Identification and Genotyping of *Mycobacterium tuberculosis* Complex Species by Use of a SNaPshot Minisequencing-Based Assay[▽]

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The aim of the present study was to investigate the use of the SNaPshot minisequencing method for the identification of *Mycobacterium tuberculosis* complex (MTBC) isolates to the species level and for further genotyping of *M. tuberculosis* isolates. We developed an innovative strategy based on two multiplex allele-specific minisequencing assays that allowed detection of eight species-specific and eight lineage-specific single nucleotide polymorphisms (SNPs). Each assay consisted of an eightplex PCR amplification, followed by an eightplex minisequencing reaction with the SNaPshot multiplex kit (Applied Biosystems) and, finally, analysis of the extension products by capillary electrophoresis. The whole strategy was developed with a panel of 56 MTBC strains and 15 negative controls. All MTBC strains tested except one *M. africanum* clinical isolate were accurately identified to the species level, and all *M. tuberculosis* isolates were successfully further genotyped. This two-step strategy based on SNaPshot minisequencing allows the simultaneous differentiation of closely related members of the MTBC, the distinction between principal genetic groups, and the characterization of *M. tuberculosis* isolates into one of the seven prominent SNP cluster groups (SCGs) and could be a useful tool for diagnostic and epidemiological purposes.

Although tuberculosis (TB) is an age-old disease, it still represents a major health problem worldwide, accounting for nearly 2 million deaths annually (World Health Organization, Tuberculosis Facts 2009 [http://www.who.int/tb/publications/factsheets/en/]). Besides the need for an improved therapy and for new vaccines, effective TB control requires the initiation of appropriate therapy as well as an increased understanding of its epidemiology.

The causative agents of TB in humans and animals, including *Mycobacterium tuberculosis*, *M. africanum*, *M. bovis*, *M. canettii*, *M. microti*, *M. caprae*, and *M. pinnipedii*, form the *Mycobacterium tuberculosis* complex (MTBC). Although they differ widely in terms of their host tropisms, phenotypes, and pathogenicities, all members of the MTBC are closely related genetically (51). *M. tuberculosis* species, the most common pathogen in humans, can be further divided into genetic groups that also show differences in their levels of virulence, immunogenicities, and geographical distributions (21). On the one hand, it is important to differentiate MTBC species to distinguish between strict human and zoonotic TB and to initiate an appropriate therapy (15). In particular, the distinction

between *M. tuberculosis* and *M. bovis* is necessary, as the latter species is naturally resistant to the antituberculous drug pyrazinamide (48). On the other hand, genotyping of *M. tuberculosis* isolates is useful as a means of addressing evolutionary questions but also as a means of surveying the transmission dynamics of this pathogen and identifying new outbreaks.

Identification of MTBC isolates to the species level is so far routinely performed by analysis of the phenotypic and biochemical characteristics of the bacteria after culture. However, this is a time-consuming and subjective process, and that is why various molecular methods have been developed in recent years. Most molecular methods for MTBC species differentiation described in the literature are based on the analysis of genomic deletions by PCR, followed by agarose gel electrophoresis (30, 40, 56), or are based on the detection of single nucleotide polymorphisms (SNPs) by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis, which consists of digestion of the PCR product with a restriction enzyme, followed by agarose gel electrophoresis (3, 13, 23, 33, 38). A commercial kit based on a DNA strip assay for the analysis of *gyrB* gene SNPs, 23S rRNA, and the RD1 deletion, named GenoType MTBC (Hain Lifescience GmbH, Nehren, Germany), has recently become available (37, 42, 50). This line probe assay enables identification of the presence of various members of the MTBC but does not differentiate *M. canettii* from *M. tuberculosis* and *M. africanum* type I from *M. pinnipedii*.

Various molecular methods for genotyping of *M. tuberculosis* have also been developed and were recently reviewed by

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Mathema and colleagues (36). The most commonly used methods include the “gold standard” analysis of highly conserved DNA fingerprinting patterns obtained by RFLP analysis of the insertion sequence IS6110 (IS6110-RFLP fingerprinting) (54), the study of variations within the genomic direct repeat region by spoligotyping (10, 32), and the typing of mycobacterial interspersed repetitive-unit-variable number of DNA tandem repeats (MIRU-VNTR) (52). They are powerful molecular epidemiological typing methods since they are based on the analysis of mobile DNA elements (IS6110-RFLP fingerprinting) or repetitive DNA elements (spoligotyping and MIRU-VNTR typing) that change quite rapidly and thus provide a high degree of discriminatory power, but they are less useful for definition of phylogenetic groupings (14, 21). Recently, two studies have revealed that SNPs are valuable phylogenetically informative markers (18, 26), and the use of a minimal number of sets of SNPs has been proposed as a means of resolving *M. tuberculosis* and *M. bovis* isolates into seven genetic groups called SNP cluster groups (SCGs) (1, 18).

