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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*, epidemiologia molecular, genotipo, ADN, polimorfismo de longitud del fragmento de restricción (*fuente: 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. turberculosis, 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* (izoniacid), *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
Kassama (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	MIRU 12 loci	32	25	7	19	0.9715	0.9491
Evans (32)	2004	UK	70	70	MIRU 12 loci	56	44	12	26	0.9930	
Godreuil (33)	2007	Burquina Faso	120	120	MIRU 12 loci	71	51	20	69	0.9600	
Chin (34)	2005	Taiwan	502	502	MIRU 12 loci	69	ND	ND	ND	0.9510	
Maes (35)	2008	Venezuela	41	41	MIRU 12 loci	15	7	8	34	0.8700	
Maes (35)	2008	Venezuela	41	41	MIRU 12 loci +spoligotyping	16	8	8	31	0.9100	0.9100
Maes (35)	2008	Venezuela	41	41	MIRU 15 loci + poligotyping	20	11	10	30	0.9300	0.9300
Maes (35)	2008	Venezuela	41	41	MIRU 24 loci	21	11	10	30	0.9500	0.9500
Maes (35)	2008	Venezuela	41	41	MIRU 24 loci + poligotyping	21	11	10	30	0.9500	0.9500
Sun (36)	2004	Netherlands	68	68	MIRU 15 loci	61	58	3	10	0.9940	0.9940
Chin (34)	2005	Taiwan	502	502	MIRU 15 loci	84	ND	ND	ND	0.9720	0.9720
Maes (35)	2008	Venezuela	41	41	MIRU 15 loci	18	9	9	32	0.9200	0.9200
Sun (36)	2004	Netherlands	68	68	MIRU-ETR ABC-RFLP-IS6110	67	66	1	2	1.0000	1.0000

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

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Sun (36)	2004	Netherlands	68	68	MIRU-ETR ABC-RFLP-IS6110-Spoligotyping	67	66	1	2	1.0000	1.0000
Sun (36)	2004	Netherlands	68	68	MIRU-ETR ABC-Spoligotyping	62	59	3	9	0.9950	0.9950
Haas (37)	1997	Sierra Leona	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	
Martínez (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	0.9982	
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	
Maes (35)	2008	Venezuela	41	41	RFLP-IS6110	18	9	9	32	0.9300	
Sun (36)	2004	Netherlands	68	68	RFLP-IS6110-Spoligotyping	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 HGID (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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