

Molecular tools for *Mycobacterium tuberculosis* genotyping

Herramientas moleculares empleadas en genotipificación de *Mycobacterium tuberculosis*

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ABSTRACT

Objective The present work studied molecular typing methods used for *Mycobacterium tuberculosis* characterization in order to learn about their advantages, disadvantages and discrimination power as regards the implementation of tuberculosis surveillance and control programs.

Methods To analyze the discrimination power of each method we studied articles that included Hunter-Gaston discrimination index (HGDI) values or data allowing their calculation.

Results The highest discrimination power was registered for LM-PCR followed by FLiP and 15-loci MIRU. The most frequently used methods showed an HGDI of 0.9491, 0.9519 and 0.8630 for 12-loci MIRU, RFLP-IS6110 and spoligotyping, respectively.

Conclusion *M. tuberculosis* isolates molecular characterization requires at least two molecular markers to discriminate non related isolates, as well as previous analysis to their implementation.

Key Words: Tuberculosis, *Mycobacterium tuberculosis*, molecular epidemiology, genotype, DNA, Polymorphism, Restriction Fragment Length (source: MeSH, NLM).

RESUMEN

Objetivo En el presente trabajo se estudiaron las metodologías de tipificación molecular empleadas para caracterizar *Mycobacterium tuberculosis* con el objetivo de conocer las ventajas, desventajas y poder discriminatorio para ser consideradas al momento de la implementación en los programas de vigilancia y control de la tuberculosis.

Métodos Para el análisis del poder discriminatorio de cada metodología se estudiaron los artículos que suministraban el valor del Hunter-Gaston discrimination index (HGDI) ó los datos que permitían su determinación.

Resultados Se documentó que el LM-PCR tiene una mayor capacidad discriminatoria seguida de FLiP y MIRU de 15 loci. Las metodologías más comúnmente empleadas

mostraron un HGDI de 0.9491, 0.9519 y 0.8630 para MIRU de 12 loci, RFLP-IS6110 y *spoligotyping* respectivamente.

Conclusión La caracterización molecular de aislamientos de *M. tuberculosis* requiere mínimo el análisis de al menos dos marcadores moleculares para discriminar aislamientos no relacionados y la necesidad de realizar análisis previos a la implementación de estas metodologías.

Palabras Clave: Tuberculosis, *Mycobacterium tuberculosis*, epidemiología molecular, genotipo, ADN, polimorfismo de longitud del fragmento de restricción (*fuentes: DeCS, BIREME*).

Tuberculosis (TB) is an infecto-contagious disease caused by *Mycobacterium tuberculosis* complex that affects around a third of the world's population (1). *M. tuberculosis* genome is markedly homogeneous. The species of *M. tuberculosis*, *M. africanum*, *M. bovis*, *M. bovis BCG*, *M. caprae*, *M. pinnipedii*, *M. microti* and *M. canettii* are genetically related. The presence of mutations in drug interaction places in genes *rpoB* (rifampicin), *inhA*, *KatG*, *ahpC* (isoniazid), *rrs*, *rpsl* (streptomycin), *embB* (ethambutol) and *gyrA* y *gyrB* (quinolones) results in resistance to these drugs producing a phenotypical change (2). This fact gave way to the development of methods capable of differentiating among this species isolates. Various techniques were used for the differential identification of *M. tuberculosis*, among them, those for determining unusual drug resistance, serotyping, multilocus enzyme electrophoresis (3), biochemical heterogeneity and phagus typing (4), being the latest one the standard method used until the late 1980's that presented however considerable disadvantages such as the low amount of identified phagotypes and its laboriousness.

The development of new molecular methods for *M. tuberculosis* genetic characterization has greatly contributed to the understanding of the transmission dynamics and pathogenesis of the disease (5,6). Today, the typing standard method for *M. tuberculosis* complex species is the restriction fragment length polymorphism (RFLP) -IS6110.

Molecular typing methods for *M. tuberculosis* complex are grouped in genomic methods for DNA studies such as RFLP IS6110 (7-10), polymorphic GC-rich sequence (PGRS) (11-13) analysis and pulsed-field gel electrophoresis (PFGE) on the one hand, and on the other hand, DNA-specific sequence amplification methods using polymerase chain reaction such as spoligotyping (spacer oligonucleotide typing) (14-18), ligation-mediated PCR (LM-PCR) (19,20), double repetitive element PCR (DRE-PCR) (21), fast ligation mediated PCR (FLiP) (22), fluorescent amplified-fragment length polymorphism (FAFLP) (23,24), my-

cobacterial interspersed repetitive unit (MIRU) (25) analysis, amplification and sequencing of single nucleotide polymorphism (SNP), amplification of exact tandem repeats (ETR) and Queen's University Belfast (QUB) polymorphism of variable number tandem repeat (VNTR) (Table 1).

