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Molecular typing of *M. tuberculosis* isolates obtained from Sethi Colony Slum area of Jaipur City

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Abstract

Globally, 9.6 million people are estimated to have fallen ill with TB in 2014. Due to high prevalence of tuberculosis in India, there is an urgent need to apply the techniques that characterize tubercle bacilli to facilitate epidemiological studies. Moreover, there are limited studies delineating transmission dynamics of the disease in high risk zones such as slum areas. Slum areas and congested localities have particularly been known to favor transmission of tuberculosis and the spread of drug resistance. This study was planned to understand the transmission of tuberculosis in the Sethi Colony slum area of Jaipur City by MIRU-VNTR typing method. All the 24 culture positive isolates were processed for MIRU-VNTR typing, of which 41.67% isolates were found in three clusters and the transmission rate of tuberculosis was found to be 29.16% in the study area.

Keywords: *Mycobacterium tuberculosis*, Molecular typing, MIRU-VNTR, Transmission, Clustering, Fingerprinting

Introduction

Epidemiological studies help in understanding transmission of tuberculosis which helps in controlling spread of TB. The advent of molecular biology has further helped in understanding molecular epidemiology of *Mycobacterium tuberculosis*. Molecular typing has been used as a powerful adjunct to TB control (Evans *et al.,* 2010). Strain diversity may also be related to differences in severity of disease, transmissibility and mortality (*Caws et al.*, 2008). Molecular typing can also help in distinguishing between members of the *M. tuberculosis* (MTB) complex, which might have vital clinical implications under certain circumstances (Allix-Beguec *et al.*, 2008; Cardoso *et al*., 2011). DNA fingerprinting of *MTB* isolates performed in combination with contact tracing by interviews helps to confirm or dispute contact tracing information (van Lambregts *et al*., 2003; de Vries *et al.*, 2009; Schurch and van Soolingen, 2012). Important aspects of epidemics such as spatio-temporal, pathogenetic and phylogeographic characteristics (Filliol *et al.*, 2003; Hirsh *et al.*, 2004; Brudey *et al.*, 2006) and the description of the distribution of bacterial genotype families can be elucidated by a

collection of bacterial typing data (Schurch and van Soolingen, 2012).Various PCR based strain genotyping methods including Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Restriction Fragment Length Polymorphism (RFLP), Direct Repeat (DR) based methods, MIRU-VNTR typing and spoligotyping are also now available (Mostrom *et al.,* 2002). MIRU-VNTR typing has been described as effective tool in strain typing of *MTB* complex isolates, representative of global diversity (Cowan *et al.,* 2002). The aim of this study was to understand the transmission and diversity in the Sethi Colony slum area of Jaipur City by MIRU-VNTR typing method.

Sample Collection:

This study was conducted in Advanced Research and TB Laboratory of Department of Microbiology and Immunology, SMS Medical College, Jaipur. A total of 98 sputum samples were collected from Sethi Colony slum area of Jaipur city. Early morning sputum samples in 2-10 ml volume were collected in sterile disposable sputum collection containers and were stored at 4 ºC until processed.

Culture for MTB:

Culture of sputum samples was done on Lowenstein Jensen (LJ) medium (Kubica and David, 1980) in BSL-II laboratory. Sputum samples were processed with 4% sodium hydroxide (Modified Petroff's method) in 1:1 ratio for 20 minutes in 50 ml falcon tubes with frequent vortexing. Tubes were filled with sterile distilled water up to the mark of 50 ml and mixed well. After centrifugation for 15 minutes at 3000g, the supernatant was discarded and the pellet was dispersed in 1 ml of distilled water and inoculated on two labelled LJ slants using 5 mm inoculating loop. The inoculated LJ slopes were incubated at 37 ºC up to 8 weeks and observed every week for growth.

