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Discriminatory Power and Reproducibility of Novel DNA Typing Methods for *Mycobacterium tuberculosis* Complex Strains

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In recent years various novel DNA typing methods have been developed which are faster and easier to perform than the current internationally standardized IS*6110* restriction fragment length polymorphism typing method. However, there has been no overview of the utility of these novel typing methods, and it is largely unknown how they compare to previously published methods. In this study, the discriminative power and reproducibility of nine recently described PCR-based typing methods for *Mycobacterium tuberculosis* were investigated using the strain collection of the interlaboratory study of Kremer et al. (J. Clin. Microbiol. 37:2607–2618, 1999). This strain collection contains 90 *M. tuberculosis* complex and 10 non-*M. tuberculosis* complex mycobacterial strains, as well as 31 duplicated DNA samples to assess reproducibility. The highest reproducibility was found with variable numbers of tandem repeat typing using mycobacterial interspersed repetitive units (MIRU VNTR) and fast ligation-mediated PCR (FLiP), followed by second-generation spoligotyping, ligation-mediated PCR (LM-PCR), VNTR typing using five repeat loci identified at the Queens University of Belfast (QUB VNTR), and the Amadio speciation PCR. Poor reproducibility was associated with fluorescent amplified fragment length polymorphism typing, which was performed in three different laboratories. The methods were ordered from highest discrimination to lowest by the Hunter-Gaston discriminative index as follows: QUB VNTR typing, MIRU VNTR typing, FLiP, LM-PCR, and spoligotyping. We conclude that both VNTR typing methods and FLiP typing are rapid, highly reliable, and discriminative epidemiological typing methods for *M. tuberculosis* and that VNTR typing is the epidemiological typing method of choice for the near future.

In the past decade, molecular typing methods have increasingly been applied to study the epidemiology and control of tuberculosis (74, 81). When DNA typing is applied to local samples, it can be used to trace outbreaks of tuberculosis and transmission within institutions. DNA typing, on a population-based scale, has provided insight on the risk factors for transmission of tuberculosis, the relative contribution of reactivation of disease compared with reinfection, and transmission between ethnic groups. On a worldwide scale, it has been shown that, in general, the genetic population structure of tuberculosis is heterogeneous in low-incidence countries and much more homogeneous in high-incidence areas. It has also been shown that certain *Mycobacterium tuberculosis* lineages or genotype families, such as the Beijing and the Haarlem genotype families, are responsible for a large part of the tuberculosis cases worldwide. DNA fingerprinting has, therefore, significantly increased our understanding of the epidemiology of tuberculosis.

M. tuberculosis can be typed by using various genetic markers. Initially, short perfect and imperfect repeat sequences as well as insertion sequences (IS) were used (19, 20, 26, 30, 46, 49, 50, 76, 77, 79, 84). However, IS*6110* restriction fragment length polymorphism (RFLP) has gained recognition as the international standard for epidemiological typing of *M. tuberculosis* (72, 78). More recently, after the genome sequences of various members of the *M. tuberculosis* complex became available (12, 21), variable numbers of tandem repeat (VNTR) typing (56, 61, 63, 65, 67), fluorescent amplified fragment length polymorphism (FAFLP) (1, 2, 23, 60), single nucleotide polymorphisms (SNPs) (6, 25), and large sequence polymorphisms (10, 22, 27, 31, 69) have been developed to differentiate strains.

As more genetic markers have become available, it has become more difficult to compare DNA typing methods and to choose the appropriate method. Typing methods should preferably be reproducible, rapid, inexpensive, easy to perform, and directly applicable to clinical material. Outbreak management requires genetic markers with a relatively high turnover,

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such as IS*6110* RFLP, variable numbers of tandem repeat (VNTR) typing, and FAFLP typing. In contrast, determination of the evolutionary relatedness between distantly related bacteria requires genetic markers that are extremely stable and do not converge, such as SNPs and chromosomal deletions. The degree of discrimination and stability of the markers used, therefore, should be appropriate to the research question addressed. To fully understand the reliability of the currently available typing methods and to compare their abilities to differentiate between isolates, it is vital that they be applied to the same set of strains.

In 1999, most of the available molecular typing methods for *M. tuberculosis* were compared with regard to discriminatory power and reproducibility in an interlaboratory study by Kremer et al. (36). The discriminatory power of each technique was assessed using a set of 90 chromosomal DNAs from strains of the *M. tuberculosis* complex. The DNA samples were sent to eight laboratories without reference to the strains from which the DNA was isolated ("blind"). To evaluate intralaboratory reproducibility, 31 duplicate DNA samples were included. All five RFLP typing methods using variability in IS*6110* (77), IS*1081* (79), the direct repeat (76), the polymorphic GC-rich sequence (76), and the GTG_5 repeat (84) were found to be fully reproducible (36). Among the PCR-based methods investigated, mixed-linker PCR (26), VNTR typing using five loci with so-called exact tandem repeats (20), and spoligotyping (30) were also highly reproducible. In contrast, double repetitive element PCR (19), IS*6110* inverse PCR (46), IS*6110* ampliprinting (50), and arbitrarily primed PCR typing (47) were poorly reproducible. IS*6110* RFLP and mixed-linker PCR were the most discriminatory methods, followed by arbitrarily primed PCR, polymorphic GC-rich sequence RFLP, double repetitive element PCR, spoligotyping, and exact tandem repeat VNTR typing (36).