Table 1. Several *M. tuberculosis* typing techniques

Métodos	Advantages	Disadvantages
RFLP-IS6110	The number of copies and their positions in the genome may vary from isolate to isolate (7) providing identical profiles in isolates from patients involved in recent transmission chains, and different genetic patterns in isolates from patients not associated by infection (8).	Large amounts of DNA (1-2 µg), technical skills, it is slow and has little discrimination power in isolates with less than six IS6110 copies(9), reproducibility problems in isolates presenting large amounts of IS6110 copies (10)
PGRS	Higher discrimination power in isolates with six or less IS6110 copies	Requiring large amounts of high quality DNA (11,12) and lower discrimination power in isolates with multiple copies (13).
PFGE	Higher discrimination power	Requiring large amounts of high quality DNA and efforts to establish a standardized protocol have resulted in considerable modifications.
PCR-based typing methods Spoligotyping	Typing isolates with less than six IS6110 copies and it has shown a 98 % identification specificity and 96 % sensitivity in clinical samples (14,-16).	It shows a 63 % sensitivity and 49% specificity compared with the standard RFLP-IS6110 method in molecular epidemiology studies (17,18)
LM-PCR	Requiring smaller DNA amounts than the RFLP-IS6110 (19) that has shown to be highly reproducible and to have great discrimination power (20).	
DRE-PCR	Discrimination power is high (21).	This method has shown to be difficult to reproduce (58 %) due to the weak intensity of the lanes obtained.
FLIP	The main advantage is that it is a quick method based on the IS6110 sequence from 1 ng DNA	low discrimination power in isolates with less than six IS6110 copies (22)
FAFLP	Especially useful for differentiating isolates with less than five IS6110 copies (23).	Its disadvantages include the need of isolates for culture, DNA digestion and initial investment required to implement the method (24).
MIRU	Results in a code system helpful in inter-lab comparisons. It has shown to be useful in the identification of polyclonal infections (25) as its discrimination power is similar to that of the RFLP IS6110 and it is more specific than spoligotyping when the number of IS6110 copies is less than six	Show a 65 % specificity and a 35 % sensitivity as compared with the RFLP-IS6110 in molecular epidemiology studies.
SNP	It has advantages such as allowing multiple polymorphisms analysis in a short time, rendering results in a binary format, and easy storage and inter-lab comparison.	It has shown similar results to those obtained with RFLP-IS6110 and spoligotyping.

METHODS

The characteristics, advantages and disadvantages of the different methods used for *M. tuberculosis* complex molecular typing always pose a problem at the time of choosing the most adequate. We undertook a review of literature by determining and analyzing the discrimination power for each method or combination of methods through the Hunter-Gaston discrimination index (HGDI) (26) as selection criterium at the time of implementing molecular methods in transmission studies because this is the first factor to consider at the initial stage of choosing a method (27).

Publications were searched in PubMed, Blackwell-synergy, ScienceDirect and EBSCOhost electronic data bases from March 2007 to August 2008 including the terms "*Mycobacterium tuberculosis* complex", "RFLP-IS6110", "spoligotyping", "MIRU", "VNTR", "Pulsed Field Gel Electrophoresis" "Direct repetition", "DRE-PCR", "Fluorescent amplifield fragment leng polymorphism", "FLip", "Genotyping AND *Mycobacterium*", "SNP", "LM-PCR", "HGDI AND *Mycobacterium*".

The selection criterium aimed at publications with titles related to *M. tuberculosis* complex genotyping and choosing those related to epidemiology and molecular characterization that included the Hunter-Gaston discrimination index value (HGDI) or data on the number of groupings and the number of isolates grouped in each of them to enable HGDI determination.

RESULTS

Our search exhibit as LM-PCR showed the highest HGID (0.9980), followed by FLiP (HGID 0.9945) and 15-loci MIRU (0.9620). RFLP-IS6110, the standard method used in *M. tuberculosis* molecular epidemiology, showed an average HGID of 0.9519. The combination of two or more methods showed higher HGDI values than those found when analyzing each method separately; these values were equal or close to one (28-54) (Table 2).

