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Molecular Diagnosis of Multidrug-Resistant *Mycobacterium tuberculosis* Isolates in and around Mumbai

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Mycobacterium tuberculosis **(MTB), resistant to both isoniazid (INH) and rifampicin (RIF) and leading to multidrug-resistant tuberculosis (MDR-TB) can be successfully identified by an 8-week long phenotypic method. However, there is a need for faster diagnostic techniques.In the present study, we evaluated the use of PCR followed by sequencing as a rapid diagnostic tool and determined the incidence of *rpoB* and *katG* resistance-associated mutations in *Mtb* isolates in and around Mumbai. Fifty-Five sputum samples from clinically suspected tuberculosis patients were decontaminated and screened for MTB using Ziehl Neelsen (ZN) staining and Lowenstein–Jensen (LJ) culturing. Extracted DNA from positive samples was subjected to conventional PCR for *rpoB* and *katG* genes. In addition, Drug Susceptibility Testing (DST) of isolates was performed by the standard proportional method. PCR products of selected samples of either type i.e., resistant and susceptible by both techniques were sent for sequencing. Fifteen samples (27.27%) were found to be resistant to both drugs phenotypically by DST and by PCR for *rpoB* and *katG* genes. Sequencing analysis revealed point mutations Ser 531 Leu for *rpoB* gene and Ser 315 Thr for *katG* gene. The results obtained by direct PCR followed by sequencing method were in accordance with phenotypic method (DST) with high sensitivity and specificity for both genes. The results suggest that the use of molecular techniques can be more suitable for rapid diagnosis of multi-drug resistance in clinical isolates of MDR-TB.**

Key words: Multi-drug Resistant tuberculosis (MDR-TB), Polymerase Chain Reaction (PCR), Sequencing, *rpoB* & *katG* genes.

In 2012, with an estimated 8.6 million new cases and 1.3 million deaths every year, tuberculosis (TB) represents one of the most serious infectious diseases worldwide[1]. The alarming emergence of multidrug-resistant TB (MDR-TB) cases, resistant to at least two drugs, including isoniazid (INH) and rifampin (RIF), and the recent emergence of extensively drug-resistant TB (XDR-TB), with additional resistance to a fluoroquinolone (FQ) and at least one of the three injectable second-line drugs, pose a significant threat to tuberculosis control[2,3]. Lack of adequate treatment, often due to irregular drug supply, inappropriate regimens, or poor patient compliance, is associated with the emergence of drug-resistant *Mycobacterium tuberculosis*[4]. In India, the incidence of all forms of TB was estimated to be 193/100,000 population in 2012[1]. In 2012, approximately 450,000 cases of MDR-TB were estimated throughout the world with 58 nations reporting at least one case of XDR-TB to the World Health Organization (WHO)[1]. Among them, India and China had the highest burden of MDR-TB, together accounting for almost half of the world's total cases[5]. Previous studies have shown that 68% of the patients for whom drug susceptibility testing

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(DST) was requested had multidrug-resistant tuberculosis. Therefore, there is an urgent need for rapid and accurate detection of MDR-TB in order to achieve immediate patient treatment and hence cure and from a public health perspective, to prevent transmission[6].

TB detection using molecular techniques namely, polymerase chain reaction (PCR), PCR based restriction fragment length polymorphism (PCR-RFLP) and PCR based DNA sequencing are very rapid, more sensitive and reliable when compared to the conventional culture[7].

There are several in-house nested PCR (nPCR) standardized for the detection of *M. tuberculosis* from clinical specimens targeting MPB64, IS6110 and 16S rRNA genes. MPB64 is an immunogenic protein produced by certain mycobacterial strains while IS6110 is an insertion element present in single or multiple copies in *M. tuberculosis* complex isolates specifically[7]. The collection of data from different countries has indicated that resistance to RIF in 90% of cases is due to mutations resulting in an amino acid substitution within the 81-bp core region of the RNA polymerase-subunit gene (*rpoB*), called the RIF resistance-determining region (RRDR)[8-11]. In contrast, INH resistance is mediated by mutations in several genes, most frequently within the *katG* gene, encoding a catalase-peroxidase which transforms INH into its active form[10,12,13] and in the promoter region of *inhA*, encoding a putative enzyme involved in mycolic acid biosynthesis. An upregulation mutation in the *inhA* promoter region results in the overexpression of InhA and develops INH resistance via a titration mechanism[9]. In the present study, we determined the incidence of resistance-associated mutations in two specific genes (*rpoB* and *katG*) of *M. tuberculosis* isolated in and around Mumbai and compared the results of sequencing with the results of phenotypic method (DST by proportional method).

MATERIALS AND METHODS

Study Groups

Fifty-five clinical samples from patients diagnosed with pulmonary tuberculosis were included in the study. Individuals more than 18 years of age, belonging to either sex and attending outdoor patient department or admitted to Government Sewree TB Hospital, Mumbai were selected for the study. Individuals less than 18 years of age and pregnant women were excluded from the study. A written informed consent was acquired from all individuals that were included in the study. Ethical approval for the study was taken previously from Institute Ethics Committee.