Recently, this well-characterized strain collection of Kremer et al. (36) has been used to propose a new evolutionary scenario for the *M. tuberculosis* complex based on chromosomal deletions (10) and to investigate the prevalence of the occurrence of mutations in putative mutator genes (52). A subset of these strains were also used to study the distribution of deletions, single nucleotide polymorphisms, and other genetic polymorphisms in the genome of *M. tuberculosis* complex (28, 44, 68, 70; unpublished data) and for virulence studies (14, 40).

Recently developed DNA typing methods have not been compared with regard to their reproducibility, discriminatory power, and specificity. In this study, we therefore evaluated these characteristics for nine novel PCR-based typing methods with the strain collection described in the study of Kremer et al. (36). We compared our results with those obtained in that study (35) and with those of two previous studies of VNTR typing based on 12 loci containing mycobacterial interspersed repetitive units (MIRUs) (65) and second-generation spoligotyping (71).

MATERIALS AND METHODS

Study design. Laboratories that specialize in the various techniques were supplied with 131 DNA samples from the study of Kremer et al. (36). To ensure that the laboratories could not identify the samples used in the original study, the numbers were coded. The laboratories were asked to subject these DNAs to the typing method for which they were expert and to analyze the results in their standard way. The results were returned to the organizing laboratory (National Institute of Public Health and the Environment [RIVM], Bilthoven, The Netherlands), where the sample numbers were decoded. The reproducibility, discriminative power, and specificity of each typing method were determined by the organizing laboratory on the basis of the conclusions drawn by the laboratory that had performed the typing.

Mycobacterial strains. The set of DNA samples comprised 90 *M. tuberculosis* complex strains with highly diverse IS*6110* RFLP patterns from 38 countries and 10 non-*M. tuberculosis* complex mycobacterial strains (36). The set also contained 31 duplicate DNA samples, which were prepared by dividing the DNA of 31 *M. tuberculosis* complex strains over two tubes (36). The species designations of 12 strains in this study do not correspond to those in the previous study (Table 1). In the previous study the species were determined on the basis of a combination of biochemical testing and DNA typing (36). However, identification of mycobacterial strains has since improved by the use of chromosomal deletions (10, 28, 44, 48), SNPs (28), and sequencing of the 16S rRNA gene DNA (8, 33). The 90 *M. tuberculosis* complex strains comprised 73 *M. tuberculosis* strains isolated in 34 countries, two *M. africanum* subtype I strains from one country (28, 44), six *M. bovis* strains from three countries, three *M. bovis* BCG vaccine strains, three *M. pinnipedii* strains (13), two *M. microti* strains (82), and one *M. canettii* strain (80). After the initial study, three strains (strain 47 [originally designated *M. bovis*] and strains 92 and 100 [originally designated *M. africanum*]) were first designated *M. africanum* subtype II by Huard et al. (28), but they have since been identified as *M. tuberculosis* (11, 44, 45). The latter designation was established after revision of the interpretative guidelines used for analysis of genetic data (44). The *M. tuberculosis* strains from this collection represent at least 17 genotype families or subfamilies, as determined by previous studies (35, 36) and a large spoligotype database described by Filliol et al. (17, 18). The set of 10 non-*M. tuberculosis* complex mycobacterial strains contained two strains of each of the species *Mycobacterium avium*, *Mycobacterium kansasii*, and *Mycobacterium smegmatis* and one *Mycobacterium gordonae* II, one *Mycobacterium phlei*, one *Mycobacterium xenopi* I, and one *M. xenopi* II strain (8, 33).

IS*6110*-based typing methods. Two PCR-based typing methods targeting IS*6110* were performed: fast ligation-mediated PCR (FLiP) (53, 54) was conducted at the University of Heidelberg, Heidelberg, Germany, and ligation-mediated PCR (LM-PCR) was carried out according to the protocol of Prod'hom et al. (51) at the Centre National de Référence des Mycobactéries, Institut Pasteur, Paris, France.

VNTR typing. VNTR typing uses primers to the flanking regions of VNTR loci to determine the number of repeats at each locus based on the sizes of the PCR products. VNTR typing was performed using six loci by estimating the size of the PCR products on agarose gels at the Queens University of Belfast (QUB), Belfast, Northern Ireland, as described by Roring et al. (57). The six QUB VNTR loci were 11a, 11b, 26, 1895, 3336, and 3232 (56, 61). In addition, VNTR typing was performed on 12 MIRU loci at the Institut Pasteur de Lille, Lille, France. In contrast to QUB VNTR typing, MIRU VNTR typing was performed using four multiplex PCRs and separation of the PCR products on a 96-well ABI 377 sequencer, followed by automated detection and allele calling as described previously by Supply et al. (65).

Second-generation spoligotyping. First-generation spoligotyping detects the presence of 43 spacer sequences in the direct repeat region by PCR with primers directed to the direct repeats followed by reversed line blot hybridization (30). Second-generation spoligotyping detects the presence of the 43 traditional spacers using optimized oligonucleotides, as well as 51 novel spacers (73). Second-generation spoligotyping was performed at the Gelre Hospitals, Apeldoorn, The Netherlands, as described by van der Zanden et al. (71).

FAFLP typing. FAFLP typing identifies sequence differences across the genome (23). In brief, genomic DNA is digested by two restriction enzymes and adaptors are ligated to the restriction fragments. PCR is performed with labeled primers directed to the adaptors, and the PCR products are separated on a denaturing polyacrylamide gel and detected on an automated sequencer.

FAFLP typing was performed by using the restriction enzymes EcoRI and MseI and primers directed to the EcoRI adaptors containing selective bases, as described previously, at the Central Public Health Laboratory, London, United Kingdom (60). The patterns obtained at the Central Public Health Laboratory were analyzed using the curve-based Pearson similarity coefficient and a dendrogram constructed using the unweighted-pair group method using average linkages (UPGMA) in BioNumerics (version 3.5; Applied Maths, Sint-Martens-Latum, Belgium). A similarity cutoff of 94% was used to determine identity between patterns.