Table 2. Comparison of several *M. tuberculosis* typing techniques

Author	Publication year	Country	Number of samples	Number of typed samples	Method or combination of methods	Number of distinct patterns	Number of single isolates	Number of groupings	Total number of grouped strains	HGDI	Average HGDI
Skuce (28)	2002	UK	100	100	ETR	ND	ND	ND	ND	0.8100	0.8100
Kasama (29)	2006	UK	44	44	FAFLP	32	24	8	20	0.9799	0.9799
Reisig (22)	2005	Germany	131	131	FLIP	94	61	33	70	0.9945	0.9945
Burger (30)	1998	Brazil	78	78	LM-PCR	72	66	6	12	0.9980	0.9980
Banu (31)	2004	Bangladesh	48	44	MIRU12 loci	32	25	7	19	0.9715	
Evans (32)	2004	UK	70	70	MIRU12 loci	56	44	12	26	0.9930	
Godreuil (33)	2007	Burkina Faso	120	120	MIRU12 loci	71	51	20	69	0.9600	0.9491
Chin (34)	2005	Taiwan	502	502	MIRU12 loci	69	ND	ND	ND	0.9510	
Maes (35)	2008	Venezuela	41	41	MIRU12 loci	15	7	8	34	0.8700	
Maes (35)	2008	Venezuela	41	41	MIRU12 loci + spoligotyping	16	8	8	31	0.9100	0.9100
Maes (35)	2008	Venezuela	41	41	MIRU15 loci + spoligotyping	20	11	10	30	0.9300	0.9300
Maes (35)	2008	Venezuela	41	41	MIRU24 loci	21	11	10	30	0.9500	0.9500
Maes (35)	2008	Venezuela	41	41	MIRU24 loci + spoligotyping	21	11	10	30	0.9500	0.9500
Sun (36)	2004	Netherlands	68	68	MIRU15 loci	61	58	3	10	0.9940	0.9940
Chin (34)	2005	Taiwan	502	502	MIRU15 loci	84	ND	ND	ND	0.9720	0.9720
Maes (35)	2008	Venezuela	41	41	MIRU15 loci	18	9	9	32	0.9200	0.9200
Sun (36)	2004	Netherlands	68	68	MIRUETR ABC-RFLP- IS6110	67	66	1	2	1.0000	1.0000

Table 2. Comparison of several *M. tuberculosis* typing techniques

Author	Publication year	Country	Number of samples	Number of typed samples	Method or combination of methods	Number of distinct patterns	Number of single isolates	Number of groupings	Total number of grouped strains	HGDI	Average HGDI
Sun (36)	2004	Netherlands	68	68	MIRU-ETR ABC-RFLP-IS6110-Spoiligtotyping	67	66	1	2	1.0000	1.0000
Sun (36)	2004	Netherlands	68	68	MIRU-ETR ABC-Spoiligtotyping	62	59	3	9	0.9950	0.9950
Haas (37)	1997	Sierra Leone	138	135	Mixed-linker PCR	71	59	12	76	0.9067	0.9067
Skuce (28)	2002	UK	100	100	QUB-VNTR	ND	ND	ND	ND	0.9100	0.9100
Skuce (28)	2002	UK	100	100	QUB-VNTR-ETR	ND	ND	ND	ND	0.9600	0.9600
Dahle (38)	2005	Norway	14	14	RFLP-IS6110	6	5	1	9	0.6044	
Durmaz (39)	2003	Turkey	320	320	RFLP-IS6110	206	155	51	165	0.9879	
Garcia (40)	2002	Spain	147	147	RFLP-IS6110	94	69	25	78	0.9874	
Gori (15)	2005	Italy	64	64	RFLP-IS6110	61	58	3	6	0.9985	
Marínez (41)	2006	Spain	154	154	RFLP-IS6110	133	122	11	32	0.9971	
Sun (36)	2004	Netherlands	68	68	RFLP-IS6110	47	43	4	25	0.9310	
van der Zanden (42)	2002	Netherlands	314	314	RFLP-IS6110	175	132	43	182	0.9845	0.9519
Asgharzadeh (43)	2006	Iran	125	105	RFLP-IS6110	81	70	11	35	0.9897	
Barlow (44)	2001	Tanzania	93	90	RFLP-IS6110	61	55	6	35	0.9693	
Jou (45)	2005	Taiwan	155	155	RFLP-IS6110	137	122	15	33	0.9882	
Lari (46)	2005	Italy	248	248	RFLP-IS6110	201	166	35	82	0.9979	
Gómez (47)	2002	Colombia	53	53	RFLP-IS6110	51	49	2	4	0.9985	
Maces (35)	2008	Venezuela	41	41	RFLP-IS6110	18	9	9	32	0.9300	
Sun (36)	2004	Netherlands	68	68	RFLP-IS6110-Spoiligtotyping	62	59	3	9	0.9950	0.9950

