Snapshot of Genetic Diversity of *Mycobacterium Tuberculosis* in Al-Sadr City, by Using Mycobacterial Interspersed Repetitive Units-Variable Number Tandem Repeats (MIRU-VNTR) Genotyping Method. A Preliminary study

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Abstract

Background: Genotyping methods of *M. tuberculosis* (MTB) isolates such as MIRU-VNTR provides informations that may help in TB control program because it help in detecting the infection, confirming lab errors, determining treatment failure, reactivation and exogenous reinfection, and to detect any epidemiological link between patients by monitoring the transmission chain and identifying successful clones of MTB isolates and especially those with multidrug resistance. Al-Sadr city is the most populated city in Iraq with majority of people are living under the poverty line. Crowdedness and poverty are two essential requirements for successful tuberculosis (TB) transmission.
Aims: To Shed light on the genetic diversity, clustering rate and transmission dynamics of TB cases while assessing the utility of 15-loci MIRU-VNTR in typing of MTB clinical isolates in Al-Sadr city.
Subjects and Methods: Twenty nine MTB culture isolates were genotyped by using MIRU-VNTR-15 typing.
Results: Twenty five distinct type were yielded, 8 isolates (27.6%) were grouped into 4 clusters (2 isolates/cluster), and 21 (72.4%) were unique. The clustering rate was (13.7%). Fourteen out of 15 loci were moderate or highly discriminative ($h > 0.6$, and $h > 0.3$, respectively) according to their allelic diversity. The mean of the allelic diversity of the 15 loci was high (0.64), indicating the high power of discrimination of MIRU-VNTR typing (HGDI was 0.99).
Conclusions: MIRU-VNTR typing has a high discriminatory power and is useful in identification of the origin and transmission of *M. tuberculosis* isolates Al-Sadr city and possibly other densely populated cities in Iraq.

Introduction

Mycobacterium tuberculosis is one of the most clinically significant infectious agent which is responsible for extensive morbidity and mortality worldwide with approximately 8-10 million new cases and 2 million deaths annually (1). Iraq is one of the countries with the highest incidence among the eastern Mediterranean region with tuberculosis (TB) accounting for 56/100000 population in 2006 (2). Al-Sadr city is the most populated city in Iraq with more than 4 millions inhabitant and number of people/meter is the highest. In addition, for the last 4 decades, most of people are living under the poverty line. Crowdedness and poverty are two essential requirements for successful TB transmission. Furthermore, there is little information about the genotyping and molecular characterization of MTB clinical isolates in Iraq. Thus the study of the genetic diversity may be helpful in demonstration of the transmission dynamics of TB in this city.
Genotyping methods have unraveled the clonal population structure of MTB which

comprises distinct geographic distribution, virulence and association with multidrug resistance TB (MDR TB). Therefore the recognition of specific MTB clones (e.g. some clones of (W/Beijing lineage) can be predictive of (multi) drug resistance TB in certain context and can provide indications for differentiation between an infection acquired abroad and clonal transmission (3,4,5,6,7,8,9)

Identification of homogeneity between strains of *M. tuberculosis* can help not only in certifying the route of infection and the pattern of spread in a nationwide outbreak, but also in recognizing whether a particular infection represents reactivation or reinfection (10). The accurate identification and studying specific clones worldwide may contribute to the development of new diagnostic, prophylactic and therapeutic tools in TB control (4).

Restriction Fragment Length Polymorphism (RFLP) based on variation in copy number of an MTB specific insertion sequence IS6110 is considered a "gold standard" genotyping method (11). However, there are several drawbacks for this method, it is labor intensive and required culturing for 3 to 4 weeks because it uses colonies, not DNA amplification (10). In addition , it has poor discriminatory power for MTB strains with low copy number of IS6110 (12, 13, 14). Moreover, it's difficult to compare IS6110 RFLP results between laboratories. This hinders global studies of MTB epidemiology and transmission (15).

Mycobacterial interspersed repetitive unit based on variable number tandem repeats (MIRU-VNTR) genotyping has become a major method for the epidemiological tracking of MTB clones because of its portable data, discriminatory power, and recently proposed standardization (16). Moreover, have been proved to be reliable and reproducible typing methods that enable a level of discrimination between MTB strains. MIRU-VNTR genotyping is performed by amplifying a panel of 12, 15, 24 loci (12, 17, 18, 19), and makes the use of length variation of independent minisatellite like loci scattered throughout the MTB genome (15). Alonso-Rodriguez *et al* demonstrated that amplification of 15 MIRU-VNTR loci is very efficient tool in genetic characterization of MTB (20). The high resolution, fast turnaround time, easy to perform, ability to compare results between labs and the possibility of high throughput analysis make MIRU-VNTR an attractive method for global study of the molecular epidemiology of MTBC isolates (10).

