Asian Journal of Advances in Basic and Applied Science
2015: Vol-1: No-1: 38-47
Original Research Article

Identification of multi drug resistant *M Tuberculosis* and corresponding changes in rpoB gene

S.Asokan*[1] and J.Sangeetha[1]

PG and Research Department of Microbiology, Marudapandiyar College, Thanjavur-- 613 403, Tamil Nadu, India

Abstract

In this investigation, identification of MDR strain and molecular characterization of *rpoB* from a clinical isolate was performed. Sputum samples obtained for the study were first identified for the presence of *Mycobacterium tuberculosis* by standard diagnostic procedures like AFB staining, Fluorescent staining, PCR using IS6110 amplifying primers and sputum culture. The *Mycobacterium tuberculosis* was confirmed by various standard biochemical tests and it was subjected to standard sensitivity test by proportional sensitivity test method. The samples which are found to be resistant by conventional method towards the drugs such as rifampicin and isoniazid were subjected for PCR amplification of *rpo B* gene. The amplified products are purified using gel extraction kit and subjected for sequencing. The obtained sequences were subjected to sequence analysis using BLASTn to find out the specificity of amplification as well as to identify the nucleotide variations in *rpoB* gene. There was deletion (point mutation) at position 8,17,28 and 29bp,addition at 20 and 22bp and substitutions at 11,18,19,19,26,27,2738 and 39 bp in *rpoB* gene. The same sequences were subjected for BLASTx to find out the amino acid changes. The amino acid changes identified are Serine(Ser) → Asparagine(Arn),Proline(Pro)→ Serine(Ser), Threonine→ Asparagine(Arn), Glutamicacid(Glu)→ Arginine(Asg), Isoleusine(Ilu) → Tryptophan(Trp), Cysteine(Cys) → Arginine(Asg),Methionine(Met) → Isoleusine(Ilu), Tyrosine (Tyr) → Histidine (His). These mutations in *rpoB* gene may be responsible for the Rifampicin drug resistance.

Key Words: *rpo*B gene, *Mycobacterium tuberculosis,* Rifampicin, Isoniazid, Primer, Mutation, BLASTx and BLASTn

Corresponding autor

S. Asokan
PG and Research Department of Microbiology, Marudapandiyar College, Thanjavur-- 613 403, Tamil Nadu, India
mbasokan@yahoo.com
Mobile: +91 9952330096

1.0 Introduction

Drug resistant *M. tuberculosis* is becoming an increasing public health problem and poses a serious threat to the control of disease. At least 50 million people are estimated to be infected with drug resistant tuberculosis, lead to higher mortality and treatment failure rates, and increases periods of transmissibility of the disease. A great deal of progress has been made in understanding the molecular basis of drug resistance in *M. tuberculosis* in the past few years. Such knowledge should facilitate the rational design of new antituberculosis drug and the development of rapid tests for the detection of drug resistance. The widespread emergence of isolates of *Mycobacterium tuberculosis* resistant to one or more antituberculous drugs represents one of the most alarming corollaries of acquired immune deficiency syndrome-related tuberculosis (TB) during recent years. Delayed detection, identification and susceptibility testing of drug-resistant isolates and failure to appropriately isolate contagious patients and to begin adequate chemotherapy, have all been identified as predisposing factors of transmission of drug-resistant *M. tuberculosis*. Drug resistance in *M. tuberculosis* is due to the acquisition of mutations in chromosomally encoded genes, and the generation of multidrug resistance (MDR) is a consequence of serial accumulation of mutations primarily caused by inadequate therapy (Ramaswamy *et.al.*, 1998). Because the development of isoniazid (INH) resistance usually precedes resistance to RIF, and resistance to RIF (Rifambicin) is considered as a surrogate marker for MDR-TB, molecular techniques for identifying resistant isolates of *M. tuberculosis* have largely focused on RIF's resistance. The reverse line blot assay reported recently by Mokrousov *et.al.*, (2004) is the first reported attempt to combine different targets in a single assay for prediction of multiple anti-TB drugs resistance. Following Mokrousov's report, Grace Lin *et.al.*,(2004) reported the use of molecular beacon to detect simultaneously INH and RIF's resistance-associated mutations in *M. tuberculosis* complex from cultures and smear positive sputa. Here, we report the development of a multiplex allele specific PCR (MAS-PCR) that simultaneously detects INH,RIF, and EMB (Ethambutol) resistance-associated genetic mutations at a lower cost, and using less demanding techniques and more economical equipment than the currently available methods. Rapid detection of drug resistance would help not only to optimize treatment of MDR-TB but also in breaking chains of transmission and identification of any hot spot regions in the country for proper implementation of the TB control programs. Resistance in rifampicin (RIF) has been attributed to mutations within an 81-bp RIF's resistance-determining region (RRDR) of the *rpo*B gene corresponding to codons 509 to 533 in 96% of RIFr (RIF resistant) strains (Telenti *et al.*, 1993). RIF's resistance serves as a surrogate marker for the detection of MDR TB because more than 95% RIFr isolates are also isoniazid (INH) resistant, the 2 most important drugs in anti-TB treatment regimen.

