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Full Length Research Paper

Study of genetic diversity in *Mycobacterium tuberculosis* by using mycobacterial interspersed repetitive unit: Variable number tandem repeat typing in Khuzestan Province, Iran

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To investigate the genetic diversity of *Mycobacterium tuberculosis* (MTB) strains MIRU-VNTR as a powerful tool was used. In this study 80 isolates from patients were analyzed using primers based on IS6110 gene fragment specific for MTB complex. Then RD typing based on regions of difference was used to differentiate MTBC members. The MTB isolates were genotyped by 12 loci based MIRU-VNTR. The discriminatory index for each loci was calculated using the Hunter Gaston Discriminatory Index (HGDI).Of these, 60 cases were identified as MTB by polymerase chain reaction (PCR) method and RD-PCR typing. Forty eight distinct patterns comprising of 11 clusters and 37 unique Patterns were identified. The discriminatory power of MIRU-VNTR typing was high (HGDI= 0.991) for these samples. Also we could detect a case of mixed infection by MIRU-VNTR. The results show that MIRU-VNTR typing is a useful method for studying genetic diversity of MTB in regional settings.

Key words: *Mycobacterium tuberculosis*, genotyping, mycobacterial interspersed repetitive unit – variable number tandem repeat, Khuzestan, tuberculosis.

INTRODUCTION

Tuberculosis (TB) is a major cause of illness and death worldwide, especially in Asia and Africa. According to WHO report (2009), 9.27 million new cases of TB (139 per 100,000) including 4.1 million new smear positive occurred in 2007. Also the prevalence of TB in Iran was 27 per 100,000 populations in 2007 (WHO, 2009).

According to available data, in some countries there is an increase incidence of disease, because of effect of HIV and deficiency of tuberculosis control plan (Godreuil et al., 2007), so scientists experienced a feeling of need for strong tools in the epidemiological studies (Mazars et al., 2001).

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Mycobacterium tuberculosis (MTB) genotyping can effectively improve TB control program (Sun et al., 2004), by controlling the disease transmission (Barnes and Cave, 2003), discrimination between relapse and re-infection (Allix-Beguec et al., 2008), identification of laboratory cross-contamination (Burgos and Pym, 2002), recognition of risk factors for transmission (Daley and Kawamura, 2003), and evaluation of drug resistance (Mathema et al., 2008).

Several molecular techniques are available to explore the genetic diversity of MTB populations (Kanduma et al., 2003). Three main methods used in molecular epidemiology studies include: IS 6110- restriction fragment length polymorphism (RFLP), Spoligotyping and Mycobacterial Interspersed Repetitive Unit – Variable Number Tandem Repeat (MIRU-VNTR) (Sola et al., 2003).

IS 6110-RFLP is a gold standard for MTB genotyping (Oelemann et al., 2007), however comprises significant disadvantages: It is time-consuming (Sun et al., 2004), a large quantities of DNA is required (Christianson et al., 2010), and in low copy number strains it has weak discriminatory power (Kwara et al., 2003). MIRU-VNTR which was introduced by Supply et al. (2000), has been suggested as a new and first line genotyping standard technique in recent decade (van Deutekom et al., 2005; Hanekom et al., 2008). This PCR-based method is faster than IS6110-RFLP and the results can be demonstrated in a digital format (Allix et al., 2004). Since the introduction of MIRU-VNTR technique, several sets of loci has been suggested, however, the 12 locus MIRU-VNTR is the most popular system among other sets of loci (Supply et al., 2006).

Identification of MTB Complex (MTBC) members including *M. tuberculosis, Mycobacterium africanum, Mycobacterium microti, Mycobacterium pinnipedii, Mycobacterium bovis, Mycobacterium bovis BCG, Mycobacterium caprae*and, *Mycobacterium canetti* (Huard et al., 2003), is important for epidemiological surveillance (Pinsky and Banaei, 2008). One of the reliable techniques for differentiation of MTBC members is region of difference (RD) typing, which is a PCR-based method rely on the presence or absence of RDs (Pinsky and Banaei, 2008).

