* Corresponding author:

Mycobacteriology Research Center (MRC), National

Mehdi Jafarian, M.Sc.,

Research Institute of

Tuberculosis and Luna

Beheshti University of

Tel: +98 21 20109505

Received: 5 Sep 2010 **Accepted:** 25 Sep 2010

Iran

E-mail:

gmail.com

Disease (NRITLD), Shahid

Medical Sciences, Tehran,

mehdijafariandormency@

Synchronous Comparison of *Mycobacterium tuberculosis* Epidemiology Strains by "MIRU-VNTR" and "MIRU-VNTR and Spoligotyping" Technique

Mehdi Jafarian^{1*}, Muayed Aghali-Merza¹, Parissa Farnia¹, Mojtaba Ahmadi¹, Mohammad Reza Masjedi², and Ali Akbar Velayati²

1. Mycobacteriology Research Center, National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

2. National Research Institute of Tuberculosis and Lung Disease (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran

Abstract

Molecular epidemiology analyses are frequently used in determining epidemiology of tuberculosis. Recently, Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat (MIRU-VNTR) and Spoligotyping has become an important method, as it allows high-through put, discriminatory and reproducible analysis of clinical isolate. The purpose of this study is to compare techniques of "MIRU-VNTR" versus "MIRU-VNTR and Spoligotyping" together for study of genetic pattern of Mycobacterium tuberculosis (M. tuberculosis) strains. Sixty M. tuberculosis (MTB) isolates were selected (30 susceptible, 30 multi-drug resistant) for this study. Thereafter, the "MIRU-VNTR and Spoligotyping" were performed to identify their genetic patterns. The frequency of unknown genetic pattern of MTB was compared using technique of "MIRU-VNTR" alone versus "MIRU-VNTR and Spoligotyping" together. The MIRU-VNTR allelic diversity at each of the loci was calculated by Hunter - Gaston Discriminatory Index (HGDI). Based on differentiation index of all strains 10, 16, 26, 31 and 40 loci were identified as the most distinctive (HGI 2 0.6) and 2, 4, 20 and 24 as the weakest distinctive locus (HGI ≤ 0.3). By using MIRU-VNTR technique 38% (n= 23) of isolates could not be typed, whereas by applying "MIRU-VNTR and Spoligotyping" together only 15% (n= 9) of isolates remained unknown (p = 0.004). For sensitive strains, the difference was significant (67% us. 90%, p = 0.028), but only marginally significant for drug resistant strains (57% vs. 80%, p = 0.052). The discrimination power of 12-locus MIRU-VNTR and Spoligotyping was equal to that of MIRU-VNTR analysis. If appropriate loci are added to the standard MIRU analysis, MIRU-VNTR genotyping could be a valuable tool for strain typing and epidemiological research of *M. tuberculosis*. With this approach a more clear understanding about genetic pattern of MTB can be achieved.

Avicenna J Med Biotech 2010; 2(3): 145-152

Keywords: Alleles, Genetic Loci, Molecular epidemiology, *Mycobacterium tuberculosis*, Restriction fragment length polymorphism

Introduction

Tuberculosis is one of the main factors of vital statistic in the third world countries. Ninety percent of all tuberculosis patients live in third world countries ⁽¹⁾. In some regions

like Saharan desert in Africa 1000 of 100,000 people are infected with tuberculosis and HIV co-infection, among which one out of three dies from HIV virus ⁽²⁾. Recent, spread and

145

rise in multi-drug resistant *M. tuberculosis* (*MTB*) isolate had worsened the matter. In addition, the worldwide development of transport and migration contributes to globalize these threats. Therefore, there is an urgent need to understand and estimate the incidence of tuberculosis in large and small biographical ranges $^{(3)}$.

In this context, effective methods for accurate identification and typing is required. However, all the current typing markers suffer from significant drawbacks. For example DNA fingerprinting method based on IS6110 is a powerful tool to study molecular epidemiology of *MTB* isolates. But RFLP based on IS6110 typing is difficult and time consuming, and cannot be applied to *MTB* strains with low or no IS6110 banding patterns ⁽⁴⁾.

Spoligotyping analyzes the Direct Repeat (DR) locus in the genome of *M. tuberculosis*, which is composed of a cluster of 36 *bp* repeat sequences interspersed with unique spacers of 35 to 41 *bp*. By using a reverse Southern blotting technique, the variability in the spacer sequences can be interrogated and recorded in a digital code ⁽⁵⁾. Although this method provides digital typing data, it is only measuring variability in a single locus and does not generally provide sufficient discrimination for outbreak investigation ⁽⁶⁾.

Another molecular technique for strain typing of *MTB* is based on Variable Number Tandem Repeats (VNTRs) of Mycobacterial Interspersed Repetitive Units (MIRUs). The repeated units are 52 to 77 nucleotides in length and the number of repeated units can be determined by the size of the entire locus. Previous studies demonstrated the importance of MIRU-VNTR method for tracking epidemiological key events such as transmission or relapse and provide non-ambiguous data which are highly portable between different laboratories ⁽⁷⁾.

The purpose of this study was to compare the frequency of unknown genetic pattern of *MTB* isolates by using single technique of "MIRU-VNTR" versus the combined techniques of "MIRU-VNTR and Spoligotyping".

Materials and Methods

Design and setting

The study was conducted in Mycobacteriology Research Center, NRITLD, Shahid Beheshti University of Medical Sciences, Tehran, Iran (2009).

Participants and sampling

Sixty *MTB* isolate along with their clinical files (30 sensitive and 30 multi-drug resistant *MTB* from Mycobacteriology bank in MRC) were used for this study.

Extraction DNA

DNA from *M. tuberculosis* isolates was extracted from growth on LJ slants by a well standized method. In brief, fifty µl of Lysozyme (Merck, Germany) was added to the mycobacterial suspension in 400 μl of water, followed by stirring and incubation at 37 $^{\circ}C$. Ten μl with 10 mg/ml of proteinase K (Merck, Germany) was added to the sample, which was incubated for 10 min at 65 \mathcal{C} , and then heated at 100 °C for 30 min. One volume of phenol/ chloroform/ isoamyl-alcohol 25: 24: 1 was added to the supernatant, mixed by inversion and centrifuged at 12,000 xg for 10 min. The aqueous phase was transferred to other tubs, and the extraction procedure was repeated. A volume of chloroform/ isoamylalcohol 24: 1 was added and mixed by inversion followed by centrifugation for 10 min at 12,000 xg. One hundred μl of 5 M Nacl and two volumes of absolute ethanol were added to the supernatant. Samples were incubated for 60 min at -20 °C and centrifuged for 15 min at 12,000 xg. The pellet was washed twice with 1 ml of 70% ethanol and re suspended in 200 μl of 0.1 x TE buffer (1 mM Tris- Hcl, 0.1 mM EDTA, Ph 8). Four μl of this sample was used for PCR⁽⁸⁾.

