare the discriminating capacity of the seven methods, we used an index of discrimination (*D*) based on Simpson's index of diversity according to the following formula described previously by Hunter and Gaston (12):

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j - 1)$$

where *N* is the total number of strains in the sample population for which data were obtained, *s* is the total number of types/subtypes described, and *n_j* is the number of strains of the *j*th type. Confidence intervals for *D* were determined by a method described previously by Grundmann et al. (10).

RESULTS

As shown in Fig. 1, a dendrogram produced from PFGE data with the results of the other typing methods distributed along it, we found a high concordance of groupings by all methods.

All isolates belonged to one of six allele profiles (AP-A through AP-F) defined by toxinotype, binary toxin PCR results, and the presence or absence of a deletion in the *tdcC* gene. AP-A (toxinotype 0, binary toxin negative, and no *tdcC* deletion) was considered to be the archetype profile. Isolates with variant APs (i.e., those differing from the archetypal allele profile) tended to group together by all methods, whereas isolates with profile A were distributed among several groups (Table 2).

All typing methods were able to type 100% of the isolates examined. With the use of thiourea in the gel, PFGE was able to type the previously described REA type "J" strains (AP-A) that were reported to have degraded with the use of earlier PFGE procedures (7, 28). Samples US32 and US35 were the same isolate submitted to participating laboratories in duplicate as a limited indicator of reproducibility, and all methods except MLVA showed identical patterns for the duplicate sample on initial testing. The initial MLVA results for the duplicate sample showed a low level of target amplification, but upon repeat test-

