INDIAN COUNCIL OF MEDICAL RESEARCH

ICMR International Fellowship

Report

February 25, 2012- July 23, 2012.

1. TITLE OF THE RESEARCH SCHEME	Molecular detection of drug resistance in *M. tuberculosis* isolates in North Delhi, India
2.NAME AND ADDRESS OF FELLOW	Dr. Mandira Varma (Associate Professor) Dept. of Microbiology V.P. Chest Institute University of Delhi Delhi - 110007.
3. NAME AND ADDRESS OF MENTOR	David Alland, MD Professor and Chief, Division of Infectious Disease Director, Center for Emerging Pathogens Assistant Dean for Clinical Research Center for Emerging Pathogens New Jersey Medical School, MSB A-920 185 South Orange Ave Newark, NJ 07101 USA
6. DATE OF VISIT	February 25, 2012
8. DURATION	5 Months

9. PURPOSE OF VISIT

Prof. David Alland has extensive experience in molecular epidemiology of tuberculosis and has also developed extensive throughput SNP assays to detect drug resistant mutations in *M. tuberculosis.* SNP assays have been developed for virtually all the mutations known to be associated with isoniazid, rifampicin, ethambutol, streptomycin and fluoroquinolone resistance. We attempted to analyze the mutations in 108 isolates of *M. tuberculosis* obtained from patients being treated at the DOTS center at RBTB hospital and Vallabhbhai Patel Chest Institute in Delhi, India, by a sloppy molecular beacon assay. Drug susceptibility assays were carried out in the Department of Microbiology at Vallabhbhai Patel Chest Institute (VPCI), Delhi. In addition, a set of 9 SNPs were also used for phylogenetic analysis of 153 *M. tuberculosis* isolates.

The data generated gives a preliminary information on the mutations prevalent in North Delhi. It also helps to validate the sloppy molecular beacon assay for use on Indian isolates as a rapid assay to detect drug resistance. Use of the sloppy molecular beacon assay would not only give us information on the drug resistance rapidly but would also be able to predict if a strain can develop multidrug resistance or extensive drug resistance and could help in instituting intervention therapy. In the present study this was highlighted by the detection of mutations in *gyrA* and *gyrB* genes in isolates which were susceptible to fluroroquinolone by the conventional drug susceptibility assay. Such strains could develop extensive drug resistance in the future. In addition, since Delhi has a diverse population of patients, phylogenetic analysis of the *M. tuberculosis* isolates from these patients would help us understand if the observed differences of SNPs in the isolates are due to an evolutionary change or have arisen as a unique event.

10. SOURCE OF SPONSORSHIP OF THE VISIT: Indian Council of Medical Research

9. OBJECTIVES OF THE PROPOSAL

i) To validate the utility of the SMB assay to detect drug resistance mutations in *M. tuberculosis* clinical isolates from India as compared to sequencing and phenotypic Drug Susceptibility Testing (DST).

ii) To determine the frequency of specific mutations that lead to drug resistance in *M. tuberculosis* isolated from patients of tuberculosis being treated in the North Delhi area.

iii) Phylogenetic analysis of clinical isolates of *M. tuberculosis* from India by SNP cluster grouping and relationship to drug resistance patterns if any.

iv) Correlation of *embB*, and *embC* mutations with Ethambutol resistance.

11. **RELEVANCE TO ICMR**

The research priorities of ICMR coincide with the National health priorities such as control and management of communicable diseases. In this context the present visit to the University of Medicine and Dentistry was in line with the priorities of ICMR. The current research was carried out to be able to use a new and rapid molecular assay to detect drug resistance in *M. tuberculosis*. The study would help in defining new methods for identification of not only *M. tuberculosis*, but other pathogens as well. The sloppy molecular beacon assay which has been developed in Prof. Alland's Lab is one such assay. This assay can also be used to detect drug resistant *M. tuberculosis* and can be used to predict the development of MDR or XDR-TB. The study was also carried out to perform phylogenetic analysis of the isolates by using a set of 9 genes for SNP cluster grouping. It is important to understand the evolution of *M. tuberculosis* to understand the epidemiology and biology of tuberculosis.

12. METHODOLOGY Annexure I

15. BENEFIT TO THE INSTITUTE

The Vallabhbhai Patel Chest Institute (VPCI) is a post-graduate Medical Institution devoted to the study of chest diseases. The main objectives of VPCI have been to conduct

research on basic and clinical aspects of chest medicine, to train post graduates in Pulmonary Medicine and allied subjects, to develop new diagnostic technology and disseminate it to other institutions in the country and to provide specialized clinical and investigative services to patients. The Department of Microbiology investigates over 40 clinical samples for tuberculosis in a day. Besides routine diagnostic services, we are working towards developing rapid molecular techniques to detect and identify *M. tuberculosis* directly from clinical specimens. Molecular epidemiology of *Mycobacterium tuberculosis*, host-pathogen interaction studies and genomics are some of the areas of intense research in our department. One of our projects is on Drug resistance profiling and molecular typing of *M. tuberculosis* isolates from different community settings in Delhi. The current proposal was an extension of the previously mentioned project. The techniques learnt in Dr. Alland's laboratory will be used in the Laboratory at Vallabhbhai Patel Chest Institute. The analysis of drug resistant mutations shall be continued on follow-up samples from patients who fail treatment to study the effect of treatment on selective development of drug resistant mutations and also to predict the development of MDR and XDR TB.

I PUBLICATIONS:

UNDER PREPARATION:

1. **Rapid detection of drug resistance and heteroresistance in *M. tuberculosis* isolates from India using sloppy molecular beacons**
2. **Phylogenetic analysis of *M. tuberculosis* isolates in North India reveals predominance of a single cluster group.**

ANNEXURE I

METHODOLOGY

Selection of Patients. Three groups of AFB smear positive patients of tuberculosis were selected for the study. Group I comprised of patients from Rajan Babu TB Hospital (RBTB hospital) that has an "on campus" DOTS center in North Delhi. Group II consisted of patients visiting the Department of Respiratory Medicine at Vallabhbhai Patel Chest Institute (VPCI), Delhi. VPCI provides referral diagnostic services and treatment to patients with chest diseases. Only adult patients over 18 years were enrolled in the study. A detailed proforma was filled for every patient including their residential address, contact telephone numbers, occupation etc. Informed consent was also taken from the patients. The study was approved by the Institutional Ethical Committee.

Sample Collection: Two consecutive sputum samples were collected from all the patients according to the RNTCP guidelines (http://www.tbcindia.org/rntcp.asp). Since BCG coverage in India is high, tuberculin skin testing was not used to monitor tuberculosis infection. Mobile population coming in for treatment at any of the centers and patients unable to expectorate sputum were not considered for the study. Records of patients were maintained at V. P. Chest Institute.

Sample size: 153 samples were collected from the DOTS center at RBTB Hospital and Vallabhbhai Patel Chest Institute (VPCI). Of these, 109 were from the DOTS center and 44 were from VPCI. Of the 109 samples obtained from RBTB, 69 were new, 35 had been previously treated and 5 were multidrug resistant. Of the 44 patients obtained from VPCI, 20 were new, 10 had been previously treated and 4 were multidrug resistant. The status of 10 patients could not be confirmed.

