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Genotyping of field isolates of *Mycobacterium bovis* and *Mycobacterium tuberculosis* using mycobacterial interspersed repetitive units (MIRUs)

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ABSTRACT

The study explored the usefulness of MIRU-VNTR genotyping in analyzing field strains of *Mycobacterium bovis* and *M. tuberculosis*. The MIRU-VNTR typing of 24 *M. bovis* strains with one reference strain resulted in 8 different profiles. While the MIRU-VNTR typing of 49 *M. tuberculosis* strains with 2 reference strains resulted in 16 different clusters. For *M. bovis*, HGD index varied from 0 to 0.18 in which, MIRU 2, 20, 24, 31 and 39 were mostly conserved while MIRU 4, 23 and 27 were moderately discriminatory, and none of the loci was highly discriminatory. HGD index for all loci were varied from 0 to 0.35 for *M. tuberculosis* strains with MIRU 39 as most discriminatory locus followed by MIRU26 and MIRU40.

Key words: *Mycobacterium tuberculosis*, *Mycobacterium bovis*, MIRU-VNTR typing

Mycobacterium bovis, member of *Mycobacterium tuberculosis* complex (MTBC) which includes *M. tuberculosis, M. africanum, M. microti* and *M. canetti* (van Soolingen *et al.* 1997), causes tuberculosis in cattle and other domestic animals. Verma and Gupta (1990) reviewed status of bovine TB in India. Although majority of cases of human TB is caused by *M. tuberculosis*, 5 – 10% of cases were reportedly due to *M. bovis* (Verma and Srivastava 2001).

Molecular approaches, amplityping (Ross and Dwyer 1993), pulsed field gel electrophoresis (PFGE) analysis (Feizabadi *et al*. 1996), ribotyping (Dandapat *et al*. 2000), restriction fragment length polymorphism (RFLP) analysis (Singh 2002), deligotyping (Fleischmann *et al*. 2002) and random amplified polymorphic DNA (RAPD) analysis (Singh, 2003) techniques, were used for typing the strains of MTBC. Spoligotyping (Kisa *et al*. 2012) and MIRU-VNTR typing (Vadwai *et al*. 2012) are recent molecular techniques.

MIRU-VNTR typing is a PCR based amplification-electrophoresis analysis of mycobacterial strains which is based on analysis of polymorphism at these loci. Main advantage of this technique is the digital format of MIRU-VNTR patterns generated which help towards understanding of molecular epidemiology. The present investigation focused to characterize the Indian field strains of *M. tuberculosis* and *M. bovis* using MIRU-VNTR typing and to know discriminatory power of 12-MIRU loci for these 2 closely related species of mycobacteria to understand the genetic relatedness among the different strains.

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MATERIALS AND METHODS

Bacterial strains: Total 75 mycobacterial strains comprising *M. tuberculosis* (43 from Mycobacteria Lab, IVRI and 6 isolated from District Tuberculosis Hospital, Bareilly), *M. bovis* (24 from Mycobacteria Lab, IVRI) and 2 reference strains (*M. tuberculosis* $H_{37}R_v$ and *M. bovis* BCG) were used. All mycobacteria used were first screened by cardinal biochemical tests, viz. niacin accumulation test, nitrate reduction test and growth on thiophen-2-carboxylic acid hydrazide media (Kubica and Wayne 1984). The genomic DNA used for the PCR analyses was isolated as described by Mazars *et al*. (2001). PCR assays for IS6110 and 12.7 kb fragments were used for confirmation and differentiation of *M. tuberculosis* and *M. bovis* (Shah *et al*. 2002).

MIRU-VNTR genotyping: The oligonucleotide primer sequences for 12 different MIRU-VNTR loci were downloaded from online website http: //minisatellites.u-psud.fr/ASPSamp/base_ms/bact.php. Each 25 µl rection mixture contained 1× PCR buffer, 1U of relevant Taq DNA polymerase, 200 µM of each dNTPs, 5pM of each primers and 0.7–1.5 mM of $MgCl_2$ with 5 µl of genomic DNA as template. The DNA amplification cycle was 10 min at 95°C followed by 30 cycle of 95°C for 1 min, 53–60°C for 45 sec

and 72°C for 1–1.5 min, and a final extension of 10 min at 72°C. Concentration of $MgCl_2$, annealing temperature and extension time were varied depending on particular MIRU locus. Gel electrophoresis of all PCR products was done on 2% agarose to calculate amplicon size for determination of repeat number at particular locus.