Due to the importance of TB, significant prevalence of the disease in Iran, importance of genotyping in studies of molecular epidemiology and it's role in construction of effective control program, we have used RD typing for differentiation of MTBC members and standard 12 MIRU-VNTR loci for genotyping MTB strains isolated from tuberculosis patients in Khuzestan Province, Iran.

MATERIALS AND METHODS

Clinical isolates

This study was performed on 80 MTB positive cultures obtained from patients referred to tuberculosis reference laboratory of Khuzestan Province, from October 2008 to July 2010. The preliminary proposal of the work has been approved in University's ethics committee under the registration number of T/8/20/D/855 dated July 2009. The positive cultures include growth in Lowenstein Jensen (LJ) medium after decontamination of sputum samples and incubation at 37°C for 4-6 weeks (Khosravi et al., 2009). For DNA extraction, a few colonies from surface of LJ medium were harvested and suspended in 500 µl 10 mM Tris-HCl/1mM EDTA [pH 7] (Mazars et al., 2001), and inactivated at 100°C for 30 min (Hosek et al., 2006). After centrifugation at 12000 rpm for 15 min, the supernatant was used for PCR.

PCR

Identification of the MTBC was performed with PCR amplification of the IS 6110 element, specific for the MTBC, using the forward primer (5´CCTGCGAGCGTAGGCGTCGG 3´) and reverse primer (5´CTCGTCCAGCGC CGCTTCGG 3´) which amplify a 123 bp fragment (Walker et al., 1992; Kocagoz et al., 1993).

Table 1. The primer sequences of RD1, RD4, RD9 and RD12.

RD1	5´AAGCGGTTGCCGCCGACCGACC 3´ 5´CTGGCTATATTCCTGGGCCCGG 3´ 5´GAGGCGATCTGGCGGTTTGGGG 3´
RD4	5´ATGTGCGAGCTGAGCGATG 3´ 5´TGTACTATGCTGACCCATGCG 3´ 5´AAAGGAGCACCATCGTCCAC 3´
RD9	5´CAAGTTGCCGTTTCGAGCC 3´ 5´CAATGTTTGTTGCGCTGC 3´ 5´ GCTACCCTCGACCAAGTGTT 3´
RD12	5´ GGGAGCCCAGCATTTACCTC 3´ 5´GTGTTGCGGGAATTACTCGG 3´ 5´AGCAGGAGCGGTTGGATATTC 3´

A reaction mixture of 50 µl containing 10x PCR buffer [50 mM KCl, 10 mM HCl (pH 8)],1 mM $MgCl_2$,0.2 mM dNTPs, 0.2 µM of each primer, 2.5 U of Taq polymerase and 10 µl of DNA template were prepared. All the reagents were purchased from Cinnagen Company, Tehran, Iran. The standard MTB H37Rv (Institute Pasteur, Iran) was used as positive control and deionized distilled water (DDW) was used as negative control.

The reaction condition were as follows: An initial denaturation at 94°C for 5 min; 35 cycles of denaturation at 94°C for 2 min, annealing at 68°C for 2 min and extension at 72°C for 2 min using a thermocycler [eppendorf, Germany] (Hosek et al., 2006). The PCR products were analyzed on 1.5% agarose gel in 1X TBE and visualized by staining with 0.5 mg/ml ethidium bromide. The product sizes were identified using a 50bp DNA ladder (MBI Fermentas, Germany).

RD-PCR typing

The PCR for RD analysis was performed on specimens having an initial PCR positive results and belonged to MTBC using the RD group primers (Table 1) (Warren et al., 2006).

PCR amplification

Each PCR reaction (12.5 µl) contained 1.25 µl 10X PCR buffer, 0.04 mM dNTPs, 2 mM $MgCl_2$, 0.08 µM of each primer, 0.5 µl DMSO (Merck), 3.1 U Taq polymerase, 5.38 µl of DNA template and was made up to final volume with DDW. The reaction condition were as follows: 95°C for 5 min, followed by 35 cycles of 94°C for 40 s, 60°C for 40 s, 72°C for 40 s, and a final extension at 72°C for 10 min. The analysis of products was similar to the initial PCR amplification, except for ladder which 100 and 50 bp ladders were used for the latter PCR.

