# MOLECULAR HETEROGENEITY IN MYCOBACTERIUM TUBERCULOSIS CLINICAL ISOLATES PREVALENT IN PUNJAB

(A Major Research Project sanctioned by the University Grants Commission)

UGC Letter No: F.37-186/ 2009 (SR); dated 12-1-2010
Effective start date: February 1, 2010
Period of project: February 1, 2010 to January 31, 2013

# Final Report

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**Objective of the project:**

- To create genomic data base of various molecular markers in *M. tuberculosis* clinical isolates prevalent in Punjab.
- To ascertain and compare MIRU-VNTR and spoligotyping based genetic diversity among the *M. tuberculosis* clinical isolates.
- To identify genetic predictors of differential pathogenicity in *M. tuberculosis* clinical isolates and their role in drug resistance.

**Achievements from the project:**

1. The *M tuberculosis* clinical isolates prevalent amongst the tuberculosis patients of Punjab at very heterogeneous and were characterized at the molecular level using MIRU-VNTR and DR repeats.
2. The isolates present in Punjab are predominantly modern based on the presence of a defined TbD1 region in contrast to the strains prevalent in southern India.
3. The project documented the clear genetic divide between Northern and southern *M tuberculosis* isolates.
4. The technology developed in the project will be used to, in the lab and outside, to characterize other populations of India.

**Work done:**

**Sample collection**

Two hundred and twenty-five (172 Pulmonary tuberculosis and 53 extra pulmonary tuberculosis-Pleural effusion) samples, in sterile containers were collected randomly from suspected tuberculosis patients visiting Sri Guru Ram Das Institute of Medical Sciences & Research and TB and Chest Hospital, Amritsar or other parts of Punjab.

M**ycobacteriological Analysis**

All the clinical samples were processed following modified Petroff's method (1915). The smear of processed sputum samples was examined microscopically with Ziehl-Neelson staining method using conventional guidelines (Bishop and Neuman 1970). A loop full of decontaminated specimen was plated onto duplicate marked Lowenstein Jenson (L-J) slants and the growth of the culture were monitored weekly, for at least 8 weeks. Niacin test was

performed on the culture positive slants as previously described by Venkataraman and Prabhakar (1977).

**DNA Isolation**

The DNA was isolated from a loop full of biomass following CTAB-NaCl method (van Solingen et al 1995) and quantified following U-V spectrophotometric analysis.

**MIRU-VNTR Typing**

DNA from biomass was typed for 12 MIRU loci using polymerase chain reaction (PCR). The 25µl optimized reaction mixture consisted of 40ng of template DNA, 200nM of each primer as published by Supply *et al (1997)*, 50µM of each dNTPs, 0.3U Taq DNA polymerase (Bangalore Genie, Bangalore), 10mM Tris-Cl (pH 9.0), 50mM KCl and 0.01% gelatin. The $MgCl_2$ concentration for MIRU 2, 4, 10, 16, 23, 26, 31 was 1.5mM; for MIRU 20, 24, 39, 40 was 2.0 mM. For all the MIRUs except MIRU 2 and 23 di-methyl sulfoxide (DMSO) at a concentration of 5% was used for amplification. The reaction mixture was initially denatured at 94ºC for 3 min followed by 35 cycles of denaturation at 94ºC for 30s, annealing {55ºC for all the MIRUs, except 10, 27 (50ºC) and 31 for which the temperature was 60ºC} for 30s, and extension at 72ºC for 30s. Amplified products were analyzed in 2% agarose gel, using 100bp and 20bp DNA ladders and visualized by ethidium bromide staining. DNA from standard strain H37Rv was used as positive control. The genotypes were expressed as a 12-digit code representing the number of MIRUs in each of the 12 loci. The isolates having same genotype based on the MIRU profile were identified as a cluster, whereas those with genotype different from other isolates were identified as unique.

**Spoligotyping**

Spoligotyping is the widely used PCR-based reverse-hybridization blotting technique that assays the genetic diversity of DR locus. The DR repeat spacers were amplified and analyzed using Ocimum Biosolution (India) spoligotyping kit as per manufacturer's instructions.

***Statistical analysis***

Statistical analysis was performed using SPSS software.

Hunter-Gaston discriminatory index (HGDI) was used as a numerical index for MIRU-VNTR discriminatory power (Hunter et al 1988).

**Salient findings:** All 225 clinical samples were processed and screened for *M tuberculosis* by conventional methods i.e. Microscopy and LJ culture. All culture positive samples were confirmed for *M. tuberculosis* isolates by using 38kDa gene.