The same enzyme combination (EcoRI/MseI), but not the primers with the selective bases, were used at the Centre for DNA Fingerprinting and Diagnostics

TABLE 1. Mycobacterial strains used in this study

Species	Strain[a]	Source, genotype, or characteristic[b]	Country of isolation	Origin
M. tuberculosis	120	Africa (T1)	Burundi	F. A. Portaels
	121		Central African Republic	F. A. Portaels
	40	Africa (family)	Burundi	F. A. Portaels
	72		Central African Republic	F. A. Portaels
	4 (125), 35 (79)		Rwanda	F. A. Portaels
	37 (94), 97		Uganda	T. Aisu
	90	Beijing (atypical)	South Korea	A. H. J. Kolk
	43	Beijing (family)	China	W. Guozi
	34, 45 (113)		Malaysia	Z. Zainuddin
	20 (22)		Mongolia	D. Enkhasaikan
	14, 30		South Africa	W. Sturm
	111		South Korea	A. H. J. Kolk
	44, 54		Thailand	A. H. J. Kolk
	58	Haarlem (H1)	Argentina	A. Alito
	1		The Netherlands	This laboratory
	87 (91)		United States	P. M. Small
	53 (61)	Haarlem (H3)	Argentina	A. Alito
	60, 86		Bolivia	M. Camacho
	50 (84), 123		Czech Republic	M. Kubin
	29		India	This laboratory
	33, 51		The Netherlands	This laboratory
	63 (99)	Haarlem (family)	Italy	A. Gori
	13 (70), 28 (114)		Sri Lanka	J. Perrera
	8		Vietnam	A. H. J. Kolk
	112	CAS1 (Delhi)	The Netherlands	This laboratory
	49	CAS1 (Kilimanjaro)	Tanzania	Z. Yang
	67	EAI	Comoro Islands	F. A. Portaels
	36, 74 (102)		India	This laboratory
	93		Tanzania	Z. Yang
	21		Zimbabwe	J. ten Have
	41 (80)	LAM3	Chile	M. Velasco
	118		Honduras	G. Dubon
	95	LAM4	Spain	S. Samper
	42 (78)	LAM9	Tahiti	B. Gicquel
	82	S	Canada	H. Hoeppner
	103 (108)	T1	China	W. Guozi
	56		Curacao	R. J. Gijsbertha
	27 (104)	T3	Ethiopia	P. W. M. Hermans
	19	CAS (family)	India	This laboratory
	16	Other	Canada	H. Hoeppner
	46		Chile	M. Velasco
	10 (59), 98 (124)		Equador	J. Mendibele
	11 (52), 17		Greenland	Z. Yang
	23 (64)		Honduras	G. Dubon
	15 (55), 31		Iran	A. Moghaddam
	7 (131)		Mongolia	D. Enkhasaikan
	88		Russia	V. Golyshevskaya
	66 (89)		Spain	S. Samper
	38 (75)		Tahiti	B. Gicquel
	47, 65 (122), **92**, 96 (105), **100**		The Netherlands	This laboratory
	12, 77		Tunisia	F. Messadi-Akrout
	18 (107)		United States	P. M. Small
	26		Zimbabwe	J. ten Have
	32 (68)	H37Rv	United States	P. M. Small
	109	H37Ra	United States	P. M. Small
M. africanum subtype I	**6, 85 (128)**	Human	The Netherlands	This laboratory
M. bovis	117, 126	Cattle	Argentina	A. Alito
	73, 130		The Netherlands	This laboratory
	24	Oryx	Saudi Arabia	J. Haagsma
	69		The Netherlands	This laboratory
M. bovis BCG	71	Vaccine strain	Japan	D. Fomukong
	2 (48)		The Netherlands	This laboratory
	83		Russia	V. Golyshevskaya
M. canettii	116 (129)	Human	Somalia	This laboratory
M. microti	25, 62	Vole	United Kingdom	P. Draper
M. pinnipedii	**76, 81, 101**	Seal	Argentina	A. Alito
M. avium	3, 57	Human	The Netherlands	This laboratory
M. gordonae II	**127**	Human	The Netherlands	This laboratory
M. kansasii	5, 115	Human	The Netherlands	This laboratory
M. phlei	**119**	ATCC 14470		
M. smegmatis	106	ATCC 10143		
	110	ATCC 607		
M. xenopi I	**39**	Human	The Netherlands	This laboratory
M. xenopi II	**9**	Human	The Netherlands	This laboratory

[a] Duplicate DNA samples are indicated between parentheses after the original DNA sample, and strains with a new species designation are indicated in bold.
[b] All *M. tuberculosis* strains were isolated from humans. CAS, Central Asian clade; EAI, East African-Indian clade; LAM, Latin American and Mediterranean clade.