Thus, SNPs can be useful markers for both the identification of MTBC species and the genotyping of *M. tuberculosis* isolates. We describe here the development of an SNP typing-based strategy that was designed to simultaneously distinguish members of the MTBC to the species level and *M. tuberculosis* lineages. Among the various SNP typing methodologies described in the literature, we selected the SNaPshot minisequencing-based approach because of its high multiplexing capacity, robustness, and extreme sensitivity (22, 49). Moreover, it is a relatively simple and affordable method that requires the use of a thermal cycler and a genetic analyzer, which are types of equipment commonly available in molecular biology laboratories. This approach is based on the single-base extension (SBE) of an unlabeled minisequencing primer that anneals one base upstream of the relevant SNP with a fluorochrome-labeled dideoxynucleotide (ddNTP). The allelic state is then determined after separation of the extension products and the detection of fluorescence by capillary electrophoresis. This SNP typing method is currently widely used in the fields of forensic and population genetics (8, 9, 25, 44, 47) and has recently raised interest in clinical research (2, 5, 17, 31, 39). It was also applied in a large-scale study for the elucidation of the *M. tuberculosis* population substructure and evolution (26, 27), and a recent study reports on its use for the identification of six common mycobacterial species (*M. tuberculosis*, *M. avium*, *M. intracellulare*, *M. chelonae*, *M. kansasii*, and *M. goodii*) (55). We propose here its application for the rapid and simultaneous identification of MTBC species and the genotypic characterization of *M. tuberculosis* isolates.

MATERIALS AND METHODS

Bacterial strains tested and reference tests. The following 56 MTBC strains were used for the development of the assays: *M. tuberculosis* H37Rv ATCC 27294; *M. bovis* CIP 102426; *M. bovis* BCG CIP 105226; and clinical isolates of *M. tuberculosis* ($n = 35$), *M. bovis* ($n = 6$), *M. bovis* BCG ($n = 4$), *M. africanum* ($n = 4$), *M. canettii* ($n = 1$), *M. caprae* ($n = 1$), *M. microti* ($n = 1$), and *M. pinnipedi* ($n = 1$) (the *M. pinnipedi* isolate was recovered from a tapir, and the others were isolated from human clinical samples). Eleven clinical isolates of mycobacteria other than members of the *M. tuberculosis* complex (MOTT), comprising *M. fortuitum* ($n = 2$), *M. kansasii* ($n = 2$), *M. abscessus* ($n = 1$), *M. avium* ($n = 3$), *M. chelonae* ($n = 2$), and *M. goodii* ($n = 1$), as well as DNA samples from four bacteria that do not belong to the genus *Mycobacterium*

(*Nocardia nova*, *Corynebacterium amycolatum*, *Staphylococcus aureus*, and *Escherichia coli*) were used as negative controls.

DNA samples of control strains *M. tuberculosis* H37Rv ATCC 27294, *M. bovis* CIP 102426, and *M. bovis* BCG CIP 105226 were provided by the Laboratory of Bacteriology of Strasbourg, Strasbourg, France. DNA extraction was performed from solid cultures by use of a MagNA Pure LC DNA III isolation kit (Roche Diagnostics, Indianapolis, IN), according to the manufacturer's protocol. Samples of clinical isolates, which were prepared by simple thermolysis, were provided by the Laboratories of Bacteriology of Strasbourg, France, and Toulouse, France, and the Centre National de Référence des Mycobactéries in Paris, France. A loop of culture (i.e., bacteria grown on Lowenstein-Jensen or Coletos medium) was suspended in 300 to 500 μ l water, and the mixture was heated at 95°C for 15 to 20 min. Mycobacterial cells were disrupted by sonication for 15 min or heat shock treatment (1 min at 95°C, followed by 1 min on ice, repeated five times). The supernatants obtained after centrifugation at full speed for 5 min contained the mycobacterial extracts used for the PCR amplifications.