The aim of this study was to investigate the clonal diversity of MTB culture isolates derived from population at a single geographic region in Iraq (Al-sadr community) by using MIRU-VNTR. This will help in identification the route of infection, transmission dynamics and highlight the most successful strain circulating in the community.

Subjects and Methods

Clinical isolates and DNA extraction:

Twenty nine *M. tuberculosis* culture isolates recovered from 29 Iraqi patients suffering from active pulmonary tuberculosis attending the National Reference Laboratory (NRL) that belonging to the National Center of Tuberculosis and chest illnesses-NTP at Baghdad/ Iraq were collected between January 2011 and July 2012.

Genomic DNA was extracted as described previously (21, 22, 23). Briefly, cultured colonies were suspended in 30 µl TE buffer (10 mM Tris-HCL (pH,8), 1 mM EDTA). Then, the mixture was heat inactivated at 95 °c for 30 min, centrifuged (4000 rpm for 2 min), and the supernatant was stored at -20 °c until use. H37Rv DNA was used as positive control.

MIRU-VNTR typing of the isolates

All of the isolates were typed by Individual PCR-amplification targeting 15 MIRU-VNTR loci (Mtub04, Mtub21, Mtub30, Mtub39, QUB11b, QUB26,

QUB4156, MIRU4, MIRU10, MIRU16, MIRU26, MIRU31, MIRU40, ETRA, ETRC) using specific primers that described in the MIRU-VNTR standard protocol (20). For each reaction, DNA from *M. tuberculosis* H37Rv was used as a positive control, and sterile water was used as a negative control. PCR products were electrophoretically separated on 2% agarose gels, using a 100-bp DNA ladder as size markers. From the gel images, the corresponding MIRU-VNTR bands were interpreted as copy numbers based on the reference table in the Supply's (2006) protocol (23).

Data analysis and construction of phylogenetic tree:

Data obtained from MIRU-VNTR were imported in the MIRU-VNTR*plus* database (http://www.miru-vntrplus.org/), using a categorical coefficient of 1 and then Neighbor Joining dendogram was constracted using MIRU-VNTR*plus* online program, (Figure 1).

Clustering rate was calculated using the formula $(n_c\text{-}c)/n$ where the n_c: is the total number of clustered cases, *c*: is the number of clusters, *n*: is the total number of cases in the study (24).

The discriminatory power and allelic diversity were calculated using *h* value and Hunter Gaston Discriminatory Index (HGDI) using the equations: $h= 1-\Sigma xi^2$ where xi is the frequency of the *i*th allel at each locus (25, 26).

HGDI= $1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j-1)$ where N: is the total number of isolates, s: is the total number of different patterns, and *nj*: is the number of isolates belonging to the *j*th pattern (27).

The mean of allelic diversity of the MIRU-VNTR method was calculated from the formula: $H=\Sigma h/n$ where the *h* is the allelic diversity and *n*: is the number of loci (28).

Results

Twenty nine MTB culture isolates from 18 (62.1%) males and 11 (37.9%) females Iraqi patients were included in this study (male to female ratio was 1.6 to 1). The mean age of patients was 34±15.67 years. The mean age of the males(36.5±16.2) and the mean age of the females(30.2±14.6).

MIRU-VNTR: strain relatedness and clustering rate

Amplification of 15 MIRU-VNTR loci was performed for all isolates to determine MTB strain relatedness, and clustering, the MIRU-VNTR genotypes. Then, data were matched with the reference strains in the MIRU-VNTR*plus* database (http://www.miru-vntrplus.org/), using a categorical coefficient of 1. Neighbor Joining dendogram was constructed from strains genotypes using the MIRU-VNTRplus online program.

A total of 25 distinct MIRU-VNTR profiles were detected, corresponding to 21(72.4%) orphan profiles and 4(27.6%) clusters containing 2 isolates/ cluster. The clustering rate was 13.7%. MIRU-VNTR typing showed high discriminatory efficiency in this study as it showed a high HGDI value (0.99). This high resolution was due to 14 out of the 15 loci were highly to moderately discriminate.