TABLE 2. Reproducibility and number of types obtained by using various DNA typing methods for differentiation of 90 *M. tuberculosis* complex strains and 10 non-*M. tuberculosis* complex mycobacterial strains

DNA target	Method used (ref.)	% Reproducibility[a] (95% CI)[b]	No. of types obtained	No. of nontypeable TB complex strains	No. of non-*M. tuberculosis* complex strains positive
12 MIRUs	VNTR typing (65)	100 (89–100)	78	0	0
IS*6110*	FLiP	97 (83–100)	81	2	0
DR locus	Second-generation spoligotyping (71)	90 (74–98)	61	0	0
Five QUBs[c]	VNTR typing	87 (70–96)	82	0	0
IS*6110*	LM-PCR	81 (63–93)	73	2	0
Four conserved loci	Amadio PCR	74 (55–88)	13	0	8
EcoRI/MseI sites	FAFLP typing (+selective primers)	7 (1–21)	ND[d]	0	10
EcoRI/MseI sites	FAFLP typing	0 (0–11)	ND	1	10
BamHI/PstI sites	FAFLP typing	0 (0–11)	ND	0	9/9

[a] Fraction of duplicates showing identical types (31).
[b] CI, confidence interval.
[c] Results indicated exclude QUB locus 3232.
[d] ND, not done.

(CDFD), Hyderabad, India (1, 2). At the CDFD the fragment analysis was performed with GeneScan analysis (version 3.1; Applied Biosystems).

Furthermore, FAFLP typing was performed with the enzyme combination BamHI/PstI at the National Center of Infectious Diseases and Parasitic Diseases, Sofia, Bulgaria, applying a modification of the original protocol developed by Vos et al. (83). BamHI and PstI restriction enzymes were selected as suitable for AFLP analysis according to the frequency of cutting. These frequencies were estimated by applying pDRAW32 software (AcaClone) for restriction analysis on the whole genome sequence of *M. tuberculosis* H37Rv. The BamHI adaptor was constructed by hybridization of partially complementary oligonucleotides BamA15, GAGCCTGATTGGATG, and BamA14, GATCCATCCAATCA. The PstI adaptor was constructed with PstA16, GTGTCACTGACGTGCA, and PstA15, CGTCAGTGACACTGC, oligonucleotides. PCR amplification was performed with a 5′ fluorescently Cy5-labeled BamHI primer, GCCTGATTGGAT GGATCC, and with an unlabeled PstI primer, GTGTCACTGACGTGCAGAG. The PstI primer had one selective G base at the 3′ end. The patterns were analyzed using the Dice similarity coefficient, and a dendrogram was constructed using UPGMA in BioNumerics (Applied Maths).

PCR of more-conserved loci (Amadio PCR). Polymorphisms in the genes *Rv3479* (encoding a hypothetical transmembrane protein), *rpfA* (possible resuscitation-promoting factor), *Rv0648* (RvD6; similar to α-mannosidases or glycosyl hydrolases), and *lppA* (lipoprotein) may be useful to differentiate *M. tuberculosis* complex isolates at the species level (3). Previously, deletion of part of the *Rv3479* gene and length variability in *rpfA* had been described for strains of *M. bovis*. Also, an insertion in *Rv0648* was observed in all isolates of *M. bovis*, *M. pinnipedii*, *M. microti*, and *M. caprae*, while this gene was polymorphic in *M. tuberculosis*. Duplication of *lppA* is found in both *M. bovis* and *M. tuberculosis* isolates. The four genes were amplified by PCR, as described previously by Amadio et al. (3). Based on the absence or presence and sizes of the PCR fragments, the isolates were assigned to different types.

Statistical analysis. The exact 95% confidence limits were calculated using the binominal distribution, while for each of the pairs of tests the Fisher exact test for equality of reproducibility was used. A P value lower than 0.05 was considered significant.

The Hunter-Gaston discriminatory index (HGI) was used to calculate the level of discrimination of each typing method (29).

RESULTS

Reproducibility of typing methods. Nine laboratories, each specializing in one or more of the typing methods, subjected the set of 131 DNAs to nine PCR-based typing methods. The organizing laboratory evaluated the results. The reproducibility of the typing methods was assessed by including 31 duplicate DNA samples of *M. tuberculosis* complex isolates.

Of the nine PCR-based typing methods, only MIRU VNTR typing was 100% reproducible (65). The reproducibility of the other eight typing methods varied between 97% and 0% (Table 2). Only one pair of the duplicate DNA samples showed discordant results by FLiP typing (97% reproducible). The respective samples had completely different patterns. Repeated analysis of these discordant samples after decoding of the set resulted in patterns identical to those in the initial experiment, thus suggesting a labeling error during the initial analysis. For second-generation spoligotyping, 28 of the 31 duplicate DNA samples were scored identical (90% reproducible). For the three duplicate samples that were scored incorrectly, the respective spoligotype patterns were highly similar, differing in one, two, and three spacers, respectively. After evaluation of the original hybridization patterns, the differences in two of these duplicates appeared to be due to an error in reading of the hybridization patterns. A similar human error in the interpretation of first-generation spoligotype patterns was observed in the previous study (36). Examination of the patterns of the remaining duplicate sample confirmed the difference of three spacers between the patterns. In this case we assume a technical failure, such as interfering air bubbles, during the hybridization or detection procedure.

The next-most-reproducible method was LM-PCR, which scored 25 of the 31 duplicates identical (reproducibility, 81%). Evaluation of the patterns of the six duplicate samples that were scored differently after decoding of the samples showed that the respective LM-PCR patterns were highly similar, differing in one or two low-intensity bands of the highest molecular weight (>800 bp). To examine the observed differences, LM-PCR typing of the respective six duplicates was repeated. On repeat, the six duplicates yielded identical patterns, suggesting that minimal technical differences were the cause of the noncorresponding patterns in the initial experiment.