MIRU – VNTR typing

PCR was performed in 25 μl volume that contained 5 to 50 ng of DNA, 0.5 μm of specific primers (Table 1) in the presence of 1.5 m MgCl₂, 100 μm of each d NTP, 70 m PCR buffer and 1.25 u recombinant DNA polymerase (Cinagen Co. Iran)⁽⁹⁾. DNA was amplified by general PCR. All PCRs were

Locus of MIRU	Primer sequence $(5^{2} \rightarrow 3^{2})$
2	TGGACTTGCAGCAATGGACCAACT F
2	TACTCGGACGCCGGCTCAAAA` R
4	GCGCGAGAGCCCGAACTGC F
4	GCGCAGCAGAAACGTCAGC R
10	GTTCTTGACCAACTGCAGTCGTCC F
10	GCCACCTTGGTGATCAGCTACCT R
16	TCGGTGATCGGGTCCAGTCCAAGTA F
10	CCCGTCGTGCAGCCCTGGTAC R
20	TCGGAGAGATGCCCTTCGAGTTAG F
20	GGAGACCGCGACCAGGTACTTGTA R
23	CAGCGAAACGAACTGTGCTATCAC F
25	CGTGTCCGAGCAGAAAAGGGTAT R
24	CGACCAAGATGTGCAGGAATACAT F
24	GGGCGAGTTGAGCTCACAGAA R
26	CCCGCCTTCGAAACGTCGCT F
-0	TGGACATAGGCGACCAGGCGAATA R
27	TCGAAAGCCTCTGCGTGCCAGTAA F
	GCGATGTGAGCGTGCCACTCAA R
31	ACTGATTGGCTTCATACGGCTTTA F
••	GTGCCGACGTGGTCTTGAT R
39	CGCATCGACAAACTGGAGCCAAAC F
	CGGAAACGTCTACGCCCCACACAT R
40	GGGTTGCTGGATGACAACGTGT F
	GGGTGATCTCGGCGAAATCAGATA R
Locus of	Primer sequence $(5^{2} \rightarrow 3^{2})$
Spoligotyping	
DR _a	Biotin- GGTTTTGGGTCTGACGAC
DRb	CCGAGAGGGGGACGGAAAC
MIRU-VNTR: Myco	bacterial Interspersed Repetitive Unit Variable

 Table 1. Primer sequence of the MIRU-VNTR loci and

 Spoligotyping in this study

MIRU-VNTR: Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat

initiated by a 10 *min* denaturizing step at 72 ^{o}C . The temperature cycles for different types of PCRs were as follow: 35 cycles of 1 *min* at 94 ^{o}C , annealing temperatures were used as follow: 61, 60, 64, 67, 65, 64, 61, 64, 58, 63, 62 and 64 for MIRU loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40, respectively. Also the MIRU copy numbers in the 12

MIRU-VNTR loci are shown in table 2⁽⁵⁾.

Analysis

Genetic analysis patterns MIRU-VNTR for each strain to a twenty digit profile from right to left indicate loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40, respectively. For example pattern of genetic strains H37RV (*M. tuberculosis* standard strain) a 2, 3, 3, 2, 2, 6, 1, 3, 3, 3, 2 and 1 is the genetic pattern that can be seen in this model in figure 1A ⁽¹⁰⁾.

Spoligotyping

Spoligotyping was performed as previously described by Kamerbeek et al (11). The DR region was amplified by PCR using primers derived from the DR sequence. Fifty μl of the following reaction mixture was used for the PCR: 10 ng of DNA, 20 pmol each of primers DR_a and DR_b, each deoxynucleoside triphosphate at 200 mM, PCR buffer, and 0.5 u of taq polymerase (Cinagen Co. Iran). The mixture was heated for 2 min at 94 °C and subjected to 30 cycles of 30 sec at 94 $^{\circ}C$, 30 sec at 55 $^{\circ}C$, and 1 min at 72 °C. The amplified DNA was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequence within the DR locus.

The sequence of the oligonucleotides used is given in table 2. These oligonucleotides were covalently bound to a hybridization membrane for hybridization. Twenty μl of the amplified PCR product was diluted in 150 μl

MIRU alias	Locus name	Location on H37RV genome	Expected length in H37RV	No. of repeat and size repeat	Size of repeat (bp)
MIRU 02	H37RV0154-53 bp	154111	508	2 (53)	
MIRU 04	H37RV0580-77 bp	580546	353	3(77) —	
MIRU 10	H37RV0959-53 bp	959868	643	3(53)	
MIRU16	H37RV1644-53 bp	1644026	671	2(53)	
MIRU20	H37RV2059-77 bp	2059429	591	2(77)	
MIRU23	H37RV2531-53 bp	2531560	873	6(53)	
MIRU24	H37RV2387-54 bp	2684427	447	1(52)	
MIRU26	H37RV2996-51 bp	2996002	614	3(51)	
MIRU27	H37RV3006-53 bp	3006875	657	3(53)	
MIRU31	H37RV3192-53 bp	3192168	651	3(53)	
MIRU39	H37RV4348-53 bp	4348401	646	2(53)	
MIRU40	H37RV0802-54 bp	802194	408	1(54)	

Table 2. MIRU locus information for M. tuberculosis H37RV

MIRU-VNTR: Mycobacterial Interspersed Repetitive Unit Variable Number Tandem Repeat

Avicenna Journal of Medical Biotechnology, Vol. 2, No. 3, July- September 2010

Strain Comparison of *M. tuberculosis* by "MIRU- VNTR" and "MIRU-VNTR+Spoligotyping"



Figure 1. A) PCR products of the various *M. tuberculosis* H37RV isolates with using primer that amplify 12 locus MIRU-VNTR. Lane M, 100-*bp* molecular marker. B) Genotypic of *M. tuberculosis* H37RV. Spoligotyping is from the MIRU-VNTR plus database MIRU-VNTR: Mycobacterial Interspersed Repetitive Units Variable Number Tandem Repeat

of 2 Saline – Sodium Phosphate – EDTA (SSPE), supplemented with 0.1% sodium dodecyl sulfate, and heat denatured. The diluted samples (130 μl) were pipetted in to the parallel channels in such a way that the channels of the miniblotter apparatus were perpendicular to the rows of oligonucleotides deposited previously. Hybridization was done for 60 *min* at 60 °*C*.

After hybridization, the membrane was washed as previously described. Detection of hybridizing DNA was done using a (chemiluminescent ammersham ECL direct nucleotide acid) detection kit. The 4 digit binary result was converted in to a 15 digit octal designation as previously described ⁽¹²⁾.

Analysis

These rational systems (Figure 1B) are based on the identification of the spoligotype pattern as a 43- digit binary number (taking a negative as 0 and positive as 1), which can be presented in a shorter format by converting to either octal or hexadecimal formats. For the octal system, the pattern is read in groups of 3, as 3 - digit binary number can be read as a single digit octal number. In this way the binary series 000, 001, 010, 011, 100, 101, 110, 111 is read respectively as 0, 1, 2, 3, 4, 5, 6, 7. Following 14 groups of 3, the 43^{rd} spacer is read on its own, simply as 0 or 1 ⁽¹³⁾.

MIRU-VNTR plus

The information was analysed by MIRU-VNTR plus website. MIRU-VNTR plus software compares and analyses the received information with the information bank in its own software.

Codes of ethics

This study was short term research project and information regarding each patient kept confidential. The study was approved by the medical board of NRITLD.

Statistical analysis

The Hunter – Gaston Discriminatory Index (HGDI) described by Hunter and Gaston (Hunter and Gaston, 2003) was used as a numerical index for MIRU-VNTR discriminatory power. HGDI was calculated by using the following formula: ⁽¹⁴⁾

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

Where, N stands for the total number of strains in the typing scheme, S stands for the total number of different MIRU-VNTR patterns, and xj stands for the number of strains belonging to the Jth pattern.

P less than 0.05 was considered as significant. Statistical package SPSS ⁽¹⁵⁾ was used for analysis of data. Chi square was used to

determine possible difference by means of unknown *MTB* strains between the single and combined techniques.