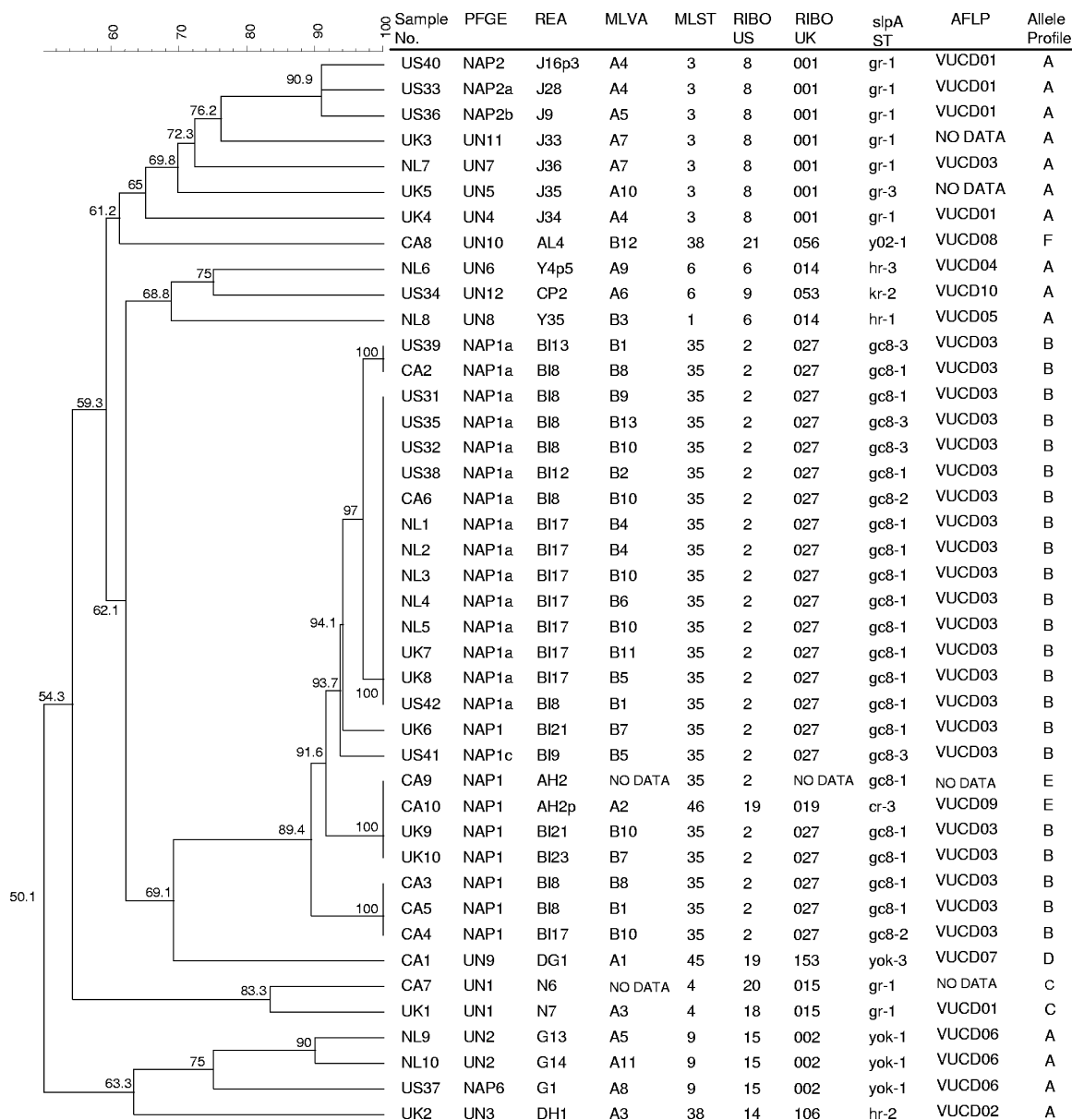


FIG. 1. PFGE dendrogram with results of all methods aligned. RIBO US, PCR-ribotyping performed in the United States; RIBO UK, PCR-ribotyping performed in the United Kingdom.

ing, the duplicate samples showed identical patterns, and the results with other samples remained unchanged.

Four methods (MLVA, REA, *slpA*ST, and PFGE) recognized subtypes under primary types, and the other three (PCR-ribotyping, MLST, and AFLP) recognized only primary types. The *D* values were considerably higher for methods that recognized subtypes (>0.900 for MLVA and REA, 0.843 for PFGE, and 0.819 for *slpA*ST) than for those that did not (0.631 for AFLP and 0.688 to 0.700 for United Kingdom and U.S. PCR-ribotyping and MLST) (Table 3).

The current international epidemic strain (BI/NAP1/027) is defined as being AP-B in this study. REA, PFGE, and PCR-ribotyping grouped each of these 22 AP-B isolates together with or without subtypes. The type names assigned to this

group by various methods did not occur in other AP groups except for the two AP-E isolates typed as NAP1 by PFGE, one AP-A isolate typed as VUCD03 by AFLP, and one AP-A isolate and one AP-F isolate typed as B by MLVA (Table 2). Among the other variant APs, profiles C and E contained two isolates each, and profiles D and F contained one isolate each. AP-C and AP-E isolates were found to have the same type by REA, PFGE, MLST, and *slpA*ST but different types by PCR-ribotyping (U.S. laboratories only).

DISCUSSION

Because of the sporadic occurrence of geographically widespread CDAD outbreaks caused by a common strain, it is