MIRU-VNTR typing

For typing the Hot Start Taq DNA polymerase Kit (Qiagen) was used. The volume of 2 µl DNA was added to a PCR mixture (final volume 20 µl) composed of 4 µl Q-solution, 2 µl PCR buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 0.4 U of Hot start DNA

Table 2. Primer sequences for 12 MIRU-VNTR loci.

MIRU locus	Primer sequences
4	5′- GCGCGAGCCCGAACTGC- 3′ 5′- GCGC AGCA GAAACGTCAGC- 3′
26	5′-TAGGTCTACCGTCGAAATCTGTGAC-3′ 5′-CATAGGCGACCAGGCGAATAG-3′
40	5′-GGGTTGCTGGATGACAACGTGT-3′ 5′-GGGTGATCTCGGCGAAATCAGATA-3′
10	5′-GTTCTTGACCAACTGCAGTCGTCC-3′ 5′-GCCACCTTGGTGATCAGCTACCT -3′
16	5′-TCGGTGATCGGGTCCAGTCCAAGTA-3 ′ 5′-CCCGTCGTGCAGCCCTGGTAC -3′
31	5′-ACTGATTGGCTTCATACGGCTTTA -3′ 5′-GTGCCGACGTGGTCTTGAT-3′
2	5′- TGGACTTGCAGCAATGGACCAACT -3′ 5′- TACTCGGACGCCCGCTCAAAAT-3′
23	5′-CTGTCGATGGCCGCAACAAAACG -3′ 5′-AGCTCAACGGGTTCGCCCTTTTGTC -3′
39	5′-CGCATCGACAAACTGGAGCCAAAC-3′ 5′-CGGAAACGTCTACGCCCCACACAT-3′
20	5′-TCGGAGAGATGCCCTTCGAGTTAG-3′ 5′-GGAGACCGCGACCAGGTACTTGTA -3′
24	5′-CGACCAAGATGTGCAGGAATACAT-3′ 5-′GGGCGAGTTGAGCTCACAGAA-3′
27	5′-TCGAAAGCCTCTGCGTGCCAGTAA-3′ 5′-GCGATGTGAGCGTGCCACTCAA-3′

polymerase and 1-3.5 mM $MgCl_2$ according to MIRU locus [http://www.miruvntrplus.org] (Weniger et al., 2010).

The sequences of primers are presented in Table 2 (Supply et al., 2001). Then reaction condition were as: 15 min initial denaturation at 95°C followed by 40 cycles of 94°C for 1 min, 59°C for 1 min and 72°C for 1.5 min. The reaction was terminated by a final extension at 72°C for 10 min. The PCR amplification products were analyzed on 1.5% agarose gel. The sizes of the amplicons were estimated by comparison with control strain H37Rv and the size of ladder (100 and 50 bp). The sizes of amplicons were used to determine the number of repetitions by reference to a table that is available at http://www.miruvntrplus.org. The results were given in the 12-number code and Dendrogram of the typing method was generated using UPGMA analysis using the www.miruvntrplus.org website (Weniger et al., 2010).

MIRU-VNTR analysis

The MIRU-VNTR allelic diversity (h) at each of the loci was calculated by the equation

$$h = 1-\Sigma\chi_i^2$$

Where , X*i* is the frequency of the *i*th allele at the locus (Sun et al., 2004). The Hunter-Gaston discriminatory index (HGDI) utilized for calculating MIRU-VNTR discriminatory power by using following formula (Hunter and Gaston, 1988).

$$HGDI = 1-\left[\frac{1}{N(N-1)}\sum_{j=1}^{s} n_j(n_j-1)\right]$$

S is the total number of different MIRU-VNTR patterns, N is the total number of strains in the typing pattern and nj is the number of strains belonging to the jth pattern.