Anti-tuberculosis drugs and the gene(s) involved in their resistance: (Tarshis *et al.*, 1953).

S.No	Drug	Gene(s) involved in drug resistance
1	Isoniazid	Enoyl acp reductase(inh*A*)
		Catalase-peroxidase (kat G)
		Alkyl hydroperoxide reductase (ahpC)
		Oxidative stress regulator(oxyR)
2	Rifampicin	RNA polymerase subunit B (rpo B)
3	Pyrazinamide	Pyrazinamidase(pncA)
4	Streptomycin	Ribosomal protein subunit 12(rpsl)
		16s ribosomal RNA(rrs)
		Aminoglycoside phosphotransferase gene (strA)
5	Ethambutal	Arabinosyl transferase (*embC*)
		Arabinosyl transferase (*embA*)

Therefore the present study was carried out to identify the nucleotide variation and the corresponding changes in amino acid sequence of the *rpoB* gene involved in drug resistance in *Mycobacterium tuberculosis*.

2.0 Materials and methods

Sample Collection

Early morning sputum samples were collected from the TB patients of Govt. Medical College of Tiruvarur, Tamilnadu and South India. The samples were aspirated using sterile needle under aseptic precaution and collected in a sterile container.

Sputum staining

A thickest purulent part of the sputum sample was taken and circular movement smeared on clean glass slide with clean broom stick and allowed to air dry and then fix the smear in hot plate (80°C). The slides were stained with Ziehl-Neelsen and fluorescent staining technique (Balir *et.al.,* 1970) after staining the slides were sloped on the hot plate to dry and examined under microscope using a 100x oil immersion objective, to ensure good coverage of the smear and were graded.

Slant Culture Preparation

About 37.24 gm of Lowenstien Jensen medium was suspended in 600ml of distilled water containing 12 ml of glycerol, this sterilized medium mixed with pre-sterilized, filtered 1 liter hen's egg emulsion. The medium was mixed well and was distributed approximately 7-10ml into each pre-sterilized Mc Cartney bottles. The medium was inspissated for 50 minutes at 90°C and stored at 37° C to check the sterility of the medium. Sputum culture was prepared on slant described by Modified Petroff's Method (Allen *et.al.*, 1968 and Petroff *et.al.*, 1915). The inoculated media was placed in the 37°c incubator.

Biochemical Tests to identify *M.tuberculosis*

Biochemical tests were used to identify and differentiate the *M.tuberculosis* based on Nitrate reduction test (Virtanen *et.al.*, 1960), Niacin Test (Venkatraman *et.al.*, 1977), Tween 80 Hydrolysis Test (Wayne *et.al.*, 1964), Catalase Test (Kubica G.P. *et.al.,* 1960) and PNB Test (Monica *et.al.,* 1994).

Mycobacterium DNA extraction and amplification

The genomic DNA extraction was performed as per the procedure of Mani *et.al.*,(2001*)* and the genomic DNA was isolated following the procedure of Shoemaker *et.al.,* (1986).The isolated template DNA was amplified using IS6110 primer in an authorized thermal cycler (Eppendorf). This confirms the template DNA is *Mycobacterium tuberculosis*.