Results

As it shown in table 3, 34% of susceptible strains could not identified by MIRU-VNTR, and the remaining 66% were identified as Haarlem family (17%), Dehli/ CAS (17%), NEW1 (14%), and LAM, Uganda and M.X families were 7%. Similarly, 44% drug resistant strains could not be identified by MIRU-VNTR technique. Using both "MIRU-VNTR and Spoligotyping" techniques together significantly reduced the percentage of unknown strains (Table 3).

As shown in table 3, thirteen strains with

Table 3. Frequency of sensitive and drug resistance strains of *MTB* by "MIRU-VNTR" assay versus "MIRU-VNTR and Spoligotyping" together

Families	MIRU-VNTR	MIRU-VNTR+
Sonsitivo str	NO. (%)	Spongotyping No. (%)
Sensitive su	20 (67) Casa	27 (00) Casa
Known	20(07) Case	27(90) Case
Haarlem	5 (16) Case	7 (23) Case
Dehli/CAS	5 (16) Case	8 (26) Case
NEW1	4 (14) Case	6 (20) Case
LAM	2 (7) Case	2 (7) Case
Uganda1	2 (7) Case	2 (7) Case
M.X	2 (7) Case	2 (7) Case
Unknown	10 (33) Case	3 (10) Case
Multi-drug	resistant strains	
Known	17 (57) Case	24 (80) Case
Haarlem	4 (14) Case	6 (20) Case
Dehli/CAS	4 (14) Case	5 (16) Case
LAM	3 (10) Case	4 (14) Case
Uganda 1	2 (7) Case	2 (7) Case
Cameron	1(3) Case	2 (7) Case
Beijing	1 (3) Case	1(3) Case
Ghana	0 (0) Case	1(3) Case
Bovis	1 (3) Case	1(3) Case
Caparie	1 (3) Case	1(3) Case
M.X	0 (0) Case	1(3) Case
Unknown	13 (43) Case	6(20) Case
All strains		
Known	37 (62) Case	51(85) Case
Unknown	23 (38) Case	9(15) Case

unknown sample (by "MIRU-VNTR" technique) decreased to 6 strains by using "MIRU-VNTR and Spoligotyping" technique. On the other hand strains (samples specified) increased by using the two techniques; because of the setting of the family samples for some of the unknown strains (Table 3).

Overall by using "MIRU-VNTR" technique, 38% of isolates could not be identified whereas by applying "MIRU-VNTR and Spoligotyping" together only 15% of the isolates remained unknown (p<0.05).

In table 4, the allelic differences for MIRU loci had accounted based on HGDI statistic formula; that is loci 20, 24 and 27 had lower allele difference (HGDI \leq 0.3), loci 31, 39 and 40 were in middle (0.3 \leq HGDI \leq 0.6) and loci 10, 16, 23 and 26 had the most allele difference among the locus MIRU (HGDI \geq 0.6).

Based on table 4 in both sensitive and resistant drug loci 10, 16 and 26 had the most

Lo	cus	1	2	3	4	5	6	7	8	9	HGDI
•	М	1	60	-	-	-	-	-	-	-	0
2	S	-	33	-	-	-	-	-	-	-	0
	Μ	-	61	-	-	-	-	-	-	-	0
4	S	-	31	-	-	-	-	-	-	-	0
10	Μ	1	18	14	11	13	4	-	-	-	70%
10	S	-	16	5		8	3	1	-	-	66%
16	Μ	4	16	32	8	1	-	-	-	-	64%
10	S	2	2	18	10	1	-	-	-	-	59%
20	М	2	59	-	-	-	-	-	-	-	10%
20	S	3	30	-	-	-	-	-	-	-	14%
23	Μ	-	-	-	-	6	55	-	-	-	16%
23	S	-	-	-	1	-	32	-	-	-	10%
24	Μ	61	-	-	-	-	-	-	-	-	0.02%
24	S	33	-	-	-	-	-	-	-	-	0
26	М	-	2	2	6	24	13	8	5	1	78%
20	S	-	-	-	7	10	1	9	6	-	76%
77	М	-	3	3	55	-	-	-	-	-	17%
21	S	-	-	1	30	2	-	-	-	-	14%
31	М		1	1	40	8	11	-	-	-	51%
51	S	-	-	-	20	6	7	-	-	-	45%
39	М	-	-	44	17	-	-	-	-	-	42%
•	S	-	1	20	12	-	-	-	-	-	52%
40	М	3	5	41	12	-	-	-	-	-	50%
	S	1	4	25	3	-	-	-	-	-	38%

MIRU-VNTR: Mycobacterial Interspersed Repetitive Units Variable Number Tandem Repeat

MIRU-VNTR: Mycobacterial Interspersed Repetitive Units Variable Number Tandem Repeat; S strain (Susceptible), M strain (Resistant)

Strain Comparison of *M. tuberculosis* by "MIRU- VNTR" and "MIRU-VNTR+Spoliaotvpina"