RESULTS

Positive cultures were obtained from 80 patients, of which 44 were male and 36 were female with mean age of 40.6. Sixty out of 80 samples were positive by PCR method, so they belonged to MTBC (Figure 1). RD-PCR typing showed that all 60 isolates were MTB. By MIRU-VNTR analysis, 48 distinct patterns comprising of 11 clusters (9 clusters of 2 strains each and 2 clusters of 3 strains each) and 37 unique patterns were identified (Tables 3a and 3b). The discriminatory power of MIRU-VNTR typing was high (HGDI= 0.991) for these samples.

The allelic diversities of the 12 MIRU-VNTR locus are shown in Table 4. Based on this study, loci 10, 16, 27, 31 and 39 were highly discriminative (≥ 0.6), loci 23, 26 and 40 were moderately discriminative (≥ 0.3) and loci 2,4,20 and 24 were designated as poorly discriminative (< 0.3). Sixty clinical isolates were analyzed. The dendrogram was obtained using the Bionumerics software and the UPGMA algorithm (Unweighted Pair Group Method) was located in website (Figure 2).

DISCUSSION

In this study we used MIRU-VNTR typing to analysis MTB strains in Khuzestan Province that is located on the south western of Iran. Khuzestan is the focus of interest for transport and migration due to oil and petrochemical industry. In the other hand, this province is located in the neighborhood of Iraq, and According to WHO report (2009), the prevalence of TB in Iraq was 79 per 100,000 populations in 2007 (WHO, 2009).

We used RD typing for differentiation of MTBC members which is important for the accurate genetic diversity. According to results we had 37 unique patterns and 24 isolates included in 11 clusters. The variety of genetic patterns, and relatively high average age (40.6) probably revealed the reactivation in studied population.

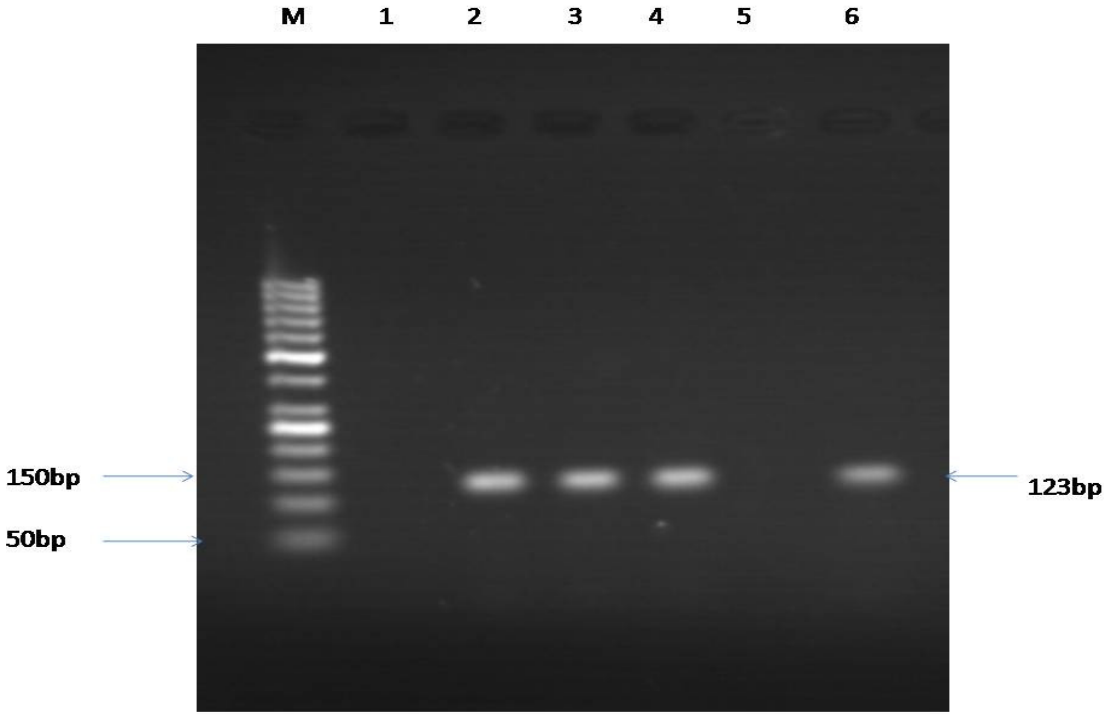

Figure 1. Gel electrophoresis of the PCR amplification using MTBC specific primers. Lanes: M, Molecular size marker; 1, negative control; 2, positive control; 3,4,6, positive samples; 5, negative sample.

