

Molecular typing methods used in studies of *Mycobacterium tuberculosis* in Iran: a systematic review

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ABSTRACT

Background and Objectives: Molecular typing methods are important and useful tools to assess the transmission, diversity of strains and differentiation between new infections and relapses which can effectively help in controlling infections. The aim of this study was to evaluate the molecular typing methods which have been used in Iran. By evaluating the results and discriminatory power of each method, we can assign appropriate weight to each technique and ultimately offer a common strategy for future epidemiological studies.

Methods: We searched several databases to identify studies addressing *Mycobacterium tuberculosis* molecular epidemiology in Iran. Hunter-Gaston discrimination index (HGDI) was used to evaluate the discriminatory power in each method. Relevant articles were selected and analyzed; HGDI index was calculated for each technique.

Results: The most common genotyping methods used in the articles were RFLP, MIRU-VNTR, spoligotyping, PFGE and RAPD-PCR. The most frequently techniques were IS6110-RFLP, MIRU-VNTR and spoligotyping alone or in combination. The highest discrimination power (average HGDI: 0.9916) was obtained by RFLP followed by MIRU-VNTR (average HGDI: 0.9638) and spoligotyping (average HGDI: 0.9041) respectively.

Conclusion: Combination of MIRU-VNTR with spoligotyping can be recommended for large-scale genotyping in Iran. It seems appropriate to consider spoligotyping as the first technique for screening followed by other techniques with higher discrimination power such as MIRU-VNTR or IS6110-RFLP.

Keywords: *Mycobacterium tuberculosis*, Molecular epidemiology, Genotyping, HGDI, RFLP, MIRU-VNTR, Spoligotyping

INTRODUCTION

Mycobacterium tuberculosis is one of the most important pathogens in the world (1). The emergence of Multi-Drug Resistant strains (MDR) and co-infection with the HIV virus is considered one of the biggest health problems (2). According to the World Health Organization reports, in 2013, nine million

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people developed TB out of which 1.5 million lost their lives due to this disease. The WHO report in 2014 showed the number of people with TB was on the rise; the report also emphasized that the number of MDR-TB had increased in the past two decades (3). As Iran is in close proximity to high-prevalence countries, prevention of the spread of tuberculosis cases particularly MDR-TB is one of the priorities of the country (4).

The use of methods that can identify and track TB transmission is helpful in controlling the disease. Planning for TB control needs an identification of the sources of infection and the spread of disease. With the development of molecular epidemiology in recent years, the possibility of studying the epidemiology of infectious diseases has increased significantly. For understanding the path for disease transmission, molecular epidemiology studies are essential in order to prevent the spread of disease (5). Therefore, genotyping techniques are powerful tools for identifying the outbreak, contact tracing, and studying the diversity of strains. Also, increasing knowledge in this area may be considered as an effective way to prevent transmission (6).

Many different molecular epidemiology techniques have been proposed for identifying the genetic relationship between different strains of *Mycobacterium tuberculosis* and *bovis* (6-8). *M. tuberculosis* has very conserved genome; as little nucleotide diversity was reported in their genome, this organism is genetically monomorphic (9). Although genome of *M. tuberculosis* complex is highly conserved in comparison to other bacterial pathogens, some variation does exist (10, 11).

Today, there are various methods for genotyping of *M. tuberculosis* which is called fingerprinting techniques. Genotyping methods can be briefly classified as follows:

1) Sequence-based standard methods such as whole genome sequencing.

2) Non Sequence-based methods: these methods are generally classified into two categories:

A) Non-amplified methods (gel- based techniques) such as Pulsed Field Gel Electrophoresis (PFGE) and Restriction Fragment Length Polymorphism (RFLP)

B) Amplified methods (PCR-based genotyping methods) such as spoligotyping, Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU- VNTR), Random Amplified Polymorphic DNA Polymerase chain reaction (RAPD-

PCR), Repetitive element palindromic PCR (REP-PCR).