ND: no data, case in which HGDI was obtained directly from the publication under study

DISCUSSION

The different typing methods show discrimination powers expressed in a wide range of HGDI values that go from 0,6044 to 0,9985. A base HIGD value of >0,95 is required to differentiate among related organisms (26), and previous studies of each method should be undertaken in the development of *M. tuberculosis* complex molecular epidemiology research.

The analysis of HGDI average values for each method under study showed that spoligotyping has an HGDI average of 0.8630; we found values ranging from 0.6582 to 0.9817, the lowest of them resulting from the analysis of isolates obtained in Nigeria and the highest in the Netherlands, which shows the diversity of circulating strains in these places probably due to population migrations in each area.

Kremer *et al* (55) studied a group of 90 *M. tuberculosis* complex strains and determined the discrimination power based on the amount of different patterns obtained by typing them using the methods under study (RFLP-IS6110, mixed-linker PCR (LM-PCR), and arbitrarily primed PCR (APPCR), RFLP-PGRS, DRE-PCR, spoligotyping, VNTR, RFLP y RFLP-IS1081). They reported that for epidemiological research the methods of choice should be RFLP-IS6110 and LM-PCR, as they show a higher number of distinct patterns (84 and 81 patterns, respectively) (23).

Clinical isolates characterization at the Instituto Nacional de Salud de Colombia resulted in 11 % of them with less than four IS6110 copies (47), contrasting with the finding reported by Asgharzadeh 2006, who found 8.6 % of isolates with less than six copies (43). Due to the low number of IS6110 copies, a clear disadvantage of this method, complementary methods have appeared such as MIRU, that combined with spoligotyping, provide a higher discrimination power than that one obtained by using a single method (56). MIRU is a recently developed molecular typing method that has the greatest acceptance at the moment, as it shows an adequate balance between variability, an essential feature to differentiate among non related isolates, and molecular marker stability (57) with intermediate sensitivity and specificity as compared with spoligotyping sensitivity and RFLP-IS6110 specificity (58). Like spoligotyping (59), this method has shown to be useful for typing *M. bovis* species that generally present few copies of IS6110 sequence, and it has a greater discrimination power as compared with spoligotyping (60). However, its standardization has taken several years in a process where

main changes refer to the primers used to enhance amplified product specificity, the magnesium chloride concentration in PCR mixture, the annealing temperature and even the loci analyzed (61), including a 24 loci, that have shown the same or greater discrimination power than RFLP-IS6110. This method has been the only one enabling discrimination of isolates with no IS6110 insertion sequences, excelling, therefore, spoligotyping and PGRS (62).

It is important to note that some methods are seldom used in genotyping and there are few publications reporting results from their implementation: FAFLP and LM-PCR data are reported in only one publication complying with our inclusion criteria. Analysis of HGDI average values in methods included in more than one publication shows that 15-loci MIRU presents the highest discrimination power (0.9620), followed by RFLP-IS6110 (0.9519), 12-loci MIRU (0.9491) and spoligotyping (0.8630) (Table 2). The average HGDI for RFLP-IS6110, excluding the 0.6044 HGDI reported by Dahle 2005, is 0.9808, this being the highest discrimination power agreeing with results reported by Kremer 2005 who found the highest discrimination power in RFLP-IS6110 (HGDI: 0.997) followed by MIRU (HGDI 0.995). Spoligotyping was not included in the analysis done by Kremer 2005 (20).

Our review methodology enabled us to establish the low number of publications determining discrimination power of molecular methods used in *M. tuberculosis* complex characterization (28 publications from 1,352). This was a limitation in the development of the present study as it has been in other review (44).

