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Artículo científico

Comparative study of the genetic diversity of *Mycobacterium tuberculosis* Complex by Simplified Amplified Fragment Length Polymorphism and Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat Analysis

Estudio comparativo de la diversidad genética de *Mycobacterium tuberculosis* complex mediante análisis de Polimorfismo de Longitud de Fragmentos Amplificados y Número variable de Repeticiones en Tándem de Unidades Repetitivas Interespaciadas de micobacterias

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ABSTRACT.- Species within the *Mycobacterium tuberculosis* (MTBC) Complex are genetically monomorphic, hence the need for genotyping methods for a comprehensive understanding of the disease's epidemiology. The genetic diversity of a Spainish collection of sixty-three, GenoType MTBC® -confirmed *Mycobacterium tuberculosis* clinical isolates was assessed by simplified AFLP and 15-loci MIRU-VNTR. AFLP results showed 7 patterns (P1-P7); Dice's coefficient was 71% for P1 vs P7 and 96% for P1 vs P2 and P2 vs P4. MIRU-VNTR showed 25 unique patterns and 14 clusters. Lineages found were as follows: Haarlem (23, 36.51%), Cammeroon (2, 3.17%), LAM (12, 19.05%), West African (6, 9.52%) and EAI (1, 1.59%). Discrimination indexes were 0.61 for AFLP and 0.92 for MIRU-VNTR. In conclusion, MIRU-VNTR is robust and reproducible for MTBC genotyping. Simplified AFLP is a relatively easy-to-perform approach that might be useful for the screening of isolates or in low resource settings.

KEYWORDS: *Mycobacterium tuberculosis,* MIRU-VNTR, AFLP, clonal.

RESUMEN.- Las especies dentro del complejo *Mycobacterium tuberculosis* (MTBC) son genéticamente monomórficas, por lo tanto, existe una gran necesidad de métodos confiables de genotipificación para la comprensión de la epidemiología de esta enfermedad. Esta investigación evalúa la diversidad genética de una colección española de sesenta y tres aislados mediante el uso de polimorfismo de longitud de fragmentos amplificados (AFLP) y número variable de repeticiones en tándem de unidades repetitivas interespaciadas (15-loci MIRU-VNTR). Los resultados obtenidos mostraron 7 patrones de AFLP (P1-P7) cuyos coeficientes de Dice fueron: 71% para P1 vs P7 y 96% para P1 vs P2, y P2 vs P4. MIRU-VNTR demostró 25 patrones únicos y 14 clusters. Los linajes encontrados fueron: Haarlem (23**,** 36.51%), Cammeroon (2, 3.17%), LAM (12, 19.05%), West African (6, 9.52%) y EAI (1, 1.59%). Los índices de discriminación para AFLP fueron de 0.61 y 0.92 para MIRU-VNTR. En conclusión, este estudio demostró que MIRU-VNTR es robusto y reproducible para genotipificar MTBC. Adicionalmente, AFLP simplificado es relativamente sencillo de realizar y puede ser útil en el análisis de aislados con recursos limitados.

PALABRAS CLAVES: *Mycobacterium tuberculosis,* MIRU-VNTR, AFLP, clonal.

INTRODUCTION

In its 2017 Global Tuberculosis Report, the World Health Organization (WHO) reported an estimated 10.4 million tuberculosis (TB) cases worldwide with 1.3 million deaths from the disease plus 374000 deaths among HIV-positive people, in 2016. In Spain, the incidence of TB+VIH per 100 000 population is 8.7–12, in 2016. Although the number of TB deaths fell by 22% between 2000 and 2015, TB remained one of the top ten causes of death worldwide in 2017 (WHO 2017).

The *Mycobacterium tuberculosis* complex is composed of the closely related organisms *M. tuberculosis, M. africanum, M. bovis, and M. bovis BCG, M. caprae, M. microti, M. canettii, and M. pinnipedii* (Brosch et al. 2002). Rapid and reliable identification of the members of the M. tuberculosis complex is critical in guiding public health and primary care decision-making. This is because each organism exhibits a different epidemiology, host spectrum, geographic range, pathogenicity, and drug susceptibility profile (Van Soolingen et al. 1997).
Mycobacterium species related to the *Mycobacterium tuberculosis* complex (MTBC) are considered genetically monomorphic bacterial pathogens, due to the fact that they present, on average, a single nucleotide variation per 200bp (Achtman 2008). Consequently, understanding the this disease's epidemiology by genotyping requires different technical approaches.