A			DIE				G				RIF	INF	I MIRU-VNTR	Spoligotyping	
			RIF	INI	1 MIRU-V	NIK	Spongotyping			3610	S	S	2232613332		
	Caparie	3343	R	R 223342615343		5343			NEW 1	3628	s	5	2232614332		
	Bovis	3555	R	R	277777777	3?2?			NEW.I	3606	5	5	4232614332		
=	x	3637	R	R	22??2617	3322				3600	s	5	2232613332		
	Cameron	3463	R	R	22342515	3323				3641	s	5	2232614332		
	Ghana	3568	R	R	22112111	3 / 3 4	······································	-	M.X	3635	s	5	2232614332		
	Beijing	3515	R	R	22132617	3533	3			3647	s	5	2812614842		
	Uganda 1	3461	R	R	22?22515	332?			Uganda 1	2634	-		1021612220		
	- 8	3621	R	R	223225?6	5?6??23					1	-			
n.		3413	R	R	R	12322615	3224				3020	-	-	2252615552	
ra.	LAM	3433	R	R	22332615	3324		50	Hoorlow	3040	2	3	4232615552		
Sti		3645	R	R	22322615	3523		Ξ.	naarien	3629	2	3	2232015552		
e	Dehli/CAS	3309	R	R	22542514	3323		a.		3623	2	3	2232615352		
tanc		3538	R	R	22422617	3423		E		3617	2	5	2252614552		
		3477	R	R	77537615	2272		e	LAM	3607	S	S	2232613332		
sis		3517	P		22512615	2272		N.		3636	S	5	2232614332		
š	Haarlem	3637	P	0	33532610	2272		it		3643	5	5	2542617343		
50		3555	P.	0	22552615	2472		ũ		3644	s	5	2542615343		
n.		3303			22332017	3423		Se	Dehli/CAS	3611	S	s	2642617343		
ã		3469	ĸ	ĸ	22532615	1533				3639	S	5	2542615452		
		3627	R	R	22322616	3323				3630	S	5	2542617355		
		3382	R	R	22632614	1523				3602	s	5	2322516331		
		3648	R	R	22212615	2322				3612	s	5	2641613343	GTTEFFTEFTTEFTTEFTTEFTTEFTTEFTTEFT	
		3562	R	R	22212615	2322				3622	s	s	2532615353	GTEFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF	
		3402	R	R	22532618	3333				3640	S	5	2552514333	STITUTITITITITITITITITITITITITITITITITIT	
	Unknown	3365	R	R	22632617	3534				3651	s	s	2541615353	ATTENDED ATT	
		3432	R	R	22?32613	3523			Unknown	3601	5		2282618882		
		3491	R	R	22332618	3523				3614	-	-	2742617353		
		3395	R	R	22222??5	3324				3624	5	5	2322615432	Internet and a second s	
		3561	R	R	22432614	1523				3647	\$	-	2311613332		
		3510	R	R	22321615	2321		3		3620	<	-	2542617353		
		3358	R	R	22442618	3423					-				
											DIF	TNII	MIDII WATD	Spaligatuning	
R			RIF	INH	MIRIL-VN	TR	Spaligatyping			7640	RIF	INH	MIRU-VNTR	Spoligotyping	
В	Canarie	2242	RIF	INH	MIRU-VN	TR	Spoligotyping			3610	RIF S	INH S	MIRU-VNTR 2232613332	Spoligotyping	
B	Caparie Bovis	3343	RIF R	INH R 2	MIRU-VN 2354261	TR 534	Spoligotyping		NEW.1	3610 3628	RIF S S	INH S S	MIRU-VNTR 2232613332 2232614332	Spoligotyping	
B	Caparie Bovis X	3343 3555 3637	RIF R R		MIRU-VN 2554261 2777777	TR 534 5727	Spoligotyping		NEW.1	3610 3628 3606	RIF S S S	INH S S	MIRU-VNTR 2232613332 2232614332 4232614332	Spoligotyping	
В	Caparie Bovis x Cameror	3343 3555 3637 3463	RIF R R R R		MIRU-VN 2334261 2???????? 2??2617 2842515	TR 534 5727 5527	Spoligotyping		NEW.1	3610 3628 3606 3600	RIF S S S	S S S S	MIRU-VNTR 2252613332 2232614332 4232614332 2232613332	Spoligotyping	
В	Caparie Bovis x Cameror Ghana	3343 3555 3637 3463 3568	RIF R R R R R R		MIRU-VN 2334261 2???2617 2342515	TR 534 5727 5321 3321 332			NEW.1	3610 3628 3606 3600 3641	RIF S S S S S	INH S S S S	MIRU-VNTR 2252613352 2252614352 4252614332 2252614332 2252613352 2252614332	Spoligotyping	
B	Caparie Bovis x Cameroi Ghana Beiiin	3343 3555 3637 3463 3568 3515	RIF R R R R R R R R R		MIRU-VN 2334261 2772617 2342515 127727277	TR 534 5727 3521 3521 352 5754	Spoligotyping		NEW.1 M.X	3610 3628 3606 3600 3641 3635	RIF S S S S S S	INH S S S S S S	MIRU-VNTR 2 2 5 2 6 1 3 5 3 2 2 2 3 2 6 1 4 3 5 2 4 2 5 2 6 1 4 3 5 2 2 2 3 2 6 1 4 3 3 2 2 2 3 2 6 1 3 3 3 2 2 2 3 2 6 1 4 3 3 2 2 2 3 2 6 1 4 3 3 2	Spoligotyping	
В	Caparie Bovis x Cameror Ghana Beijin	3343 3555 3637 3463 3568 3515	RIF R R R R R R		MIRU-VN 2334261 2332617 2342515 237237 2132617	TR 534 5727 332 332 332 3734 353	Spoligotyping		NEW.1 M.X	3610 3628 3606 3600 3641 3635 3642	RIF S S S S S S S	INH S S S S S S S	MIRU-VNTR 2 2 5 2 6 1 3 5 3 2 2 2 3 2 6 1 4 3 5 2 4 2 3 2 6 1 4 3 5 2 4 2 3 2 6 1 4 3 5 2 2 2 3 2 6 1 4 3 5 2 2 2 3 2 6 1 4 3 5 2 2 2 3 2 6 1 4 3 5 2 2 3 1 2 6 1 4 3 4 2	Spoligotyping	
В	Caparie Bovis x Cameror Ghana Beijin Uganda]	3343 3555 3637 3463 3568 3515 3461 2624	RIF R R R R R R R R R R R R		MIRU-VN 2334261 2772617 2342515 1277277 2132617 2722515	TR 534 5727 532 332 5734 353 353			NEW.1 M.X Uganda	3610 3628 3606 3600 3641 3635 3642 3634	RIF S S S S S S S	INH S S S S S S S	MIRO-VNTR 2252613332 2252614352 4252614352 2252614352 2252614352 2252614352 2252614352 2252614552	Spoligotyping	
В	Caparie Bovis x Cameror Ghana Beijin Uganda]	3343 3555 3637 3463 3568 3515 3461 3621	RIF R R R R R R R R R R R		MIRU-VN 2354261 277277 2342515 1277277 2152617 2722515 12722515	TR 534 532: 332: 332 5734 353 353 332			NEW.1 M.X Uganda	3610 3628 3606 3600 3641 3635 3642 3634 3634 3626	RIF S S S S S S S S S S	INH S S S S S S S S S S	MIRU-VNTR 2252613532 2252614352 2252614352 2252613532 2252614352 2252614352 2312614342 225261352 2252615552	Spoligotyping	
B	Caparie Bovis x Camerou Ghana Beijin Uganda]	3343 3555 3637 3463 3568 3515 3461 3621 3413	RIF R R R R R R R R R R R R R R	N	MIRU-VN 2554261 2??????? 2??2617 2342515 !2??2??? 2152617 2?22515 !23225?6 2322615	TR 534 5727 332 332 5734 353 353 352 272 322			NEW.1 M.X Uganda	3610 3628 3606 3600 3641 3635 3642 3634 3626 3646	RIF S S S S S S S S S S S S S	INH S S S S S S S S S S	MIRU-VNTR 2252613352 232614352 2232614352 2232614352 2232614352 2232614352 2232614352 23261352 232615352 232615352 232615352	Spoligotyping	
B	Caparie Bovis x Cameron Ghana Beijin Uganda] LAM	3343 3555 3637 3463 3568 3515 3461 3621 3413 3433	RIF R R R R R R R R R R R R R R R R R R R	R R R R R R R R R R R R R	MIRU-VN 2334261 2??????? 2??2617 2342515 12??2??? 2132617 2?22515 123225?6 2322615 2332615	TR 534 5727 352 3754 353 353 352 772 322 322 332	Spoligotyping	US	NEW.1 M.X Uganda	3610 3628 3606 3600 3641 3635 3642 3634 3626 3646 3646	RIF S S S S S S S S S S S S S S S S	S S S S S S S S S S S S S	MIRU-VNTR 2252613352 2252614352 2232614352 2232614352 2232614352 2232614352 2232614352 2232614342 2232615352 2252615552 2232615552	Spoligotyping	
rains B	Caparie Bovis x Cameron Ghana Beijin Uganda LAM	3343 3555 3637 3463 3568 3515 3461 3621 3413 3433 3645	RIF R R R R R R R R R R R R R R R R R R R		MIRU-VN 23542617 2772617 2542515 1277277 2122615 12322575 2322615 2322615 2322615	TR 534 532 332 332 353 353 353 352 322 352 352	Spoligotyping	ains	NEW.1 M.X Ugandai Haarlen	3610 3628 3606 3600 3641 3635 3642 3634 3626 3646 3646 3629 3623	RIF S S S S S S S S S S S S S S S	S S S S S S S S S S S S S S S	MIRU-VNTR 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 231261452 231261452 2352615352 2252615552 4252615352 2252615352	Spoligotyping	
Strains	Caparie Bovis x Cameron Ghana Beijin Uganda] LAM Dehli/CAS	3343 3555 3637 3463 3568 3515 3461 3621 3413 3433 3645 3309	RIF R R R R R R R R R R R R R R R R R R R		MIRU-VN 23342617 2??????? 2?22617 2342515 12??2??? 2132617 2?22515 2322615 2322615 2322615 2322615 2322615	TR 534 532; 332; 332; 332; 332; 352; 332; 352; 35	Spoligotyping	trains	NEW.1 M.X Ugandai Haarlen	3610 3628 3606 3600 3641 3635 3642 3634 3626 3646 3629 3623 3617	RIF S S S S S S S S S S S S S S S S	S S S S S S S S S S S S S S S S S S S	MIRU-VNTR 2252613532 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2312614342 2252615352 4252615352 2252615352 2252615352 2252615352 2252615352	Spoligotyping	
e Strains B	Caparie Bovis x Cameron Ghana Beijin Uganda] LAM Debli/CAS	3343 3555 3637 3463 3568 3515 3461 3621 3413 3433 3645 3309 3538	RIF R R R R R R R R R R R R R R R R R R R		MIRU-VN 2354261 277777 2842515 1277277 2132617 2722515 12522576 2322615 2322615 2322615 2322615 2322615 2542514	TR 534 5727 332 5734 355 352 322 322 352 352 352 352 342	Spoligotyping	Strains	NEW.1 M.X Ugandai Haarlen	3610 3628 3606 3600 3641 3635 3642 3634 3626 3646 3646 3629 3623 3617 3607	R1F S S S S S S S S S S S S S S S S S S S	S S S S S S S S S S S S S S S S S S S	MIRU-VNTR 2252613352 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252615352 2252615352 2252615352 2252615352 2252615352 2252615352 2252615352	Spoligotyping	