Often these techniques are based on the repetitive sequences (6). There are two types of repetitive units, interspersed repeats (IR) (Direct Repeats [DR], insertion sequence-like repeats [IS]) and tandem repeats (TR) (variable-number tandem repeats [VNTR]) (10, 11). Each method has some advantages and disadvantages (6, 9, 12, 13).

OBJECTIVES

The aim of this study was to evaluate the molecular typing methods used in Iran. By evaluating discriminatory power of each method and comparing the results, we can assign appropriate weight to each technique and propose a common strategy for future epidemiological studies. Hunter-Gaston Discrimination Index (HGDI) was used to evaluate the Discriminatory Power for each method (12).

METHODS

Search strategy. We searched several databases such as PubMed, Web of Science, Scopus, Iran Medex, Google Scholar, and Scientific Information Database (SID) to identify studies addressing *M. tuberculosis* molecular epidemiology in Iran. Keywords that were selected for this research were: molecular epidemiology, tuberculosis and Iran. Moreover, to search for articles that were published in Persian, the corresponding Persian keywords were used.

Having the search conducted, 25 articles in English and 9 Persian articles were shortlisted; while others were excluded from the study due to lack of relevance or unavailability. Papers either contained HGDI index or the information necessary to calculate this index; we reviewed the selected articles and used the already existing indexes or calculated the index based on the data extracted from the articles. The indexes were calculated according to the following equation:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j(n_j - 1).$$

Where N is the total number of strains in the sample population, s is the total number of types described, and n_j is the number of strains belonging to the j th type. D can take any figure between 0-1 while

the lowest and largest discriminatory power indexes are represented by 0 and 1, respectively. Assessing and determining the discriminatory power of the molecular epidemiology is important because based on the results of this study, a more powerful tool can be selected for the genotyping research.

RESULTS

After evaluating all articles, 34 relevant articles (Published from 2000 to 2014) were selected for analysis (Diagram 1). All 34 articles contained information necessary to calculate HGDI index. As presented in Diagram 1, molecular typing techniques frequently used in Iran were RFLP (*IS6110*, PGRS and DR),

MIRU-VNTR, spoligotyping, PFGE and RAPD-PCR. The majority of the methods used in the literature were spoligotyping with 18 studies followed by *IS6110*-RFLP and MIRU-VNTR with 9 and 7 cases respectively.

Our search demonstrated that *IS6110* - RFLP had the highest HGID (an average HGID of 0.9897), followed by Polymorphic GC-rich Repetitive Sequences (PGRS) - RFLP (average HGID: 0.9904) and MIRU-VNTR (average HGID: 0.9638) and spoligotyping (average HGID: 0.9433) and the lowest discrimination power (average HGDI: 0.6974) was obtained for PFGE. In most studies, a combination of two or three methods was used. The combination of two or more methods showed higher HGDI values than single method. HGDI values in each method were almost