Based on the data analyzed in our study we can conclude that *M. tuberculosis* complex isolates molecular characterization requires at least two molecular markers to discriminate non related isolates as reported by Barlow 2001, who with combination of RFLP-IS6110 and VNTR- ETR obtained a higher HGDI (0.988) than the one reported for each method taken separately. Spoligotyping reaches a higher discrimination power (HGDI 0.97) in the characterization of *M. tuberculosis* complex members by increasing spacing sequences from 43 to 65 (63), as happens with RFLP-IS6110 that provides greater evidence of epidemiological relation between two isolates when combined with other methods.

Implementation of molecular methods for *M. tuberculosis* complex typing requires an analysis of their advantages and disadvantages as regards tuberculosis surveillance and control programs because their use allows for an extra 52 % detection of epidemiological relation among patients (64) and contributes to better understanding TB transmission dynamics.

The results obtained by our review evidence that laboratories should determine the discrimination power for each molecular method to enable a better selection, implementation and combination according to the specific conditions of each laboratory and the particular features of the geographic region and to guarantee their reproducibility and discrimination power ♣

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REFERENCES

1. Organización mundial de la salud OMS. Plan mundial para detener la tuberculosis 2006-2015. Actuar para salvar vidas. Hacia un mundo sin tuberculosis. Ginebra: Organización Mundial de la Salud; 2006. p. 35.
2. Musser JM. Antimicrobial agent resistance in mycobacteria: molecular genetic insights. Clin Microbiol Rev 1995;8:496-514.
3. Feizabadi MM, Robertson ID, Cousins DV, Hampson DJ. Genomic analysis of *Mycobacterium bovis* and other members of the *Mycobacterium tuberculosis* complex by isoenzyme analysis and pulsed-field gel electrophoresis. J Clin Microbiol 1996;34:1136-42.
4. Kalantri S, Pai M, Pascopella L, Riley L, Reingold A. Bacteriophage- based tests for the detection of *Mycobacterium tuberculosis* in clinical specimens: a systematic review and meta-analysis. BMC Infect Dis 2005;5:59.
5. Kato-Maeda M, Small PM. How molecular epidemiology has changed what we know about tuberculosis. West J Med 2000;172:256-9.
6. van Soolingen D, De Haas, Kremer K. Restriction fragment length polymorphism (RFLP) typing of mycobacteria. National Institute of Public Health and Environment. Bilthoven. The Netherlands; 2002.
7. van Embden JD, Cave MD, Crawford JT, Dale JW, Eisenach KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. J Clin Microbiol 1993;31:406-9.
8. Aranaz A, Liebana E, Gomez-Mampaso E, Galan JC, Cousins D, Ortega A, et al. *Mycobacterium tuberculosis* subsp. caprae subsp. nov.: a taxonomic study of a new member of the *Mycobacterium tuberculosis* complex isolated from goats in Spain. Int J Syst Bacteriol 1999;493:1263-73.
9. Gutacker MM, Smoot JC, Migliaccio CA, Ricklefs SM, Hua S, Cousins DV, et al. Genome-wide analysis of synonymous single nucleotide polymorphisms in *Mycobacterium tuberculosis* complex organisms: resolution of genetic relationships among closely related microbial strains. Genetics 2002;162:1533-43.
10. Nakamura Y, Obase Y, Suyama N, Miyazaki Y, Ohno H, Oka M, et al. A small outbreak of pulmonary tuberculosis in non-close contact patrons of a bar. Intern Med 2004;43:263-7.
11. Asgharzadeh M, Kafil HS. Current trends in molecular epidemiology studies of *Mycobacterium tuberculosis*. Biotechnol. Mol Biol Rev 2007;2:108-15.
12. Doroudchi M, Kremer K, Basiri EA, Kadivar MR, Van SD, Ghaderi AA. IS6110-RFLP and spoligotyping of *Mycobacterium tuberculosis* isolates in Iran. Scand J Infect Dis 2000;32:663-8.
13. Bauer J, Andersen AB, Kremer K, Miorner H. Usefulness of spoligotyping To discriminate IS6110 low-copy-number *Mycobacterium tuberculosis* complex strains cultured in Denmark. J Clin Microbiol 1999;37:2602-6.
14. Kodmon C, Niemann S, Lukacs J, Sor E, David S, Somoskovi A. Molecular epidemiology of drug-resistant tuberculosis in Hungary. J Clin Microbiol 2006;44:4258-61.