Table 3a. MIRU-VNTR patterns of strains from Khuzestan Province of Iran at different loci.

**Strains	*L2	L4	L10	L16	L20	L23	L24	L26	L27	L31	L39	L40
2	3	2	2	2	3	2	1	1	1	3	4	2
1	1	2	2	3	4	2	6	1	5	4	6	2
1	1	2	2	3	3	2	6	1	5	2	6	2
2	2	2	2	4	3	2	7	1	5	2	6	2
1	2	2	2	3	3	2	7	1	5	3	6	2
1	2	2	2	4	3	2	7	1	5	3	5	2
3	3	2	2	3	3	2	5	1	5	3	3	3
2	2	2	2	3	3	2	5	1	5	3	3	2
1	3	2	2	3	4	2	5	1	5	3	4	3
1	3	2	2	3	5	2	5	1	5	4	3	3
1	3	2	2	3	4	2	5	1	5	2	3	3
1	2	2	2	3	4	2	5	1	5	4	4	3
1	2	3	2	3	4	2	6	1	4	4	4	3
1	2	2	2	4	4	2	5	1	5	4	4	3
1	2	2	2	3	3	2	5	1	4	3	4	3
2	3	2	2	7	4	2	5	1	5	3	5	3
2	2	2	2	4	4	2	5	1	5	3	5	2
1	1	2	2	7	3	2	6	1	5	3	5	3
1	3	2	2	7	3	2	5	1	5	3	3	3
2	3	2	2	3	5	2	5	1	9	4	6	3
2	3	2	2	3	3	2	5	1	9	3	6	3
1	3	2	2	3	5	2	6	1	5	2	6	4
3	3	2	2	6	4	2	5	1	5	4	6	4
2	3	2	2	6	3	2	5	1	5	3	6	3

*Locus, ** No. of strains.

Table 3b. MIRU-VNTR patterns of strains from Khuzestan Province of Iran at different loci.

**Strains	*L2	L4	L10	L16	L20	L23	L24	L26	L27	L31	L39	L40
1	2	2	2	6	3	2	5	1	5	4	6	4
1	3	1	2	6	3	2	5	1	5	4	5	4
2	3	2	2	7	4	2	5	1	5	4	6	4
1	3	2	2	7	3	2	5	1	5	3	4	4
1	1	2	2	7	3	2	5	1	5	4	6	4
1	3	1	2	7	5	2	5	1	5	4	6	4
1	2	2	2	4	1	2	5	1	5	1	3	2
1	2	2	2	4	4	2	6	1	1	4	4	3
1	2	1	2	4	4	2	6	1	4	4	4	3
1	2	1	2	3	4	2	7	1	5	4	4	3
1	4	2	2	4	4	2	5	1	5	4	2	2
1	3	2	2	7	4	2	6	1	5	2	6	3
1	2	3	2	4	1	2	5	1	4	1	3	2
1	2	2	2	3	1	2	5	1	5	1	4	2
1	3	2	2	4	1	2	5	1	5	1	5	3
1	3	2	2	2	1	2	5	1	5	1	5	2
1	3	3	2	3	3	2	5	1	5	3	5	3
1	2	3	2	3	3	2	7	1	5	3	5	3
1	4	1	2	3	4	2	7	1	5	4	4	2
1	4	1	2	3	4	2	7	1	5	3	4	3
1	2	2	2	3	3	2	6	1	4	5	4	3
1	3	2	2	3	3	2	5	1	4	5	4	3
1	2	2	2	3	3	2	6	1	4	4	4	2
1	3	2	2	3	4	2	5	1	5	3	6	3

* Locus; ** No. of strains.