**Table1. Microscopic and Culture examination of the clinical samples**

| Method employed | | No. of samples (n) | AFB +ve (%) | AFB -ve (%) |
|---|---|---|---|---|
| Microscopy (225) | Pulmonary TB | 172 | 69 ( 40.1) | 103 (59.9) |
| | Extra Pulmonary TB | 73 | 3 (4.1) | 70 ( 95.9) |
| Culture (225) | Pulmonary TB | 172 | 90 (52.3) | 82 (47.7) |
| | Extra Pulmonary TB | 73 | 16 (21.9) | 57 (78.1) |

All culture positive samples tested positive for 38kDa gene and were confirmed to be *M. tuberculosis* isolates.

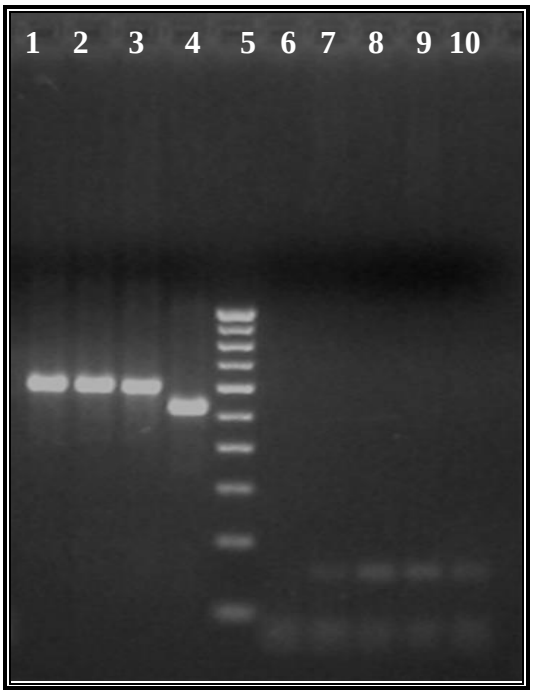
To enhance the typing of extra pulmonary tuberculosis samples, modified 7H9 broth with indicator was tested. Of the 10 samples analysed using this broth, 6 samples have yielded positive result, thus showing a marked improvement in the detection of *M tuberculosis* in pleural effusion samples. All six samples were PCR positive for 38kDa gene.

**MIRU typing**

PCR was performed on isolated DNA from clinical isolates using primers specific to 12 MIRU loci. Different reaction conditions for MIRU loci were used as published by Supply *et al* (2001). Initially, majority of samples failed to amplify for MIRUs, except 2 and 23.

Therefore, MIRU-PCR for these loci was further optimized based on annealing temperature, $MgCl_2$ concentration and addition of DMSO in amplification reaction.

**Fig1. Effect of DMSO on the amplification of MIRU-20 locus.**




**Lane 1-4, sample amplified with 5% DMSO;**
**Lane 7-10, were samples amplified without DMSO;**
**Lane 5, 100bp ladder and Lane 6, Negative control**

MIRU 26 was found to be the most discriminatory with HGCI value of (0.668) and MIRU 2 was found to be monomorphic and hence least discriminatory. The combined HGDI based on the 12 MIRU loci was found to be 0.996.

The technique of spoligotyping was standardized in the laboratory and the samples were analyzed for their spoligotype patterns.

CAS1_Delhi was found to be the most frequent spoligotype (54.7%) followed by CAS (8.5%), EAI5 (7.5) and Beijing (6.6). LAM and T1 types were also recorded were in less than 5% cases. There were around 22% cases that were orphans.

In addition to this, DNA samples isolated from the clinical isolates were typed also for the presence and absence of TbD1 region. It is pertinent to mention that the presence of TbD1 region allows strains to be classified as "ancient strains" and those without this region are termed as "modern" strains. Out of the 56 strains typed from Punjab 73.2% were found to be modern. The higher number of TB patients living in rural regions of Punjab were infected with ancient strains as compared to the patients from the urban regions (Prakash *et al* 2014). The findings of the project clearly revealed that clinical *M tuberculosis* isolates prevalent in Punjab are predominantly modern and very heterogeneous, an observation that goes against the popular belief that not much heterogeneity exists in *M tuberculosis* strains. The findings will also help in designing molecular approached for the detection of M tuberculosis isolates using molecular tools.