The reproducibility of QUB VNTR typing varied significantly by locus. Four loci were highly reproducible: loci 11b and 1895 were 100% reproducible, and loci 11a and 26 were 97% reproducible. For locus 3336, 29 of the 31 duplicates were scored correctly (reproducibility, 94%). Locus 3232 exhibited the lowest reproducibility, 74%, since eight duplicate samples yielded different results with this locus. Combining the results of the six QUB VNTR loci yielded 21 duplicate samples which were scored identically (reproducibility, 68%). Excluding locus 3232, QUB VNTR typing scored 27 duplicate samples identical (reproducibility, 87%) (Table 2). As was found for loci 11a and 26, all the PCR products from loci 3336 and 3232 of the

duplicate samples which were not scored identically showed a difference of a single repeat in length. To reexamine the observed differences in PCR product length, the PCRs and the electrophoretic analysis of 7 of the 12 duplicates that yielded discrepant results were repeated. On repeat all seven duplicates yielded identical patterns, suggesting that the original error was either caused by the human interpretation of the patterns by eye, by failure of the agarose gels to sufficiently separate the PCR fragments, or by difficulties of the PCR itself.

The Amadio PCR, a method to differentiate subspecies within the *M. tuberculosis* complex, determines the polymorphism at four independent genes (3). The reliability of the PCR amplification and analysis varied significantly per gene. *Rv0648* yielded 100% reproducible results. For *rpfA* and *lppA*, 28 of the 31 duplicate samples were classified as the same (90% reproducible). The PCR for *Rv3479* identified 27 of the 31 duplicate samples (87% reproducible). The overall reproducibility of the Amadio PCR was 74% (23/31). Reexamination of the pictures of the PCR results of, in total, the 10 erroneous duplicates revealed that three errors occurred because of PCR failure and six were because of wrong interpretation of the obtained results which were, in fact, identical. For the remaining duplicate sample, truly different patterns were obtained, and we expect a mix-up of samples caused this error.

Finally, the reproducibility of FAFLP typing was evaluated. This method was performed in three different laboratories with minor differences in protocol. DNA was digested by restriction enzymes EcoRI and MseI in two laboratories. One of these laboratories amplified the digested DNA by using selective primers, and the other laboratory used standard primers. The third laboratory used BamHI and PstI to digest the DNA, followed by ligation of adaptors to the restriction sites and PCR with a BamHI fluorescently labeled primer and PstI-unlabeled primer. All three methods yielded poorly reproducible results, although the use of selective primers was slightly superior (Table 2).

EcoRI/MseI FAFLP typing with selective primers yielded identical patterns for only 2 of the 31 duplicates (reproducibility, 7%). This result was obtained by interpreting a dendrogram based on the similarities of the FAFLP patterns, using a similarity cutoff for identity of >94%. In this dendrogram the patterns of an additional 11 duplicate samples were less than five positions apart, indicating that these patterns had the majority of their bands in common. When higher similarity cutoffs were used to determine pattern identity, none of the duplicate samples was scored identical. A lower similarity cutoff yielded too many clustered isolates (data not shown).

Using EcoRI/MseI FAFLP typing without selective primers, none of the duplicate samples was scored identical (reproducibility, 0%). With this method, a maximum of 42 fragments were detected per pattern, and the reproducibility of these individual fragments was investigated (data not shown). Four to 32 of the fragments were scored differently within the various duplicate pairs. Furthermore, none of the 42 fragments was reproducible for all duplicates; for 7 to 20 of the 31 duplicate DNA sets there was no concordance in the presence or absence of the respective fragments.

Also with BamHI/PstI FAFLP typing, none of the duplicate samples was scored identical (reproducibility, 0%). When the banding patterns of the duplicate DNA samples were compared, it was found that the majority of the bands in these banding patterns were reproducible. However, most patterns differed in a few bands that were scored differently for the duplicate samples. Low-intensity bands were sometimes scored as such, but sometimes as present or absent. Shifted banding patterns due to insufficient normalization of the computerized banding patterns also caused errors. Figure 1 depicts duplicate samples of BamHI/PstI FAFLP typing and is illustrative of the patterns found in all three FAFLP methods.

The reproducibilities of the four most reliable methods, MIRU VNTR, FLiP, second-generation spoligotyping, and QUB VNTR using five loci, ranging from 100 to 87%, were not significantly different. The reproducibility of LM-PCR (81%) was significantly less than that of MIRU VNTR ($P = 0.024$), but not significantly different from any of the other reproducible methods. The Amadio PCR was significantly less reproducible than MIRU VNTR ($P = 0.005$) and FLiP ($P = 0.026$), but it did not differ significantly from the other reproducible methods. The three FAFLP methods exhibited a significantly lower reproducibility compared to all other methods ($P < 0.001$).

Degree of differentiation of the various typing methods. The abilities of the methods to differentiate between strains of the *M. tuberculosis* complex were determined by inclusion of 90 different isolates in the set of 131 DNAs. The discriminatory power of the three FAFLP typing methods was not determined, considering the low reproducibilities of these methods. Thus, in total, six methods were available for this evaluation.

QUB VNTR typing and FLiP showed the highest levels of differentiation, discriminating 82 and 81 types, respectively (Table 2). This level of differentiation is comparable to the best level of differentiation obtained by IS*6110* RFLP (84 types) in the previous study (36). FLiP yielded exactly the same number of types as mixed-linker PCR in the previous study (36). This is not surprising, as both typing methods use the mixed-linker approach and rely on the same restriction enzyme and IS*6110*-specific primer (26, 53, 54). The next-most-discriminative method was MIRU VNTR typing, yielding 78 types, followed by LM-PCR (73 types) and second-generation spoligotyping (61 types). Surprisingly, the discriminatory power of second-generation spoligotyping was the same as that observed previously for standard spoligotyping (36). The addition of 51 novel spacers did not contribute to the overall discriminatory power of the method of typing this set of mainly *M. tuberculosis* strains. However, although the absolute discrimination of the two spoligotyping methods was the same, some samples were differentiated differently as a consequence of the redesigned oligonucleotides (71).