Processing of samples: On the spot and follow-up sputum samples were transported to the Microbiology laboratory at VPCI for culture and antimicrobial susceptibility testing. Ziehl Neelsen staining was performed for direct smear examination and the smears were graded according to WHO criteria. Cultures for *M. tuberculosis* were performed for every case after modified Petroff's method of decontamination. Sputum specimens were cultured on Lowenstein-Jensen medium in duplicate after decontaminating the specimens by modified Petroff's method. Positive culture isolates were identified to be *Mycobacterium tuberculosis* by niacin (Konno *et al*, 1966) , nitrate reduction (Lutz , 1992) and catalase tests (Kubica *et al*,1966).

Drug susceptibility testing: Drug susceptibility testing to isoniazid (INH), rifampicin (RIF), streptomycin (SM) and ethambutol (EMB) was performed by the **proportion method** in the Department of Microbiology at VPCI (Canetti *et al* 1969). The drug concentrations tested were as per WHO recommendations (4mg/l for streptomycin, 0.2 mg/l for isoniazid, 40mg/l for rifampicin, 2mg/l for ethambutol) (Mondal and Jain, 2007). The LJ slants were incubated at 37°C and observed at the 28^{th} and 42^{nd} day of incubation.
Minimum Inhibitory Concentration: A subset of the strains were also tested for their MIC by the Microplate Alamar Blue Assay at VPCI.

DNA Extraction: DNA was extracted from all the strains by the CTAB method or by boiling with chelex reagent and transported to UMDNJ (Chakravorty et al, 2005).

Sloppy Molecular Beacon Assay:
For *gyrA* mutations, a 107-bp fragment (nucleotides 222 to 330, with numbering based on the gene start site) containing the *gyrA* Quinolone Resistance Determining Region (QRDR) was amplified with the target primers gyrA-F (5′-CCGGTCGGTTGCCGAGACC-3′) and gyrA-R (5′-CCAGCGGGTAGCGCAGCGACCAG-3′). The two SMB sequences used were QDR1 (5′-6-carboxyfluorescein CCGTGCgcgcaccagggtgccctagatcgacacgtcGCACGG-DABCYL-3′) and QDR2 (5′-cyanine 5-CCCGAGGgItgtcgtagattgacacgtcgccgcgcggCCTCGGG-BHQ1-3′) (Chakravorty et al,

2011). For *rpoB* mutations, three probes rpo1 (5-tetramethylrhodamine-**cgacc**gCccatgaattggctcagctggctggtgAc**ggtcg**-BHQ2-3=), rpo2 (5-cyanine 5-**ggcgcg**aaccAcgacagcgggttgttctggtccatgaa**cgcgcc**-BHQ2-3) and rpo3 (5-6 carboxyfluorescein-**cgcgcg**caTcAccAacagtcggTgcttgtgggtcaacc**cgcgcg**-BHQ1-3) were used, where the underlined boldface sequences represent the stem portion of the SMB, the lowercase sequences represent the probe portion of the SMB, uppercase letters represent the mutations introduced into the probe region to obtain a stable stem-loop structure, and BHQ represents Black Hole Quencher) (Chakravorty et al, 2012). Similarly, sloppy molecular beacon probes were used to detect *embB*306, *embB*406, *embB*497, *katG, inhA, rrs, rpsL* and *eis* genes (unpublished data). *Tm* calls were performed at the end of the reaction using the automated *Tm* calling software (Light Cycler 480 software), and resulting *Tm* values for each probe were determined. A subset of the isolates which were streptomycin resistant but did not have a mutation in the *rpsL* gene were sequenced for the *gidB* gene.

SNP Cluster Grouping: For the current study, we used a set of nine SNP markers that enabled us to classify the isolates studied into seven SNP cluster groups (SCG) and five SNP cluster subgroups (Table 1). All of the study samples were then tested at the nine SNP loci by using hairpin primer assays as described previously (Alland et al, 2007) and the alleles were determined.

embCAB operon sequencing.

The PCR products of the *embCAB* locus were generated from genomic DNAs by using primer sets: Three overlapping PCR products (amplicon 1, primers 1 [5′-CGCACATAACAGCTACACCC-3′] and 2 [5′-CGAAGGTCTGATCACGAAAG-3′]; amplicon 2, primers 3 [5′-CTTTCGTGATCAGACCTTCG-3′]) and 4 [5′-ACCAGCCAGTCCAGGAACAC-3′]; amplicon 3 primers 5 [5′-GTGTTCCTGGACTGGCTGGT-3′] and 6 [5′-GATCGACCGTTCCACCAACA-3′]; were amplified from genomic DNAs to span the *embCAB* locus. Overlapping *embCAB* amplicons were purified with a QIAquick PCR cleanup column (Qiagen) and were used as templates for Sanger sequencing (Applied Biosystems). The genome coordinates of

polymorphisms were determined by BLAST analysis by using *M. tuberculosis* strain H37Rv as a reference (Safi et al, 2007).

RESULTS:

CHARACTERIZATION OF STRAINS:

Samples were collected from the DOTS center at RBTB hospital (n=109) and from the non-DOTS center at Vallabhbhai Patel Chest Institute (n=44). 58% of the patients were category I, 29.4% were category II, 5.8% were multidrug resistant and 6.5% could not be categorized (Table 1). All the isolates were confirmed to be *M. tuberculosis* by biochemical reactions and PCR restriction analysis. Some strains were also confirmed by sequencing the *hsp*65 gene (Table 2).

Table 1: Patient Profile: Categories (Revised National TB Control Program, India)

Treatment profile	Cat I (New Cases) (%)	II (Retreatment/ failure/ non-MDR) (%)	IV (MDR) (%)	Unknown (%)	Total
Non DOTS	20 (45.4)	10 (22.7)	4 (9)	10 (22.7)	44
DOTS	69 (63.3)	35 (32)	5 (4.5)	0	109
Total	89 (58)	45 (29.4)	9 (5.8)	10 (6.5)	153

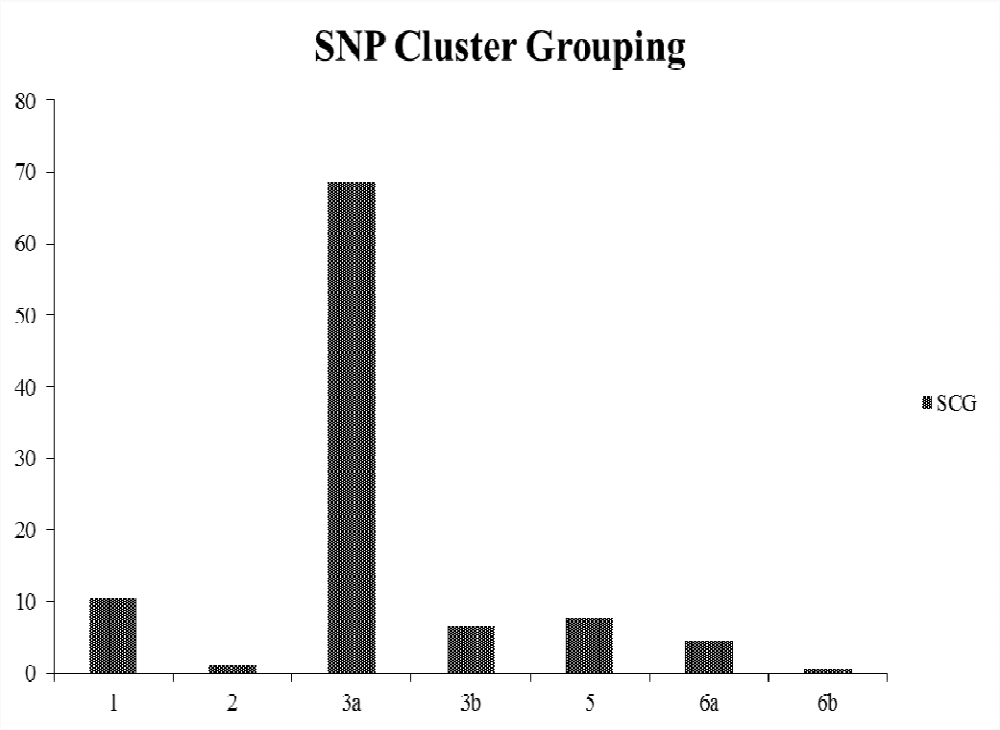
Table 2: Species typing of clinical isolates