15. Gori A, Bandera A, Marchetti G, Degli EA, Catozzi L, Nardi GP, *et al.* Spoligotyping and *Mycobacterium tuberculosis*. *Emerg Infect Dis* 2005;11:1242-8.
16. Baker LV, Brown TJ, Maxwell O, Gibson AL, Fang Z, Yates MD, *et al.* Molecular analysis of isoniazid-resistant *Mycobacterium tuberculosis* isolates from England and Wales reveals the phylogenetic significance of the *ahpC* -46A polymorphism. *Antimicrob. Agents Chemother* 2005;49:1455-64.
17. Scott AN, Menzies D, Tannenbaum TN, Thibert L, Kozak R, Joseph L, *et al.* Sensitivities and specificities of spoligotyping and mycobacterial interspersed repetitive unit-variable-number tandem repeat typing methods for studying molecular epidemiology of tuberculosis. *J Clin Microbiol* 2005;43:89-94.
18. Supply P, Magdalena J, Himpens S, Locht C. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. *Mol Microbiol* 1997;26:991-1003.
19. Prod'homme G, Guilhot C, Gutierrez MC, Vamerot A, Gicquel B, Vincent V. Rapid discrimination of *Mycobacterium tuberculosis* complex strains by ligation-mediated PCR fingerprint analysis. *J Clin Microbiol* 1997;35:3331-4.
20. Kremer K, Arnold C, Cataldi A, Gutierrez MC, Haas WH, Panaiotov S, *et al.* Discriminatory power and reproducibility of novel DNA typing methods for *Mycobacterium tuberculosis* complex strains. *J Clin Microbiol* 2005;43:5628-38.
21. Mostrom P, Gordon M, Sola C, Ridell M, Rastogi N. Methods used in the molecular epidemiology of tuberculosis. *Clin Microbiol Infect* 2002;8:694-704.
22. Reisig F, Kremer K, Amthor B, van SD, Haas WH. Fast ligation-mediated PCR, a fast and reliable method for IS6110-based typing of *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 2005;43:5622-7.
23. Sims EJ, Goyal M, Arnold C. Experimental versus in silico fluorescent amplified fragment length polymorphism analysis of *Mycobacterium tuberculosis*: improved typing with an extended fragment range. *J Clin Microbiol* 2002;40:4072-6.
24. Mortimer P, Arnold C. FAFLP: last word in microbial genotyping? *J Med Microbiol* 2001;50:393-5.
25. Garcia DV, Alonso RN, Andres S, Ruiz MJ, Bouza E. Characterization of clonal complexity in tuberculosis by mycobacterial interspersed repetitive unit-variable-number tandem repeat typing. *J Clin Microbiol* 2005;43:5660-4.
26. Hunter P, Gaston M. Numerical Index of the Discriminatory Ability of Typing Systems: an Application of Simpson's Index of Diversity. *J Clin Microbiol* 1988;26:2465-6.
27. Gaston MA, Hunter PR. Efficient selection of tests for bacteriological typing schemes. *J Clin Pathol* 1989;42:763-6.
28. Skuce RA, McCorry TP, McCarroll JF, Roring SM, Scott AN, Brittain D. Discrimination of *Mycobacterium tuberculosis* complex bacteria using novel VNTR-PCR targets. *Microbiology* 2002;148: 519-28.
29. Kassama Y, Shemko M, Shetty N, Fang Z, Macintyre G, Gant V, *et al.* An improved fluorescent amplified fragment length polymorphism method for typing *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006;44:288-9.
30. Burger M, Raskin S, Brockelt SR, Amthor B, Geiss HK, Haas WH. DNA fingerprinting of *Mycobacterium tuberculosis* complex culture isolates collected in Brazil and spotted onto filter paper. *J Clin Microbiol* 1998;36:573-6.
31. Banu S, Gordon SV, Palmer S, Islam MR, Ahmed S, Alam KM. Genotypic analysis of *Mycobacterium tuberculosis* in Bangladesh and prevalence of the Beijing strain. *J Clin Microbiol* 2004;42:674-82.
32. Evans JT, Hawkey PM, Smith EG, Boese KA, Warren RE, Hong G. Automated high-throughput mycobacterial interspersed repetitive unit typing of *Mycobacterium tuberculosis* strains by a combination of PCR and non denaturing high-performance liquid chromatography. *J Clin Microbiol* 2004;42:4175-80.
33. Godreuil S, Torrea G, Terru D, Chevenet F, Diagbouga S, Supply P, *et al.* First molecular epidemiology study of *Mycobacterium tuberculosis* in Burkina Faso. *J Clin Microbiol* 2007;45:921-7.