As expected, the Amadio PCR showed the least discrimination, i.e., 13 types (Table 2). In agreement with the previous report (3), *Rv3479* was consistently deleted in cattle-adapted *M. bovis* and BCG isolates. Analysis of the *rpfA* gene distinguished the three BCG vaccine strains from most other isolates. Also consistent with the previous report, a deletion in *Rv0648* was found in some *M. tuberculosis* strains but not in isolates of *M. bovis*, *M. pinnipedii*, and *M. microti*. Our analysis showed that this region is also intact in *M. canettii* and *M. africanum* subtype I. Four of the five *M. tuberculosis* isolates containing a deletion in *Rv0648* were of principal genetic group 3 (64), suggesting that the deletion in this gene occurred rel-

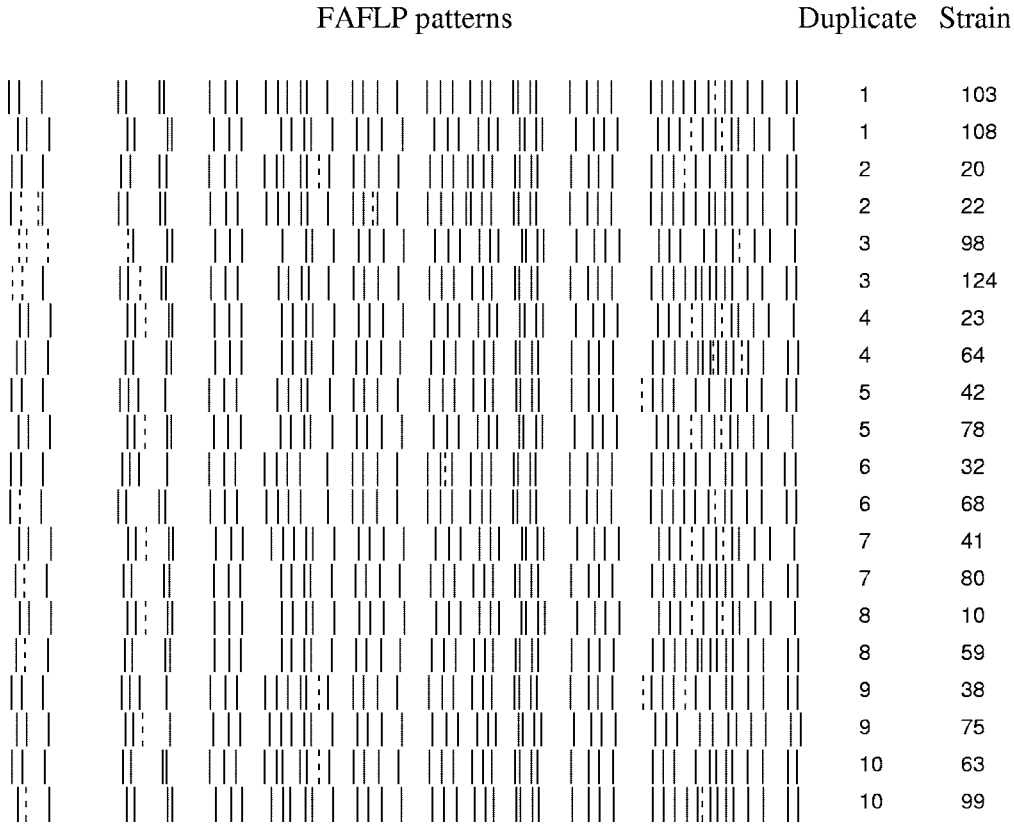

FIG. 1. Representative examples of BamHI/PstI FAFLP patterns of duplicate *M. tuberculosis* complex samples. The PCR fragments are depicted from high to low molecular weight (left to right) and ranged from 150 to 470 bp. Stippled bands indicate bands with low fluorescent intensity. Numbers of duplicate samples and the respective strain numbers are indicated on the right. Note that none of the FAFLP patterns of these duplicate samples was identical, nor were they identified correctly, mainly because of shifts in the banding patterns and differently assigned bands.

atively recently in the evolution of *M. tuberculosis*. Finally, a duplication of the gene *lppA* was present in *M. canettii*, *M. africanum* subtype I isolates, oryx- and cattle-adapted *M. bovis*, and in the BCG isolates, but not in *M. pinnipedii*, *M. microti*, and about two-thirds of the *M. tuberculosis* isolates. This pattern of occurrence suggests that the duplication of this gene was present in the common ancestor of the *M. tuberculosis* complex and was lost in at least two independent lineages.

Besides looking at the total number of types, the discriminatory power of typing methods can also be expressed by using the HGI (29). This index measures the average probability that a typing system will assign a different type to two unrelated strains randomly sampled in a population. For a set of completely unrelated isolates, the HGI is 1. QUB VNTR typing and MIRU VNTR typing yielded the highest HGIs, 0.998 and 0.995, respectively (Table 3). The two methods based on polymorphism in IS*6110*, FLiP and LM-PCR, showed a somewhat lower HGI, due to the failure of these methods, similar to all other IS*6110*-based methods, to discriminate between low-IS*6110* copy strains. Spoligotyping had the lowest discriminatory index (Table 3). For comparison, the HGI and the parameters used to calculate this index of IS*6110* RFLP are also mentioned in Table 3. This method was the most discriminative method in the previous study (36).