Table 4. Allelic diversity of each MIRU-VNTR locus.

MIRU locus	Allele number									Allelic diversity
	1	2	3	4	5	6	7	8	9	
2*	6	51	4							0.28
4		60								0.02
10		3	29	11		7	10			0.68
16	5		28	22	5					0.63
20		60								0.02
23	2				41	9	8			0.48
24	60									0.02
26*	3			7	47				4	0.36
27	5	6	26	21	5					0.67
31		1	10	16	11	22				0.73
39		19	30	11						0.61
40	4	21	32	3						0.58

*Total number of 61 because of mixed infection.

Comparing the patterns between our study and a previous study in East Azarbaijan (Asgharzadeh et al., 2007), the province located in North Western of Iran, showed that there were not any similarity between them.

The average of allelic diversity of the 12MIRU-VNTR in our samples (0.47) was higher than that reported earlier for strains from America (0.29) (Cowan et al., 2002), but it was near to Singapore (0.42) (Sun et al., 2004), and France (0.4) (Mazars et al., 2001). The discriminatory power (0.991) was near to the discriminatory power that

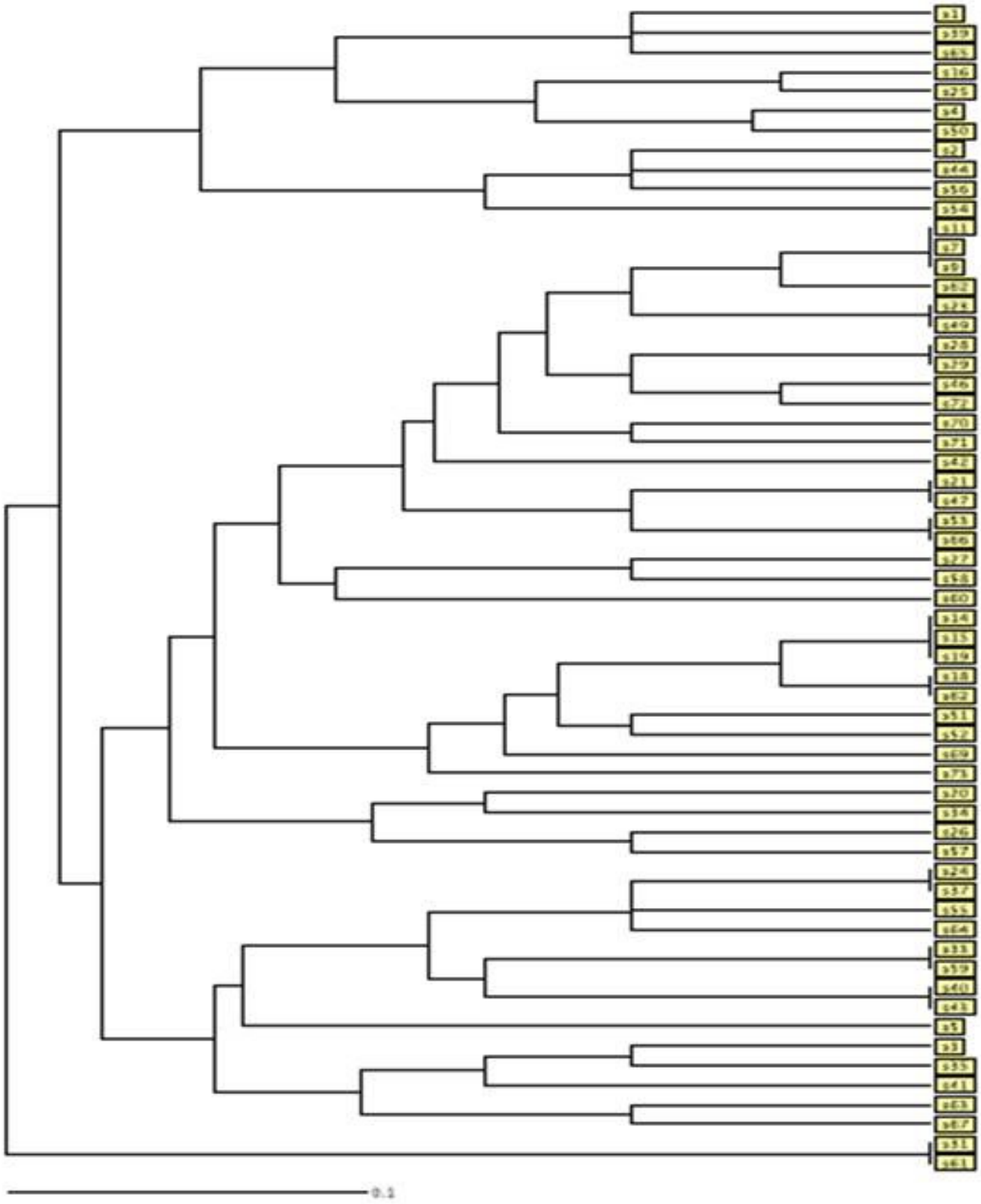

Figure 2. The dendrogram obtained for analyzed clinical isolates using the Bionumerics software and the UPGMA algorithm (Unweighted Pair Group Method).

was reported for 12 MIRU-VNTR loci in previous studies as (0.978) (Alonso-Rodriguez et al., 2008), (0.951) (Chin and Jou, 2005), (0.993)(Asgharzadeh et al., 2007).

In our samples, loci 10, 16, 27, 31 and 39 were highly discriminative, they were similar to reported loci of 10, 16, 26, 27, 31, 39 and 40 in previous study from Pakistan (Ali et al., 2007). Our difference with the East Azarbaijan study was in loci 26 and 40 (Asgharzadeh et al., 2007). Also in this study, loci 2, 4, 20 and 24 were designated as poorly discriminative. In a similar study from Iran, loci 2, 4, 20 and 24 were identified with concordant to our study (Jafarian et al., 2010). The effect of MIRU-VNTR Loci, for differentiation between strains in various populations is different, therefore, determination of the loci with high discriminative power in each population is important.. Due to the presence or absence of an *M. tuberculosis* specific deletion (TbD1), it is possible to divide *M. tuberculosi*s strains into ancestral and “modern” strains. The major clusters that the latter includes are Beijing, Haarlem and African *M. tuberculosis* (Brosch et al.,2002).