34. Chin PJ, Jou R. A modified automated high-throughput mycobacterial interspersed repetitive unit method for genotyping *Mycobacterium tuberculosis*. *Diagn Microbiol Infect Dis* 2005;53: 325-7.
35. Maes M, Kremer K, van Soolingen D, Takiff H, de Waard J. 24-Locus MIRU-VNTR genotyping is a useful tool to study the molecular epidemiology of tuberculosis among Warao Amerindians in Venezuela. *Tuberculosis* 2008;88:490-4.
36. Sun YJ, Bellamy R, Lee AS, Ng ST, Ravindran S, Wong SY, *et al.* Use of mycobacterial interspersed repetitive unit-variable-number tandem repeat typing to examine genetic diversity of *Mycobacterium tuberculosis* in Singapore. *J Clin Microbiol* 2004;42:1986-93.
37. Haas WH, Bretzel G, Amthor B, Schilke K, Krommes G, Rusch-Gerdes S, *et al.* Comparison of DNA fingerprint patterns of isolates of *Mycobacterium africanum* from east and west Africa. *J Clin Microbiol* 1997;35:663-6.
38. Dahle UR, Nordtvedt S, Winje BA, Mannsaaker T, Heldal E, Sandven P, *et al.* Tuberculosis in contacts need not indicate disease transmission. *Thorax* 2005;60:136-7.
39. Durmaz R, Gunal S, Yang Z, Ozerol IH, Cave MD. Molecular epidemiology of tuberculosis in Turkey. *Clin Microbiol Infect* 2003;9:873-7.
40. Garcia MR, Rodriguez JC, Navarro JF, Samper S, Martin C, Royo G. Molecular epidemiology of tuberculosis in Elche, Spain: a 7-year study. *J Med Microbiol* 2002;51:273-7.
41. Martinez M, Garcia D, Alonso VM, Andres S, Bouza E, Cabezas T, *et al.* Impact of laboratory cross-contamination on molecular epidemiology studies of tuberculosis. *J Clin Microbiol* 2006;44:2967-9.
42. van der Zanden AG, Kremer K, Schouls LM, Caimi K, Cataldi A, Hulleman A, *et al.* Improvement of differentiation and interpretability of spoligotyping for *Mycobacterium tuberculosis* complex isolates by introduction of new spacer oligonucleotides. *J Clin Microbiol* 2002;40:4628-39.
43. Asgharzadeh M, Shahbadian K, Majidi J, Aghazadeh AM, Amini C, Jahantabi AR, *et al.* IS6110 restriction fragment length polymorphism typing of *Mycobacterium tuberculosis* isolates from East Azerbaijan Province of Iran. *Mem Inst Oswaldo Cruz*. 2006;101:517-21.
44. Barlow RE, Gascoyne-Binzi DM, Gillespie SH, Dickens A, Qamer S, Hawkey PM. Comparison of variable number tandem repeat and IS6110-restriction fragment length polymorphism analyses for discrimination of high- and low-copy-number IS6110 *Mycobacterium tuberculosis* isolates. *J Clin Microbiol* 2001;39:2453-7.
45. Jou R, Chiang CY, Huang WL. Distribution of the Beijing family genotypes of *Mycobacterium tuberculosis* in Taiwan. *J Clin Microbiol* 2005;43:95-100.
46. Lari N, Rindi L, Sola C, Bonanni D, Rastogi N, Tortoli E, *et al.* Genetic diversity, determined on the basis of katG463 and gyrA95 polymorphisms, Spoligotyping, and IS6110 typing, of *Mycobacterium tuberculosis* complex isolates from Italy. *J Clin Microbiol* 2005;43:1617-24.
47. Gomez JE, Leon CI, Inirida M, Leen R, Françoise P. IS6110 fingerprinting of sensitive and resistant strains (1991-1992) of *Mycobacterium tuberculosis* in Colombia. *Mem Inst Oswaldo Cruz* 2002;97:1005-8.
48. Cadmus S, Palmer S, Okker M, Dale J, Gover K, Smith N, *et al.* Molecular analysis of human and bovine tubercle bacilli from a local setting in Nigeria. *J Clin Microbiol* 2006;44:29-34.
49. Gazouli M, Ikonomopoulos J, Koundourakis A, Bartos M, Pavlik I, Overduin P, *et al.* Characterization of *Mycobacterium tuberculosis* complex isolates from Greek patients with sarcoidosis by Spoligotyping. *J Clin Microbiol* 2005;43:4858-61.
50. Jou R, Chen HY, Chiang CY, Yu MC, Su IJ. Genetic diversity of multidrug-resistant *Mycobacterium tuberculosis* isolates and identification of 11 novel rpoB alleles in Taiwan. *J Clin Microbiol* 2005;43:1390-4.
51. Goguuet de la Salmoniere YO, Li HM, Torrea G, Bunschoten A, van EJ, Gicquel B. Evaluation of spoligotyping in a study of the transmission of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1997;35:2210-4.