TABLE 3. Comparison of the discriminatory power of various DNA typing methods to differentiate 90 *M. tuberculosis* complex strains

Method used (reference)	HGI[a]	No. of unique types (%)	No. of clusters	No. of clustered isolates (%)	Maximum no. of isolates in a cluster	Avg cluster size
QUB VNTR, 5 loci[b]	0.998	74 (82.2)	8	16 (17.8)	2	2.0
IS*6110* RFLP (36)	0.997	81 (90.0)	3	9 (10.0)	5	3.0
MIRU VNTR, 12 loci (65)	0.995	70 (77.8)	8	20 (22.2)	6	2.5
FLiP	0.994	77 (85.6)	4	13 (14.4)	7	3.3
LM-PCR	0.987	65 (72.2)	8	25 (27.8)	10	3.1
Second-generation spoligotyping (71)	0.982	47 (52.2)	14	43 (47.8)	8	3.1

[a] Hunter-Gaston discriminative index (29).
[b] Results indicated exclude QUB locus 3232 results.

Specificity of typing methods for *M. tuberculosis* complex. The specificity of the typing methods was determined by inclusion of 10 DNAs of non-*M. tuberculosis* complex mycobacterial strains in the set of 131 DNAs. QUB VNTR typing, second-generation spoligotyping, and the IS*6110*-based typing methods, FLiP and LM-PCR, did not give a PCR product for any of the non-*M. tuberculosis* complex mycobacterial strains (Table 2). In contrast, in the previous study four other IS*6110*-based PCR methods were not completely specific for the *M. tuberculosis* complex (36). The three FAFLP typing methods yielded banding patterns for the non-*M. tuberculosis* complex mycobacterial strains that were quite distinct from the ones obtained with strains of the *M. tuberculosis* complex. For MIRU VNTR typing, the non-*M. tuberculosis* complex mycobacterial strains yielded either no PCR product or the amplified fragments had sizes that did not correspond to any of the *M. tuberculosis* complex VNTR alleles. Finally, with the Amadio speciation PCR, failure of the amplification of the *lppA* gene marked the non-*M. tuberculosis* complex strains. Thus, all the typing methods were able to distinguish between *M. tuberculosis* complex strains and other mycobacteria.

DISCUSSION

The method most frequently used for molecular typing of *M. tuberculosis* strains is IS*6110* RFLP, and its application has significantly increased our understanding of the epidemiology of tuberculosis (74, 81). However, it takes several weeks before a strain is sufficiently grown for RFLP typing, and the method is labor-intensive and complicated. Furthermore, RFLP typing results are often obtained long after contact tracing has been initiated, restricting its use for outbreak management (38). PCR-based alternative typing methods are especially advantageous for outbreak management, early detection of laboratory cross-contamination, and directing contact tracing. In recent years various novel PCR-based typing methods have been developed for *M. tuberculosis*. We have evaluated the reproducibility and discriminatory power of these novel methods in this study. Laboratories specializing in the various techniques analyzed a set of samples from the study of Kremer et al., including 90 *M. tuberculosis* complex isolates and 31 duplicate samples, by nine PCR-based typing methods. These samples were previously used to asses the reproducibility and discriminatory power of 12 other typing methods (36).

Samples of chromosomal DNA were sent to each laboratory without reference to the strain from which they were isolated ("blind"), and by using the duplicate samples we were able to measure the reproducibility of each technique. In this study we found that MIRU VNTR typing and FLiP analysis were highly reproducible, followed by second-generation spoligotyping, LM-PCR, QUB VNTR typing, and the Amadio speciation PCR. Among the previously evaluated typing methods, RFLP typing using various probes, mixed-linker PCR, VNTR typing, and first-generation spoligotyping were also highly reproducible (36). In contrast to previous reports (1, 2, 4, 5, 62), we found that FAFLP typing, performed by three different protocols at different laboratories, was poorly reproducible. We have previously shown that other PCR-based typing methods, such as IS*6110* inverse PCR, IS*6110* ampliprinting, double repetitive element PCR, and arbitrarily primed PCR, are also poorly reproducible (36).

FAFLP typing has been applied successfully to various organisms, such as *Clostridium*, *Bacillus*, *Acinetobacter*, *Pseudomonas*, and *Escherichia* (5, 62). In *Escherichia coli*, comparison of in vitro FAFLP to in silico analysis showed that the technique was extremely accurate (4, 5). However, in the same laboratory a similar analysis, carried out on *M. tuberculosis* strains H37Rv and CDC1551, appeared to be, at most, only 66% accurate (60). In our study, the accuracy for the identification of 31 duplicates ranged from 7 to 0%. Background peaks, variation in the relative peak heights, and unexpected fragments were sometimes observed in the analysis of H37Rv and CDC1551 (60). These unexpected fragments may be generated by incomplete digestion of DNA caused by secondary structures in the GC-rich *M. tuberculosis* genome or by failure to amplify the digested fragments because of their secondary structure (60). Alternatively, it is possible that modification of restriction sites might also be a cause of the poor reproducibility of the technique (75). The reproducibility of the method might increase when deionized formamide is used in the digest step, dITP instead of dGTP is used in the PCR amplification (60), or when other restriction enzyme combinations are used.