nce Strains B	Caparie Bovis x Cameroi Ghana Beijin Uganda] LAM Dehli/CAS	3343 3555 3637 3463 3568 3515 3461 3621 3413 3645 3309 3538 3477	RIFRRRRRRRRRRRRRRR		MIRU-VN 23542617 2372617 2342515 2372617 2722515 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2322615	TR 534 5727 332 332 5734 353 352 352 352 352 352 352 352 352 352	Spoligotyping	ve Strains	NEW.1 M.X Ugandai Haarlen LAM	3610 3628 3606 3641 3635 3642 3634 3626 3646 3646 3629 3623 3617 3607 3636	RIF S S S S S S S S S S S S S	S S S S S S S S S S S S S S S S S S S	MIRU-VNTR 225261332 2252614352 2252614352 2252614352 2252614352 2252614352 2312614352 2312614342 2252615352 2232615552 2252615352 2252615352 2252615352 2252614352	Spoligotyping	
ance Strains	Caparie Bovis x Cameroi Ghana Beijin Uganda] LAM Dehli/CAS	3343 3555 3637 3463 3568 3515 3461 3621 3413 3645 3309 3538 3477 3517	RIFRRRRRRRRRRRRRRRR		MIRU-VN 2334261 2334261 2332251 2322515 1292272 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2542514 2422617 2532615	TR 534 5727 352 352 352 352 352 352 352 352 352 352	Spoligotyping	tive Strains	NEW.1 M.X Uganda Haarlen LAM	3610 3628 3606 3600 3641 3635 3642 3624 3626 3629 3623 3617 3607 3636	RIF S S S S S S S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 225261352 2352614352 2252614352 2252614352 2252614352 2352614352 2352614352 2252615352 2252615352 2252615352 2252615352 2252615352 2252614532 2252614532 2252614352 2254435 225445 225455 225455 225455 225455 225455 225455 225455 225455 225455 225455 225455 225455 225455 225455 225455 225455 22555 22555 22555 22555 22555 22555 22555 22555 22555 22555 22555 22555 22555 2255	Spoligotyping	
istance Strains	Caparie Bovis x Camero Ghana Beijin Uganda LAM Dehli/CAS	3343 3555 3637 3463 3515 3461 3621 3413 3645 3309 3538 3477 3517 3632	RIF		MIRU-VN 2534261 2772777 2342515 2342515 2322515 2322615 2322615 2322615 2322615 2322615 2342514 2422617 2532615 2542514	TR 534 5727 5522 532 5754 555 552 552 552 552 552 552 552 552 5	Spoligotyping	sitive Strains	NEW.1 M.X Uganda Haarlen LAM	3610 3628 3606 3600 3641 3635 3642 3634 3626 3646 3629 3623 3617 3607 3636 3643	RIF S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252615352 2252615352 2252615352 2252615352 2252615352 2252614352 2252614352 2252614352	Spoligotyping	
esistance Strains	Caparie Bovis x Camerot Ghana Beijin Uganda LAM Dehli/CAS	3343 3555 3637 3463 3515 3461 3621 3413 3645 3309 3538 3477 3517 3632 3565	RIF R R R R R R R R R R R R R R R R R R		MIRU-VN 2334261 2737377 272617 2342515 1232257 2322617 232255 2322615 2322615 2322615 2322615 2542514 2422617 2532615 2512615 2532615	TR 534 5727 332 5734 353 352 772 352 352 352 352 352 352 352 352 352 35	Spoligotyping	ensitive Strains	NEW.1 M.X Uganda Haarlen LAM	3610 3628 3606 3600 3641 3635 3642 3634 3646 3646 3646 3629 3623 3617 3607 3636 3643 3644	RIF S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 2252613332 2352614352 2252614352 2252614352 2252614352 2312614532 2352615352 2252615352 2252615352 2252615352 2252615352 2252614352 2252614352 2252614352 2252614352 2552614554 2552614554 2552614554 2552614554 2552614 255261 2554 2554 2554 2554 2554 2554 2554 255	Spoligotyping	
Resistance Strains	Caparie Bovis x Cameroi Ghana Beijin Uganda] LAM Dehli/CAS Haarlem Unknown	3343 3555 3637 3463 3568 3515 3461 3621 3413 3645 3309 3538 3477 3632 3565 3469	RIF R R R R R R R R R R R R R R R R R R R	N R R R R R R R R R R R R R R R R R	MIRU-VN 2334261 2334261 234261 234261 234261 234261 232261 232261 232261 232261 232261 232261 232261 232261 252261 252261 252261 252261 252261 252261 252261 252261 252261 252261	TR 534 5727 332 5754 352 5754 352 352 352 352 352 352 352 352 352 352	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM	3610 3628 3606 3641 3635 3642 3634 3646 3646 3623 3617 3607 3636 3643 3644 3644	RIF S S S S S S S S S S S S S	S S S S S S S S S S S S S S S S S S S	MIRU-VNTR 225261332 2352614352 2252614352 2252613532 2252614332 2312614342 2352615352 2252615352 2252615352 2232615352 2232615352 2232615352 2232615352 2232614322 2232614352 2232614352 2232614352 2232614352 22542615343 2642617343	Spoligotyping	
g Resistance Strains	Caparie Bovis x Cameron Ghana Beijin Uganda] LAM Dehli/CAS Haarlem Unknown NEW.1	3343 3555 3637 3463 3515 3461 3413 3433 3645 3309 3538 3477 3517 3632 3565 3629 3629	RIF R R R R R R R R R R R R R R R R R R R		MIRU-VN 2334261 2772777 2342515 2342515 2322515 2322615 2322615 2322615 2322615 2322615 2342514 2422617 2532615 2512615 2532619 2532619 2532619	TR 554 5727 3522 352 352 352 352 352 352 352 352 35	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA	3610 3628 3606 3600 3641 3635 3642 3644 3646 3643 3623 3617 3607 3636 3643 3644 3644 3644	RIF 5 5 5 5 5 5 5 5 5 5 5 5 5	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 2252613352 2252614352 2252614352 2252614352 2252614352 2252614352 225261535 2252615 2252615 225615 225615 225615 225615 225615 225615 225615 225615 225615 225615 225615 225615 225615 225615 2257 2255615 2257 225615 2257 2257 2257 2257 2257 2257 2257 22	Spoligotyping	
rug Resistance Strains	Caparie Bovis x Camerol Ghana Beijin Uganda] LAM Dehli/CAS Haarlem Unknown NEW.I Unknown	3343 3555 3637 3663 3568 3568 3461 3621 3413 3645 3645 3538 3477 3537 3538 3469 3469 3469 3538	RIF R R R R R R R R R R R R R R R R R R		MIRU-VN 2334261 2733737 2722617 2342515 12322515 12322515 2322615 2322615 2542514 2522615 2512615 2532615 2532615 2532615 2532615 2532615 2532615	TR 554 5727 3522 352 352 352 352 352 352 352 352 35	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA:	3610 3628 3606 3600 3641 3635 3642 3642 3646 3643 3646 3643 3644 3644	RIF 5 5 5 5 5 5 5 5 5 5 5 5 5	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 225261332 2252614352 2252614352 2252614352 2252614352 2252614352 2252615352 2252615352 2252615352 2252615552 2252615552 2252614552 2252614552 2252614552 2542617345 2542615452 2542617555	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x Cameron Ghana Beijin Uganda LAM Dehli/CAS Haarlem Unknown NEW.1 Uuknown Haarlem	3343 3555 3637 3463 3568 3515 3461 3621 3621 3621 3623 3645 3433 3645 3437 3555 3469 3557 3382 3648	RIFRRRRRRRRRRRRRRRRRRRRRR		MIRU-VN 2354261 2354261 23722617 2342515 1272275 2322617 2322615 2322615 2322615 2522615 2522615 2532615 2532615 2532615 2532615 2532616 2532616	TR 554 5727 3322 3754 355 355 355 355 352 352 352 352 352 352	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA:	3610 3628 3606 3600 3641 3635 3642 3646 3643 3646 3643 3646 3643 3644 3645 3645	RIF S S S S S S S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 225261332 2252614352 2252613332 2252613332 2252614352 2252614352 232615352 232615352 2232615352 2232615352 2232615352 2232615352 2232615352 2232615352 2232615352 2232614352 2232614352 2352614352 2352615352 2352614352 2352614554 2452617343 254261	Spoligotyping	
Drug Resistance Strains	Caparici Bovis x Cameror Ghana Beijin Uganda J LAM Dehli/CAS Haarlem Unknown NEW.1 Unknown NEW.1 Haarlem Haarake	3343 3555 3637 3463 3568 3515 3461 3613 3413 3433 3645 3339 3538 3477 3517 3562 3632 3632 3562 3469 3622 3382 3562	RIF ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~		MIRU-VN 2334261 2772777 2342515 2342515 2322515 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2522615 2522616 2532616 2532612 2532615 2552615 2552615 2552615 2552615 2552615 2552615 2552615 2552615 2552615 255261	TR 554 5727 3322 3754 355 3754 355 352 352 352 352 352 352 352 352 352	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA3	3610 3628 3606 3600 3641 3635 3642 3634 3644 3629 3623 3644 3617 3607 3636 3643 3644 3611 3630 3630 3630 3630 3630	RIF S S S S S S S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 225261352 2252614352 2252614352 2252613532 2252614352 2252614352 2252613532 225261352 2312614342 232615332 225261532 225261532 225261532 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 252615343 2542615343 2542615453 2542615453 2542615452 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 2542615453 25426153543 25426153543 25426153	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x camero Ghana Beijin Uganda] LAM Dehli/CAS Haarlem Unknown Haarlem Unknown	3343 3555 35637 3463 3568 3515 3461 3413 3645 3309 3538 3477 3532 3555 3565 36627 3682 36627 3682 3662 3682 3662	RIF ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~	N	MIRU-VN 2334261 2733737 232617 2342515 12322515 12322515 2322615 2322615 2322615 2322615 2542514 2512615 25326	TR 534 522 3322 5734 353 332 332 332 332 332 332 332 332 332	