Given the evolution of DNA-based assays in recent decades, many genotyping methods have been used effectively in taxonomic and identification studies of a range of bacterial genera, including M. tuberculosis. DNA fingerprinting of M. tuberculosis isolates is a helpful technique for the study of recent transmission episodes in a population, as well as for identifying potential risk factors. Fingerprinting has also made it possible to characterize earlier unsuspected transmission paths, to screen for the transmission of drug-resistant strains, and to confirm laboratory cross contamination (Cowan et al. 2002).

For routine laboratory work, different automated methods such as the GenoType MTBC assay (Hain Lifescience GMbH, Nehren) have been implemented for species-level identification of *M. tuberculosis* complex. The assay is a multiplex PCR-based, solid-phase reverse hybridization that relies on the detection of single nucleotide polymorphisms of the *gyr*B gene, and on the presence or absence of RD1 (Richter et al. 2003; Richter et al. 2004). The GenoType MTBC assay provides a rapid and accurate method for the detection of various members of the *M. tuberculosis* complex when used on grown-cultures (Richter et al. 2003; Richter et al. 2004; Gómez et al. 2007; Neonakis et al. 2007; Somoskovi et al. 2008).

Several alternative PCR-based methods have been developed and are currently widely used as molecular tools for the characterization of Mycobacterium tuberculosis isolates, mostly for TB epidemiology studies (Lambregts-Van Weezenbeek et al. 2003; Cattamanchi et al. 2006; Clark et al. 2006; Iñigo et al. 2007; Allix-Béguec et al. 2008). Restriction fragment length polymorphism (RFLP) analysis, based on the IS6110 sequence, is the reference genotyping method for *M. tuberculosis* (Van Soolingen et al. 1997). However, this method is technically demanding and time-consuming, thus making it unsuitable for real-time epidemiological intervention. A method variation, an amplified-fragment length polymorphism (AFLP) simplified test, can be carried out by using only one restriction enzyme (*Xho*I), one double strand adapter, and one PCR primer (Viader-Salvadó et al. 2009).

Currently, one of the most promising PCR-based genotyping methods is a variable number of tandem repeats (VNTR) assay that makes use of mycobacterial interspersed repetitive units (MIRU). This technique is based on the variability found in 12 specific MTB loci interspersed throughout the mycobacterial genome (Supply et al. 2001; Kremer et al. 2005; Supply 2005; Supply et al. 2006). Recently, MIRU-VNTR genotyping approaches with 15 or 24 loci (Supply et al. 2006; Alonso-Rodriguez et al. 2008; Alonso-Rodriguez et al. 2009) have been evaluated and applied in molecular epidemiological typing in mycobacteria.

The purpose of this study was to determine genetic variability, circulating the lineages and discrimination power of two PCR-based techniques: AFLPs and MIRU-VNTR (15 loci) on GenoType® MTBC-confirmed MTB clinical isolates.

MATERIALS AND METHODS

Clinical isolates.- Sixty-three clinical isolates were collected. Fifty-seven isolates, corresponding to 53 patients, were identified as belonging to the *Mycobacterium tuberculosis* complex. Isolates were part of the Microbiology Department collection at the Hospital General Universitario de Valencia, España. The collection included six *M. africanum* isolates from three patients. Isolates for this study were collected from 2008 to 2011. Clinical samples were classified as respiratory (n=35; 55.6%) and extra-pulmonary (n=28; 44.4%). For each assay, the *Mycobacterium tuberculosis* ATCC 25177 reference strain was used as a control. Clinical and epidemiological data from patients are shown in Supplementary Information 1.

Molecular species-level identification of isolates by GenoType® MTBC.- Molecular identification was performed for the entire collection as well as for the Mycobacterium tuberculosis ATCC 25177 control strain. Isolates were inactivated using heat-ultrasound methods, and their processing was as follows: 1) DNA extraction from the clinical isolates; 2) multiplex-PCR with biotin labeled primers; and, 3) reverse hybridization.

PCR procedure.- For the amplification assay, 5 µl of the inactivated bacterial supernatant was mixed with 5 µl of amplification buffer (10X), 2 µl of $MgCl^2$ (25mM), 3 µl of sterile distilled water, 0.2 µl of HotStart-Taq®DNA polymerase and 35 µl of PMN Mix (Primer/Nucleotide mix). The thermocycler (A®TEFACTindustriekulture) program consisted of: i) 95 °C for 15 minutes; ii) ten cycles of 30 seconds at 95 °C and 2 minutes at 58°C; iii) twenty cycles of 25 seconds at 95 °C, 40 seconds at 53 °C and 40 seconds at 70 °C; and iv) one final step at 70 °C for 8 minutes.