All isolates were previously identified to the species level by phenotypic and biochemical characterization methods after culture (43) or a gene probe assay, according to the manufacturer's protocol (GenoType MTBC; Hain Lifescience GmbH). Ten samples, including seven clinical samples of *M. tuberculosis*, one clinical sample of *M. africanum*, *M. tuberculosis* H37Rv, and *M. bovis* control strains, were further genotyped by 24-locus MIRU-VNTR typing (52). The person who performed the MIRU-VNTR typing was blinded to the species identification and to the results of SNP typing.

SNP selection. A set of 16 well-characterized SNPs was chosen from the literature (1, 18, 19, 24, 29, 33, 38, 51). The following eight SNPs were selected for use for the differentiation of MTBC species: *hsp65*⁵³¹ (C $\rightarrow$ T in *M. canettii*), *gyrB*(675) (numbers in parentheses refer to the SNP position on the gene) (C $\rightarrow$ T in *M. microti*), *gyrB*(756) (G $\rightarrow$ A in *M. caprae* and *M. bovis*), *gyrB*(1410) (C $\rightarrow$ T in *M. bovis* and *M. bovis* BCG), 16S rRNA¹²⁴⁹ (T $\rightarrow$ C in *M. pinnipedi*), and the three SNPs that determined the so-called principal genetic groups (PGGs), which are *katG*²⁰³ (ACT $\rightarrow$ ACC for PGG 1a and PGG 1b differentiation), *katG*⁴⁶³ (CTG $\rightarrow$ CGG for PGG 2), and *gyrA*⁹⁵ (ACC $\rightarrow$ AGC for PGG 3). For genotyping of the *M. tuberculosis* isolates, the minimal SNP set that has recently been proposed for use by Alland and coworkers was chosen (the bases at the following SNP positions in H37Rv: 1977, 3352929 [instead of 54394], 74092, 105139, 2460626 [instead of 144390], 232574, 311613, and 913274) (1). These SNPs define the seven prominent SCGs that were first described by Filliol et al. (18). The sequence variation and lineage defined by them can be obtained from previously published reports (1, 18). It is important to note that the SNP at position 2154724, which also belongs to the minimal set, corresponds to *katG*⁴⁶³, which was already selected for use in the first SNP set. In our study, the first eight SNPs were qualified as “species specific,” whereas the last eight were “lineage specific” or “genotype specific.”

Design and validation of PCR and minisequencing primers. Primer design was performed according to the recommendations of Sanchez et al. (45) so that the species-specific and lineage-specific SNPs were typed in two different multiplex assays: multiplex PCR 1 (mPCR 1) followed by SBE 1 and mPCR 2 followed by SBE 2, respectively. The sizes of the amplicons ranged from 72 to 150 bp with mPCR 1 and 81 to 141 bp with mPCR 2. The minisequencing primers were tailed at the 5' end with a nonhomologous sequence (34) and poly(C), if necessary, to produce extension products that ranged in length from 28 to 76 nucleotides (nt) with SBE 1 and from 31 to 73 nt with SBE 2 and that differed in length from each other by 6 nt to allow sufficient separation by capillary electrophoresis. The sequences of the PCR and minisequencing primers used in this study are shown in Tables 1 and 2, respectively. All primers were tested in singleplex reactions with 1 ng template DNA under the conditions outlined by Sanchez et al. (45). Since the *M. tuberculosis* genome has a high GC content, 1 M betaine (Sigma-Aldrich, St. Louis, MO) was added to the singleplex PCR mixtures in order to reduce the formation of secondary structures in GC-rich regions (28). The amplification products were then verified by classical sequencing.

Multiplex PCR amplifications and purification of PCR products. Each mPCR was performed with 1 ng template DNA or 1 to 10 μ l mycobacterial extract for clinical samples in a 50- μ l final volume composed of 1 $\times$ PCR Gold buffer (Applied Biosystems [AB], Foster City, CA), 8 mM MgCl₂, 400 μ M each deoxynucleoside triphosphate (dNTP), 0.2 to 0.5 μ M each primer, and 2 U of AmpliTaq Gold DNA polymerase (AB). The concentrations of the PCR primers used in each reaction mixture are specified in Table 1. The thermal cycling consisted of a first denaturation step at 94°C for 5 min, followed by 33 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 65°C for 30 s and with a final extension at 65°C for 7 min. Excess PCR primers and dNTPs were removed by using a NucleoSpin Extract II kit (Macherey-Nagel,