TABLE 2. Grouping of isolates by AP

AP and sample ^a	Isolate type by ^b :							
	REA	PFGE	MLVA	MLST	PCR-ribotyping		<i>slpAST</i>	AFLP
					United States	United Kingdom		
AP-A								
US34	CP2	UN12	A6	6	9	053	kr-2	VUCD10
UK2	DH1	UN3	A3	38	14	106	hr-2	VUCD02
US37	G1	NAP6	A8	9	15	002	yok-1	VUCD06
NL9	G13	UN2	A5	9	15	002	yok-1	VUCD06
NL10	G14	UN2	A11	9	15	002	yok-1	VUCD06
US33	J28	NAP2a	A4	3	8	001	gr-1	VUCD01
US36	J9	NAP2b	A5	3	8	001	gr-1	VUCD01
US40	J16p3	NAP2	A4	3	8	001	gr-1	VUCD01
UK3	J33	UN11	A7	3	8	001	gr-1	No data
UK4	J34	UN4	A4	3	8	001	gr-1	VUCD01
UK5	J35	UN5	A10	3	8	001	gr-3	No data
NL7	J36	UN7	A7	3	8	001	gr-1	VUCD03
NL8	Y35	UN8	B3	1	6	014	hr-1	VUCD05
NL6	Y4p5	UN6	A9	6	6	014	hr-3	VUCD04
AP-B								
US38	BI 12	NAP1a	B2	35	2	027	gc8-1	VUCD03
US39	BI 13	NAP1a	B1	35	2	027	gc8-3	VUCD03
UK7	BI 17	NAP1a	B11	35	2	027	gc8-1	VUCD03
UK8	BI 17	NAP1a	B5	35	2	027	gc8-1	VUCD03
NL1	BI 17	NAP1a	B4	35	2	027	gc8-1	VUCD03
NL2	BI 17	NAP1a	B4	35	2	027	gc8-1	VUCD03
NL3	BI 17	NAP1a	B10	35	2	027	gc8-1	VUCD03
NL4	BI 17	NAP1a	B6	35	2	027	gc8-1	VUCD03
NL5	BI 17	NAP1a	B10	35	2	027	gc8-1	VUCD03
CA4	BI 17	NAP1	B10	35	2	027	gc8-2	VUCD03
UK6	BI 21	NAP1	B7	35	2	027	gc8-1	VUCD03
UK9	BI 21	NAP1	B10	35	2	027	gc8-1	VUCD03
UK10	BI 23	NAP1	B7	35	2	027	gc8-1	VUCD03
CA2	BI 8	NAP1a	B8	35	2	027	gc8-1	VUCD03
CA3	BI 8	NAP1	B8	35	2	027	gc8-1	VUCD03
CA5	BI 8	NAP1	B1	35	2	027	gc8-1	VUCD03
CA6	BI 8	NAP1a	B10	35	2	027	gc8-2	VUCD03
US31	BI 8	NAP1a	B9	35	2	027	gc8-1	VUCD03
US32	BI 8	NAP1a	B10*	35	2	027	gc8-3	VUCD03
US35	BI 8	NAP1a	B13*	35	2	027	gc8-3	VUCD03
US42	BI 8	NAP1a	B1	35	2	027	gc8-1	VUCD03
US41	BI 9	NAP1c	B5	35	2	027	gc8-3	VUCD03
AP-C								
CA7	N6	UN1	No data	4	20	015	gr-1	No data
UK1	N7	UN1	A3	4	18	015	gr-1	VUCD01
AP-D								
CA1	DG1	UN9	A1	45	19	153	yok-3	VUCD07
AP-E								
CA9	AH2	NAP1	No data	46	18	No data	cr-3	No data
CA10	AH2p	NAP1	A2	46	19	019	cr-3	VUCD09
AP-F								
CA8	AL4	UN10	B12	38	21	056	y02-1	VUCDO8
D Index	0.933	0.843	0.964	0.699	0.700	0.688	0.815	0.631

^a AP-A, toxinotype 0, binary toxin negative, and no *tdcC* gene deletion; AP-B, toxinotype III, binary toxin positive, and an 18-bp *tdcC* gene deletion; AP-C, toxinotype 0, binary toxin negative, and an 18-bp *tdcC* gene deletion; AP-D, toxinotype III, binary toxin positive, and no *tdcC* gene deletion; AP-E, toxinotype IX, binary toxin positive, and no *tdcC* gene deletion; AP-F, toxinotype XII, binary toxin positive, and no *tdcC* gene deletion.