Hybridization assays.- Auto-LiPA (Innogenetics N.V., Ghent, Belgium) was the automated system used for amplicon hybridization. The instrument requires 20uL of sample, and the protocol includes chemical denaturation, a hybridization step for biotin probes binding to a membrane, and a stringent wash step and acid phosphatase staining (AP). Interpretation of results was performed according to the manufacturer's instructions.

Simplified Amplified Fragment Length Polymorphism (AFLP).- AFLP was performed with the entire collection and the *Mycobacterium tuberculosis* ATCC 25177 control strain, following the protocols published by Viader-Salvadó (2009), with modifications based on Gaafar (2003).

DNA Extraction.- Two full bacterial loops were dissolved in 400 µL TE (1X) buffer in 2ml microfuge tubes, followed by heat inactivation at 80 °C for 20 minutes. A 50µL (10mg/ml) lysozyme volume was added, and the tubes were incubated at 37 °C for 18 hours. Then, 75µL of SDS 10% (w/v) and 20µL of proteinase K (20 mg/ml) were added to the mix and the tubes were heated at 65 °C for 10 minutes. Next, 100µL of 5M NaCl and 100µL of CTAB/NaCl were added and the solution was incubated at 65 °C for 10 minutes. For DNA precipitation, 750µL of chloroform: isoamyl alcohol (24:1) was used; the vial was then vigorously shaken and centrifuged at 13000 rpm for 8 minutes. The aqueous phase was removed into a new tube, cold isopropyl alcohol in 1:0.6 (V/V) ratio was added and the mixture was homogenized by inversion, stored at -20 °C for 30 minutes, centrifuged at 13000 rpm for 15 minutes, and the supernatant removed. Finally, the obtained pellet was washed with 1mL of cold 70% ethanol, followed by inversion mixing and centrifugation at 13000 rpm for 5 minutes (twice). The pellet was left to dry, and 50µL of TE (1X) buffer was then added. The elution was incubated at 37 °C for 1 hour and stored at -20 °C.

Enzymatic Digestion.- Genomic DNA (200ng) was digested with 10 U of XhoI (Roche) for 2 hours at 37 °C in a 25µL reaction containing 2.5µL 10X buffer and sterile ultra-pure water. Digested DNA was maintained at 4 °C. Oligonucleotides: XA-1 (GTAGACTGCGTACATGCA) and XA-2 (TCGATGCATGTACGCAGT) at a stock concentration of 25µM were diluted in PCR 10X buffer to a final volume of 50µL. For hybridization, this mixture was heated at 90 °C for 5 minutes and cooled down to 4° C in a thermal cycler (A®TEFACT industriekulture) with a ramp rate of 1 °C/min. Double strand adaptors obtained were preserved at 4 °C for a week.

Adaptors Ligation.- Digested DNA (15µL) was mixed with 2.5 µM adaptors and 1U of T4 DNA ligase (Roche) in a final volume of 25µL, and incubated at 12 °C for 17 hours. Next, T4 DNA ligase was thermally inactivated at 65 °C for 10 minutes. Finally, 40µL of distilled water was added and eluted DNA was stored at -20 °C.

PCR.- PCR reactions of 25µL were assembled as follows: PCR buffer 1X, 1.5 mM MgCl2, 200µM dNTPs, 6µM XP-G primer (TGCGTACATGCATCGAGG), 2 U Hot Star Taq DNA polymerase (HAIN Lifescience) and 4, 2µL of the digested-ligated DNA solution. The thermocycler program consisted of: i) initial denaturation at 72 °C for 2 minutes; ii) sixteen cycles of 20 seconds at 94 °C, 30 seconds at 65 °C and 2 minutes at 72°C; iii) nineteen cycles of 30 seconds at 94 °C, 30 seconds at 50 °C and 2 minutes at 72 °C; and iv) a final extension step of 30 minutes at 60 °C with a subsequent permanent hold at 4 °C. Amplicons were visualized by agarose horizontal electrophoresis in a 1.5% (w/v) agarose gel with TBE 1X buffer. Electrophoresis runs were set at 55 volts for 2h30min, and products were visualized by staining with a 0.5µg/mL ethidium bromide solution (Sigma-Aldrich Co) for 20 minutes. A molecular weight marker (1Kb) was used in all assays. Identification of the bands was performed with the automated system CHEMI HR (SYNGENE). Two negative controls were used: i) DNA without digestion-ligation control, in order to identify any band related to non-specific hybridization of the primer into genomic DNA; and, ii) blank PCR reaction (negative control) without DNA, to recognize contamination or primer-related artifacts.

Relative size of the fragments was calculated with the software Bio-1D++ and similarity of the isolates was determined with Dice's coefficient in order to obtain ho-

mology matrixes and dendograms. For AFLP analysis, genetically identical isolates were expected to have similar patterns in terms of number and size of fragments; dissimilar isolates were expected to show different patterns (Gaafar et al. 2003).