The PCR reaction mixture was set up as follows:

Content	Req.
Milli Q water	31.0µl
10x Buffer	5.0µl
Mgcl2	2.0µl
Taq Polymerase	1.0µl
dNTPs	2.0µl
Mtb(10pmol/µl)	2.0µl
Mtb (10pmol/µl)	2.0µl
DMSO	3.0µl
Template DNA	2.0µl

The PCR cycling parameters were 94°Cfor 5 minutes, followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 74°C for 1 minute; and a final extension of 74°C for 5 minutes. The PCR was then kept at hold at 4°C for 15 minutes.

Amplification of *rpoB* gene from clinical isolated strain

Extracted Mycobacterial DNA and the Taq polymerase, dNTPs, $Mgcl_2$,Milli Q water,10x Buffer, DMSO, template DNA, forward and reverse *rpoB* primers were used for amplification of each respective gene as mentioned in the below table .

Master Mix preparation for each target gene amplification:

Content	**Req.**
Milli Q water	31.0µl
10x Buffer	5.0µl
$Mgcl_2$	2.0µl
Tag Polymerase	1.0µl
dNTPs	2.0µl
F-Primer (10pmol/µl)	2.0µl
R-Primer(10pmol/µl)	2.0µl
Template DNA	1.0µl

The PCR cycling parameters were 94°Cfor 5 minutes followed by 40 cycles of 94°C for 1 minute, 57°C for 1 minute and 74°C for 1 minute and a final extension of 74°C for 5 minutes. The PC3R was then kept at hold at 4°C for 15 minutes.

Agarose gel electrophoresis and Gel cleanup

The amplified PCR product was withdrawn from thermal cycler and run on a 2% Agarose gel in TAE buffer. The Ethidium bromide stained gels were observed in a UV Trans illuminator and photographed using a Geldoc. The gel cleanup method was followed as per the procedure of Chen *et.al.,* (1980) for sequencing analysis.

DNA sequencing analysis

The purified PCR product was directly sequenced in an automated DNA Sequencer at MGWAG biotech at UK. The nucleotide sequence obtained was analyzed using BLASTn Bioinformatics tool available at National Center for Biotechnology Information (Altschul *et al,* 1997.) to know the specificity of PCR amplification and to identify the nucleotide variation. The sequence was further subjected for BLA

STx to know the amino acid changes in comparison with the wild type *Mycobacteruim tuberculosis* ($H_{37}Rv$).

3.0 Results and discussion

Small, rough and cream colored colonies were observed on the inoculated L.J.slants after 4 weeks of incubation, indicating the presence of *Mycobacterium tuberculosis* in the isolate *(Fig 1)*. Various biochemical tests were performed to confirm the M*ycobacterium tuberculosis* in the LJ medium slants and the results are tabulated in table 1.

Polymerase Chain Reaction:

Mycobacterial DNA was isolated from the L.J.medium slant and was subjected to PCR amplification using species specific primers, targeting the insertion sequence IS6110 (*M. tuberculosis* 5' GTGAGGGCATCGAGGTGG 3') & (*M.tuberculosis* 5'CGTAGGCGTCGGTCACAAA 3') for conforming the *M. tuberculosis*. The PCR product was run on a 2% agarose gel . A clear band was formed at 123bp region confirming the presence of *M. tuberculosis* in the culture *(Fig 2)*.