^b *, identical upon repeat testing. "No data" indicates that isolates were not tested because they were nonviable for the test laboratory.

important to have tools to detect and track these strains. Since the discovery of the "J" strain in the 1990s, typing methods have relied increasingly on a strain's genotypic characteristics; however, only a few studies have compared the typing perfor-

mances of these newer techniques. Early in this decade, BI/NAP1/027 emerged as the single most important epidemic strain causing CDAD in North America and Europe (16, 21, 24). Our study provides the most comprehensive comparison

TABLE 3. Discrimination indices of compared methods^a

Method	No. of types	No. of subtypes	<i>D</i>	95% CI
MLVA	2	24	0.964	0.94–0.99
REA	10	27	0.933	0.88–0.98
PFGE	15	19	0.843	0.75–0.93
<i>slpAST</i>	7	13	0.815	0.72–0.91
PCR-ribotyping (United States)	10	NA	0.700	0.56–0.83
PCR-ribotyping (United Kingdom)	10	NA	0.688	0.55–0.83
MLST	9	NA	0.699	0.56–0.83
AFLP	10	NA	0.631	0.46–0.80

^a CI, confidence interval; NA, not applicable.

of typing techniques since the emergence of the BI/NAP1/027 strain and is the first to compare the discrimination and typing capabilities of some of the newer genotypic techniques.

To be useful, a strain typing method should cluster isolates that appear to be related (e.g., similar antibiotic resistance profiles, toxin production levels, and epidemiologic links) and exclude those that do not. Most of the techniques in this study were able to do this. Although those methods that distinguish subtypes of *C. difficile* had higher *D* values than those that did not, the groupings of isolates by primary type were very similar for all methods tested, and all methods appeared to be adequate for detecting an outbreak strain in a particular institution. However, only REA and MLVA were able to distinguish NA isolates of AP-B organisms from European (EU) isolates. REA separated the 10 EU AP-B isolates into three types, including seven classified as being type BI 17, whereas only 1 of 12 NA AP-B isolates was identified as being BI 17. REA separated the NA isolates into five types, with type BI 8 accounting for 8 of 12 of the NA AP-B isolates and none of 10 EU AP-B isolates. MLVA separated the NA and EU AP-B isolates into six and seven types, respectively, with only two types (type B5 [one isolate] and type B10 [three isolates]) occurring in both NA and EU isolates. Using *slpAST*, all EU AP-B isolates tested here were typed into gc8-1, while three subtypes (gc8-1, gc8-2, and gc8-3) were found in NA AP-B isolates. All of the other techniques showed little to no type variation between NA and EU AP-B isolates. Although several typing methods grouped the isolates into subtypes, the subtypes were not necessarily congruent (Table 2).

While MLVA showed a high level of discrimination and separated the AP-B isolates from the non-AP-B isolates, the dendrogram based on MLVA results (Fig. 2) is so discriminating that it obscures strain similarities that are evident in dendrograms based on PFGE (Fig. 1) and AFLP results (not shown). Using different MLVA targets and different analytic methods, others have found MLVA to be a useful tool in tracking health care-related *C. difficile* infections (20).

PFGE typing of *C. difficile* has proven to be problematic in the past because of the DNA degradation observed with some isolates, particularly those of the REA “J” type (28) or serogroup G (7). Bidet et al. (4) previously found good concordance between results of PFGE and PCR-ribotyping. We found that no sample was untypeable by PFGE, including samples of the epidemic “J” strain (28). The use of thiourea in

the gel has proven effective in alleviating typing problems attributable to DNA degradation (7).

Non-sequence-based methods rely on some form of band size comparison in agarose or acrylamide gels. Some of these techniques, such as REA, are very labor-intensive and may require analysts to compare isolates on the same gel, making interlaboratory data comparisons very difficult. The standardization of PFGE gel runs and the use of computer-assisted gel analyses have made interlaboratory data comparisons somewhat easier, but PFGE is still labor-intensive.