MIRU-VNTR. Based on its high discrimination power (Comas et al. 2009) a 15 loci MIRU-VNTR assay (4, 26, 40, 10, 16, 31, 42, 43, ETR A, 47, 52, 53, QUB-1 1b, 1955, QUB-26) was selected based on the protocols recommended by Supply (2005).

Individual PCR reactions (final volume: 20µL) were assembled for each locus as follows: PCR 1X buffer, 3mM MgCl2 (MIRU 4, 26, 40, 47, 52, 53), 2mM MgCl2 (MIRU 10, 16, 31), 1.5mM MgCl2 (MIRU 42, 43, ETRA, QUB-11b, 1955, QUB-26), 0.2mM dNTPs, 1M betaine, 0.4 pmol primers, 0.5 U Platinum Taq DNA Polymerasa (Invitrogen) and 2µL of genomic DNA (5 ng/µL). Amplicons were separated in a 1.5% (w/v) agarose gel electrophoresis with TBE 1X and a 0.5 µg/mL ethidium bromide solution staining step. The size of the fragments for each locus was calculated thorough visual comparison with the molecular weight marker. Allelic assignment was based on the methodology proposed by Supply (2005), with a 15-digit numeric code for each isolate. This code was then entered into an on-line database platform (http://www.miru-vntrplus.org/) to establish clonal complexes and lineages. The criterion for a clonal complex in a clinical isolate was the absence of variation in more than two loci.

Discrimination index.- Discrimination power analysis of this methodology was performed according to Simpson's diversity index (Dillon et al. 1993). A value of 0.9 is considered satisfactory in order to establish appropriate conclusions (Hunter and Gaston 1988).

RESULTS

Molecular species-level identification using Genotype® MTBC showed the following (Figure 1): two clinical isolates (3.17%) were identified as *M. bovis* BCG (positive probes 4, 7, 9, 10 and 13), and six isolates (9.52%) as *M. africanum* (positive probes 4, 5, 6, 7 and 10). Control strain ATCC 25177 plus 55 clinical isolates (87; 30%) were identified as *M. tuberculosis* (positive probes 4, 5, 6, 7, y 8).

Simplified AFLP showed polymorphic restriction profiles with the XP-G primer. Consequently, restriction patterns and classification of isolates according to their homology was performed in order to establish AFLP profiles for the entire collection. Restriction fragments obtained with XP-G primer fluctuated from 7 to 12, with sizes from 200 pb to 750 pb. Electropherogram analysis allowed for identification of seven patterns (P1 to P7) (Figure 2), in which the most prevalent were P1 and P2

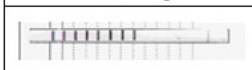
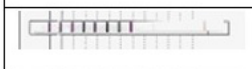
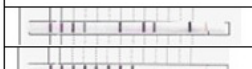
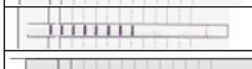
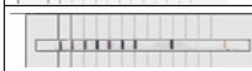
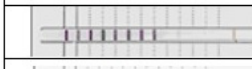
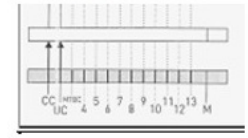
Panel Image	*Positive Probes*	*Identification Result*
	1,2,3,4,5,6,7,8	*M. tuberculosis*
	1,2,3,4,5,6,7,8	*M. tuberculosis*
	1,2,3,4,7,9,10,13	BCG
	1,2,3,4,5,6,7,8	*M. tuberculosis*
	1,2,3,4,5,6,7,10	*M. africanum*
	1,2,3,4,5,6,7,8	*M. tuberculosis*
	1,2,3,4,5,6,7,8	*M. tuberculosis*
	1,2,3,4,5,6,7,8	*M. tuberculosis*
		

Figure 1. Interpretation of GenoType® MTBC´s results

with 35 (55.6%) and 17 (26.9%) isolates, respectively. Out of the total isolate number, 52 (82.5%) belonged to P1 and P2, followed by patterns P3, P4 and P5 with three isolates (4.8% each) and P6 y P7 with one isolate (1.59%). The *M. tuberculosis* ATCC 25177 control strain restriction pattern was not present in the previously described patterns and was not included in the analysis. However, the control profile was consistently present in all assays. Reproducibility of the method was evaluated with AFLP (duplicate) characterization of five random isolates, which showed the same band pattern in each experiment.

Dice's coefficient establishes the level of genetic similarity among isolates that have different restriction patterns. For the group P5 vs. P7, the Dice's coefficient was 71%, while for P1 vs. P2 and P2 vs. P4, it was 96%. Data obtained allowed for the construction of a homology dendogram as represented in Figure 3.