Culture Isolate Control

Fig 1 Growth of Mycobacterium *tuberculosis* on L.J.medium slants

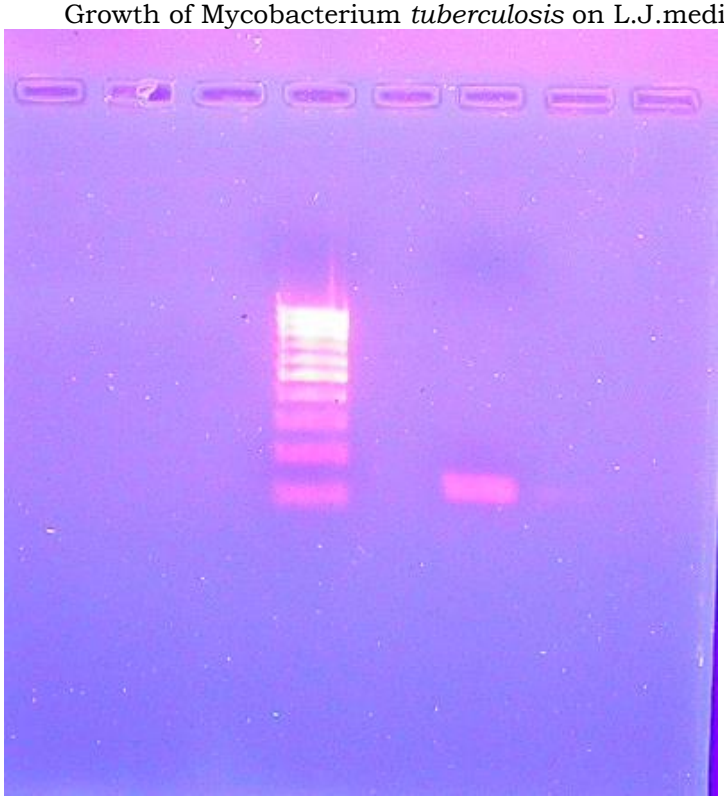

Fig 2 123 bp products amplified with IS6110 primer. Lane 4: 100 bp ladder, Lane 6: suspected clinical isolate

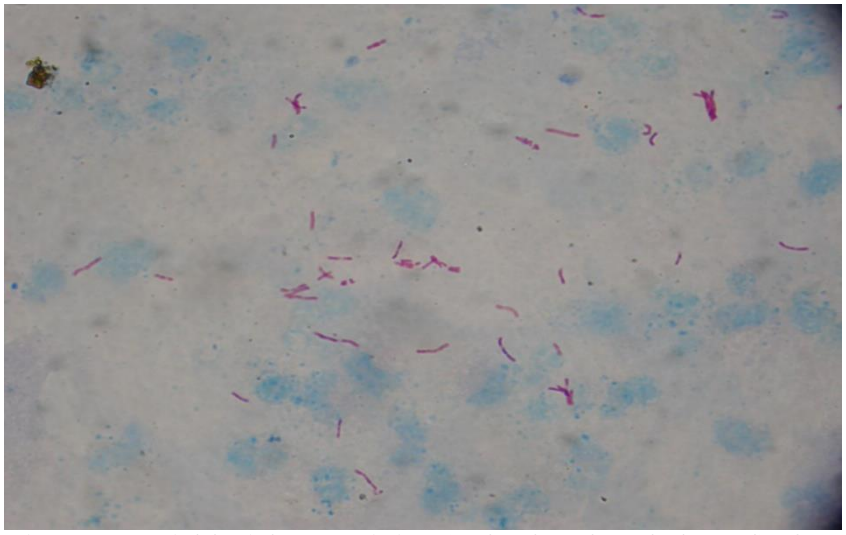

Fig 3 Ziehl Nielsen staining (red colored, rod shaped) of *Mycobacterium tuberculosis.*