Once the reactions are optimized, AFLP and PCR-ribotyping both require relatively little labor. In this study, we found that PCR-ribotyping results had very good interlaboratory agreement; even though the laboratories involved used two different primer sets, they disagreed on only one isolate. The sequence-based methods MLST and *slpAST* also performed well, although MLST is much more labor-intensive because it sequences multiple loci, whereas *slpAST* sequences only one locus. One advantage of sequenced-based techniques is that their data are highly transportable from laboratory to laboratory.

The limitations of the study include a small sample size and minimal data with which to evaluate intralaboratory and interlaboratory reproducibility. Only one isolate was included in duplicate to check intralaboratory reproducibility. A larger sample size may have better defined the concordance of test methods among the APs other than AP-A and AP-B. An evaluation of interlaboratory reproducibility was not possible because no two laboratories used exactly the same method. A nonrandomized sample selection was used to determine the predominant *C. difficile* types in different geographic regions and to increase strain variety for testing the discrimination of each method. The inclusion of more isolates of variant APs other than AP-B might have better defined the concordance of the methods with variant APs.

The epidemiology of *C. difficile* is changing, due in part to the emergence of more virulent CDAD in North America and Europe (16, 21, 24) and the emergence of CDAD in the community (1). Additionally, *C. difficile* appears to be a growing problem in food production animals (25, 30) and has even been isolated from food products (26). Thus, CDAD is no longer strictly a health care-associated phenomenon, and risk factors other than antimicrobial exposure might be important as we begin to understand the broader scope of CDAD. MacCannell et al. (19) previously reported the frequent occurrence of BI/NAP1/027 in community-associated isolates, emphasizing the need for good clinical histories and typing methods to define the epidemiology of CDAD in community and health care populations. Sorting out the answers to these questions will require the ability to discriminate among strains from all of these sources even if they share AP characteristics and the same type designations.

Our results showed that the methods evaluated can identify the current BI/NAP1/027 epidemic strain, the “J” strain, and would likely recognize a new outbreak strain if one occurred in an institution. However, because some typing techniques do not distinguish all APs, in the event of an outbreak, it may be beneficial to do a complete allele profile characterization. If one wishes to track outbreak strains geographically and do intratypic discrimination, one should consider the use of one of