	DOTS	Non DOTS	TOTAL
No. of Isolates	**109**	**44**	**153**
Confirmed by Biochemical Reactions	**99**	**38**	**137**
Confirmed by PCR restriction analysis	**109**	**44**	**153**
Confirmed by sequencing of *hsp*65 gene	**16**	**6**	**22**

SNP CLUSTER GROUPING

Each isolate was tested for the presence of nine SNP markers, and an SCG or SC subgroup was assigned to each isolate based on the pattern of its SNP alleles. The SNP alleles are shown in Figure 1. The study set was found to include members of all SCGs except SCG 7 (which contains primarily *Mycobacterium bovis*). SCG 3a was the most frequent and had 69 isolates. This was followed by SCG 1 with 10 isolates (Figure 1). There were no isolates in SCG 3c, 4 and 7.

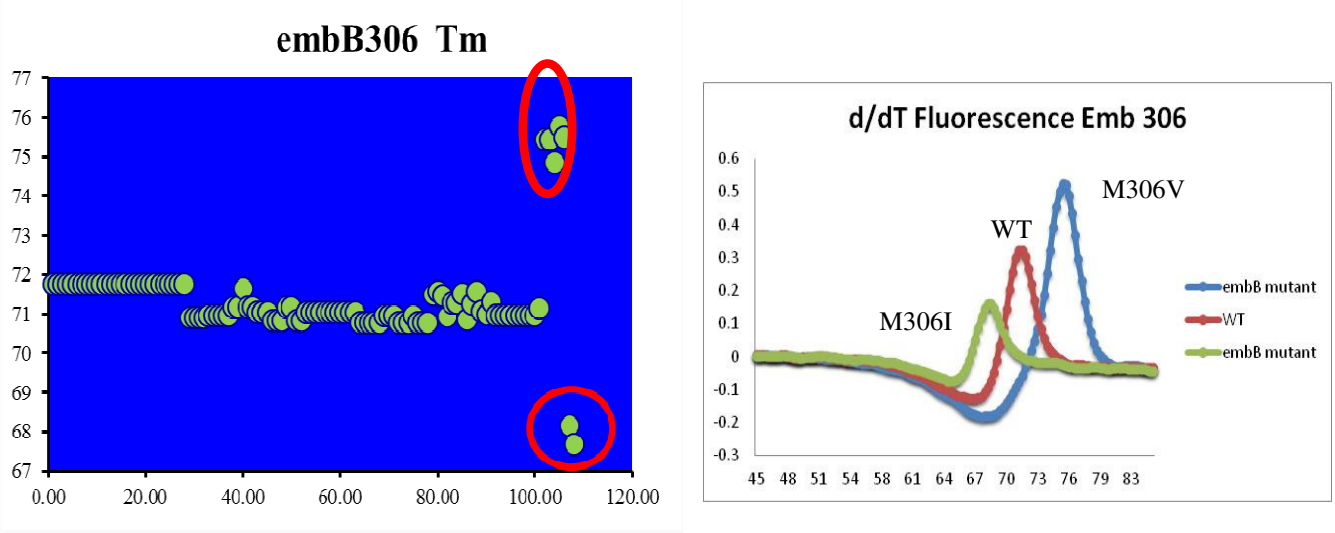
Figure 1



Sloppy Molecular Beacon Assay

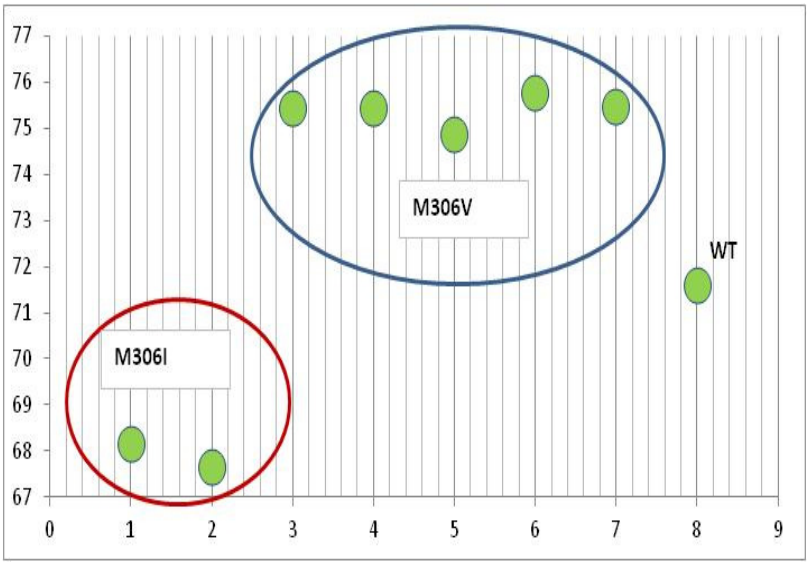
The Sloppy Molecular Beacon Assay was carried out to detect resistance to ethambutol (*embB306, 406, 497* codons), fluoroquinolones (*gyrA* and *gyrB*), rifampicin (*rpoB*), isoniazid (*katG* and *inhA*) and aminoglycosides (*rrs*, *rpsL*, *eis*). Seven isolates were found to have mutations at the *embB306* codon (Figure 2 A). The mutations were confirmed by sequencing and found to be M306I in 2 isolates and M306V in 5 isolates (Figure 3). None of the isolates had a mutation at the embB406 codon, while one had a mutation at the *embB497* codon. The sensitivity of the assay was found to be 63.6% and the specificity was 100% when compared with Proportion method of drug susceptibility. The sensitivity was 100% when compared with results of sequencing (Table 3).

Figure 2:



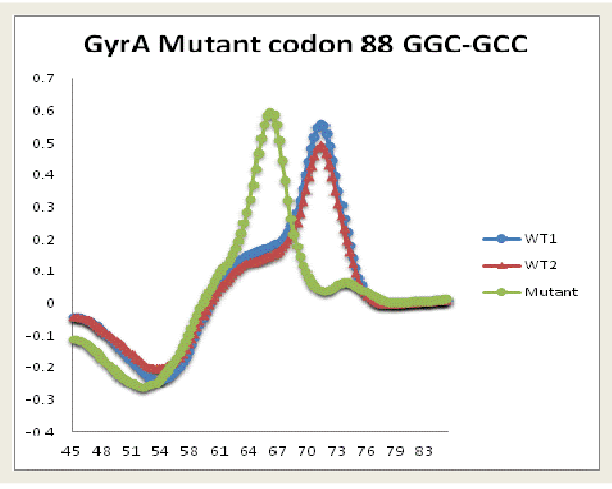
A. Scatter plot of mutations at embB 306 codon in the SMB assay.B. Real time graph showing a shift to the left for M306I mutant and a shift to the right for M306V mutant.