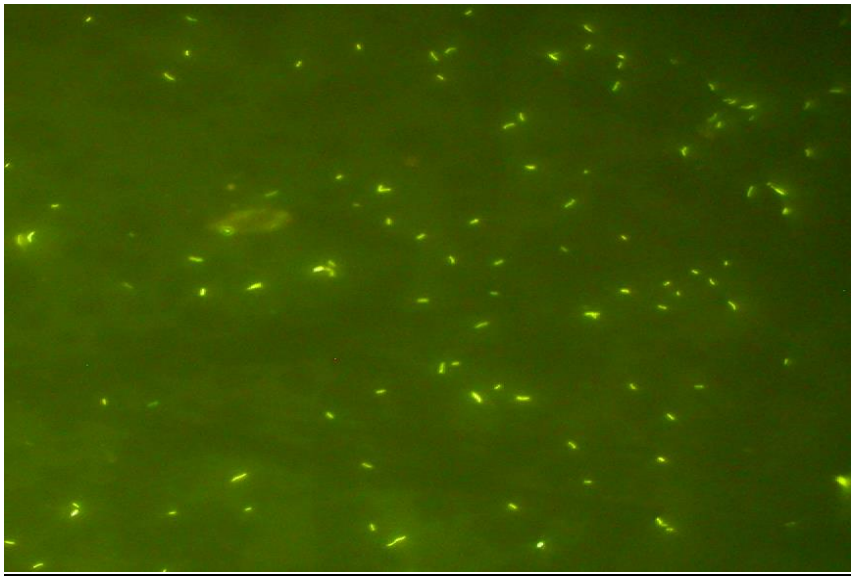

Fig 4 Fluorescent staining (golden yellow colour) of *Mycobacterium tuberculosis*

.

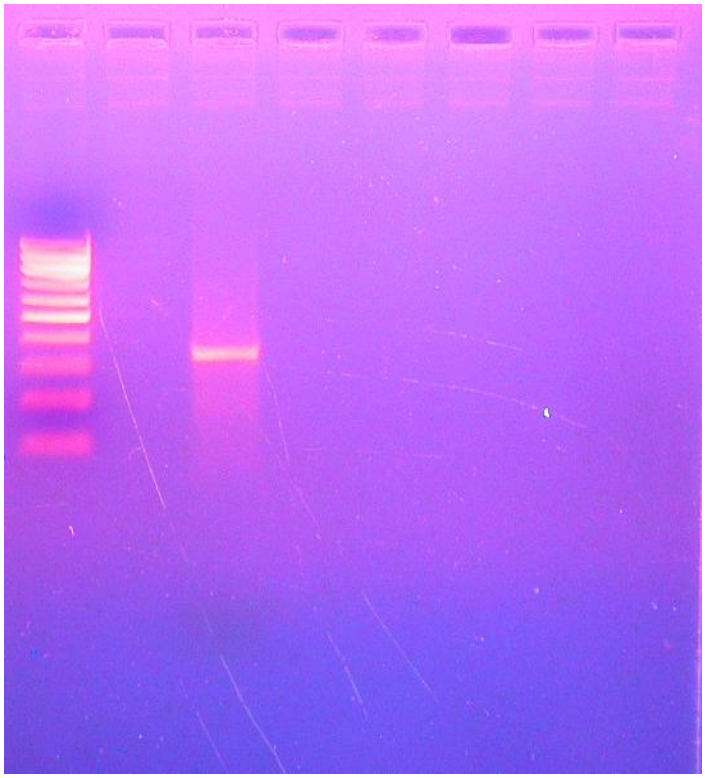

Fig 5 329 bp products amplified *with rpoB* primers. Lane 1: 100 bp ladder, Lane 3: suspected clinical isolate

S.No.	Name of the tests	Results
1	Ziehl Nielsen staining	Appearance of pink colored bacilli with beaded in blue back ground *(Fig 3)*
2	Fluorescent staining	Appearance golden yellow colored bacilli in dark back ground *(Fig 4)*
3	Niacin test	Pink to red colour formation
4	Nitrate reduction test	Appearance of deep red colour
5	Catalase test	Appearance of bright effervescence
6	Tween hydrolysis	Amber to pink colour formation
7	PNB test	Growth on L.J medium supplemented with PNB

Table: 1 Biochemical tests for identification of *M.tuberculosis*

Amplification of *rpo B* gene

DNA was isolated from the suspected isolates on L.J.medium slant and H_{37}RV wild type strain and was subjected to PCR amplification using *rpo B* primers : *rpo*95 5'-CCACCCAGGA CGTGGAGGCGATCACAC-3 'and *rpo*397 5'-CGTTTCGATGAACCCGAACGGGTTGAC-3'.The amplified PCR product of H_{37}Rv and suspected clinical isolates were run on a 2% agarose gel with 100bp DNA ladder. A clear band was formed at 329bp region and confirms the molecular size of the amplified product is 329bp *(Fig 5)*.