Analysis: START software, version 1.0.5 was used to perform lineage assignment by the UPGMA algorithm and by using alleles as categorical characters. Allelic profile frequencies and allele frequencies were calculated. The Hunter-Gaston diversity index (HGDI) was calculated as per Hunter *et al*. (1988). The discriminatory power of individual and combined MIRU-VNTR loci were assessed through allelic diversity (h), calculated using the equation $h = 1 - [\Sigma x_i^2/n(n-1)]$ where, n is the number of strains and x_i the frequency of the i^{th} allele at the locus (Selander *et al*. 1986).

RESULTS AND DISCUSSION

The respective mycobacterial cultures biochemical tests given the confirm results specific for individual species of mycobacteria. In IS6110 PCR assay all strains showed 445 bp amplification band indicating that they belong to *M. tuberculosis* complex. 12.7 kb multiplex PCR assay yielded 389 bp band specific for *M. tuberculosis* and 823 bp band for *M. bovis*.

The MIRU-VNTR typing of 49 *M. tuberculosis* strains with 2 reference strains resulted in 16 (M1 to M16) different clusters. Largest of this includes M1 comprised 27 strains with 52.94% of dataset. Next big cluster (M2) contained 7 strains with 13.21% of dataset and all of them were isolated from human sputum in the year 1994. Other 2 strains isolated from the same year were placed in different clusters (M8 and M9) and both were closely related with the M2. Third large cluster (M3) contained total 4 strains (2 strains from human sputum and 1 each from buffalo lung and calf lymph node). Remaining all clusters (M4 to M18) comprised 1 strain each. Six *M. tuberculosis* strains from District Tuberculosis Hospital, Bareilly showed considerable diversity among the repeat units and were placed differently.

For 24 field strains of *M. bovis* with BCG 8 clusters (M1 to M8) were obtained in which M1 was the biggest and comprised 17 strains followed by M2 with only 2 strains namely 3/87 and 3/86. Remaining 6 clusters (M3 to M8) comprised 1 strain each. *M. bovis* isolated from camel from NRC Camel, Bikaner was placed in M8 and was found to be related with the 324/96, isolated from Cattle Farm, IVRI (UP) present in very distant place from Bikaner. Allelic frequencies of 12 MIRU-VNTR loci for all mycobacterial strains are shown in Tables 1 and 2.

MIRU-VNTR typing for *M. tuberculosis* yielded the highest allelic diversity of 0.35 for MIRU 39 and 0.34 for MIRU26 and MIRU40. This was followed by 0.26 for MIRU 16, while MIRU20 and MIRU27 showed no any polymorphism. For *M. bovis* strains, MIRU 2, 20, 24, 31 and 39 showed no any allelic diversity. Most discriminatory locus found was MIRU 4 having highest allelic diversity of 0.18. This was followed by MIRU 23 and 27 having 0.12 HGDI. Details of allelic diversity for *M. tuberculosis* and *M. bovis* are shown in Tables 1 and 2.

In India, Sharma *et al*. (2008) characterized prevalent genotypes of *M. tuberculosis* on a collection of 97 isolates in rural area of Kanpur, North India by MIRU-VNTR with spoligotyping. All isolates were amplified on the basis of six MIRU loci in which MIRU 26 was most discriminatory loci followed by other loci. Velji *et al*. (2009) conducted a large prospective population-based analysis of 265 serial isolates from UK by using a standardized 15 MIRU-VNTR panel, out of 12 MIRU loci five (MIRU 10, 16, 23, 26 and 40) showed high discriminative power.