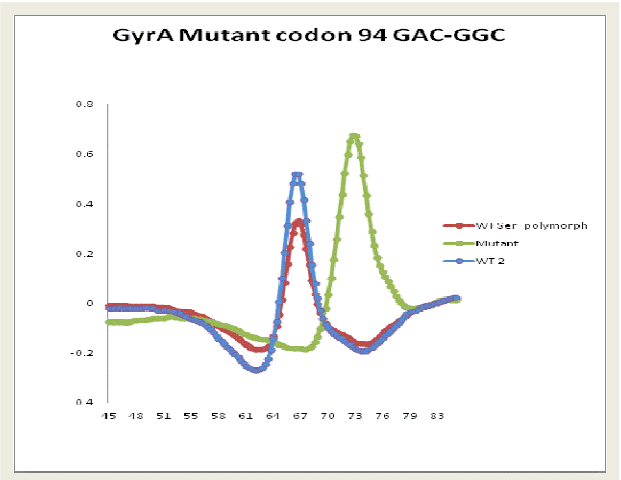
Figure 3: Results of sequencing for embB306 mutants



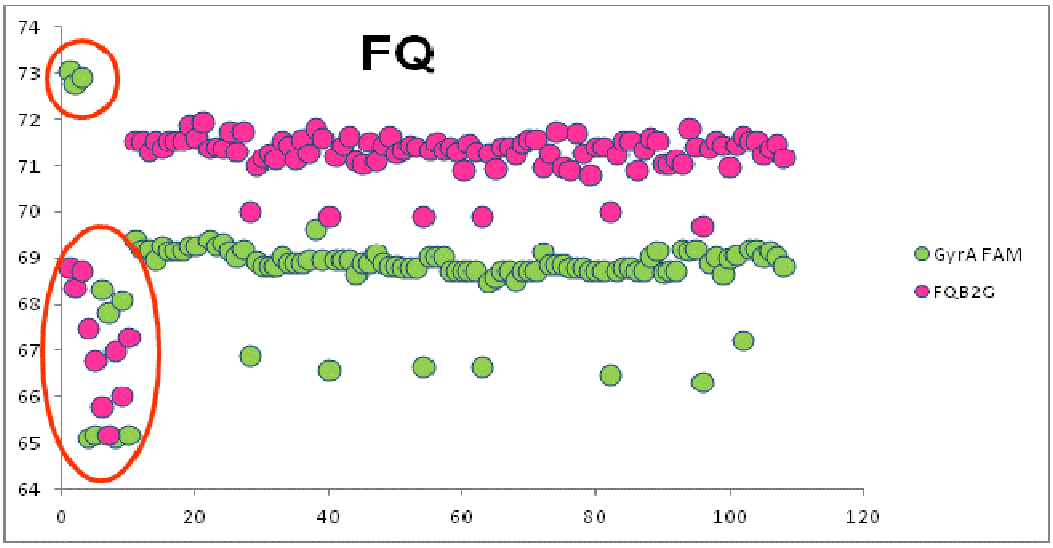
In the *gyrA* assay, 10 isolates had mutations. (Figure 4). The mutations were confirmed by sequencing. The most common mutation was A90V, seen in 40% of isolates. The sensitivity of the assay was found to be 82% and the specificity was 99% when compared with Proportion method of drug susceptibility. The sensitivity and specificity were 100% when compared with results of sequencing (Table 3).

Figure 4: A. Real time graph for *GyrA* mutants

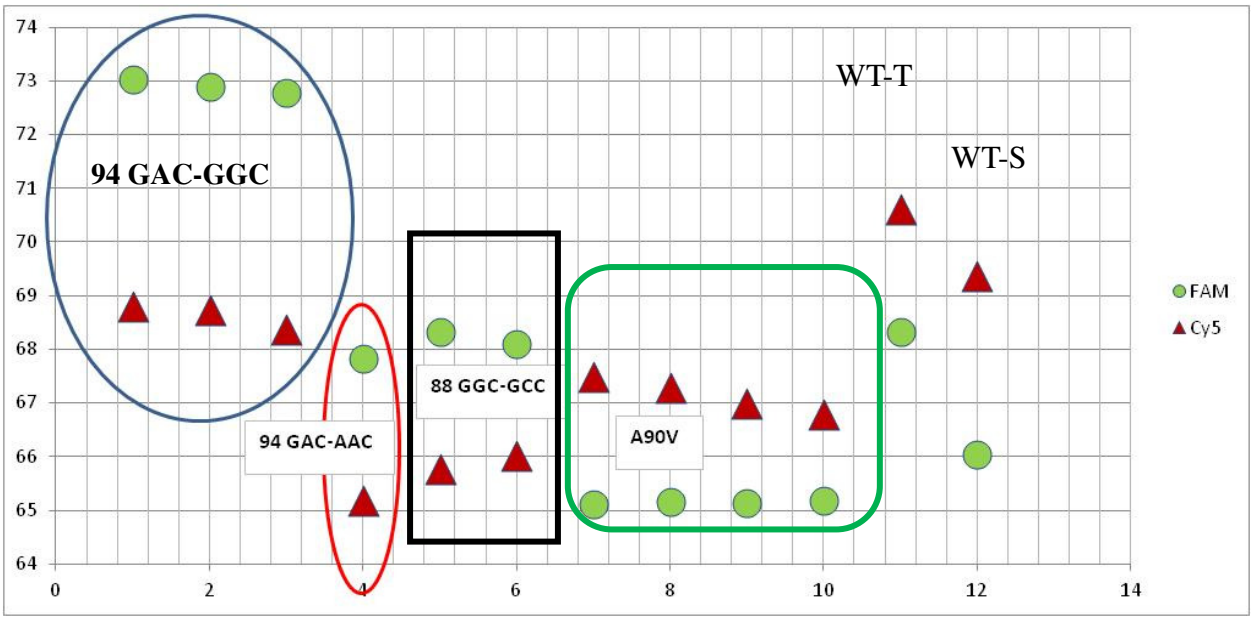




B. Scatter plot of mutations at *gyrA*. The *gyrA* FAM probe spanned *gyrA* codons 89 to 98, while the FQB2G probe spanned *gyrA* codons 86 to 95