Sample Collection and Processing

A total of 55 sputum samples were collected from subjects during one year period from 2011 to 2012. The samples were transported to the laboratory and stored at 4ºC within 4 h of collection and processed immediately for decontamination of sputum sample by NALC-NaOH Method.

Phenotypic Method

Detection of *Mycobacterium tuberculosis* was done by using Ziehl Neelsen (ZN) staining (Microscopy) and culturing on to Lowenstein–Jensen (LJ) medium[14]. Drug Susceptibility Testing (DST) of isolates was performed by the standard proportional method on Lowenstein–Jensen (LJ) medium containing 0.2 mg Isoniazid per mL and 40.0 mg Rifampicin per mL, as described in RNTCP guidelines[14].

Extraction of DNA from MTB

DNA from MTB processed sputum sample was extracted by Qiagen QIAmp DNA mini kit (Qiagen, Inc., Valencia, CA) method as per manufacturer's instructions[15]. Briefly, 200.0 µL of sputum sample warmed to room temperature (15-20°C) and 180.0 µL of lysis solution i.e ATL was added in 1.5 mL microcentrifuge tube. After briefly vortexing, it was incubated in dry bath (Benchmark Instruments) at 37°C for 3 hours. After addition of proteinase K and AL solution, the mixture was further incubated at 56°C for two hours in dry bath. A volume of 200.0 µL of 96% ethanol was added, mixed and the mixture was transferred on to a spin column. Following intermittent centrifugation and washing steps, 100.0 µL of elution buffer pre-warmed at 70°C was added to elute purified DNA. DNA yield from all the samples of extraction was determined on a NanoDrop Spectrophotometer (Thermo Scientific) by measuring the absorption at 260 nm, and purity was calculated based on 260/280 ratio.

Detection of MTB by Conventional Nested PCR Using Suitable Primers

Nested Polymerase Chain Reaction was used to amplify the IS6110 sequence of

Mycobacterium tuberculosis using primers as mentioned in Table 1. The outer and inner rounds of PCR were carried out using a PCR Master Mix (Fermentas Inc., Maryland, USA) as per manufacturer's instructions with primer concentration being 0.4 µM and 0.7 µM respectively in a 25.0 µL reaction. The cycling conditions used were: 94°C/20s, 60°C/20s, 72°C/30s for 35 cycles to give a product size of 556 bp and 285 bp for outer and inner rounds respectively in a M/s. Takara PCR Thermal Cycler Dice (TP600 System) Instrument[16]. Amplified products were detected on 1.5% agarose gel infused with SYBR®Safe dye for visualization.

Detection of Rifampicin & Isoniazid Resistance Gene by Conventional PCR Using Suitable Primers

The conventional PCR was used to amplify *rpoB* gene sequence and *katG* gene sequence of *Mycobacterium tuberculosis* using primer as mentioned in Table 2. PCR primers were designed to amplify the regions between nucleotides 2335 and 2492 of the *rpoB* gene (codon 531 TCG?TTG and 526 CAC?GAC) and nucleotides 2759 and 2967 (codon 315AGC?AAC) of the *katG* gene[11,17]. PCR was carried out using a PCR Master Mix (Fermentas Inc., Maryland, USA) as per manufacturer's instructions with primer concentration being 0.5 µM for both the genes and concentration of $MgCl_2$ being 5.0 mM in a 25.0 µL reaction. The cycling conditions used were 95°C/45 s followed by 38 cycles of 95°C/15 s, 53°C (annealing temperature for the *rpoB* gene) and 60°C (annealing temperature for the *katG* gene) for 15 s, and 72°C/30 s to give a product size of 158 bp for the *rpoB* gene and 209 bp for the *katG* gene in a M/s. Takara PCR Thermal Cycler Dice (TP600 System) Instrument. Amplified products were detected on 1.5% agarose gel infused with SYBR®Safe dye for visualization. PCR products were purified by using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA Catlog No: 28106) as per manufacturers' Instructions.

DNA sequencing

Direct sequencing of PCR products of selected samples and standard strains was outsourced to Ocimum Biosciences Ltd. The sequencing was performed with a 96-capillary 3730xl DNA Analyzer.