Table 1. Allele frequencies and allelic diversity of MIRU loci for *M. tuberculosis*

Allele	2	4	10	16	20	23	24	26	27	31	39	40
0	-	-	-	-	-	-	-	-	-	-	-	4
1	1	-	-	6	-	-	47	3	-	-	1	40
2	50	5	2	42	51	-	4	1	-	-	40	4
3	-	46	49	2	-	-	-	40	51	47	1	1
4	-	-	-	1	-	1	-	2	-	2	9	-
5	-	-	-	-	-	2	-	4	-	2	-	-
6	-	-	-	-	-	48	-	1	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	1
11	-	-	-	-	-	-	-	-	-	-	-	1
Total	2	2	2	4	1	3	2	6	1	3	4	6
Allelic diversity	0.02	0.13	0.02	0.26	0	0.06	0.09	0.34	0	0.13	0.35	0.34

Table 2. Allele frequencies and allelic diversity of MIRU loci for *M. bovis*

Allele	2	4	10	16	20	23	24	26	27	31	39	40
1	-	-	-	-	-	-	-	-	-	-	-	-
2	25	22	24	-	25	-	25	-	1	-	25	24
3	-	3	-	24	-	-	-	-	23	25	-	-
4	-	-	-	1	-	23	-	1	1	-	-	-
5	-	-	-	-	-	1	-	24	-	-	-	-
6	-	-	-	-	-	-	-	-	-	-	-	-
7	-	-	1	-	-	1	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-	-	-	-	1
Total	1	2	2	2	1	3	1	2	3	1	1	2
Allelic diversity	0	0.18	0.04	0.04	0	0.12	0	0.04	0.12	0	0	0.04

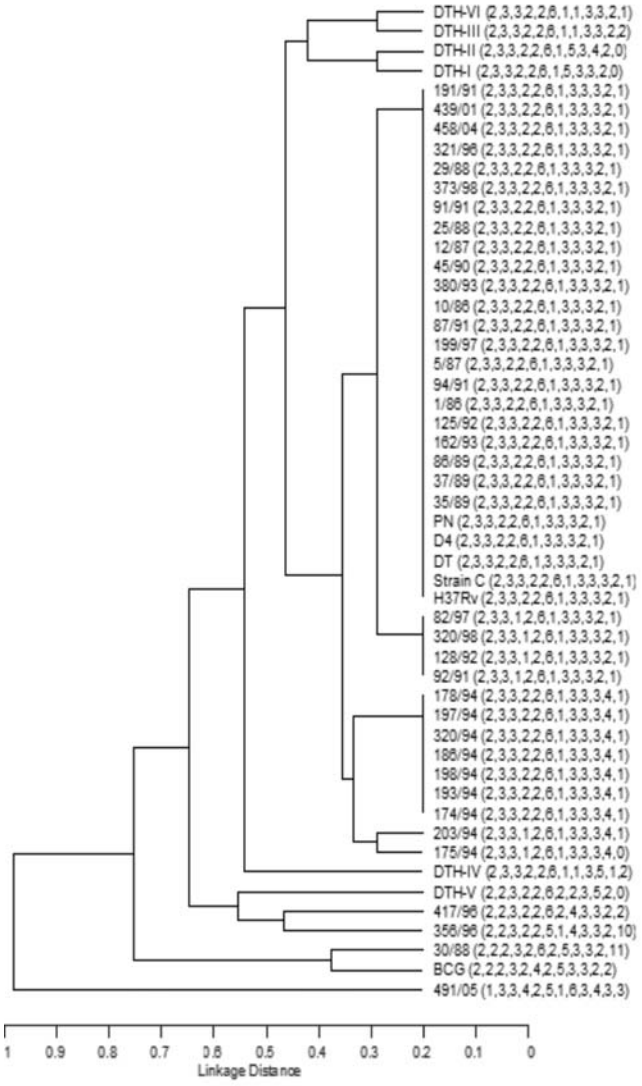

Fig. 1. UPGMA phylogenetic tree for *M. tuberculosis* strains.