TABLE 1. PCR primers for mPCR 1 and mPCR 2 used in this study

mPCR and marker	PCR primer sequence (5'→3')		Amplicon size (bp)	Concn (μM)
	Forward	Reverse		
mPCR 1				
<i>katG</i> ⁴⁶³	CGGTCGAACTAGCTGTGAGACAGT	AAGCCGAGATTGCCAGCCTTA	72	0.2
<i>hsp65</i> ⁶³¹	GTCTCAAACGCGGCATCGAAAAG	AATCTGCTCCTTGGTCTCGACCTC	89	0.3
<i>katG</i> ²⁰³	GTTACAGTAGATCAGCCCCATCTG	ATGAGGTCTATTGGGGCAAGGAAG	113	0.2
16S rRNA ¹⁴²⁹	TGTCCAGGGCTTCACACATGC	TTCACGGGGTCGAGTTGCAG	120	0.2
<i>gyrA</i> ⁹⁵	CGAGACCATGGGCAACATCCA	ATTGCCTGGCGAGCCGAAGT	130	0.5
<i>gyrB</i> (1410)	TCAAATCGTTTGTGAGAAGGTCTGT	CACCAACTCTCGTGCCTTACGTG	143	0.3
<i>gyrB</i> (675 + 756)	AGATCAAGCGCGACGGGTA	CGAAGTCGTATTCCGTGGTTTCG	150	0.2
mPCR 2				
232574	CCACGGCGGGGACAAGAT	AGAAAGGCGCCGCTGTAGG	81	0.3
74092	GACGGTCCGAATTGCCTTGG	GACCAGGAGAAGGCCATCAAAGAG	88	0.5
105139	ATAACGTCGGGCACTGACAAAGAG	TCCCGTATCAACTCGTAGGATCTGG	94	0.5
3352929	TCGACGTCCGGGTAGCATTC	GCGTCGCAAGCATCTGACATT	108	0.2
311613	CACCACTGTTGCCACGATGTTCTT	GGCGACTTGCTACGCGTCCTAC	114	0.3
2460626	GGGCTCGCAGCCAGACTTC	ATGATCACGGCGACCCAGAC	120	0.5
1977	TGAGAAGCTCTACGGTTGTTGTTTCG	TTTACCTCACGATGAGTTTCGATCC	131	0.3
913274	GCAATCGCCGTGCAACC	CTGCATGTTATGGGTGACGATGAC	141	0.4

Düren, Germany), according to the manufacturer's recommendations. The PCR products were eluted from the filter in 50 μl sterile water.

Multiplex SBE reactions and purification of extension products. Each SBE reaction was carried out in a 10 μl final volume containing 4 μl of SNaPshot multiplex ready reaction mixture (AB), 20 mM ammonium sulfate, 3 μl cleaned PCR products, and 0.13 to 0.58 μM each minisequencing primer. The concentrations of primers in the reaction mixture are specified in Table 2. Extension was performed for 25 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 30 s. Unincorporated ddNTPs were removed by addition of 1 U of shrimp alkaline phosphatase (GE Healthcare, Little Chalfont, United Kingdom) to the SBE mixtures and incubation at 37°C for 1 h, followed by incubation at 75°C for 15 min.

Capillary electrophoresis and data analysis. Two microliters of the purified SBE products was mixed with 17.6 μl Hi-Di formamide (AB) and 0.4 μl of GeneScan-120 LIZ internal size standard (AB), and the mixture was further analyzed by capillary electrophoresis on an ABI Prism 3100 genetic analyzer (AB) with 36-cm capillary arrays and performance-optimized polymer 4 (POP-4) (AB). The data were analyzed and the alleles were automatically called by the use of GeneMapper ID (version 3.2.1) software (AB).

RESULTS

Multiplex reactions for species differentiation within MTBC. The eight species-specific SNPs selected in this study were successfully genotyped for all 56 MTBC samples tested except *M. canettii*, for which the *gyrA*⁹⁵ locus failed to be amplified. The genotype data for the 56 MTBC samples are given in Table 3. For each sample, the MTBC species and PGG inferred from the genotype data were always concordant with the previous identification by use of a reference test and data from the literature (29). The reference strain, *M. tuberculosis* H37Rv, was identified as *M. tuberculosis* PGG3. The clinical *M. tuberculosis* samples were found to belong to PGG1b (*n* = 7), PGG2 (*n* = 23), and PGG3 (*n* = 5). All *M. bovis* and *M. bovis* BCG samples tested were found to belong to PGG1a, and only those samples had both the A allele for SNP position 756 and the T allele for the SNP position 1410 on the *gyrB* gene. *M. caprae* was easily distinguished from *M. bovis* because it had the A allele for SNP position 756 and the ancestral C allele for SNP position 1410 on the *gyrB* gene. *M. microti* and *M. pinnipedii* samples were assigned to PGG1a, and *M. canettii* was assigned to PGG1b. These three species were easily distin-