Diagram 1. Number of methods and articles reviewed in the study

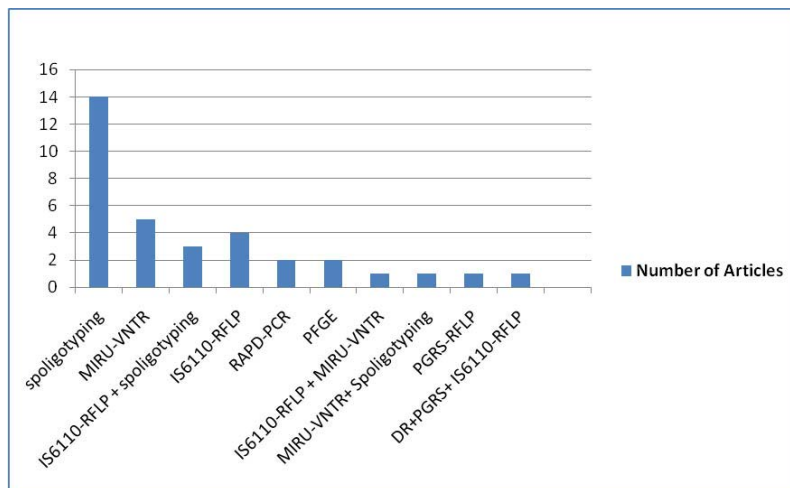


Diagram 2. Number of methods used in the articles reviewed here

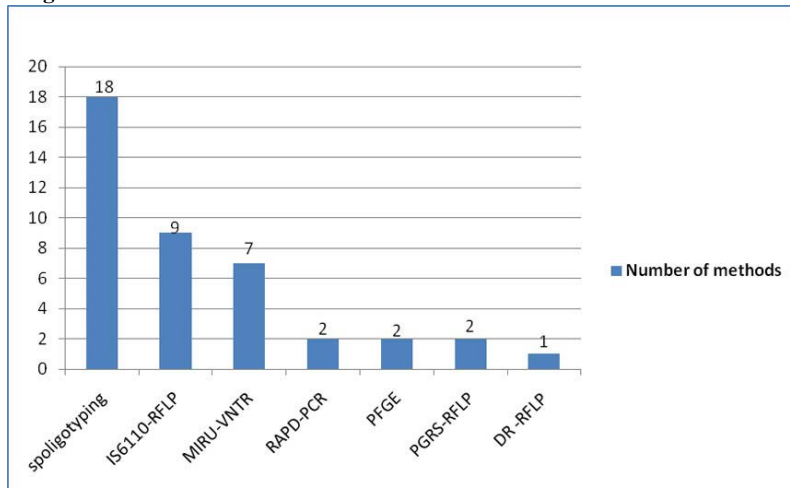


Table 1. Comparative analysis of genotyping methods of *Mycobacterium tuberculosis* published by Iranian scientists.