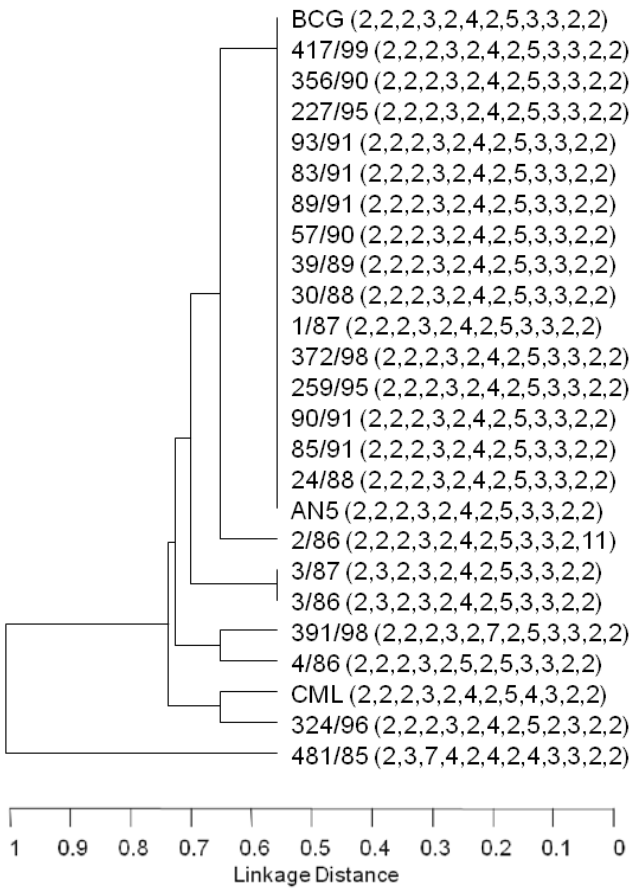

Fig. 2. UPGMA phylogenetic tree for *M. bovis* strains.

MIRU-VNTR typing method has shown more discriminatory than spoligotyping for *M. bovis* when used in limited number of isolates from Northern Ireland (Roring *et al*. 2002) or from Chad (Hilty *et al*. 2005). Few reports on the use of MIRU-VNTR genotyping of *M. bovis* isolates are

from Korea (Jeon *et al*. 2008), Belgium (Allix *et al*. 2006), Italy (Lari *et al*. 2006), Chad (Hilty *et al*. 2005) and Ireland (Roring *et al*. 2004) but there is no report available from India as far *M. bovis* genotyping is concerned.

All MIRU-VNTR loci used in the present study were divided into highly ($h > 0.25$), moderately ($0.11 < h < 0.25$) and poorly ($0.01 < h < 0.11$) discriminative and conserved i.e. no polymorphism based on HGD index. For *M. tuberculosis*, four MIRU loci (MIRU16, 26, 39 and 40) were highly polymorphic while MIRU 20 and 27 were found conserved. MIRU 4, 23 and 27 found to be moderately discriminatory for *M. bovis* while none of the loci was found to be highly discriminatory. Index of association was calculated by formula $I_A = (V_o/V_e) - 1$, where V_o = observed variance and V_e = expected variance. Index of association for present study was 2.436 for *M. tuberculosis* and 0.784 for *M. bovis*. Clustering rate of 12 MIRU-VNTR genotyping for *M. tuberculosis* was 0.70 and 0.68 for *M. bovis*. The genotyping data obtained were used to construct a phylogenetic tree based upon the UPGMA method. Dendrograms of genetic relationships among 49 strains of the *M. tuberculosis* with reference strains and 24 strains of *M. bovis* with one reference strains are shown in Figs 1 and 2, respectively.

It is to be concluded that, MIRU-VNTR a PCR based method, has the advantage of speed, reproducibility with manual analysis of 12 individual MIRU-VNTR loci PCR amplifications followed by gel electrophoresis and can be performed directly on crude DNA obtained from the cell lysate without any further DNA purification. MIRU-VNTR genotyping was found highly reliable and discriminative. MIRU 26, 39 and 40 for *M. tuberculosis* while MIRU 4, 23 and 27 for *M. bovis* was the most discriminatory. MIRU-VNTR analysis of large number of *M. tuberculosis* and *M. bovis* strains would provide more information with regard to molecular status of these strains of mycobacteria helping towards understanding the spread of infection.

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