**References:**


1. Bishop PJ, Neuman G. The history of the Ziehl-Neelsen stain. *Tubercle* 1970; **51**: 196.
2. Hunter PR, Gaston MA. Numerical index of discriminatory ability of typing systems: an application of Simpson's index of diversity. *J Clin Microbiol* 1988; **26**: 2465-2466.
3. Prakash O, Sharma R and Sehajpal PK (2014) TbD1 based genetic diversity in *M. tuberculosis c*linical isolates from North India. Int J Tub Lun Dis 18: 196-7.
4. Supply P, Magdalena J, Himpens S, Locht C. Identification of novel intergenic repetitive units in a mycobacterial two-component system operon. *Mol Microbiol* 1997; **26**: 991–1003.
5. Supply P, Lesjean S, Savine E, Kremer K, van Soolongen D, Locht C. Automated high throughput genotyping of study of global epidemiology of mycobacterium tuberculosis based on mycobacterial interspersed repetitive units. *J Clin Microbiol* 2001; **39**: 3563-3571.
6. van Soolingen D, Qian L, deHaas PEW, et al. Predominance of single genotype of Mycobacterium tuberculosis in countries of East Asia. *J Clin Microbiol* 1995; **33**: 3234-3238.
7. Vankataraman P, Prabhakar R. Niacin productions test in Mycobacterium replacement of benzidine cynogen bromide reagent by o-tolidine-cynogen bromide. *Ind J Tubercle* 1977; **24**: 153.


**NO. OF PUBLICATIONS OUT OF THE PROJECT**: One


1. Prakash O, Sharma R and Sehajpal PK (2014) TbD1 based genetic diversity in *M. tuberculosis c*linical isolates from North India. Int J Tub Lun Dis 18: 196-7.

# GURU NANAK DEV UNIVERSITY, AMRITSAR

*(Established by the State Legislature Act No. 21 of 1969)*

*Accredited at "A" grade level by NAAC and awarded "University with Potential for Excellence" status by UGC*

## UTILISATION CERTIFICATE

Certified that an amount of Rs. 11,24,077/- (Rupees Eleven Lac Twenty Four Thousand Seventy Seven Only) out of the grant of Rs. 11,52,129/- sanctioned to Guru Nanak Dev University, Amritsar by the University Grants Commission, New Delhi vide letter Nos.:-

| S. No. | Sanction Letter No. | Amount (Rs.) |
|---|---|---|
| 1. | F.37-186/2009(SR), dated 12-01-2010 | 5,92,800.00 |
| 2. | F.37-186/2009(SR), dated 31-03-2013 | 5,59,329.00 |
| | **Total** | **11,52,129.00** |

pertaining to Research Project entitled, " **Molecular heterogeneity in mycobacterium tuberculosis clinical isolates prevalent in Punjab"** undertaken by **Dr. P.K. Sehajpal, Department of Molecular Bio & Bio-Chemistry**, has been utilized for the purpose for which it was sanctioned and in accordance with the terms and conditions laid down by the Commission.

The unspent balance of Rs. 28,052/- have been refunded in your favour by way of RTGS in your A/c No. 0157101017339 on 25-03-2014 with Canara Bank UGC, New Delhi.

**Deputy Controller** 17/4/14
**(Local Audit Deptt., Punjab)**
**Guru Nanak Dev University,**
**Amritsar.**

**Registrar**
**Guru Nanak Dev University**
**Amritsar**

ਗੁਰੂ ਨਾਨਕ ਦੇਵ ਯੂਨੀਵਰਸਿਟੀ, ਅੰਮ੍ਰਿਤਸਰ
**Guru Nanak Dev University, Amritsar**
*(Established by the State Legislature Act No. 21 of 1969)*

# Expenditure Statement

Statement showing expenditure incurred out of the grant sanctioned by **University Grants Commission, New Delhi** for major research project entitled, **"Molecular heterogeneity in mycobacterium tuberculosis clinical isolates prevalent in Punjab"** undertaken by **Dr. P.K. Sehajpal, Department of Molecular Bio & Bio-Chemistry**, Guru Nanak Dev University, Amritsar **for the period 01-02-2010 to 30-06-2013.**

| Grant received | Amount (Rs.) |
|---|---|
| 2010-11 | 5,92,800/- |
| 2012-13 | 5,59,329/- |
| Total | 11,52,129/- |

| S. No. | Particulars | Expenditure (Rs.) |
|---|---|---|
| A. | **NON-RECURRING** | |
| 1. | Books & Journals | 50,000.00 |
| | **Total** | **50,000.00** |
| B | **RECURRING** | |
| 1. | Project Fellow | 5,19,130.00 |
| 2. | Contingency | 30,474.00 |
| 3. | Chemicals | 3,83,600.00 |
| 4. | Travel/Field work | 32,767.00 |
| 5. | Hiring Services | 21,806.00 |
| 6. | Overhead Charges | 86,300.00 |
| | **Total** | **10,74,077.00** |
| | **Grand Total of (A) and (B)** | **11,24,077.00** |

**(Rupees Eleven Lac Twenty Four Thousand Seventy Seven Only)**

**Deputy Controller (Local Audit Deptt., Punjab)**
**Guru Nanak Dev University,**
**Amritsar**

19/2/14

**Registrar**
**Guru Nanak Dev University**
**Amritsar**