Reference	First author	Enrollment time	Provinces and research centers	Methods used (single, dual)	Number of isolates	Non-clustered isolates	No. of clusters	No. of clustered isolates	HGDI	Average HGDI
(15)	P. Ravan	ND	5 provinces of Iran	IS6110-RFLP	258	197	30	61	0.9990	
(34)	B. Nasiri	2008-2009	Tehran	IS6110-RFLP	291	231	25	60	0.9987	
(35)	M. Doroudchi	1995-1996	Fars	IS6110-RFLP	62	50	6	12	0.9968	
(19)	Asgharzadeh M,	2004-2005	North west of Iran	IS6110-RFLP	154	107	16	47	0.9939	
(36)	Farnia, P.	2001	Tehran	IS6110-RFLP	129	73	94	56	0.9927	0.9897
(37)	Asgharzadeh M,	2002-2003	East Azerbaijan	IS6110-RFLP	105	70	11	35	0.9897	
(38)	Farazi, A.	2011-2012	central province	IS6110-RFLP	95	20	28	75	0.9827	
(39)	Farnia, P.	2006 - 2007	NRITLD	IS6110-RFLP	258	193	29	65	0.9793	
(40)	NedaAlikosarzadeh	2010-2011	Pasteur Institute	IS6110-RFLP	100	19	25	81	0.9751	
(16)	Rafiee, B.	2010-2011	central province	PGRS(PVU II)-RFLP	57	44	6	50	0.9949	
(16)	Rafiee, B.	2010-2011	central province	PGRS(AIU I)-RFLP	57	37	8	45	0.9918	0.9904
(38)	Farazi, A.	2011-2012	central province	PGRS-RFLP	95	22	28	73	0.9847	
(38)	Farazi, A.	2011-2012	central province	DR	95	17	28	78	0.9807	0.9807
(19)	Asgharzadeh M,	2004-2005	Northwest of Iran	IS6110-RFLP + MIRU(12 loci)	154	122	13	32	0.9974	
(19)	Asgharzadeh M,	2004-2005	Northwest of Iran	IS6110-RFLP + ETR	154	121	14	33	0.9979	0.9980
(19)	Asgharzadeh M,	2004 - 2005	Northwest of Iran	IS6110-RFLP + MIRU-ETR(15loci)	154	127	13	27	0.9987	
(19)	Asgharzadeh M,	2004-2005	Northwest of Iran	ETR	154	4	17	150	0.8959	0.8959
(24)	Ahmadi, M	2009-2010	central province	MIRU-ETR(12 loci)	53	40	5	13	0.9869	
(19)	Asgharzadeh M,	2004-2005	Northwest of Iran	MIRU-ETR(15 loci)	154	103	22	51	0.9966	
(2)	Zamani, S.	2010	3 provinces of Iran	MIRU(15 loci)	121	46	20	75	0.9724	0.9845
(19)	Asgharzadeh M,	2004-2005	Northwest of Iran	MIRU(12 loci)	154	95	21	59	0.9932	
(23)	Asgharzadeh M,	2002-2003	East Azerbaijan	MIRU(12 loci)	127	72	21	55	0.9932	
(41)	Vatani,S.	2008-2010	Khuzestan	MIRU(12 loci)	61	37	11	24	0.9816	0.9510
(42)	Jafarian, M	2009	Tehran	MIRU(12loci)	60	23	10	37	0.9745	
(20)	Jafarian, M	2006-2007	Tehran	MIRU(12 loci)	140	0	9	140	0.8126	
(42)	Jafarian, M	2009	Tehran	MIRU(12 loci) + spoligotyping	60	9	11	51	0.9468	0.9468
(36)	Farnia, P.	2001	Tehran	Spoligotyping	129	33	14	96	0.9917	
(43)	Ramazanzadeh, R.	2003-2004	NRITLD	Spoligotyping	195	101	25	94	0.9849	
(44)	Rohani, M.	2004 - 2005	Mashhad	Spoligotyping	113	44	17	69	0.9688	
(45)	Haeili, M	2010-2012	5 provinces of Iran	Spoligotyping	291	40	35	251	0.9518	
(35)	Doroudchi, M.	1995-1996	Fars	Spoligotyping	97	27	7	70	0.9467	
(46)	Velayati, A. A.	2000 - 2005	NRITLD	Spoligotyping	1385	63	71	1679	0.9281	0.9433
(47)	Merza, Muayad A.	2000-2005	NRITLD	Spoligotyping	1742	63	71	1679	0.9232	
(48)	Torkaman, M. R.	2009 - 2010	NRITLD	Spoligotyping	102	25	10	77	0.911	
(49)	Farnia, P.	2000 - 2005	NRITLD	Spoligotyping	263	10	17	253	0.9066	
(50)	Mozafari, M.	2010-2011	Tehran	Spoligotyping	212	27	17	185	0.9051	
(51)	Mozafari, M.	2010-2011	24 provinces of Iran	Spoligotyping (*)	1242	77	59	1165	0.8901	
(39)	Farnia, P.	2006 - 2007	NRITLD	Spoligotyping (*)	258	27	9	231	0.882	
(52)	Mozafari, M.	No Data	NRITLD	Spoligotyping (*)	105	14	12	91	0.8619	
(53)	Amirmozafari, N	2004-2005	NRITLD	Spoligotyping (*)	220	39	9	181	0.8424	Incomplete
(54)	DerakhshaniNezhad Z	2010-2011	NRITLD	Spoligotyping (*)	106	3	9	103	0.8122	data
(55)	Sharifipour, E	2011-2012	Tehran	Spoligotyping (*)	190	52	4	138	0.8089	
(56)	Taj, aldinE	2007-2008	Tehran	Spoligotyping (*)	238	0	9	238	0.8076	
(57)	Hashemi, A.	2006-2008	Khuzestan	RAPD PCR	96	16	15	80	0.9186	0.9413
(58)	Haghighi, M.A	1996-1998	Fars	RAPD-PCR	44	15	13	29	0.964	
(31)	Khosravi, A. D.	2008-2010	Khuzestan	PFGE (<i>DraI,XbaI</i>)	60	19	17	41	0.979	0.6974
(30)	Poyeede ,M.	2010-2011	Pasteur Institute	PFGE(<i>XbaI</i>)	100	0	2	100	0.4159	

NRITLD: The National Research Institute of Tuberculosis and Lung Diseases (Tehran, Iran)

close to 100%. Results are presented in Table 1.