guishable from other species. Indeed, for all MTBC samples tested, the allele at the *hsp65*⁶³¹ locus was always C except in *M. canettii* (in which it was T), the allele at 16S rRNA¹²⁴⁹ locus was always T except in *M. pinnipedii* (in which it was C), and *gyrB* gene SNP position 675 was always C except in *M. microti* (in which it was T). Finally, three *M. africanum* samples were differentiated from the other species because the ancestral allele was detected for all SNPs, which also enabled us to determine that they belonged to PGG1a and thus probably belonged to *M. africanum* subtype Ia, as referred to by Huard and colleagues (29). The fourth *M. africanum* sample tested was assigned to PGG1b since it had a C allele at the *katG*²⁰³ SNP locus and thus probably belonged to *M. africanum* subtype Ib. However, the alleles detected from this *M. africanum* isolate and all PGG1b *M. tuberculosis* isolates tested were identical for all SNP loci analyzed in this study, showing that this assay does not allow discrimination between *M. africanum* subtype Ib and *M. tuberculosis* PGG1b. All negative controls always failed to amplify all loci except for, sometimes, *hsp65*⁶³¹ and/or 16S rRNA¹²⁴⁹. Examples of electropherograms obtained from each MTBC species tested are shown in Fig. 1.

Multiplex reactions for genotyping of *M. tuberculosis* isolates (SCG identification). The laboratory strain *M. tuberculosis* H37Rv, the *M. bovis* control strain, all clinical isolates of *M. tuberculosis* (*n* = 35), and one clinical *M. africanum* strain were further successfully characterized into one of the seven SCGs by means of the second multiplex assay: SCG1 (*n* = 2), SCG2 (*n* = 3), SCG3a (*n* = 2), SCG3b (*n* = 9), SCG5 (*n* = 14), SCG6a (*n* = 5), SCG6b (*M. tuberculosis* H37Rv), and SCG7 (*M. bovis*). All samples assigned to SCGs 1, 2 and 3a were found to belong to PGG1b, those assigned to SCGs 3b and 5 belonged to PGG2, and finally, those assigned to SCGs 6a and 6b belonged to PGG3, which is consistent with the results reported in the literature (18). Genotype data for the eight lineage-specific SNPs and the MIRU-VNTR typing results obtained for seven clinical *M. tuberculosis* samples, one clinical sample of *M. africanum*, *M. tuberculosis* H37Rv, and the *M. bovis* control samples are shown in Table 4. The SCGs inferred

from SNP typing were concordant with the lineages inferred from MIRU-VNTR typing (21). For example, clinical strain *M. tuberculosis* 1 was assigned to the Beijing clade by MIRU-VNTR and to SCG2 by SNP typing. According to the literature, Beijing clade isolates are exclusively present in SCG2 (18, 21). Negative controls were always negative for all SNPs except some MOTT, for which position 232574 was amplified.

DISCUSSION

The work presented here describes the successful development of two simple, rapid, and specific assays for the differentiation of MTBC members and for the further classification of *M. tuberculosis* isolates into previously described phylogenetic groups. These assays are based on the SNaPshot minisequencing approach, which was found to be a reliable and reproducible method for SNP typing. Indeed, the SNP genotyping results were always confirmed by at least two independent PCRs for each mycobacterial sample, and the results were always in complete accordance with the results obtained by previous reference identification tests.