All isolates classified as *M. africanum* belonged to the pattern P1, two isolates identified as M. bovis ssp. BCG were included in the P5 group, and the remaining M. tuberculosis isolates were distributed along all seven patterns.

Temporal distribution of clusters showed that the most prevalent cluster P1 was found in the years 2008, 2009, 2010 and 2011. Meanwhile, clusters P6/P7 were found in the year 2009. Geographic scattering of clusters revealed the presence of the P1 pattern in isolates from the three study sites (Manises, Hospital General Universitario de Valencia and Picassent´s Detention Centre). Patterns P3, P4, P5, P6 and P7 were not found in the Detention Centre.

Patterns from isolates recovered from patients who contributed to this study with several samples showed identical restriction profiles, with only one exception showing two different patterns in the P1 and P6 groups.

MIRU-15 data from 63 clinical isolates showed the presence of 25 unique patterns. Thirty-eight isolates (60.32%) were grouped into 14 clonal complexes (CC1 to CC14), each of them with a minimum of two isolates/cluster or a maximum of six isolates/cluster. An identical MIRUtype was found in three strains/isolates of CC4 and two isolates of CC5. Clinical isolates/strains within the study belonged to the following lineages: Haarlem (36.51%; n= 23); Cammeroon (3.17%; n=2); S (30.16%; n=19); LAM (19.05%; n=12); West African I (9. 52%; n=6); EAI (1.59%; n=1).

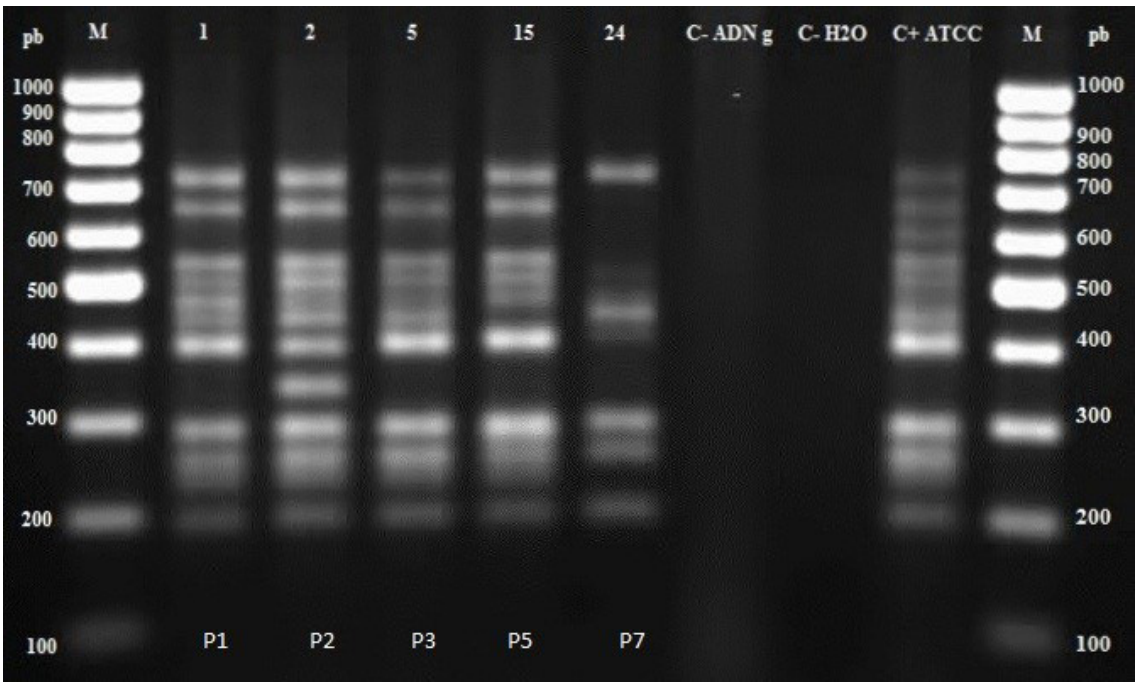


Figure 2. Restriction AFLP profiles obtained with the primer XP-G. Lane 1 and 10: M, 1Kb molecular weight marker. Lane 2 to 6: Clinical isolates restriction pattern. Lane 7: Negative control, DNA without digestion-ligation. Lane 8: Negative control without DNA. Lane 9: Restriction profile of *M. tuberculosis* ATCC 25177.