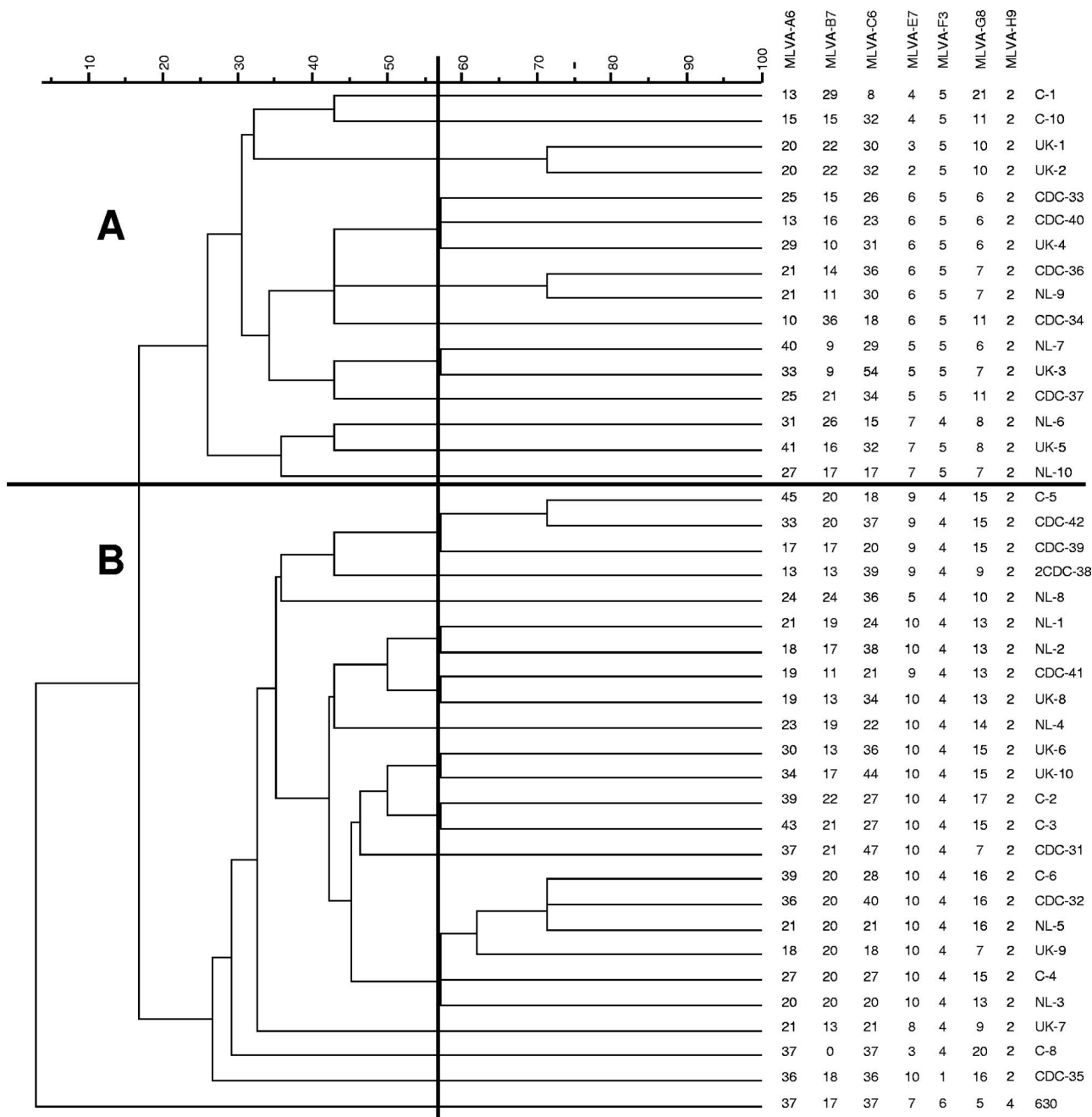


FIG. 2. MLVA dendrogram and assigned types. The dark vertical line is the cutoff value separating MLVA types A and B; the dark horizontal line separates MLVA types A and B.

the more discriminating techniques such as REA or MLVA. Recently, Pasqualotto et al. (23) found that there might be size differences of up to six bases between sequencing data and sizing data produced by capillary electrophoresis and size differences of up to three bases among data produced by different capillary electrophoresis instruments. This would make the use of MLVA in strain tracking problematic and interlaboratory comparisons less reliable unless, as suggested by Pasqualotto et al., correction factors are used (23). Because REA is not widely used, its interlaboratory reproducibility is unknown. In short, although many methods can detect an outbreak strain within

an institution, there is currently no method for interinstitutional strain tracking and intratypic discrimination that has proven laboratory-to-laboratory reproducibility.