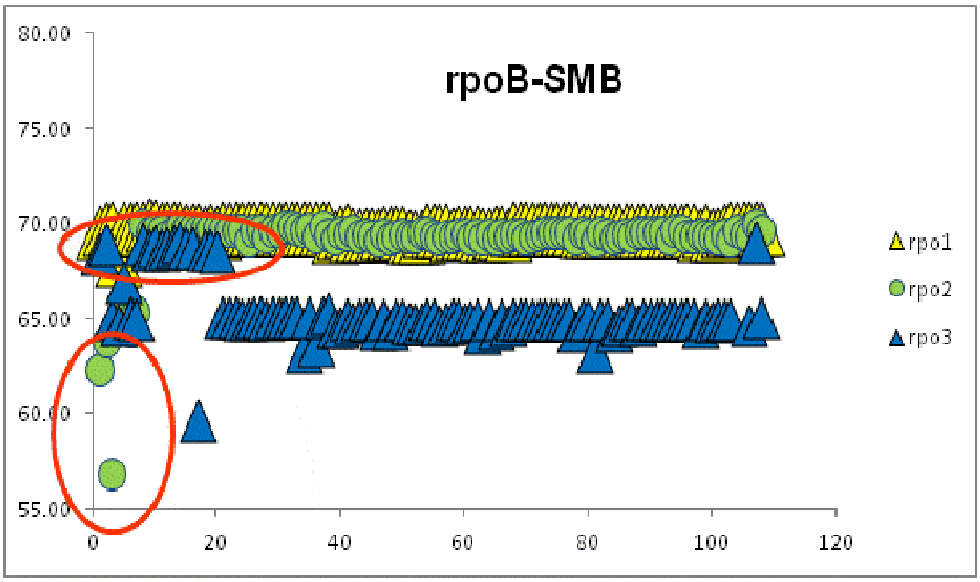
C. Results of Sequencing:

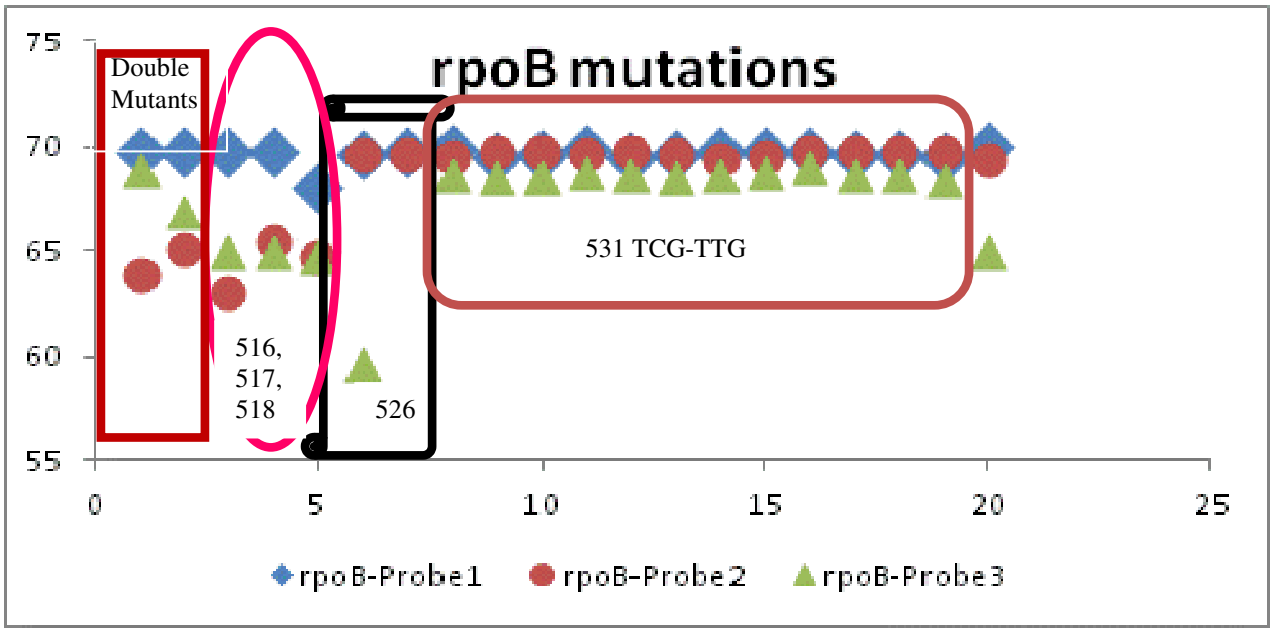


In the *rpoB* assay, 19 isolates had mutations. (Figure 5). The mutations were confirmed by sequencing. The most common mutation was at codon 531 (TCG-TTG), seen in 63% isolates. Two isolates had a double mutation. One isolate had a mutation at 516 (GAC-

GTC) and 533 (CCG) codons. The other had mutations at 517 (CAG-CAC) and 531 (TCG-TTG) codons. Three hetero-resistant isolates were also identified in the assay. The sensitivity of the assay was found to be 81% and the specificity was 97% when compared with Proportion method of drug susceptibility. The sensitivity and specificity were 100% when compared with results of sequencing (Table 3).

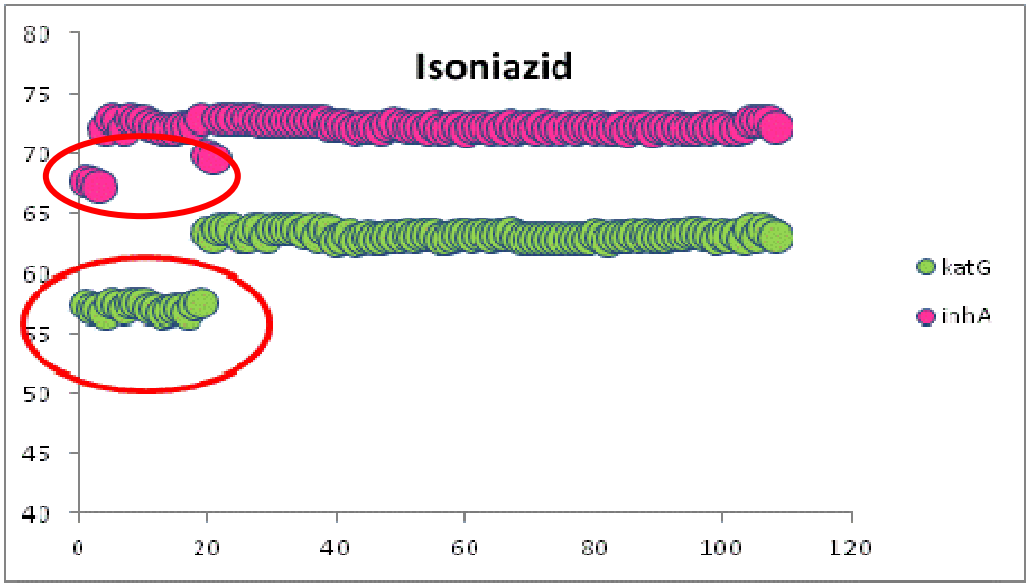
Figure 5: A. Scatter plot for *rpoB* mutants. B. Results of sequencing