DISCUSSION

Different typing methods demonstrated a wide range of discrimination power. HGDI values were from 0.4159 up to 0.9990. We used HGDI index to assess the discriminatory power of molecular typing techniques. Each genotyping method has a different value for this index in different articles depending on various parameters such as number and distribution of samples (14). A minimum of HGDI value more than 0.90 is desired for a test to distinguish among related organisms (12). The analysis of HGDI values for each typing method in this study showed that IS6110-RFLP had the highest HGDI value of 0.9990 and the lowest HGDI belonged to PFGE with an average value of 0.4159. The analysis of HGDI values for IS6110-RFLP showed an average of 0.9897 ranging from 0.9990 to 0.9751. The highest value was achieved by the analysis of isolates obtained from five provinces of Iran (15), and the lowest of them was obtained from Tehran Province (16). The major advantage of this method is the higher discriminatory power it provides but it suffers from the long time required and technical difficulties in conducting. Furthermore, IS6110-RFLP has low discriminatory power in strains with fewer than 6 copy number of IS6110 sequences (13, 17).

PGRS fingerprinting in terms of technical and other conditions were similar to IS6110-RFLP. HGDI values are also similar to IS6110-RFLP. Also, it has been proven that PGRS fingerprinting is useful for differentiating *M. tuberculosis* strains with less than six copies of IS6110 that cannot be successfully examined by IS6110 fingerprinting (1, 18).

In the present study, MIRU-VNTR showed high HGDI average value of HGDI = 0.9638 after RFLP. In this study, maximum HGDI value for MIRU-VNTR was 0.9966 (19) and the lowest was 0.8126 (20). The discriminatory power of MIRU-VNTR technique depends on the number and type of selected loci, in addition to the number and distribution of samples (21). The highest HGDI value for MIRU-VNTR in Iran has been reported by Asgharzadeh and colleagues who used a 15-locus MIRU-VNTR typing method (19). Barlow et al. believe that the use of 12 appropriate loci in MIRU-VNTR will provide high discriminatory power (22). Asgharzadeh et al. (23) and Ahmadi et al. (24) who used the 12 loci MIRU-VNTR, obtained

HGDI indexes 0.9932 and 0.9869, respectively. In both articles, ETR and MIRU loci have been used. It seems that little difference in the discriminatory power of these two studies was related to sample size (127 samples versus 53 samples).

The advantages of MIRU-VNTR are high discriminatory power, high reproducibility (25) and the ability to create a numeric code for each isolate which facilitate its tracing in the database as well as ease and cost-effectiveness. Due to these advantages, this method has become a favorite method for epidemiological and phylogenetic researchers. Thus, Supply et al. proposed a 15-locus system as a new standard for routine epidemiological discrimination of *M. tuberculosis* isolates and a 24-locus system as a high-resolution tool for phylogenetic studies (21). The simultaneous use of two techniques enhances the discriminatory power. In a relevant study, Asgharzadeh et al. showed that the combination of IS6110 and MIRU-VNTR had the greater discriminatory power than either method alone (19). In another study, Barlow et al. obtained similar findings (22).

The third most widely used technique in the articles we reviewed was spoligotyping, and the average HGDI value for spoligotyping was 0.9041, the highest HGDI average value was 0.9917 and the lowest was 0.8076. However, because the results of some papers were incomplete, or they were identified only up to the super family level, the value of HGDI was lower in these articles (marked with * in Table 1). Therefore, considering only the articles with full data on the calculations, the average HGDI for this technique will be 0.9433.