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REFERENCES

1. Barbut, F., B. Gariazzo, L. Bonné, V. Lalande, B. Burghoffer, R. Luiuz, and J.-C. Petit. 2007. Clinical features of *Clostridium difficile*-associated infections and molecular characterization of strains: results of a retrospective study, 2000–2004. *Infect. Control Hosp. Epidemiol.* **28**:131–139.
2. Bartlett, J. G., T. W. Chang, M. Gurwith, S. L. Gorbach, and A. B. Onderdonk. 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. *N. Engl. J. Med.* **298**:531–534.
3. Bidet, P., F. Barbut, V. Lalande, B. Burghoffer, and J.-C. Petit. 1999. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. *FEMS Microbiol. Lett.* **175**:261–266.
4. Bidet, P., V. Lalande, B. Saluaze, B. Burghoffer, V. Avesani, M. Delmee, A. Rossier, F. Barbut, and J.-C. Petit. 2000. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. *J. Clin. Microbiol.* **38**:2484–2487.
5. Brazier, J. S., M. E. Mulligan, M. Delmee, S. Tabaqchali, and the International *Clostridium difficile* Study Group. 1997. Preliminary findings of the International Typing Study on *Clostridium difficile*. *Clin. Infect. Dis.* **25**(Suppl. 2):S199–S201.
6. Clabots, C. R., S. Johnson, K. M. Bettin, P. A. Mathie, M. E. Mulligan, D. R. Schaberg, L. R. Peterson, and D. N. Gerding. 1993. Development of a rapid and efficient restriction endonuclease analysis typing system for *Clostridium difficile* and correlation with other typing systems. *J. Clin. Microbiol.* **31**:1870–1875.
7. Corkill, J. E., R. Graham, C. A. Hart, and S. Stubbs. 2000. Pulsed-field gel electrophoresis of degradation-sensitive DNAs from *Clostridium difficile* PCR ribotype 1 strains. *J. Clin. Microbiol.* **38**:2791–2792.
8. Delmee, M., M. Homel, and G. Wauters. 1985. Serogrouping of *Clostridium difficile* strains by slide agglutination. *J. Clin. Microbiol.* **21**:323–327.
9. Fawley, W. N., and M. H. Wilcox. 2002. Pulsed-field gel electrophoresis can yield DNA fingerprints of degradation-susceptible *Clostridium difficile* strains. *J. Clin. Microbiol.* **40**:3546–3547.
10. Grundmann, H., S. Hori, and G. Tanner. 2001. Determining confidence intervals when measuring genetic diversity and the discriminatory abilities of typing methods for microorganisms. *J. Clin. Microbiol.* **39**:4190–4192.
11. Heard, S. R., S. O'Farrell, D. Holland, S. Crook, M. J. Barnett, and S. Tabaqchali. 1986. The epidemiology of *Clostridium difficile* with use of a typing scheme: nosocomial acquisition and cross-infection among immunocompromised patients. *J. Infect. Dis.* **153**:159–162.
12. Hunter, P. R., and M. A. Gaston. 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* **26**:2465–2466.
13. Kato, H., J. Cavallaro, N. Kato, S. L. Bartley, G. Killgore, K. Watanabe, and K. Ueno. 1993. Typing of *Clostridium difficile* by Western immunoblotting with 10 different antisera. *J. Clin. Microbiol.* **31**:413–415.
14. Kato, H., T. Yokoyama, and Y. Arakawa. 2005. Typing by sequencing the *slpA* gene of *Clostridium difficile* strains causing multiple outbreaks in Japan. *J. Med. Microbiol.* **54**:167–171.
15. Killgore, G. E., and H. Kato. 1994. Use of arbitrary primer PCR to type *Clostridium difficile* and comparison of results with those by immunoblot typing. *J. Clin. Microbiol.* **32**:1591–1593.
16. Kuijper, E. J., B. Coignard, P. Tüll, and the ESCMID Study Group. 2006. Emergence of *Clostridium difficile*-associated disease in North America and Europe. *Clin. Microbiol. Infect.* **12**(Suppl. 6):2–18.
17. Kuijper, E. J., J. H. Oudbier, W. N. H. M. Stuijbergen, A. Jansz, and H. C. Zanen. 1987. Application of whole-cell DNA restriction endonuclease profiles to the epidemiology of *Clostridium difficile*-induced diarrhea. *J. Clin. Microbiol.* **25**:751–75313.