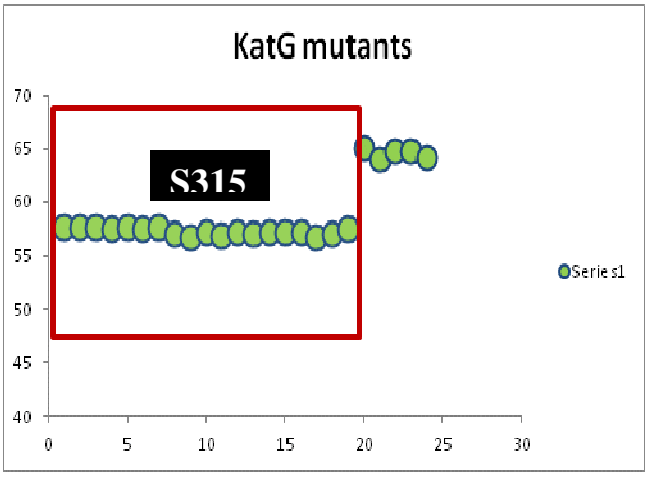


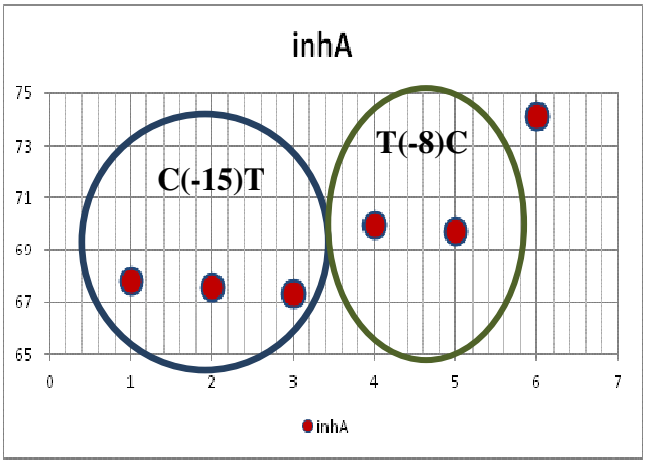


In the *katG* and *inhA* assays, 19 and 5 isolates had mutations respectively (Figure 6). The mutations were confirmed by sequencing. The mutation in *katG* was seen at codon 315. Mutations in *inhA* were seen at the *inhA* promoter region at -8 and -15. The sensitivity of the assay to detect isoniazid resistance was found to be 91% and the specificity was 99% when compared with Proportion method of drug susceptibility. The sensitivity and specificity were 100% when compared with results of sequencing (Table 3).

Figure 6: A. Scatter plot for *inhA* and *katG* mutants B. Results of Sequencing

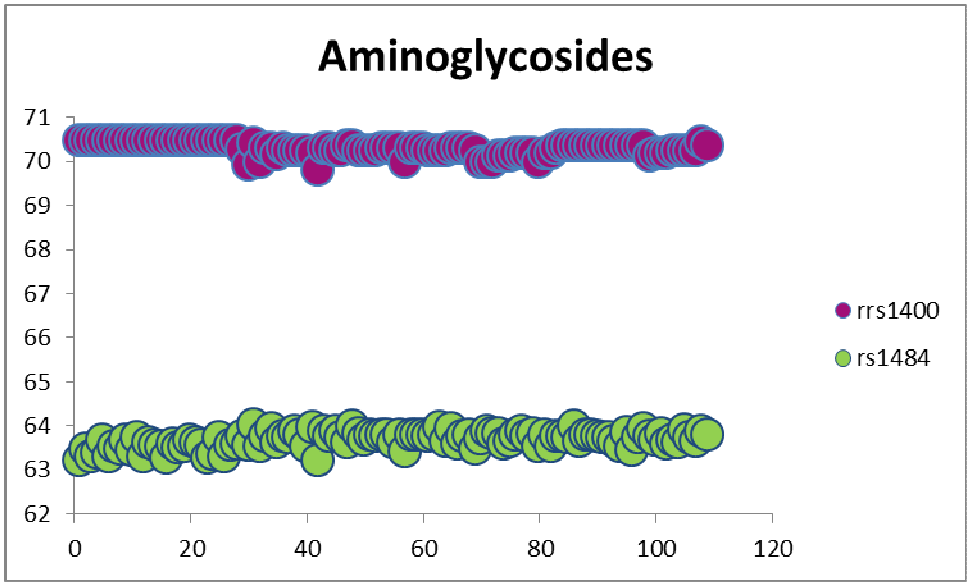


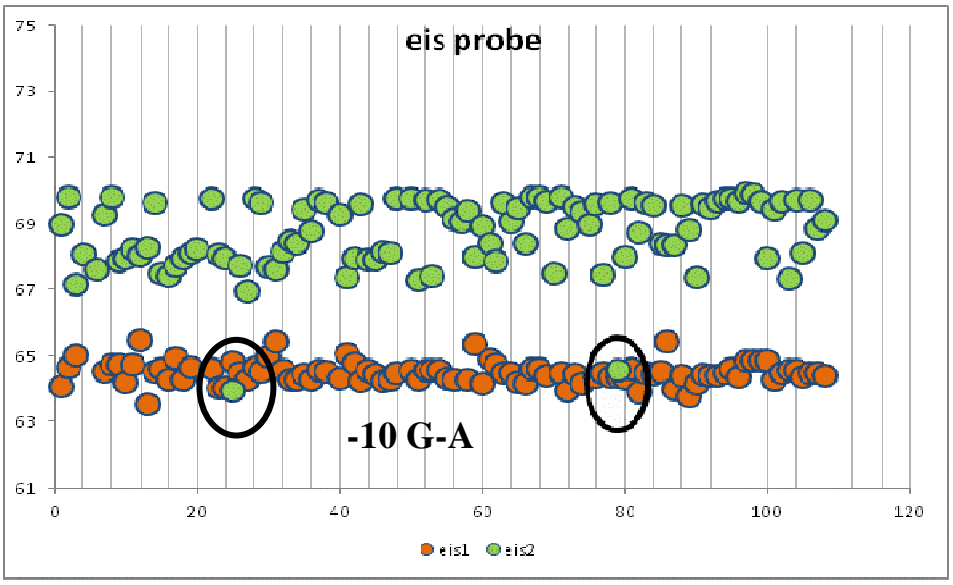


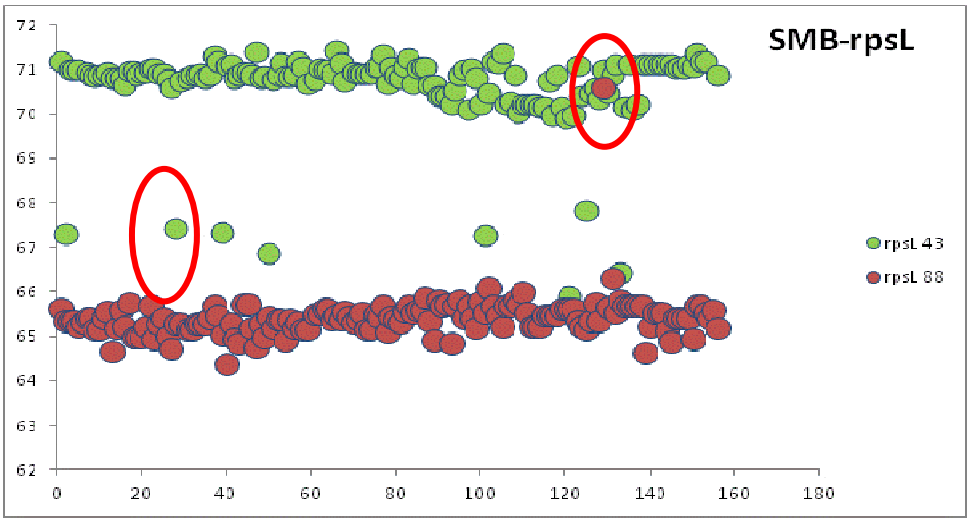


For aminoglycoside resistance, mutations at *rrs*, *rpsL* and *eis* genes were studied. No mutations were seen in the *rrs* gene (Figure 7). Two -10 mutations were seen in the *eis* gene. Eight isolates had mutations at *rpsL* 43 and one isolate had a mutation at *rpsL* 88 codon. The sensitivity of the assay was found to be 80% and the specificity was 97% when compared with Proportion method of drug susceptibility. The sensitivity and specificity were 100% when compared with results of sequencing (Table 3).