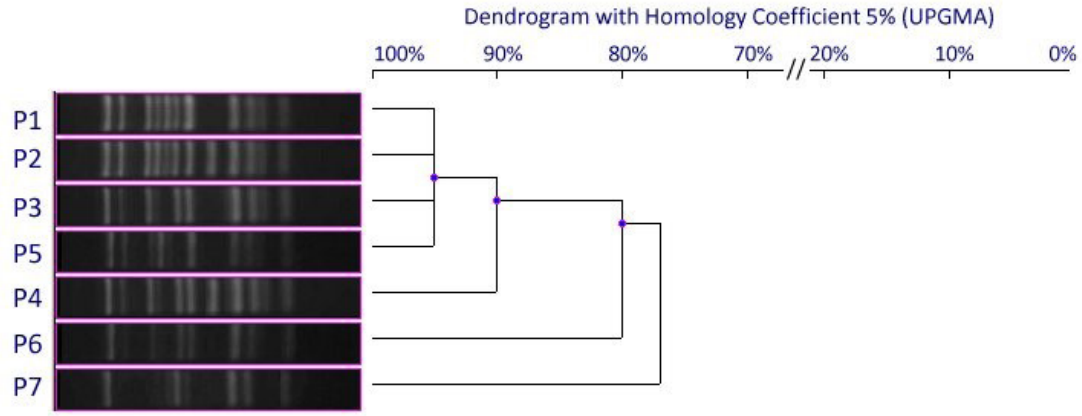


Figure 3. Homology dendogram obtained with the primer XP-G.

Simpson's diversity index was calculated in order to establish the discriminatory power of the genotyping methods. The values for AFLP and MIRU-VNTR were 0.6211 and 0.9785, respectively.

DISCUSSION

In this study, 63 clinical isolates of *Mycobacterium tuberculosis* complex were molecularly characterized and identified to species level by an automated commercially available assay (Genotype MTBC). Genotyping of the entire collection was then performed through a simplified AFLP assay as well as by a 15-loci MIRU-VNTR platform. Clustering, MIRUtypes and lineages were defined, and efficiency indexes were calculated for both AFLP and MIRU-VNTR.

Microbiological diagnosis at species level is important for targeted therapy. All *M. bovis* isolates are intrinsically resistant to pyrazinamide. *M. bovis*-BCG is used as an immunogenicity stimulator in some diseases due to its high antigenicity and immunogenic activity. In these cases, it is essential to know if the species isolated is *M. bovis*-BCG or another species within the M*ycobacterium tuberculosis* complex, which will require immediate treatment.
In 2008, Somoskovi used the automated GenoType® MTBC technique to assess its discriminatory power for Mycobacterium tuberculosis complex isolates. Thirty-five reference strains, along with 157 clinical isolates and 79 positive smear samples, were included in the study. The results showed high sensitivity and specificity for the MTBC assay, with 93.2% of the samples belonging to the *Mycobacterium tuberculosis* complex, with the exception of *M. canetti* and *M. africanum* type 2 (Richter et al. 2004) .

In our study, DNA from all clinical isolates and *M. tuberculosis* 25177 control strain hybridized to specific probes corresponding to specific *Mycobacterium tuberculosis* 87,3%), *M. bovis* BCG (3,17 %), or *M. africanum* 9,5 %) AFLP profiles. The technique is relatively easy to perform once optimized. Visualization and interpretation of band profiles are straightforward and, in our opinion, suitable for routine mycobacteriology lab work. Controls are included in the test package which allow for proper efficiency monitoring. This test has been evaluated by other authors, such as Richter, with results that support the use of the assay as a easy routine diagnostic tool for species identification of the *Mycobacterium tuberculosis* complex.

In the AFLP assay, *Xho*I restriction endonuclease (Viader-Salvadó et al. 2009) cuts the insertion sequence IS6110, which results in a fragment pattern that depends on sequence variability. In general, the typing scheme is robust, and the restriction patterns (7 to 12 bands) obtained were stable over time. Similar results were found in Viader-Salvadó's study. Distribution of clinical isolates in different profiles confirmed that *M. tuberculosis* was present in all patterns; *M. africanum* was present in the P1 group, while *M. tuberculosis* BCG was present in the P5 group. AFLP's Simpson's diversity index of 0.621 limits the assay's value as a full genotyping tool (Hunter and Gaston 1988). In our opinion, the technique, due to its capability to show clonality, its relative straightforwardness, and its highly discriminating XP-C primer, would be suitable for *M. tuberculosis* isolates screening rather than for full, in-depth genotyping. It is worth noting that the XP-C primer could be combined with XP-G (Goulding et al. 2000) for polymorphisms identification.