DNA isolation:

The DNA from cultures was extracted by standard physico-chemical method (van Soolingen *et al.,* 1994). Briefly, the culture isolates were heated at 95ºC in boiling water bath for 7 minutes and were snap chilled for 30 minutes followed by addition of 40 µl of lysozyme, incubation for 2 hours at 37ºC , followed by 5 µl of Proteinase K (10 mg/ml) and 56 µl of 10% SDS and incubation at 65ºC for 30 minutes. 80 µl of 5M sodium chloride was added along with 64 µl of CTAB-NaCl and incubated at 65ºC for 30 minutes. Equal volume of chloroform-isoamyl alcohol (24:1) was added and centrifuged at 8000 g for 5 minutes. Upper layer was transferred to fresh tube. 0.6 volume of isopropanol was added. Tubes were incubated at -20ºC for 1 hour and centrifuged at 8000 g for 15 minutes at room temperature. The supernatant was discarded.150 µl of ice-cold 70% ethanol was added gently side-by-side to DNA pellet and centrifuged at 8000 g for 5 minutes. Supernatant was discarded and pellet air dried at room temperature. DNA was finally dissolved in 30 µl of TE buffer.

MIRU-VNTR analysis:

MIRU analysis was performed to study the MIRU loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39 and 40. PCR was performed in 25 µl volume using 10 ng of *MTB* DNA, 1X Q solution (Qiagen), 2 mM of $MgCl_2$ for MIRU loci 2, 4, 10, 16, 27, 31, 39 and 40; 2.5 mM $MgCl_2$ for MIRU loci 26, 1 U of *Taq* polymerase enzyme using the profile of 40 cycles and 0.4 µM of both forward and reverse primers for the MIRU loci studied (Supply *et al.,* 2002). The amplicons were analyzed on 1% TBE/agarose gel for the presence of MIRU loci and visualized on gel documentation system. The numbers of repeats present were calculated.

Results

Sputum samples were collected from the pulmonary tuberculosis confirmed and

suspected patients. The data obtained from the samples collected, revealed that males are more prone to tuberculosis than females A total of 98 sputum samples were collected, out of which 73 (74.4%) were of males and 25 (25.51%) were of females between the age group of 16 to 85 years. The tuberculosis suspect was found higher in the individuals of age groups 16 – 35 with 44.89% both in male and female sexes (Table 1).

All the 98 sputum samples were processed for culture on LJ medium. Out of them 24 (24.49%) were culture positive, three (3.06%) were contaminated, and 71 (72.44%) samples were culture negative for *MTB* on the basis of their growth characteristics, pigmentation and by biochemical identification (Vestal *et al.,* 1977).

All the 24 MTB isolates were analyzed for the 12 set of MIRU-loci using standardized protocol (Supply *et al.,* 2002). MIRU-VNTR pattern were analyzed by 1% TBE/agarose gel electrophoresis and number of repeats were calculated by using Quantity-One software (Bio-Rad) against the 50 bp DNA ladder (G Biosciences, India).

The allelic diversity (h) calculated for each of these 12 loci using Hunter Gaston Index using the formula

$$HGDI = 1 - [1/N(N-1) \Sigma_{j=1}^{S} nj (nj-1)]$$

where,

N = Total number of strains in the typing scheme,

S = Total number of different MIRU-VNTR patterns, and

nj = Number of strains belonging to J^{th} pattern

The allelic diversity by MIRU-VNTRs were analysed. The MIRU 26 was found to have highest allelic diversity at 0.77, followed by MIRU 10, MIRU16 and MIRU31 with allelic diversity of 0.76, 0.78 and 0.73 respectively. The least diversity was found in MIRU20 followed by MIRU02 with 0.04 and 0.11 respectively.

The 12 set MIRU-VNTR analysis was done for all 24 MTB isolates, 72% isolates fell under unknown lineage, followed by the Delhi/CAs lineage in 16% isolates. The Beijing, multiple matches and LAM lineage were found in 4.0% isolates each.

The clustering analysis of MIRU-VNTRs revealed 10 (41.67%) isolates in three clusters and 14 (58.33%) unique isolates in the Sethi Colony (Figure 1). The transmission of tuberculosis in Sethi Colony was 29.16% and diversity was calculated as 70.84%.

Table 1: Distribution of cases according to age and sex.