As supported by our results, the first assay for the typing of species-specific SNPs allows the accurate differentiation of all MTBC species except *M. africanum* subtype Ib and *M. tuberculosis* PGG1b. The differentiation of these two species could be done by adding the *gyrB*^{atr} SNP (SNP position 1450 of the *gyrB* gene, G → T) to the species-specific SNP panel chosen for use in this study (29). Thus, the assay described here differentiates *M. canettii* from *M. tuberculosis* and *M. africanum* type I from *M. pinnipedii*, which is not feasible by use of the commercial GenoType MTBC DNA strip assay (Hain Lifescience GmbH). It also presents many technical advantages with respect to other PCR-based methods previously developed for MTBC species differentiation. In contrast to the previous methods, which are mostly based on an approach involving agarose gel electrophoresis (e.g., analysis of genomic deletions and PCR-RFLP assays for analysis of SNPs of the *gyrB* gene) or DNA hybridization (e.g., commercial GenoType MTBC DNA strip assay [Hain Lifescience GmbH]), the assay developed here is based on SNP detection by a multiplex minisequencing reaction, followed by capillary electrophoresis. First, this technique does not need the skilled and subjective interpretation of bands required for the other methods. Indeed, the SNaPshot minisequencing method always produced precise, unambiguous, and objective results, since allele calling was done automatically by the GeneMapper ID analysis software (AB). Second, there is no need to interpret negative results, since the SNaPshot minisequencing approach is not based on the presence or the absence of a specific signal on the resulting electropherograms. For each SNP locus investigated, the specific SBE primers must incorporate one fluorochrome-labeled ddNTP, the one that is complementary to the nucleotide present at the position of interest. On the contrary, molecular identifications based on the analysis of genomic deletions rely on the success or failure of PCR amplification in the case of a specific deletion and are thus limited by the necessity of interpreting negative results. Lastly, high-throughput analysis is possible because the process can be automated: the detection platform (i.e., the genetic analyzer) is automated, the data generated by capillary electrophoresis are automatically stored

TABLE 2. Minisequencing primers for SBE 1 and SBE 2 used in this study

Marker	No. of poly(dC) copies	Neutral sequence (5'→3')	Target specific sequence (5'→3')	Orientation ^a	Size (nt)	Concn (μM)
SBE 1						
<i>hsp65</i> ⁶³¹		AAGTCTGACAA	GTCTGACCTCCTTGGC	R	28	0.35
<i>katG</i> ⁴⁶³		CGTGAAGAAGTCTGACAA	GCCTTAAGAGCCAGATCC	R	34	0.15
<i>gyrA</i> ⁹⁵		CCACGTCGTGAAGAAGTCTGACAA	ACGCGTCGATCTAGACA	F	40	0.5
<i>katG</i> ²⁰³		ACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	GCTCATCGCCGACCA	F	46	0.14
16S rRNA ¹⁴²⁹		ACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	GGCCGGTACCAAGGGC	F	55	0.25
<i>gyrB</i> (675)	2	AACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	TCTCATAAACCTGAGACCACTC	R	64	0.14
<i>gyrB</i> (756)	13	AACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	AAGACGGGGTCAACGGT	F	70	0.4
<i>gyrB</i> (1410)	15	AACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	GTTTGTGCAGAAAGTCTGTAA	F	76	0.13
SBE 2						
1977		GTGAAGAAGTCTGACAA	GACTGCCAACGACGAA	R	31	0.3
3352929		TCGTGAAGAAGTCTGACAA	CTGACATTGGTGACACAAAC	R	37	0.2
74092		GGTGGCACGTCGTGAAGAAGTCTGACAA	GGCGTGAACCCGTAAT	R	43	0.58
105139		ACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	AGTTTCAACGGTTATCAGCG	R	49	0.58
2460626		ACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	GGACCGAAGGCTCTGG	F	55	0.58
232574	4	AACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	ACAGGGAATCATTCTG	F	61	0.2
311613	11	AACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	CCGTTGCTAGCCGACG	F	67	0.2
913274	16	AACTGACTAAACTAGGTGCCACGTCGTGAAGAAGTCTGACAA	TCGCTGGACCCCAATCTC	F	73	0.5

^a Orientation of primer according to the genomic sequence of *M. tuberculosis* H37Rv. R, reverse; F, forward.

TABLE 3. Genotypes obtained for the eight species-specific SNPs typed for the 56 MTBC strains

MTBC species (n = 56)	Nucleotide at SNP:								PGG ^a
	<i>hsp65</i> ⁶³¹	<i>katG</i> ⁴⁶³	<i>gyrA</i> ⁹⁵	<i>katG</i> ²⁰³	16S rRNA ¹²⁴⁹	<i>gyrB</i> (675)	<i>gyrB</i> (756)	<i>gyrB</i> (1410)	
<i>M. canettii</i> (n = 1)	T	T		C	T	C	G	C	1b
<i>M. tuberculosis</i> (n = 7) and <i>M. africanum</i> (n = 1)	C	T	C	C	T	C	G	C	1b
<i>M. tuberculosis</i> (n = 23)	C	G	C	C	T	C	G	C	2
<i>M. tuberculosis</i> (n = 6 including H37Rv)	C	G	G	C	T	C	G	C	3
<i>M. africanum</i> (n = 3)	C	T	C	T	T	C	G	C	1a
<i>M. pinnipedii</i> (n = 1)	C	T	C	T	C	C	G	C	1a
<i>M. microti</i> (n = 1)	C	T	C	T	T	T	G	C	1a
<i>M. caprae</i> (n = 1)	C	T	C	T	T	C	A	C	1a
<i>M. bovis</i> (n = 7) and <i>M. bovis</i> BCG (n = 5)	C	T	C	T	T	C	A	T	1a

^a Inferred from the genotyping results for *katG*⁴⁶³, *gyrA*⁹⁵, and *katG*²⁰³ and results published previously (19, 51).

in a portable format, and alleles can be automatically called by the *ad hoc* software.