18. Lemece, L., A. Dhalluin, M. Pestrel-Carson, J.-F. Lemeland, and J.-L. Pons. 2004. Multilocus sequence typing analysis of human and animal *Clostridium difficile* isolates of various toxigenic types. *J. Clin. Microbiol.* **42**:2609–2617.
19. MacCannell, D. R., T. J. Louie, D. B. Gregson, M. Laverdiere, A.-C. Labbe, F. Laing, and S. Henwick. 2006. Molecular analysis of *Clostridium difficile* PCR ribotype 027 isolates from Eastern and Western Canada. *J. Clin. Microbiol.* **44**:2147–2152.
20. Marsh, J. W., M. M. O'Leary, K. Shutt, A. W. Pasculle, S. Johnson, D. N. Gerding, C. A. Muto, and L. H. Harrison. 2006. Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in hospitals. *J. Clin. Microbiol.* **44**:2558–2566.
21. McDonald, L. C., G. E. Killgore, A. Thompson, R. C. Owens, Jr., S. V. Kazakova, S. P. Sambol, S. Johnson, and D. N. Gerding. 2005. An epidemic, toxin gene-variant strain of *Clostridium difficile*. *N. Engl. J. Med.* **353**:2433–2441.
22. Mohammadi, T., H. W. Reesink, R. N. Pietersz, C. M. Vandembroucke-Grauls, and P. H. Savelkoul. 2005. Amplified-fragment length polymorphism analysis of Propionibacterium isolates implicated in contamination of blood products. *Br. J. Haematol.* **131**:403–409.
23. Pasqualotto, A. C., D. W. Denning, and M. J. Anderson. 2007. A cautionary tale: lack of consistency in allele sizes between two laboratories for a published multilocus microsatellite typing system. *J. Clin. Microbiol.* **45**:522–528.
24. Pépin, J., L. Valiquette, M.-E. Alary, P. Villemure, A. Pelletier, K. Forget, K. Pépin, and D. Chouinard. 2004. *Clostridium difficile*-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *Can. Med. Assoc. J.* **171**:466–472.
25. Rodriguez-Palacios, A., H. R. Stämpfli, T. Duddield, A. S. Peregrine, L. A. Trotz-Williams, L. G. Arroyo, J. S. Brazier, and J. S. Weese. 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. *Emerg. Infect. Dis.* **12**:1730–1736.
26. Rodriguez-Palacios, A., H. R. Staempfli, T. Duffield, and J. S. Weese. 2007. *Clostridium difficile* in retail ground meat, Canada. *Emerg. Infect. Dis.* **13**:485–487.
27. Rupnik, M., V. Avesani, M. Janc, C. von Eichel-Streiber, and M. Delmee. 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. *J. Clin. Microbiol.* **36**:2240–2247.
28. Samore, M., G. Killgore, S. Johnson, R. Goodman, J. Shim, L. Venkataraman, S. Sambol, P. DeGirolami, F. Tenover, R. Arbeit, and D. Gerding. 1997. Multicenter typing comparison of sporadic and outbreak *Clostridium difficile* isolates from geographically diverse hospitals. *J. Infect. Dis.* **176**:1233–1238.
29. Sell, T. L., D. R. Schaberg, and F. R. Fekety. 1983. Bacteriophage and bacteriocin typing scheme for *Clostridium difficile*. *J. Clin. Microbiol.* **17**:1148–1152.
30. Songer, J. G. 2004. The emergence of *Clostridium difficile* as a pathogen of food animals. *Anim. Health Res. Rev.* **5**:321–326.
31. Stubbs, S. L. J., J. S. Brazier, G. L. O'Neill, and B. I. Duerden. 1999. PCR targeted to the 16S-23S rRNA gene intergenic spacer region of *Clostridium difficile* and construction of a library consisting of 116 different PCR ribotypes. *J. Clin. Microbiol.* **37**:461–463.
32. Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff. 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. *FEMS Microbiol. Lett.* **186**:307–312.
33. van den Berg, R. J., I. Schapp, K. E. Templeton, C. H. W. Klaassen, and E. J. Kuijper. 2007. Typing and subtyping of *Clostridium difficile* isolates using multiple-locus variable-number tandem-repeat analysis. *J. Clin. Microbiol.* **45**:1024–1028.
34. Wust, J., N. M. Sullivan, U. Hardegger, and T. D. Wilkins. 1982. Investigation of an outbreak of antibiotic-associated colitis by various typing methods. *J. Clin. Microbiol.* **16**:1096–1101.