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CAS Beijing	3610 3628 3600 3641 3635 3642 3634 3642 3643 3644 3629 3623 3644 3644 3644 3644 3644 3644 3644	RIF 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5	INH S S S S S S S S S S S S S S S S S S S	MIRU-VNTR 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252615352 2252615352 2252615352 2252615352 2252615352 2252614352 2252614352 2252614352 2542617345 2542617345 2542617345 2542617345 2542615343 254	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x Cameroo Ghana Beijin Uganda J LAM Dehli/CA? Haarlem Haarlem Unknown Haarlem	3343 3555 3637 3463 3568 3461 3461 3413 3461 3413 3461 3433 3565 3588 3538 3538 3538 3538 3538 35	RIF		MIRU-VN 2334261 2334261 2372737 232617 2322515 2322617 2322515 2322615 2322615 2322615 2322615 2532615 2532615 2532615 2532615 2532615 2532615 2212615 2212615 2212615	TR 534 5727 352: 352 352 352 352 352 352 352 352 352 352	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA3 Beijing Unkaown	3610 3628 3600 3641 3635 3642 3645 3646 3646 3646 3643 3644 3647 3667 3643 3644 3644	RIF S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S	NH S S S S S S S S S S S S S S S S S S S	MIRU-VNTR 225261332 2252614352 2252614352 2252614352 2252614352 2252613532 2252613532 2312614352 2312614352 232561352 231261432 2252615352 2252615352 2252615352 2252615352 2252615352 2252615352 2252615352 2252614352 2542617345 2542617345 2542617345 2542617345 2542617345 2542617345 2542617345 2542617345 2542617345 2542617345 254261755 252514555 252514555 2552514555	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x Camerou Ghana Beijin Uganda] LAM Debli/CAS Unknown NEW.1 Unknown Haartem Haaratem Haaratem Jushaown Debl/CAS	3343 3555 3637 3663 3565 3461 3621 3641 3645 3309 3538 3645 3632 3565 3632 3565 3632 3565 3648 3562 3648 3562 3648 3562 3648 3648 3648 3648 3648 3648 3648 3648	RIF		MIRU-VN 2334261 2772777 2342515 2342515 2322515 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2532616 2532616 2532616 2632614 2212615 253262 255262 255262 255262 255262 255262 255262 255262 255262 255262 2552	TR 534; 522; 523; 523; 523; 522; 522; 532; 532	Spoligotyping	Sensitive Strains	NEW.1 M.X Ugandai Haarlen LAM Dehli/CA: Beijing Unkaown Unkaown	3610 3628 3600 3641 3635 3642 3644 3629 3623 3647 3664 3664 3664 3664 3664 3664 366	RIF S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 225261332 2252614352 2252613332 225261332 225261332 225261332 2312614342 23261532 232261532 23261532 23261532 23261532 23261532 23261532 23261532 23261532 23261532 23261532 23261532 23261532 23261532 23261533 2542617343 2542617343 2542617343 2542615352 2542617343 2542617343 2542615352 2542617353 2542615351 2641613343 2552514333 2552514333 2544615355	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x Cameroo Ghana Beijin Uganda LAM Dehli/CAS Dehli/CAS Dehli/CAS NEW-I Unknown NEW-I Unknown Unknown Dehli/CAS	3343 3555 3637 3461 3568 3568 3568 3568 3461 3413 3645 3309 3558 3469 3557 3469 3557 3469 3562 3469 3562 3469 3562 3462 3562 3462 3562 3462 3562 3462 3562 3462 3562 3462 3562 3462 3462 3562 3462 3462 3462 3462 3462 3462 3462 34	RIF		MIRU-VN 2534261 2772727 2342515 2342515 2322515 2322617 2322515 2322615 2322615 2322615 2342514 2422617 2532615 2532615 2532615 2532615 2322616 2532616 2532616 2532616 2532616 2532616 2532616	TR 534 527 332 332 352 352 352 352 352 352 352 352	Spoligotyping Image: Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA: Beijing Unknown Unknown NEW.2	3610 3628 3606 3600 3641 3635 3642 3634 3626 3643 3623 3644 3623 3644 3623 3644 3623 3644 3644	RIF S S S S S S S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 225261332 2252614352 2232614352 2232614352 2232614352 2232614352 2232614352 2232614352 2232614352 2232614352 2232615352 2232615352 2232615352 2232615352 2232614352 2232614352 232617343 242617343 2542617343 2542617343 2542615343 2542615343 2542615343 2542615343 2542615343 2542615343 2542615343 2542615343 2542615343 2542615343 2552514333 2552514353 2544615353 2542615353 2552514333 2544615353 2544615353 2544615353 2544615353 2544615353 2544615353 2544615353 254461	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x Cameroo Ghana Beijin Uganda I LAM Haarlem Haarake Haarake Haarake Unknown Baarke Unknown Baarke LaAM	3343 3555 3637 3565 3515 3515 3461 3621 3413 3433 3433 3437 3538 3477 3632 3565 3469 3517 3627 3562 3469 3527 3462 3462 3462 3462 3462 3462 3462 3462	RIF		MIRU-VN 2334261 27372617 2342515 12722757 2132617 2722515 2322615 2322615 2322615 2322615 2532615 2532615 2532615 2532615 2532615 2532615 2532615 2532615 2532616 2532617 2532615 25565 255656 255656 25565656 2556565656	TR 534 5727 3522 3523 352 352 352 352 352 3	Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA: Beijing Unknown Unknown NEW.2 Dehli/CA:	3610 3628 3600 3641 3635 3642 3644 3644 3644 3644 3644 3644 3647 3647	RIF S <	NH S	MIRU-VNTR 225261332 2252614352 2252614352 2252614352 2252614352 2252613532 2252613532 2312614352 2312614352 2312614352 232615332 2232615332 2232615332 2232615332 2232615332 2232615332 2232615332 223261332 223261332 223261332 223261332 223261332 223261332 223261332 232614332 23261333 23261333 23261333 232261333 232261333 2322516331 2641613353 2532514333 2532514333 2542613352 2532514333 2542613352 2532514353 2532514353 254261352 254261352 2542613552 2542613552 <td>Spoligotyping Spoligotyping Spoligotyping</td>	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x Cameroo Ghana Beijin Uganda J LAM Haarlem Haarlem Haarlem Haarlem Haarlem Haarlem Laknown Dehli/CAS Dehli/CAS	3343 3555 3637 3463 3568 3568 3568 3433 3645 3309 3538 3538 3545 3469 3555 3469 3562 3469 3562 3469 3562 3469 3562 3469 3562 3469 3562 3461 3565 3469 3565 3469 3567 3568 3568 3568 3568 3568 3568 3568 3568	RIF		MIRU-VN 2334261 2772777 2342515 1272277 2132617 2722515 1322576 2322615 2322615 2322615 2322615 2322615 2322615 2532615 2532616 2632614 2212615 2532616 2632614 2212615 2532618 2632614 2122615 2532618 2632614 2122275 2432614	TR 534 5527 3527 3527 352 352 352 352 352 352 352 352 352 352	Spoligotyping Sector	Sensitive Strains	NEW.1 M.X Ugandai Haarlen LAM Dehli/CA: Beijing Unknown Unknown NEW.2 Dehli/CA:	3610 3628 3606 3600 3641 3635 3642 3644 3623 3644 3623 3644 3643 3644 3643 3644 3651 3602 3602 3602 3602 3602 3602 3602 3602	RIF S S S S S S S S S S S S S S S S S S S	INH SSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSSS	MIRU-VNTR 225261332 225261332 225261332 225261332 225261332 223261332 223261332 23261332 23261332 23261332 23261332 23261332 23261532 23261532 23261532 23261332 23261332 23261332 23261332 23261332 23261332 23261332 23261332 23261332 23261332 23261333 242615343 2542617343 2542617343 2542617343 2542617353 252514334 2542615353 2552514333 254163353 254163353 254163353 254163353 254163353 254163353 254163353 2542617353 2542617353 254163353<	Spoligotyping	
Drug Resistance Strains	Caparie Bovis x Cameroo Ghana Beijin Uganda LAM Dehli/CAS Haarlem Unknown NEW.1 Unknown Unknown Unknown Unknown Unknown Unknown	3343 3555 3637 3463 3568 3568 3568 3568 3621 3413 3645 3563 3563 3565 3462 3565 3462 3565 3462 3565 3462 3565 3462 3627 3382 3648 3568 3402 3652 3432 3565 3432 3565 3432 3565 3432 3565 3561 3452 3561 3452 3561 3452 3561 3561 3561 3562 3561 3562 3562 3562 3562 3562 3562 3562 3562	RIF	N	MIRU-VN 2534261 277277 2342515 2322515 2322617 2322515 2322615 2322615 2322615 2322615 2322615 2322615 2322615 2542514 2422617 2532615 2552615 2552615 2552615 2552615 2552615 2552615 2552615	TR 534 532: 535: 535	Spoligotyping Image: Spoligotyping	Sensitive Strains	NEW.1 M.X Uganda Haarlen LAM Dehli/CA Dehli/CA Unknown NEW.2 Dehli/CA Unknown NEW.2	3610 3628 3606 3600 3641 3632 3634 3634 3632 3633 3632 3633 3633	RIF S	NH S	MIRU-VNTR 225261332 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252614352 2252615352 2252615352 2252615352 2252615352 2252614352 2252614352 2252614352 2252614352 2542617343 2542617343 2542617343 2552514353 2552514353 2522613553 2522613553 25225165353 2522615353 2522615353 2522516353 2522615353 2522615353 2522615353 2522615353 2522615353 2522615352 252615352 252615352 252615352 252615352 252615352 252615352 252615352 252615352 252615352 </td <td>Spoligotyping Second Second</td>	Spoligotyping Second	