DNA sequencing:

The desired PCR product (*rpo B* gene) to be sequenced was eluted from the gel by Gel cleanup kit. The purified PCR product was directly sequenced in an automated DNA Sequencer. Sequenced DNA of mutant strain was compared with H_{37}Rv (wild type) strain DNA sequence using Bioinformatics tool BLASTn available at National Centre for Biotechnology Information (Altschul *et al,* 1997.) for detecting the mutation. BLASTn and BLASTx alignments of genes of interest are indicated. The mutation and the amino acid changes pattern are shown in table - 2.

Gene	Nucleotide changes	Amino acid change
rpoB	Deletion at 8,17,28 and 29bp Substitution at 11,18,19,19,26,27,2738 and 39 bp Addition at 20 and 22bp	Serine(Ser) → Asparagine(Arn) Proline(Pro)→ Serine(Ser) Threonine→ Asparagine(Arn) Glutamicacid(Glu)→ Arginine(Asg) Isoleusine(Ilu) →Tryptophan(Trp) Cysteine(Cys) → Arginine(Asg) Methionine(Met) → Isoleusine(Ilu) Tyrosine (Tyr)→Histidine(His)

Table 2: Nucleotide and amino acid changes in drug target genes:

DNA Sequence of *rpoB* gene (+strand)

>RPOB11-RP095.scf
TAAGGAGTTCTTCGGCCAAGACCAGTAACCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTG
ACCCACAAGCGCCGACTGTCGGCGCTGGGGCCCGGCGGTCTGTCACGTGAGCGTGCCGGGCT
GGAGGTCCGCGACGTGCACCCGTCGCACTACGGCCGGATGTGCCCGATCGAAACCCCTGAGGG
GCCCAACATCGGTCTGATCGGCTCGCTGTCGGTGTACGCGCGGGTCAACCCGTTCGGGTTCAT
DNA Sequence of *rpoB* gene (-strand)
>RPOB11-RP0397.scf
GCCCCTCGGAGTTTCGCCACGGGCGTATCCGGCCGTGATGCGACGGGTGCACGTCGCGGACCT
CCAGCCCGGCACGCTCACGTGACAGACCGCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGG
GTCAACCCCGACAGCGGGTTGTTCTGGTCCATGAATTGGCTCAGCTGGCTGGTGCCGAAGAACT
CCTTGATCGCGGCGACCACCGGCCGGATGTTGATCAACGTCTGCGGTGTGATCGCCTCCACGTC
CTG

Global prevalence of *Mycobacterium tuberculosis* infection has been estimated to be 32 per cent. Eighty per cent of all incidence cases are found in 22 countries, with more than half the cases occurring in five Southeast Asian countries (India, Indonesia, Bangladesh, Philippines, and Vietnam). According to estimation, 30 per cent of world's tuberculosis patients live in India.

Treatment of MTB infection relies primarily on the use of two major first-line drugs, isoniazid and rifampicin, which are often included in a four-drug regimen that also includes ethambutol and pyrazinamide. The second-line fluoroquinolone drugs may be prescribed either when the two first-line drugs fail as a result of emergence of resistant organisms or in cases where their use is not appropriate due to hepatic problems in patients. Hence, the emergence of clinical isolates that are resistant to fluoroquinolones signals a significant compromise in the effectiveness of MTB treatment. When MTB strains exhibit resistance to both isoniazid and rifampicin, they are termed multidrug-resistant MTB (MDR-TB). These MDR-TB strains have been shown to be increasingly associated with infections in AIDS patients. Furthermore, treatment of MDR-TB infection has been complicated by the increased cost (up to 100 times higher than the treatment of diseases involving drug-susceptible organisms) and higher frequency of adverse reactions.