FAFLP typing by using primers with selective bases was slightly superior to those obtained with regular primers. This finding was confirmed at the CDFD, Hydrabad, where the samples, which were initially analyzed without selective primers, were reanalyzed using selective primers and a capillary sequencer. When 15 of the duplicate samples were repeated, the results were more reliable; 14 of the 15 duplicate samples showed similar patterns. In addition to the problems of PCR amplification, failure to correctly analyze the FAFLP patterns by computer also caused errors. At the National Center of Infectious Diseases and Parasitic Diseases, Sofia, Bulgaria, various difficulties were encountered when analyzing the FAFLP patterns. Firstly, normalization of the patterns appeared insufficient due to too few DNA markers on each gel. Secondly, variability in band intensities caused major interpretation errors. And finally, the coefficient used to calculate similarities between the patterns affected the outcome. It was found that the Pearson correlation is more appropriate to identify similarities between FAFLP patterns than the Dice coefficient; using the same patterns and the Pearson coefficient to calculate similarities, the results improved. Instead of 0, 4 out of 28 duplicate samples were identified correctly. Although on repeat the reproducibility of the FAFLP results increased, the results presented in this and a previous study (60) question the utility of an international FAFLP pattern database and comparison of large multicenter collections of FAFLP patterns (2, 42).

In contrast to MIRU VNTR typing (100% reproducible), the reproducibilities of the six QUB VNTR loci analyzed varied between 100 and 74%. All differences between the lengths of the PCR products of the duplicate samples consisted of a single repeat unit. In order to reduce this problem, the allele calling in the agarose system has been much improved by standardizing and normalizing gel conditions and image analysis (Robin Skuce, personal communication). The QUB loci that were least reproducible in this study, 3232 and 3336, were also problematic with *M. tuberculosis* in other studies; however,

they seem to work well with *M. bovis*, presumably due to differences in the number of repeats at these loci in the different species (Philip Supply, personal communication). Because the reproducibility of QUB 3232 was so much lower than that of the other five QUB VNTR loci, this locus was excluded from the evaluation of the discriminatory ability of QUB VNTR typing.

We estimated the discrimination of each technique using Hunter and Gastons' discriminative index (29). We found that QUB VNTR typing was the most discriminative method (HGI, 0.998), followed by MIRU VNTR typing, FLiP, LM-PCR, and second-generation spoligotyping (HGIs ranging from 0.995 to 0.982). The Hunter and Gastons' indices of LM-PCR, QUB VNTR and, to a lesser extent, of second-generation spoligotyping may actually be slightly overestimated, as the reproducibility of these methods was between 81 and 90%. As a comparison, IS*6110* RFLP yields an HGI of 0.997 on this same set of 90 *M. tuberculosis* complex strains. In agreement with previous comparative studies, LM-PCR was found to be more discriminatory than spoligotyping (9, 16) and slightly less discriminatory than IS*6110* RFLP (43, 58). Also consistent with previous reports, both VNTR typing methods and first- and second-generation spoligotyping were more useful than IS*6110* RFLP for discriminating strains containing few IS*6110* copies (7, 24, 37, 43, 55).

Second-generation spoligotyping includes 51 novel spacers identified by van Embden et al. (73) in the direct repeat region of *M. tuberculosis* complex strains. Those authors anticipated that addition of these novel spacers in spoligotyping would improve the discriminatory power of this method. These spacers were found to increase the ability of spoligotyping to discriminate between *M. bovis*, *M. caprae*, and *M. microti* strains and also showed polymorphisms between strains of the *M. tuberculosis* Beijing genotype (34, 71, 73). In a recent study by Brudey et al. (11) it was shown that the additional spacers enhanced the ability of spoligotyping to differentiate between *M. africanum* isolates. In the present study we found that the inclusion of these novel spacers did not improve the overall discriminatory power of the method in comparison with the traditional spoligotyping when applied to 90 isolates of mainly *M. tuberculosis*. Recently, more novel spacers were detected in strains of *M. canettii* isolated in Djibouti (15), but we suspect that these spacers will also not contribute significantly to the discriminatory power of spoligotyping of isolates of *M. tuberculosis*, as *M. canettii* is only distantly related to the rest of the *M. tuberculosis* complex.

For the few methods which were not included in this or the initial interlaboratory study, the reproducibility and the discriminatory power have been estimated elsewhere. The heminested inverse PCR has been documented to be reproducible (32, 49), and the discriminatory power of enterobacterial repetitive intergenic consensus sequences PCR was found to be higher than that of IS*6110* RFLP typing (59). These methods have not been widely used since their publication. The reliability of the recently described "deligotyping," a macroarray assay to detect large sequence polymorphisms, has only been compared to GeneChip analysis (22). In a similar manner to spoligotyping, which is highly reproducible, deligotyping uses reversed line blot hybridization. However, simultaneous PCR amplification of many loci might reduce the reproducibility of this method.

In conclusion, four techniques, IS*6110* RFLP, mixed-linker PCR, FLiP, and VNTR typing, are highly reliable, discriminative, and appropriate for epidemiological typing of strains of the *M. tuberculosis* complex. The PCR-based methods have the advantage of speed over RFLP typing, as detailed above. VNTR typing, in contrast to mixed-linker PCR and the recently described FLiP, has been extensively applied to strains of the *M. tuberculosis* complex in recent years, suggesting that VNTR typing is more easily adopted by laboratories than the mixed-linker-based methods. Furthermore, VNTR typing detects polymorphisms at multiple loci and is therefore superior for typing strains containing only a few copies of IS*6110*, the element targeted in mixed-linker PCR and FLiP. Many VNTR loci have been identified in the past few years, and these loci can vary considerably in their ability to differentiate between isolates (20, 39, 41, 56, 61, 63, 66, 67). The discriminatory power of VNTR typing, therefore, will depend on the loci used. With international standardization of the most appropriate VNTR loci, which may differ between species or genotypes, VNTR typing will be applied more widely and will further enhance our understanding of the transmission dynamics and population structure of tuberculosis.

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