Figure 2 A) Genotypic and characteristic of resistance and sensitive strains. MIRU-VNTR are from the MIRU-VNTR plus database, MIRU-VNTR; Mycobacterial Interspersed Repetitive Units Variable Number Tandem Repeat; B) Genotypic and other characteristics of resistant and sensitivity strains. Spoligotyping and MIRU-VNTR from the MIRU-VNTR plus database, MIRU-VNTR; Mycobacterial Interspersed Repetitive Units Variable Number Tandem Repeat; RIF: Rifampicin, INH: Isoniazid

allelic profile between the studied loci. Also loci 2, 4, 20 and 24 had the least allelic difference between all loci.

Discussion

This study showed that "MIRU-VNTR" had low discriminatory power when it is used alone in comparison to when it is used together with Spoligotyping. Using both techniques together decreased the unknown genetic strains to about 10% in sensitive strains and 20% in drug resistant strains. Therefore, for epidemiological studies we suggest to use "MIRU-VNTR and Spoligotyping" techniques together.

DNA fingerprinting of *M. tuberculosis* isolates is useful for determining the extent of recent transmission in a community and the potential risk factors for recent transmission for identifying previously unsuspected trans-

mission, monitoring the transmission of drugresistant strains, and confirming laboratory cross contamination ⁽¹⁶⁾.