Currently, the detection of drug resistance in *Mycobacterium tuberculosis* is primarily based on phenotypic drug susceptibility testing, which involves time-consuming culture of the slow-growing *M. tuberculosis* bacilli in the presence of antibiotics (Canetti *et al.,* 1969, Laszlo *et al.,* 1997). With the increased understanding of the genetic mechanisms of *M. tuberculosis* drug resistance and the advancement of molecular technologies in recent years, a number of more rapid molecular methods to detect mutations in genes implicated in *M.tuberculosis* drug resistance.

Rifampicin

Rifampicin is the most potent sterilizing antibiotic used for the treatment of TB. In the case of drug-susceptible strains of *M. tuberculosis*, the combined use of the first-line anti-TB drugs,

rifampicin, isoniazid, ethambutol, and pyrazinamide, will most often result in successful cures. Rifampin and isoniazid are the most active of the first-line anti-TB drugs, and *M. tuberculosis* strains that are resistant to both of these antibiotics are considered MDR. In general, multidrug resistance is acquired in two steps, with the first step being the evelopment of isoniazid resistance rather than rifampin resistance (Nikolayevsky, V *et.al.,2004*), suggesting that rifampin resistance can be used as a surrogate marker for the detection of MDR *M. tuberculosis*. Since 91% of the rifampicin resistant strains examined were also resistant to isoniazid, this indicates that rifampin resistance is a good predictor for multidrug resistance (Yuen, *et al.,* 1999).
Rifampicin resistance is an excellent marker for multi drug resistant tuberculosis because all of these strains are resistant to rifampicin (Telenti A.*et al.,* 1997). Therefore, a screening assay does not need to test susceptibility to all antituberculosis drugs. rifampicin resistance is particularly amenable to detection by rapid genotypic assays because 95% of all rifampicin-resistant strains contain mutations localized in an 81-bp region of the bacterial RNA polymerase gene (*rpoB*) which encodes the active site of the enzyme (Musser, J. M. *et al.,* 1995). Moreover, all mutations that occur in this region result in rifampicin resistance. By contrast, all rifampicin susceptible *M. tuberculosis* isolates have the same wild-type nucleotide sequence in this region (Watterson.S *et al.,* 1998). Thus, it is only necessary to detect a mutation in the *rpoB* core region to know that the bacilli are rifampicin resistant.

4.0 Discussion

The clinical *M.tuberculosis* isolated in this study had mutation in the *rpoB* gene, which was detected by direct sequencing in an automatic sequencer. The obtained sequences were subjected to sequence analysis-using BLASTn to find out the specificity of amplification as well as to identify the nucleotide variations in *rpo B* gene. The same sequences were subjected for BLASTx to find out the amino acid changes. These mutations in *rpoB* gene may be responsible for the rifampicin drug resistance.

References

1. Allen, B., W. and Baker, F., J. 1968. Mycobacterial isolation, identification and sensitivity testing. Butter worths. 10.
2. Altschul, Stephen, F. Thomas, L. Madden. Alejandeo, A. Schaffer, Jinghui zhang, Zheng Zhang, Webb miller and David. J. Lipman. 1997. Gapped Blast and PSI Blast- a new generation of protein database search programme, Nucleic Acid resistance. 25: 3387 – 3402.
3. Blair, E., B. Weiser, O., L. and Tull, N., H. 1970. Micro bacteriology laboratory methods – Lab report No. 32s U.S. Army Medical Research and Nutrition Laboratory. Fitrzsimmoun General Hospital, Denver, Colorado, USA.
4. Canetti, G. Fox, W. Khomenko, A. Mahler, H., T. Menon, N., K. and Mitchison. D.A., *et al.,* 1969. Advances in techniques of testing Mycobacterial drug sensitivity, and the use of sensitivity tests in the tuberculosis control programmes. Bull World Health Organ. 41*:* 21-43.
5. Chen, C., W. and Thomas, C., A. 1980. Recovery of DNA segment from Agarose gel. Analytical Biochemistry. 101: 339 – 341.
6. Grace Lin, S., Y. William Robert, Melanie Lo, and Edward Microbial Diseases Laboratory, California Department of Health Services, Richmond, California 94804 Received 29 October 2003/Returned for modification 20 February 2004/Accepted 12 May 2004
7. Kubica, G., P. and Pool, G., I. 1960. Studies on the catalase activity of acid. fast bacilli. American Review of Respiratory Diseases. 81: 387 – 391.
8. Laszlo, A. Rahman, M. Raviglione, M. and Bustreo, F. 1997. Quality assurance program for drug susceptibility testing of *Mycobacterium tuberculosis* in the WHO/IUATLD Supranational Laboratory Network: first round of proficiency testing. Int. J. Tuberc. Lung Dis. 1: 231–238.Desmond
9. Mani Cheruvu, Selvakumar, N. Narayanan Sujatha and Narayanan. P., R. 2001. Mutations in the *rpoB* Gene of Multidrug-Resistant *Mycobacterium tuberculosis* clinical isolates from India". J Clin Microbiol. 39: 2987-2990.