As observed, the discriminatory power of spoligotyping was less than the two other techniques. The reason for the lower discriminatory power of this method is that it targets only a single genetic locus, included less than 0.1% of the *M. tuberculosis* complex genome (26). An important advantage of spoligotyping is its sensitivity which can be performed by 10 fg of chromosomal DNA, equivalent to DNA from 2-3 bacterial cells (27), so that the method can be directly performed on clinical samples, without the need for prior culture. Moreover, spoligotyping has proven to be practical for typing on nonviable samples such as paraffin-embedded tissue sections or Ziehl-Neelsen stained slides (28, 29).

Other techniques that we encountered to be used in the articles were RAPD-PCR and PFGE. As observed, their discrimination powers were less than aforemen-

tioned techniques. The lowest discrimination power was represented by PFGE (average HGDI= 0.6974). In this review, the discrimination power of PFGE was low because in one manuscript, Poyide et al. used only one restriction enzyme (*XbaI*) and obtained extremely low discrimination power (HGDI value = 0.4159) (30). Although in another study conducted by Khosravi et al., they used two restriction enzymes (*DraI* and *XbaI*) and the discrimination power was higher (HGDI value = 0.9790) (31).

In general, each typing system has its own advantages and disadvantages, and it is difficult to decide clearly which of them is superior to others. But an ideal molecular typing method must comply with the needs of researchers in terms of performance feasibility as well as analytical standards (26). The performance parameters included technical simplicity or ease of implementation, repeatability, robustness, time and cost-effectiveness. Another special advantage of the method was that it can be standardized and the results would be easily interpretable. The results should simply be comparable with the results of other laboratories as well as global databases. Another attractive advantage of a technique is its capability to be performed directly on clinical samples (26).

Analytical parameters include the level of genetic differentiation and stability of markers. A general rule is that the higher discriminatory power of the method, the more reliable the results obtained. It has been confirmed that discriminatory power of a molecular marker relates directly to its stability. Half-life of IS6110-RFLP profile is much shorter than the profile of spoligotyping (about 3 to 8 years in RFLP compared to spoligotyping with more than 50 years). The half-life of MIRU-VNTR is slightly longer than RFLP profiles and shorter than spoligotyping. The half-life of a desirable molecular marker should be short enough to separate unrelated samples from each other and on the other hand be long enough to be able to find the relationship between epidemiological samples (26).

Selecting a genotyping method, in compliance with good discriminatory power, also depends on the type of research. A technique with high discriminatory power with short half-life of genetic pattern (e.g. RFLP and MIRU-VNTR techniques) is more useful to distinguish reactivation from reinfection, while a method with a long half-life of genetic pattern (such as spoligotyping) is more useful for global strain tracking and evolutionary studies. Therefore, it is better to perform typing with a combination of these techniques.

Gold standard for *M. tuberculosis* genotyping was IS6110-RFLP, but it needs to change to MIRU-VNTR technique because of its similar discriminatory power in addition to feasibility, time and cost-effectiveness as well as the interpretation of results. More importantly, the PCR-based typing methods require fewer bacteria and can be performed in a shorter period of time. Thus, many researchers have focused on the MIRU-VNTR method as a standard technique.

Spoligotyping method has attracted the attention of researchers because of simplicity and cost-effectiveness in addition to the advantage that the results are expressed as positive or negative, according to the presence or absence of the spacers (digital format) (32). However spoligotyping will not be sufficient to be used alone in the epidemiological studies. Therefore, spoligotyping is recommended to be used as a first-line screening test, followed by techniques with higher discriminatory power such as MIRU-VNTR or IS6110-RFLP (33). Also, it should be emphasized that the present study was limited to techniques reported in Iran. Therefore, the number of articles relating to some methods was too low. There are other genotyping techniques used by researchers around the world which were not included in this article.

CONCLUSION

According to the present study, combination of MIRU-VNTR with spoligotyping can be recommended for large-scale genotyping in Iran. It seems appropriate to consider spoligotyping as the first techniques for screening followed by other techniques with higher discrimination abilities such as MIRU-VNTR or IS6110-RFLP.

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