Allelic diversity:

The allelic diversity of each of the 15 MIRU-VNTR loci was calculated. Twelve loci were highly discriminate (*h>0.6)*, two moderately discriminate (Mtub30, Mtub39, MIRU31; $0.3\leq h\leq 0.6$) and one loci was less polymorphic or poorly discriminate (MIRU4; *h<0.3)*, when ranked according to the method of Sola *et al.* (tables, 1). QUB4156 was found to be the most discriminatory loci (*h*=0.87), whereas, MIRU4 was found to be the least discriminatory one (*h*=0.04). The mean of the allelic diversity of 15 loci was 0.64. HGDI was calculated (0.99) and this result revealed that the discriminatory power of the MIRU-VNTR was high.

Table 1: Allelic diversity of each MIRU-VNTR locus

loci	Number of isolates with each locus									Allelic* diversity	Conclusion
	1	2	3	4	5	6	7	8	9		
MIRU4	1	28								0.04	Poorly discriminate
MIRU10		1	4	9	7	6	2			0.8	Highly discriminate
MIRU16	1	4	10	11	1	2				0.74	Highly discriminate
MIRU26	1		3	1	9	1	11	1	2	0.76	Highly discriminate
MIRU31		1	14	12	2					0.66	highly discriminate
MIRU40		5	14	9						0.66	Highly discriminate
ETRA	1	11	8	7		2				0.75	Highly discriminate
ETRC		14	7	8						0.65	Highly discriminate
Qub11b		16	1	8	1	1	1			0.63	Highly discriminate
Qub26		3	11	14	1					0.63	Highly discriminate
Qub4156			6	2	3	5	7	4	2	0.87	Highly discriminate
Mtub04		7	9	1	8					0.78	Highly discriminate
Mtub21	1	4	7	8	3	6				0.82	Highly discriminate
Mtub30		23	1	5						0.37	Moderately discriminate
Mtub39	1		4	19	3					0.56	Moderately discriminate
H value‡	0.64										

* Allelic diversity *(h)* was defined as h= 1-Σxi2 where xi is the frequency of the ith allel at each locus (26, 27). ‡ H value is the mean of allelic diversity(29).

Discussion

We have used MIRU-VNTR typing method to study the genetic diversity and clonal population structure of MTB clinical isolates. This may highlight the most successful strains circulating within population in specific geographical region. Data generated from this assay were used to construct phylogeny. There was high diversity of MIRU-VNTR patterns among our isolates; a total of 25 distinct profile were detected (Figure 1), corresponding to 21(72.4%) orphan profiles and 4 (26.4%) clusters each with 2 isolates/ cluster. A cluster was defined as two or more patients strains with identical genetic patterns. Clustered were assumed to have arisen from recent transmission, and the clustering rate was used to determine recent transmission of MTB (29). The clustering rate in the current study was (13.7%), implying that the minimum estimate of disease related to recent transmission was (13.7%). The impact of the presence of low transmission rate of disease in this community could be due to reactivation of MTB infection.

HGDI calculation revealed that the discriminatory power of the MIRU-VNTR was high 0.99. This high resolution was due to 14 out of the 15 loci were highly to moderately discriminate. Twelve loci were highly discriminate (*h>0.6)*, two moderately discriminate (Mtub30, Mtub39, MIRU31; $0.3 \leq h \leq 0.6$) and one loci was less polymorphic or poorly discriminate (MIRU4; *h<0.3)*, (tables 1). QUB4156 was found to be the most discriminatory loci (*h*=0.87), whereas, MIRU4 was found to be the least discriminatory one (*h*=0.04). The mean of the allelic diversity of 15 loci was 0.64. Indeed, loci with high discriminatory index show more allelic diversity than others, which may be due to different selection pressures acting at different loci. The molecular clocks of these loci may be faster than others. On the other hand, loci with low discriminatory index may tolerate more polymorphisms than others (30). This is because that The stepwise trend seen among discrepant isolates suggests that evolution is bidirectional in that the

number of repeat copies either increases or decreases over time. MIRU-VNTR high variability may be a way for the bacteria to adapt to a new environment. Changes in the copy number of tandem repeats may affect gene regulation (31) and have been shown to affect the expression of genes involved in adaptive responses (32). Taking into account the high degree of clonal stability of MIRU-VNTR loci and evidence available from well-defined epidemiologic studies (23, 33, 34, 35), differences of two or more loci and even, to a lesser extent, of a single locus remain strongly predictive of absence of a direct transmission link (i.e., of infection by independent strains). This reflects the high genetic diversity of MTB strains in Al-Sadr community which could be due to reactivation of latent MTB infection, and diversity in host genetics (36).