10. Mokrousov, I. Bhanu, NV. Suffys, P., N. Kadival, G., V. Yap, S., F. Cho, S., N. Jordaan, A., M. Narvskaya, O. Singh, U., B. Gomes, H., M. Lee, H. Kulkarni, S., P. Lim, K., C. Khan, B.K. Van Soolingen, D. Victor, T., C. and Schouls, L., M. 2004. Multicenter evaluation of reverse line blot assay for detection of drug resistance in Mycobacterium tuberculosis clinical isolates. J Microbiol Methods. 57:323–335.
11. Monica cheesbrough, 1994. Medical Laboratory Manual for Tropical Countries. Butter worth – Heinmann Limited, Cambridge. 415: 32-38, 59-66.
12. Musser, J., M. 1995. Antimicrobial agent resistance in *Mycobacterial* molecular genetics insight. Clin. Microbiol. Rev. 8: 496-514.
13. Nikolayevsky, V. Brow, T. Balabanova, Y. Ruddy, M. Fedorin, I. and Drobniewski, F. 2004. Detection of mutations associated with isoniazid and rifampin resistance in *Mycobacterium tuberculosis* isolates from Samara region, Russian Federation. J Clin Microbiol. 42: 4498–4502
14. Petroff, S., A. 1951. A new and rapid method for isolation and cultivation of *Tubercle bacilli* directly from sputum and feces. Journal of Experimental medicine (USA). 21: S8.
15. Ramaswamy, S., V and Musser, J., M. 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis. Tuberculosis* and Lung Diseases. 79: 3–29.
16. Shoemaker, S., A. Fisher, J., H. Jones Jr J., D and Scoggin, C., H. 1986. Restriction fragment analysis of chromosomal DNA defines different strains of *Mycobacterium tuberculosis.* Am. Rev. Respir. Pis. 121: 210-213.
17. Tarshis. M., S, and Weed, W., A, 1953 Lack of significant in vitro sensitivity M. tuberculosis to pyrazinamide on three different solid media. Am.Rev.Tuberc. 67: 391–395.
18. Telenti, A., N. Honore, C. Bernasconi, J. March. A. Ortega. B. Heym. H., E. Takiff and Cole, S., T. 1997. Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. Journal of Clinical Microbiology. 35(3): 719–723.
19. Venkatraman, P. and Alexander, C. 1987. Manual of laboratory methods in bacteriology. 4:5.
20. Virtanen, S. 1960. A study of Nitrate reduction by *Mycobacterial tuberculosis.* Acta Scandinavia. 48: 119-123
21. Watterson, S. Wilson. S. and Yates. M., *et al.* 1998. Comparison of three molecular assays for rapid detection of rifampin resistance in *Mycobacterium tuberculosis*. J. Clin. Microbiology. 36(7): 1969-73.
22. Wayne, L., G. Douber J., K and Russel R., L. 1964. Classification and identification of *Mycobacterial Sp.* American Review of Respiratory Disease. 90: 588-597.
23. Yuen, L. Leslie. D. and Coloe, P. 1999. Bacteriological and molecular analysis of rifampin-resistant *Mycobacterium tuberculosis* strains isolated in Australia. J. Clin. Microbiology. 37(12): 3844-50