According to study of Thorne et al. (2007), all TbD1+ strains (ancestral) contained two copies of MIRU24, while all TbD1- strains (modern) contained one copy. In our study, all the strains contained one copy, therefore it can be concluded that probably all of the strains belong to modern genotype. Also in their study, the ancestral genotype comprised of predominantly five copies in MIRU4, however in Haarlem and Beijing genotypes 2 copies were predominant, while in our study all the strains comprised of two copies.

According to Rao et al. (2006), MIRU26 locus is appropriate for differentiating between Beijing and non-Beijing strains. The Beijing strains have 7 copies but non-Beijing ones have more or less than 7 copies. In Throne et al. study (2007) in MIRU26, in the Beijing and Haarlem genotypes respectively 7 and 5 copies and in our study 5 copies were predominant. Also we could detect a case of mixed infection by MIRU-VNTR and simultaneous occurrence of 2 different alleles in 2 loci of one sample was identified. To examine the reproducibility of this situation, each of the 12 loci of this strain were analyzed several times. This patient referred as a treatment control. There are other reports of identification of mixed infection by MIRU-VNTR analysis (Allix et al., 2004; Shamputa et al., 2006).

In conclusion, MIRU-VNTR is a reproducible method and has a potential for tracking epidemiological events such as transmission or relapse. This technique allows direct comparison of results between laboratories. Besides, it can detect mixed infection which can facilitate management of treatment and disease control. MIRU-VNTR typing may be accepted as a first line method for molecular epidemiology of MTB.

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