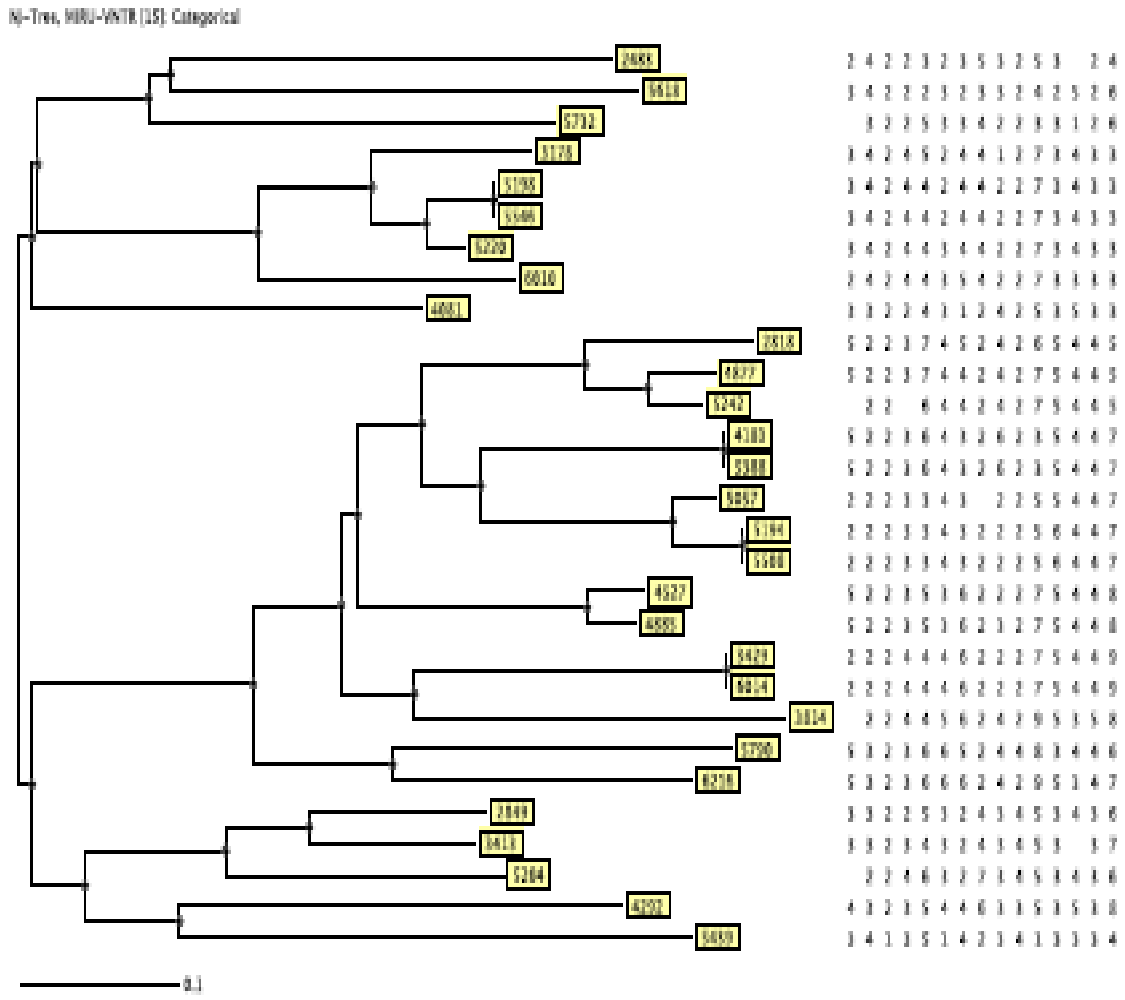

Figure 1: Neighbor Joining dendogram showing clustering among the genotype of MTB isolates.

Conclusions

MIRU-VNTR is a high-throughput, reproducible method, can differentiate MTB isolates, have a high discriminatory value for molecular epidemiological investigations, as demonstrated in this study. the high genetic diversity of MTB strains and the presence of low transmission rate in Al-Sadr community could be due to reactivation of latent MTB

infection rather than recent transmission, and/or due to diversity in host genetics. This will be highly influential with regards to infection control measures and disease management. Probably, in future, treatment of latent MTB infections should be considered as a control strategy.

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