Besides the differentiation of MTBC species, the first assay presented here allows the simultaneous classification of MTBC isolates into three distinct genotypic groups, termed PGGs (PGGs 1a/b, 2 and 3), which is not achieved by previously developed MTBC species identification methods. All MTBC strains can be assigned to one of the three PGGs on the basis of the comparative analysis of three SNPs (*katG*²⁰³, *katG*⁴⁶³, and *gyrA*⁹⁵) (19, 29, 51). To date, *M. tuberculosis* strains have been found to segregate to PGG1b, PGG2, and PGG3, while other MTBC members are restricted to PGG1a and PGG1b, which was also illustrated by our results (29). In the evolutionary pathway proposed by Sreevatsan et al., *M. tuberculosis* PGG1 strains are ancestral to PGG2 and PGG3 strains (51). Moreover, those authors observed through their study that PGG3 organisms are rather associated with sporadic TB cases, while PGG1 and PGG2 organisms are associated with clustered TB cases, suggesting differences in transmissibility or virulence between these genotypic groups (51). Thus, the determination of these major genetic groups, which is currently widely used, can be useful for both evolutionary and epidemiological applications.

In recent years, several additional comparative genomic studies of human-adapted members of the MTBC have identified other phylogenetically informative polymorphisms that define discrete strain lineages (1, 4, 10, 18, 20, 26). Because of the highly clonal population structure of the MTBC, the phylogenetic groupings recognized in the different studies are relatively concordant, even though they are based on the analysis of different markers (21). Although molecular epidemiological informative markers like MIRU-VNTR loci have been proposed for use in phylogenetic and population genetic analyses of MTBC, a recent comparative study conducted by Comas et al. revealed that SNPs and large sequence polymorphisms (LSPs) are the most robust and appropriate phylogenetically informative markers (14). The classification of MTBC strains into these distinct lineages is relevant for evolutionary purposes but also for TB control. Indeed, these discrete lineages are associated with particular human populations or geographical regions and show differences in virulence and disease outcomes (12, 20, 21, 35, 41, 53).

The second assay presented here allows the further accurate and unambiguous classification of *M. tuberculosis* isolates into six more resolved genotypic groups called SCGs. Because these

SCGs are congruent with the lineages defined on the basis of other phylogenetically informative markers (21), interlaboratory comparisons can be done even if different markers are studied. This second assay can be run after the first assay, if the PGG determination is not sufficient, or it can be simultaneously performed.

The main advantage of the two-step strategy presented here is that it allows the reliable simultaneous identification of MTBC species and the classification of *M. tuberculosis* lineages into distinct lineages. It is easy to perform, gives unambiguous results, and can be easily introduced in a bacteriology laboratory with an automated sequencer, a detection platform that is also needed for other common applications like DNA sequencing and MIRU-VNTR typing.

Comas et al. recently suggested a combined SNP typing–MIRU-VNTR typing scheme to generate more accurate data for epidemiological and evolutionary applications (14). Indeed, on the one hand, SNPs are robust phylogenetic markers but offer insufficient discriminatory power for routine molecular epidemiological identifications, and on the other hand, the standard 24 MIRU-VNTR loci are powerful epidemiological markers but are unable to detect all strain lineages and their discriminatory powers vary by strain lineage. The new approach proposed by Comas et al. (14) consists of the identification of the main strain lineages by SNP typing, followed by further molecular epidemiological discrimination by the use of lineage-specific sets of the most discriminatory MIRU-VNTR markers for the lineage of interest. The SNaPshot minisequencing assay used in the present study seems to be an ideal method for SNP typing for such an approach because it uses the same equipment as MIRU-VNTR typing (i.e., a thermal cycler and a genetic analyzer). Furthermore, our study shows that the reliable identification of MTBC species can also be achieved by the SNaPshot minisequencing assay. This suggests that the identification of MTBC species could easily be added as a first step to the scheme proposed by Comas et al. (14).