52. Niobe-Eyangoh SN, Kuaban C, Sorlin P, Cunin P, Thonnon J, Sola C, *et al.* Genetic biodiversity of *Mycobacterium tuberculosis* complex strains from patients with pulmonary tuberculosis in Cameroon. *J Clin Microbiol* 2003;41:2547-53.
53. Zozio T, Allix C, Saribas Z, Alp A, Durmaz R, Fauville-Dufaux M, *et al.* Genotyping of *Mycobacterium tuberculosis* clinical isolates in two cities of Turkey: description of a new family of genotypes that is phylogeographically specific for Asia Minor. *BMC Microbiol* 2005;5:44.
54. Singh UB, Suresh N, Bhanu NV, Arora J, Pant H, Sinha S, *et al.* Predominant tuberculosis spoligotypes, Delhi, India. *Emerg Infect Dis* 2004;10:1138-42.
55. Kremer K, van SD, Frothingham R, Haas WH, Hermans PW, Martin C, *et al.* Comparison of methods based on different molecular epidemiological markers for typing of *Mycobacterium tuberculosis* complex strains: interlaboratory study of discriminatory power and reproducibility. *J Clin Microbiol* 1999;37:2607-18.
56. Gopaul KK, Brown TJ, Gibson AL, Yates MD, Drobniewski FA. Progression toward an improved DNA amplification-based typing technique in the study of *Mycobacterium tuberculosis* epidemiology. *J Clin Microbiol* 2006;44:2492-8.
57. Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, *et al.* Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006;44:4498-510.
58. Lee AS, Tang LL, Lim IH, Bellamy R, Wong SY. Discrimination of single-copy IS6110 DNA fingerprints of *Mycobacterium tuberculosis* isolates by high-resolution minisatellite-based typing. *J Clin Microbiol* 2002;40:657-9.
59. Zanini MS, Moreira EC, Lopes MT, Oliveira RS, Leao SC, Fioravanti RL, *et al.* *Mycobacterium bovis*: polymerase chain reaction identification in bovine lymphonode biopsies and genotyping in isolates from Southeast Brazil by spoligotyping and restriction fragment length polymorphism. *Mem Inst Oswaldo Cruz* 2001;96:809-13.
60. Murray M, Alland D. Methodological problems in the molecular epidemiology of tuberculosis. *Am J Epidemiol* 2002;155:565-71.
61. Sarmiento OL, Weigle KA, Alexander J, Weber DJ, Miller WC. Assessment by meta-analysis of PCR for diagnosis of smear-negative pulmonary tuberculosis. *J Clin Microbiol* 2003;41:3233-40.
62. National TB Controllers Association/CDC Advisory Group on Tuberculosis Genotyping. Guide to the Application of Genotyping to Tuberculosis Prevention and Control. Atlanta, GA: US Department of Health and Human Services, CDC; 2004.
63. Brudey K, Gutierrez MC, Vincent V, Parsons LM, Salfinger M, Rastogi N, *et al.* *Mycobacterium africanum* genotyping using novel spacer oligonucleotides in the direct repeat locus. *J Clin Microbiol* 2004;42:5053-7.
64. McNabb SJ, Kammerer JS, Hickey AC, Braden CR, Shang N, Rosenblum LS, *et al.* Added epidemiologic value to tuberculosis prevention and control of the investigation of clustered genotypes of *Mycobacterium tuberculosis* isolates. *Am J Epidemiol* 2004;160:589-97.