Figure 7: A. Scatter plot for *rrs* 1400 and *rrs* 1484 mutants B. Scatter plot for *eis* mutants C. Scatter plot for *rpsL* mutants







Observed Tm of the two rpsL SMB probes.

rpsL 43:

	Tm (mfold)	Observed Tm	Codon 43
WT	71.6°C	70.87°C	AAG
Mutant	66.9°C	67.45°C	AGG

rpsL 88:

	Tm (mfold)	Observed Tm	Codon 88
WT	66.6°C	65.6°C	AAG
Mutant 1	70.6°C	70.58°C	CAG
Mutant 2	77.7°C	-	ACG
Mutant 3	77.4°C	-	AGG

Sequencing the embCAB region in susceptible and resistant isolates revealed a mutation Arg738Gln mutation in two ethambutol susceptible and three ethambutol resistant isolates. This mutation is known to be a phylogenetic mutation of CAS strains. We have yet to perform spoligotyping on our isolates, which will be performed in India. The H37Rv SNP Arg927Arg was found in 83% isolates, irrespective of their ethambutol susceptibility. The mutations Asn394Asp and Thr270Ile were observed in a single ethambutol resistant isolate. Certain mutations were observed in the promoter region of embA which have to be analysed further (Tables 4a and 4b).

DISCUSSION

The *M. tuberculosis* genome is highly conserved, with only 1,075 single nucleotide polymorphisms (SNPs) discovered between the genomes of *M. tuberculosis* strains H37Rv and CDC1551 and only 2,437 SNPs discovered between the genomes of H37Rv and *M. bovis* strain AF2122/97 (Fleischmann et al, 2002; Garnier et al, 2003)**,** making phylogenetic analysis by multilocus sequencing of housekeeping genes uninformative and impractical. Instead, *M. tuberculosis* has been genotyped by measuring genetic variation in the number of insertion elements, such as IS*6110* (Eisenach KD et al, 1988), repetitive genetic elements in the direct repeat region (spoligotyping) (Kamerbeek et al, 1997), mycobacterial interspersed repetitive unit [MIRU] analysis (Frothingham and Meeker-O'Connell, 1998; Supply et al, 2001), and large sequence polymorphism analysis (Brosch et al, 2002). These techniques have succeeded in identifying large groups of isolates that each appear to be related through a common ancestor. However, these methods have not been measured against a single gold standard. These approaches also have a common drawback in that the rate of change of each phylogenetic marker is unlikely to be uniform across all markers. The diversity of markers used can further complicate analysis (Filliol et al, 2006). These limitations have made it difficult to estimate evolutionary distances among and between *M. tuberculosis* strains using current techniques.

SNPs are likely to be a more exact tool for phylogenetic studies. SNP-based analysis is less prone to distortion by selective pressure than genetic markers such as large sequence polymorphisms, and SNPs are also unlikely to converge, as can be the case with spoligotype or MIRU markers. In addition, selectively neutral SNPs may accumulate at a uniform rate and thus can be used to measure divergence (i.e., they can act as molecular clocks). Only a limited number of SNP-based studies have been performed in *M. tuberculosis* to date. The *M. tuberculosis* species was initially divided into three "major genetic" groups using a combination of two alleles at *katG463* and *gyrA95*. Subsequently, the complete genome sequences of two *M. tuberculosis* strains were used to identify initially 12 and later 77 SNPs to provide additional phylogenetic detail to a larger sample of isolates. Filliol et al used a combination of previously and newly identified SNPs based on whole-genome comparisons of *M. tuberculosis* strains H37Rv, CDC1551, 210 and *M. bovis* AF2122/97 to investigate *M. tuberculosis* evolution and phylogeny. This group of investigators finally used 9 SNP sets to cluster *M. tuberculosis* into 10 SNP clusters groups (SCG: 1, 2, 3a, 3b, 3c, 4, 5, 6a, 6b, 7). SNP 7 belongs to *M. bovis.* SCG 3 has three subsets and SCG 6 has two subsets. This limited SNP set was recommended as it could easily be used by international laboratories to perform SNP-based studies without repeating the large-scale SNP analysis (Filliol et al, 2006).

We performed the SCG assay on 153 strains of *M. tuberculosis* and found that the study set included members of all SCGs except SCG 7 (which contains primarily *Mycobacterium bovis*). SCG 3a was the most frequent and had 69 isolates. This was followed by SCG 1 with 10 isolates. In our previous study (Varma-Basil et al, 2011) CAS was found to be the most common spoligotype in our region. Although spoligotyping is yet to be performed on the isolates taken up for SCG, the fact that SCG 3a which mostly represents CAS strains was the most predominant corroborates our previous study.

A number of molecular methods have been described to detect mutations leading to drug resistance in *M. tuberculosis*, including line probe assays, locked nucleic acid probes, multiplex PCR amplimer conformation analysis (MPAC), denaturing high-performance liquid chromatography (HPLC) heteroduplex analysis, and direct sequencing. However, most of these methods are technically challenging and involve handling of PCR

amplicons in open tubes, which can lead to amplicon cross-contamination and diminished assay specificity. The extensive washing steps and on-membrane hybridization required by some of the most commonly used techniques also complicate assays and increase assay time. A melting temperature (*Tm*)- based method that uses asymmetrical PCR in conjunction with sloppy molecular beacons (SMBs) to identify hundreds of different target sequences has recently been described (El-Hajj et al, 2009). We used this approach to investigate whether it could be used to detect drug resistance mutations in *M. tuberculosis* isolates from India. The SMB assays were performed on 108 samples and were found to be simple, robust and rapid. The assays had high specificities for *M. tuberculosis* isolates from North India. In addition, the approach could also detect mixtures of wild-type and mutant DNA that had heteroresistance in their clinical cultures.

In *M. tuberculosis*, Fluoroquinolone (FQ) resistance appears to be principally caused by single-nucleotide polymorphisms in the *M. tuberculosis gyrA* gene. Between 60 and 90% of FQ-resistant clinical *M. tuberculosis* isolates have mutations in a short 21-bp "quinolone resistance-determining region" (QRDR) of *gyrA*, particularly in codons 90, 91, and 94. Mutations in the *M. tuberculosis gyrB* gene are also associated with FQ resistance, but at a much lower frequency and usually in association with *gyrA* mutations. While most *M. tuberculosis* strains with *gyrA* QRDR mutations are FQ resistant, virtually all strains that are wild type in this region are FQ susceptible. The exception to this rule is a known polymorphism at *gyrA* codon 95. FQ-susceptible *M. tuberculosis* strains can have either a threonine (T) or a serine (S) allele at this location (95S or 95T, respectively). Consequently, a molecular test that can differentiate between the two wild-type QRDR sequences and any other possible QRDR mutation is able to identify FQ resistance with high sensitivity and specificity. In fact, the *gyrA* QRDR has emerged as an important biomarker for rapid detection of FQ resistance in *M. tuberculosis* and is increasingly being used as a target for rapid molecular drug susceptibility tests. The sensitivity of the *gyrA* assay was found to be 82% and the specificity was 99% when compared with Proportion method of drug susceptibility. The sensitivity and specificity were 100% when compared with results of sequencing. Thus, 18% of patients had *gyrA*/*gyrB* non-QRDR mutations or mutations in some other genes which need to be

explored. The most common mutation detected was A90V, followed by D94G. Since mutations were detected even though patients were not on XDR therapy, there is a possibility that the patients were on FQ treatment. The SMB assay, can therefore, predict predisposition of the isolates to FQ resistance which could be used to guide second line drug therapy.