It is also important to note that this two-step strategy was initially developed for paleomicrobiological analyses, i.e., investigation of ancient human tissues showing lesions suggestive of TB that are suspected to contain low levels of degraded mycobacterial DNA. That is why PCR primers were carefully designed in order to obtain amplicons of less than 150 bp. This feature could be of special interest in a clinical context when laboratories must deal with samples containing small amounts of degraded DNA (e.g., fixed tissues), since short DNA frag-

FIG. 1. Electropherograms obtained from the seven MTBC species by the multiplex assay for MTBC species identification. Each plot was obtained with GeneMapper ID (version. 3.2.1) software (AB) and shows the relative fluorescence units (RFUs) versus the measured size (in nucleotides) of the SBE products relative to the GeneScan-120 LIZ internal size standard (AB). Mutated alleles are indicated by arrows.

TABLE 4. SNP and MIRU-VNTR genotyping results for 10 MTBC strains

MTBC species	Nucleotide at base:										PGG ^a	SCG ^b	24-locus MIRU-VNTR profile ^c	Lineage inferred from MIRU- VNTR analysis ^d
	1977	3352929	74092	105139	2460626	232574	311613	913274	2154724 (<i>katG</i> ⁴⁶³)	<i>gyrA</i> ⁹⁵				
Clinical <i>M. tuberculosis</i> 1	G	G	C	A	C	G	T	C	A	C	1b	2	273335444434746253213423	Beijing
Clinical <i>M. tuberculosis</i> 2	G	G	C	C	C	G	T	C	A	C	1b	3a	273-654242425253213423	Dehli/CAS
Clinical <i>M. tuberculosis</i> 3	G	G	C	C	C	G	T	C	C	C	2	3b	2535325343353625221423	Haarlem
Clinical <i>M. tuberculosis</i> 4	G	C	C	C	C	G	T	C	C	C	2	5	26443324221233622213421	LAM
Clinical <i>M. tuberculosis</i> 5	G	C	C	C	C	G	T	C	C	C	2	5	284334442252323252212423	Undetermined
Clinical <i>M. tuberculosis</i> 6	G	C	C	C	C	G	T	C	C	C	2	5	2513332442-2535252113423	Cameroon
Clinical <i>M. tuberculosis</i> 7	A	C	C	C	C	G	T	C	C	G	3	6a	2513-324223-425252213423	Undetermined
<i>M. tuberculosis</i> H37Rv	A	C	C	C	C	G	G	C	C	G	3	6b	3831323243252525262213433	H37Rv
<i>M. bovis</i> CIP 102426	G	G	T	C	C	G	T	C	A	C	1a	7	342223256421224242223352	<i>M. bovis</i>
Clinical <i>M. africanum</i>	G	G	C	C	C	G	T	C	A	C	1a	1	261725257453444242223343	West African 2

^a Inferred from the genotyping results for *katG*⁴⁶³, *gyrA*⁹⁵, and *katG*²⁰³ and results published previously (19, 51).

^b Inferred from the genotyping results and the study of Alland and coworkers (1).

^c The order of the 24 MIRU-VNTR loci (position on the *M. tuberculosis* H37Rv genome) is as follows: 0580, 2996, 0802, 0960, 1644, 3192, 0424, 0577, 2165, 2401, 3690, 4156, 2163b, 1955, 4052, 0154, 2531, 4348, 2059, 2687, 3007, 2347, 2461, and 3171.

^d Identification on the basis of the MIRU-VNTRPlus database (<http://www.miru-vntrplus.org/MIRU/index.faces>).

ments are amplified more efficiently than long fragments in such cases. We are currently testing the use of this two-step strategy with ancient human remains. However, in recent studies describing the development and validation of a similar assay for the typing of human SNPs designed for the screening of ancient human remains, we already reported on the sensitivity and robustness of the SNaPshot minisequencing approach for SNP typing (6, 7). These advantages of this SNP typing method were also reported by other authors, who successfully used it to evaluate ancient human samples or archival clinical samples (6, 7, 11, 16, 22, 31, 46).

To conclude, the present study describes the successful development of reliable SNaPshot minisequencing-based SNP assays for the identification of MTBC species, determination of the principal genetic groups (PGGs 1a/b, 2, and 3), and the further classification of *M. tuberculosis* isolates into more resolved phylogenetic groups called SCGs. SNaPshot minisequencing for SNP genotyping is currently used for various applications; here, we propose the use of this technique as a complement to current methods for the clinical diagnosis of TB and epidemiological investigations.

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