In this study, based on MIRU-VNTR analysis the obtained genetic patterns of sensitive and resistance strains were as follows: 13.3% and 16.6% of them belonged to Haarlem, 10% and 16.6% of the second group belonged to Dehli/ CAS (Figure 2A). When both "MIRU-VNTR and Spoligotyping" techniques were used together, the patterns of Haarlem family reduced about 23% and Dehli/CAS was increased about 26% (Figure 2B). These strains are the most frequent patterns in Iranian population, as shown by Farnia et al in 2004 ⁽¹⁷⁾.

In another study by Dou et al in 2002, out of 356 isolates, 66 could not be identified by Spoligotyping, but when he applied both "MIRU-VNTR and Spoligotyping", the number reduced to 30 isolates ⁽¹⁸⁾. In a study conducted by Goyal and co-workers (on 167 patients), Spoligotyping technique identified only genetic pattern of 67 strains but when they used RFLP and Spoligotyping together, the genetic patterns of 135 strains were identified ⁽¹⁹⁾.

In this study the allele differences were accounted too. According to Sola et al loci 10, 16, 23, 26 and 40 were introduced as loci with the most allele polymorphism and loci 4, 20, 24 and 27 as the most poorly discriminated loci ⁽²⁰⁾. Another study conducted by Roring et al in 2002 demonstrated that by using another locus [(VNTR) which their polymorphism is more], we can identify genetic patterns of strains and their families more carefully. In a study by Romano et al, RFLP and Spoligotyping were used to determine the molecular epidemiology of the strains ⁽²¹⁾. In the research performed by Skuce et al it was shown that techniques like MIRU-VNTR can be replaced by IS6110 in molecular epidemiology studies ⁽²²⁾.

Conclusion

The results showed that in molecular epidemiologic studies of *MTB*, using the two techniques of "MIRU-VNTR and Spoligo-

typing" simultaneously could disseminate the genetic patterns of *MTB* strains in a much better manner as compared to using only "MIRU-VNTR" technique.

Acknowledgement

This study was supported by the Mycobacteriology Research Center of Shahid Beheshti University of Medical Sciences.

References

- World Health Organization. 2001. Global tuberculosis control. WHO report 2001. WHO/CDS/TB/ 2001.287. World Health Organization, Geneva, Switzerland.
- Raviglione MC, Snider DE, Kochi A. Global epidemiology of tuberculosis: morbidity and mortality of a worldwide epidemic. JAMA 1995;273(3):220-226.
- Drobniewski F, Balabanova Y, Ruddy M, Weldon L, Jeltkova K, Brown T, et al. Rifampin- and multidrug-resistant tuberculosis in Russian civilians and prison inmates: dominance of the Beijing strain family. Emerg Infect Dis 2002;8(11):1320-1326.
- Supply P, Lesjean E, Savine K, Kremer D, van Soolingen J, Locht C. Automated high-throughput genotyping for study of global epidemiology of Mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. J Clin Microbiol 2001;39(10):3563-3571.
- Groenen PM, Bunschoten EA, van Soolingen D, van Errtbden JDA. Nature of DNA polymorphism in the direct repeat cluster of Mycobacterium tuberculosis: application for strain differentiation by a novel typing method. Mol Microbiol 1993;10(5): 1057-1065.
- Kremer K, van Soolingen D, Forthingham 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(8):2607-2618.
- Mazors E, Lesjean S, Banuls L, Gilbert M, Vincent V, Gicquel B, et al. High resolution minisatellitebased typing as a portable approach to global analysis of Mycobacterium tuberculosis molecular epidemiology. PNAS 2001;98(4):1901-1906.
- Supply P, Magdalena J, Himpens S, Locht C. Identification of novel intergenic repetitive unit in a mycobacterial two-component system operon. Mol Microbiol 1997;26(5):991-1003.

Downloaded from http://www.ajmb.org

- Hawkey PM, Smith EG, Evan JS, Philip M, Bryan G, Mohamed HH, et al. Mycobacterial interspersed repetitive unit typing of Mycobacterium tuberculosis compared to IS6110 –based restriction fragment length polymorphism analysis for investigation of apparently clustered cases of tuberculosis. J Clin Microbiol 2003:41(8):3514-3520.
- Supply P, Mazars E, Lesjean S, Vincent V, Gicquel B, Locht C. Variable human mini satellite-like regions in the mycobacterium tuberculosis genome. Mol Microbiol 2000;36 (3):762-771.
- Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of Mycobacterium tuberculosis for diagnosis and epidemiology. J Clin Microbiol 1997;35(4):907-914.
- Dale JW, Brittain D, Cataldi AA, Cousins D, Crawford JT, Driscohl J, et al. Spacer oligonucleotide typing of bacteria of the Mycobacterium tuberculosis complex: recommendation for standardized nomenclature. Tuber Lung Dis 2001;5(3):216-219.
- 13. Sola C, Filliol I, Legrand E, Mokrousov I, Rastogi N. Mycobacterium tuberculosis phylogeny reconstruction based on combined numerical analysis with IS1081, IS6110, VNTR, and DR-based spoligotyping suggests the existence of two new phylogeographical clades. J Molecular Evol 2001; 53(6):680-689.
- 14. Mokrousov I, Narvskaya O, Limeschenko E, Vyazovaya A, Otten T, Vyshnevskiy B. Analysis of the allelic diversity of the mycobacterial interspersed repetitive units in Mycobacterium tuberculosis strains of the Beijing family: practical implications and evolutionary considerations. J Clin Microbiol 2004;42(6):2438-2444.

15. Kremer K, Arnold C, Cataldi A, Gutierez 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(11):5628-5638.

- Cowan LS, Mosher L, Diem L. Massey JP, Crawford JT. Variable-number tandem repeat typing of Mycobacterium tuberculosis isolates with low copy numbers of IS6110 by using mycobacterial interspersed repetitive units. J Clin Microbiol 2002;40 (5):1592-1602.
- Farnia P, Mohammadi F, Masjedi MR, Varnerot A, Zarifi A, Tabatabee J, et al. Evaluation of tuberculosis transmission in Tehran: using RFLP and spoligotyping methods. J Infect 2004;49(2):94-101.
- Dou HY, Tseng FC, Lin CW, Chang JR, Sun JR, Tsai WS, et al. Molecular epidemiology and evolutionary genetics of Mycobacterium tuberculosis in Taipei. BMC Infect Dis 2008;8:170.
- Goyal M, Saunders NA, van Embedn JD, Young DB, Shaw RJ. Differentiation of Mycobacterium tuberculosis isolates by spoligotyping and IS6110 restriction fragment length polymorphism. J Clin Microbiol 1997;35(3):647-651.
- 20. Sola C, Filliol I, Legrand E, Lesjean S, Locht C, Supply P, et al. Genotyping of the Mycobacterium tuberculosis complex using MIRUs: association with VNTR and spoligotyping for molecular epidemiology and evolutionary genetics. Infect Genet Evol 2003;3(2):125-133.
- 21. Romano MI, Amadio A, Bigi F, Klepp L, Etchechoury I, Nato Liana M, Morsella C, et al. Further analysis of VNTR and MIRU in the genome of Mycobacterium avium complex, and application to molecular epidemiology of isolates from South America. Vet Microbiol 2005;110(3-4):221-237.
- Skuce RA, McCorry TP, McCarroll JF, Roring M, Scott AN, Brittain D, et al. Discrimination of Mycobacterium tuberculosis complex bacteria using novel VNTR-PCR targets. Microbiology 2002;148: 519-528.