Age group	Male n=73 (74.4%)	Female n=25 (25.5%)	Total, n=98 (%)
16-25	18	05	**23 (23.47%)**
26-35	14	07	**21 (21.42%)**
36-45	13	02	**15 (15.30%)**
46-55	10	04	**14 (14.29%)**
56-65	09	04	**13 (13.27%)**
66-75	07	03	**10 (10.20%)**
76-85	02	00	**2 (2. 04%)**
Total	**73**	**25**	**98 (100%)**

Fig. 1: Dendrogram showing distribution pattern in MTB isolates from Sethi colony using MIRU-VNTR.

Discussion

The lack of polymorphism associated with low copy numbers limits the discriminatory power and the epidemiological inferences that can be drawn with IS*6110* typing method. Therefore, additional or secondary typing systems have to be used to discriminate between strains with few copy numbers or zero copy numbers (van Soolingen, 1993) as seen in significant number of the *MTB* isolates from Asia (Das *et al.,* 1995, Yang *et al.,* 1995). Empirical evidence demonstrates that the certainty with which epidemiological link can be inferred between patients infected with *MTB* is markedly reduced if the strains involved yield less than five IS*6110* hybridizing bands, but can be improved using an additional marker (Sola *et al.,* 2001; Yang *et al.,* 2001; Warren *et al.,* 1996).

Thus secondary marker helps in determining whether transmission has occurred in these cases. The importance of secondary typing for low copy IS*6110* strains has been reported by many authors and is well accepted (Soini *et al.,* 2000; Cronin *et al.,* 2001). Various techniques, including PGRS profiling, DR probes, spoligotyping and MIRU-VNTR analysis have been used most extensively for this purpose (van Soolingen *et al.,* 2001).

In the present study, MIRU-VNTR analysis was performed for 12 MIRU loci of *MTB* (Supply *et al.,* 2001). The transmission of tuberculosis was 29.16% and diversity was calculated as 70.84% in Sethi Colony. With MIRU-VNTR typing profile, allelic diversity was calculated for each of these 12 loci and MIRU locus 26 was found to be most discriminatory with an index of 0.84. This was also reported by various previous studies that MIRU locus 26 was most discriminatory locus (Supply *et al.,* 2001). MIRU locus 2 and 20 was found to be least discriminatory. MIRU locus 26 has been promoted as largely as a "Beijing – discriminating" locus for some time (Rao *et al.,* 2005). According to previous studies the presence of seven allele copies at MIRU locus 26 and a spoligotype signature (hybridization corresponding to spacer 35 to 43) should together constitute a definite identification of the Beijing genotype. The discriminatory ability of the MIRU typing has gained an insight on global phylogeny of *MTB* and has found apparent advantage of this approach. Beijing, EAI and Haarlem families are known to be major worldwide epidemic strains. EAI strain also called ancestral Tb DI+ strains because of the presence of a specific region called the Tb DI region, in their genome. This region is also found in other *MTB* complex species, such as *M. canettii*, *M. bovis, M. africanum,* and *M. microti*, but has been lost in other modern *MTB* strains, which have been shown to include member of the Beijing, Haarlem and Africa families (Ahmed *et al.,* 2003). It is reported that the use of locus 24 alone has an almost equal performance to the use of the Tb DI region as a marker for identifying ancestral and modern *MTB* strains (Gutierrez *et al.,* 2006). Interestingly, isolates that contain at least two alleles in locus 24 are included in the ancestral group

and the isolates that contain one allele in locus 24 are included in the modern group. This support few previous contentions that the selective use of some less variable loci, such as this one may be especially informative for evolutionary studies (Supply *et al.*, 2001).

The discriminatory power of MIRU genotyping is almost as great as that of IS*6110* based genotyping (Mazar *et al.*, 2001; Supply *et al.*, 2001). Unlike IS*6110* based genotyping, MIRU analysis can be automated and can thus be used to evaluate large numbers of strains, yielding intrinsically digital results that can be easily cataloged on a computer data base (Supply *et al.*, 2001).

Currently, VNTR patterns are compared in international databases. Because of the ease of use and the improved performance of the latest version, VNTR typing has become the new standard in public health applications of *MTB* in the USA, Europe and other parts of the world. Limitation of the present study was that only 12 locus analysis was carried out, but such studies can be planned in future.

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