In the EMB assay, the codons *embB*306, 406 and 497 were targeted. Real Time assays were used for *embB*406 and 497. The SMB assay was used for *embB*306. The sensitivity of the EMB SMB assay was found to be 63.6% and the specificity was 100% when compared with Proportion method of drug susceptibility. The sensitivity was 100% when compared with results of sequencing. 36% of EMB resistant isolates could not be detected by the SMB assay. EMB resistance is known to be multigenic. It is also possible that there are some hitherto unknown genetic loci which need to be investigated.

Since 95% of RIF resistant isolates have mutations in the *rpoB* core region, this region was targeted using three SMB probes. Sensitivity of the *rpoB* assay when compared to proportion method of drug susceptibility was 81%. Although some mutations could be outside the *rpoB* core region, the susceptibility test has to be repeated for the discordants. The specificity of the assay was very high (97%). The most common mutation observed was 531 (TCG-TTG). This mutation has been commonly seen in several studies. Two of our strains had double mutations, one at 516 (GAC-GTC)/533 (CCG) and the other at 517 (CAG-CAC)/531 (TCG-TTG). These mutations could be easily detected in the SMB assay. The SMB assay could also detect two heteroresistant strains.

The SMB assay had a very high sensitivity and specificity for detecting isoniazid resistance. However, inclusion of both *katG* and *inhA* loci for detection of INH resistance could improve the sensitivity of assays.

Similarly, for testing aminoglycosides, inclusion of several loci could improve the sensitivity of the assay. In the present study, no mutations were seen in the *rrs* gene in the Indian isolates of *M. tuberculosis*. This was not surprising since the patients had not been exposed to Kanamycin and Amikacin. Two isolates had a -10 G/A promoter mutation in

the *eis* gene. These were Category II patients and had been exposed to Streptomycin, but not to Kanamycin or Amikacin. The SMB assay could prove valuable in being able to predict the development of XDR and in modifying the treatment of patients much before the availability of results of conventional drug susceptibility assays. However, further studies are needed on larger samples to assess the genetic loci involved in Aminoglycoside resistance.

For the EMB sequencing study, further analysis on isolates with high MIC is needed to understand the involvement of various mutations observed in ethambutol resistance.

Further studies are therefore needed to validate not only the SMB assay, but also to find out the causes of resistance to EMB.

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Signature of ICMR-IF

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Signature of Prof. Mridula Bose (Head, Dept. of Microbiology, V. P. Chest Institute, University of Delhi, Delhi-110007)

Rapid molecular detection of drug resistance in *M. tuberculosis* isolates from India using sloppy molecular beacons

ICMR-International Fellowship

Report

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CONTENTS

Comments of Officer in Charge (Dept. of Microbiology, V. P. Chest Institute, University of Delhi, Delhi-110007)

UMDNJ
NEW JERSEY MEDICAL SCHOOL
University of Medicine & Dentistry of New Jersey

Department of Medicine
Division of Infectious Diseases

Indian Council of Medical Research
REPORT

Name of Host Institute: University of Medicine and Dentistry
Name of Professor: Professor David Alland
Duration of the Fellowship: 5 months (February 25, 2012-June 23, 2012)

Highlights of the work conducted:

Dr. Varma-Basil used a sloppy molecular beacon assay that has been developed in our laboratory for molecular detection of drug resistance in *M. tuberculosis* isolates. The final objective was to validate the utility of the sloppy molecular beacon (SMB) assay to detect mutations in genes associated with drug resistance in 108 clinical isolates of *M. tuberculosis* in India and to determine the frequency of mutations in *rpoB, katG, inhA, embB, gyrA, gyrB, rrs* and *rpsL* genes in *M. tuberculosis* isolated from patients of tuberculosis being treated in the North Delhi area. The SMB assays were simple, robust and rapid and had high specificities for *M. tuberculosis* isolates from North India. Assay sensitivity as compared to conventional method of drug susceptibility testing ranged from 64%-91% for all the drugs tested and was 100% for all the assays when compared to sequencing. The discordant isolates will be retested for their drug susceptibility profile at V.P. Chest Institute. We anticipate that this work shall continue further and that we will be able to use the SMB assay on a larger number of isolates. We are particularly interested in looking at the causes of streptomycin and ethambutol resistance.

In addition, Dr. Varma-Basil performed experiments to look for mutations in the *embC*, *embA* promoter and Rv3806 genes and to correlate these with ethambutol resistance by direct sequencing. She was able to detect certain new mutations in the *embA* promoter. This part of the work shall be continued on a larger sample and the importance of the new mutations detected shall be explored further.

Dr. Varma-Basil also performed a phylogenetic analysis on 153 *M. tuberculosis* clinical isolates from India by SNP cluster grouping. She found that a majority of the North Indian isolates belonged to the SNP cluster group 3a. She would now like to extend this study and make a comparison of the MIRU and spoligotypes of these isolates.

In summary, Dr. Varma-Basil's current visit has been highly productive which will result in multiple publications and has also laid down a base for future collaboration and research.

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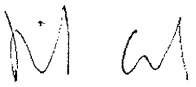
Assessment of the Candidate:

Dr. Varma-Basil is a talented scientist with outstanding critical reasoning facilities and experimental technique. She rapidly picked up both the theory and the practical skills related to her project and accomplished a tremendous amount in her short time here. Her final laboratory presentation was masterful and illustrated her command of the subject. Her work will certainly lead to several publications. I hope that she will be able to continue her research upon her return to India. We will certainly welcome collaborations with her.

Any other comments:

Dr. Varma-Basil is as pleasant a person as she is skilled as a scientist. She was a pleasure to have in the laboratory and is welcome back any time.



David Alland, MD
Professor and Chief, Division of Infectious Disease
Director, Center for Emerging Pathogens
Assistant Dean for Clinical Research
New Jersey Medical School - UMDNJ

Table 3: Sensitivity and Specificity of the SMB assay

	EMB 306		GyrA		rpoB		katG/inhA		rrs/rpsL/eis	
	P	**S**	**P**	**S**	**P**	**S**	**P**	**S**	**P**	**S**
Sensitivity	**63.6**	**100**	**82**	**100**	**81**	**100**	**91**	**100**	**80**	**100**
Specificity	**100**	**100**	**99**	**100**	**97**	**100**	**99**	**100**	**97**	**100**

Proportion method of Drug Susceptibility: P
Sequencing: S