MIRU-VNTR (15 loci) is a PCR-based methodology

that allows full genotyping of *M. tuberculosis* complex isolates. It is more efficient and has shorter turnaround times than AFLP. Also, MIRU-VNTR has a better discrimination index (HGDI 0.9785) and allows for the identification of related lineages. Analysis of MIRUtypes showed 25 ungrouped isolates and 14 clonal complexes or clusters, based on the criterion of 2 loci variation. Clonal complex 1 was comprised of isolates 55, 58, 57, 52, 59 and 56 (as defined by the GenoType® MTBC assay). M. africanum , as depicted in the phylogenetic tree of this complex, corresponds to the 6 AFRI 1 lineage, which is in agreement with other studies (Comas et al. 2009).

Clonal complex 5 comprised isolates 61 (CFS) and 62 (gastric juice), with identical MIRU type from one neonate. This result points to the possible presence of the same genotype in different bodily sites. Isolate 60, which grew from a sample from the patient's father, showed the same MIRU type, which supports a direct transmission path. Isolates 20 (sputum) and 33 (BAL), were grouped into the clonal complex 13. Both isolates corresponded to the same patient with different sample collection dates. Isolates identified as *Mycobacterium bovis* BCG present different MIRU types within clonal complexes 6 and 12. According to the AFLP assay, these isolates group to the P5 pattern.

Lineage 4 was the most prevalent, with the following distribution: Haarlem (36.51%); Cammeroon (3.17%); S (30.16%); and LAM (19.05%). Lineage 6 (West African I 9.52%) and lineage 1 (EAI1, 59%) were also present. MIRU-VNTR displayed 14 clusters while AFLP assays showed only seven. For instance, *M. africanum* isolates are present in cluster 1 and pattern group P7. This could valuable and of interest for lineage-level typing and epidemiology studies.

Based on a classical epidemiology point of view, this study's limitation is the lack of isolates, which reduces the level of genetic variation identification in a population. However, it is appropriate for the technical validation of genotyping tools. Additionally, the used of AFLP and MIRU-VNTR have advantages and disadvantages that affect the genotypification process. AFLP has lower costs and requires time (laborious) and trained personal, while MIRU VNTR is more expensive, but more accurate than AFLP.

CONCLUSION

MIRU-VNTR (15 loci) showed a better discrimination index than the simplified AFLP assay. Since MIRU-VNTR might be refined for better discrimination (24 loci system) and its sensitivity and specificity may readily improve by coupling the assay to spoligotyping, a comprehensive typing of MTBC isolates might be feasible. Simplified AFLP would be useful for initial isolates screening or in low resources settings.

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Supplementary information

Supplementary information 1

Patient								
Case	Age	Gender	Area	Diagnosis	Type of sample	Date	Sample No.	Code
1	47	M	G	Disseminated TB - HIV	Urine	5/2/2010	9496177	MTB 01
2	32	M	G	Pulmonary tuberculosis	Sputum	11/11/2010	14174751	MTB 02
3	23	F	G	Pulmonary tuberculosis	Sputum	30/9/2010	14127700	MTB 03
4	78	F	G	Unknown	Lymph node biopsy	10/6/2010	14044424	MTB 04
5	35	M	G	Pulmonary tuberculosis	Sputum	23/10/2010	14153418	MTB 05
6	85	F	G	Disseminated TB	Wound exudate	17/7/2009	9470934	MTB 06
7	30	F	G	Pulmonary tuberculosis	BAL	10/9/2009	9477086	MTB 07
8	31	F	G	Disseminated TB	Adenopathy	15/10/2009	9481517	MTB 08
9	35	F	G	Disseminated TB	Sputum	14/1/2011	14235729	MTB 09
10	35	M	G	Pulmonary tuberculosis	Sputum	30/12/2010	14222957	MTB 10
11	0	F	G	Pulmonary tuberculosis	Gastric Juices	3/9/2009	9476264	MTB 11
12	0	F	G	Contact with TB-bacillus	Gastric Juices	15/7/2009	9470679	MTB 12
13	69	M	G	Pulmonary tuberculosis with COPD	BAL	13/11/2009	9485304	MTB 13
14	74	M	G	Hydronephrosis	Urine	18/9/2009	9478063	MTB 14
15	31	M	G	Lymph node tuberculosis	FNAB	28/8/2009	9475664	MTB 15
16	34	M	G	Lymph node tuberculosis	FNAB	14/7/2009	9470491	MTB 16
17	42	M	G	Nódulo pulmonar	Lung biopsy	21/12/2009	9490687	MTB 17
18	33	F	G	Pulmonary tuberculosis	Sputum	14/12/2009	9489825	MTB 18
19	30	M	G	Disseminated TB	Feces	26/9/2009	9479042	MTB 19
20	45	M	G	Pulmonary tuberculosis	Sputum	6/8/2010	14087416	MTB 20
21	40	M	G	Quality Control SEIMC	Sputum	31/3/2010	236	BCG 01
22	31	M	G	Pulmonary tuberculosis	Sputum	3/7/2009	9469182	MTB 21
23	51	F	G	Pulmonary tuberculosis	Sputum	7/10/2009	9480524	MTB 22
24	38	F	G	Diarrhea- HIV	Feces	20/7/2009	9471285	MTB 23
25	31	F	G	Pulmonary tuberculosis	Sputum	9/7/2009	9469914	MTB 24
26	38	M	G	TB meningitis	CSF	4/3/2009	9454044	MTB 25
27	72	F	G	Mycobacterial cervical lymphadenitis	Wound exudate	8/7/2009	9469823	MTB 26
28	66	M	G	Paravertebral abscess	FNAB	2/7/2009	9469079	MTB 27
29	63	M	G	Pulmonary tuberculosis	Sputum	16/2/2010	9497624	MTB 28
30	108	F	G	Pulmonary tuberculosis	Sputum	16/9/2009	9477732	MTB 29
31	79	M	G	Pulmonary tuberculosis	Sputum	22/12/2009	9490819	MTB 30
32	33	F	G	Lymph node tuberculosis	FNAB	28/4/2010	14003097	MTB 31
33	45	M	G	Pulmonary tuberculosis	BAL	13/7/2010	14071550	MTB 32
34	18	M	G	Pulmonary tuberculosis	Sputum	24/10/2010	14153513	MTB 33
35	43	F	G	Urinary tract tuberculosis (UTB)	Urine	12/3/2010	4600656	MTB 34
36	29	M	G	Pulmonary tuberculosis	Sputum	1/6/2010	14035630	MTB 35
37	41	M	G	Lymph node tuberculosis -HIV	Pus	23/3/2010	4601738	MTB 36
38	85	M	G	Disseminated bacillus Calmette-Guérin (BCG)	Blood	3/9/2009	9476339	BCG 02
39	39	M	G	Pulmonary tuberculosis	Sputum	18/6/2010	14052210	MTB 37
40	43	F	G	Lymph node tuberculosis	Lymph node biopsy	21/5/2010	14026279	MTB 38
41	52	M	G	Pulmonary tuberculosis	Sputum	20/5/2010	14024949	MTB 39
42	17	M	G	Diarrhea- HIV	Feces	26/6/2009	9468328	MTB 40
43	40	F	G	Psoas Abscess	Abdominal biopsy	18/3/2009	9455833	MTB 41
44	33	F	G	Lymph node tuberculosis	Ulcer exudate	25/3/2010	4602201	MTB 42
45	25	M	G	Mycobacterial cervical lymphadenitis	Surgical exudate	6/3/2009	9454335	MTB 43
46	45	F	G	Pulmonary tuberculosis- HIV	Sputum	10/7/2010	14069689	MTB 44
47	65	M	G	Pleural effusion	Pleural fluid	5/7/2010	14064998	MTB 45
48	45	M	G	Diarrhea- HIV	Feces	11/10/2010	14138743	MTB 46
49	30	F	G	Lymph node tuberculosis	Lymph node biopsy	18/5/2010	14022448	MTB 47
50	23	M	G	Pulmonary tuberculosis- HIV	Sputum	28/10/2010	14159216	MTB 48
51	31	M	G	Pulmonary tuberculosis	Sputum	17/4/2009	9250266	MTB 49
52	35	M	C	Pulmonary tuberculosis	Sputum	1/3/2009	9055525	AFRI 01
53	51	M	G	Pulmonary tuberculosis	Sputum	26/1/2010	9494804	MTB 50
54	79	M	G	Pulmonary tuberculosis	Gastric Juices	27/5/2009	9464327	MTB 51
55	21	F	C	Pulmonary tuberculosis	Sputum	1/11/2008	8116631	AFRI 02
56	35	M	C	Pulmonary tuberculosis	Sputum	1/4/2009	9064005	AFRI 03
57	44	F	C	Abdominal mass	Peritoneal fluid	1/5/2010	1083364	AFRI 04
58	21	F	C	Pulmonary tuberculosis	Sputum	1/11/2008	8116629	AFRI 05
59	21	F	C	Pulmonary tuberculosis	Sputum	1/11/2008	8116630	AFRI 06
60	24	M	G	Pulmonary tuberculosis	Sputum	5/4/2011	14323266	MTB 52
61	0	M	G	Disseminated TB	CSF	1/4/2011	14321197	MTB 53
62	0	M	G	Disseminated TB	Gastric Juices	2/4/2011	14321259	MTB 54
63	24	F	G	Pulmonary tuberculosis	BAL	23/4/2010	736	MTB 55