RESULTS

A total of 55 sputum samples from patients suspected of tuberculosis were subjected to preliminary screening by simple microscopic detection using the Acid Fast Bacilli (AFB) -ZN staining technique and culturing on LJ medium, of which 52 (94.55%) samples were found to be smear positive & 3 (5.45%) samples were found to be smear negative. However, 50 (90.91 %) samples

Table 1. Amplification primer sequences of IS6110 gene for detection of *M. tuberculosis* by Conventional Nested PCR

	Primer Name	Position of IS6110	Sequences (5'→3')
Outer	TB1-F	555 - 572	CTCAAGGAGCACATCAGC
	TB2-R	1111 - 1084	TCATAGGAGCTTCCGACC
Inner	TB3-F	590 - 609	CTACGGTGTTTACGGTGCCC
	TB4-R	874 - 855	CTACGGTGTTTACGGTGCCC

Table 2. Amplification primer sequences for Detection of Rifampicin (rpoB) and Isoniazid (katG) of *M. tuberculosis* resistance gene by Conventional PCR

Name	Sequences (5'→3')	Product size(bp)	Annealing temperature(⁰C)	Melting temperature(⁰C)
rpoB-FTR8	GTGCACGTCGCGGACCTCCA	158	55	68.4
rpoB-RTR9	TCGCCGCGATCAAGGAGT	158	55	61.0
katG-FTB86	GAAACAGCGGCGCTGATCGT	209	60	64.3
katG-RTB87	GTTGTCCCATTTCGTCGGGG	209	60	62.8

were found to be culture positive & 5 (9.09%) samples were found to be culture negative. Conventional PCR was carried out using the primers targeting IS6110 sequence for detection of *Mycobacterium tuberculosis* (Table 1), 52 (94.55%) samples were found to be positive and 3 (5.45%) samples negative by PCR.

Drug susceptibility testing (DST) revealed 35 (70%) samples out of the total 50 culture positive samples were susceptible while 15 (30%) samples were resistant to both Isoniazid and Rifampicin. A total of 17 (34%) samples were found to be mono-resistant while 33 (66%) samples were found to be susceptible to isoniazid. Likewise, 18 (36%) samples were found to be mono-resistant and 32 (64%) samples were found to be susceptible to rifampicin phenotypically by DST. The results were considered as 'true results' when comparing with molecular methods, as DST is considered the gold standard for determination of drug resistance (Table 3).

Amplification of specific gene segments for detection of rifampicin and isoniazid resistance of MTB was done using suitable primers targeting the 158-bp fragment of *rpoB* gene spanning codons 526 and 531 and the 208-bp fragment of KatG gene spanning codon 315 of MTB (Table 2). Direct sequencing of PCR products of selected samples and standard strain was performed with a 96-capillary 3730xl DNA Analyzer on a third party commercial basis with M/s Ocimum Biosciences Ltd. PCR products of selected 15 samples of either type i.e., resistant and susceptible by both techniques (DST & Conventional PCR) were sent for sequencing.

Table 3. Drug Susceptibility Testing (DST) Result for Isoniazid and Rifampicin Drug

Drug Susceptibility profile	No of samples	Percentage
Susceptible to Isoniazid	33	66%
Resistant to Isoniazid	17	34%
Susceptible to Rifampicin	32	64%
Resistant to Rifampicin	18	36%
Susceptible to both drug	35	70%
Resistance to both drug(MDR)	15	30%

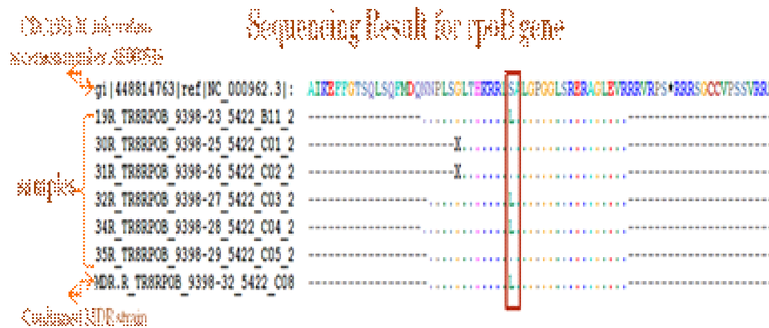

Fig. 1. *M. tuberculosis* katG gene amino acid sequence showing single nucleotide polymorphism

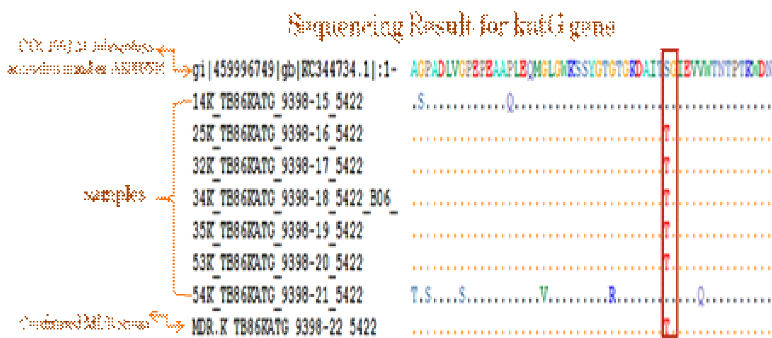

Fig. 2. *M. tuberculosis* rpoB gene amino acid sequence showing single nucleotide polymorphism

Of these, seven samples failed during sequencing due to low template yield. The sequences for remaining eight samples were aligned with standard reference strain CDC1551 of *M. tuberculosis* (Gene Bank accession number AE000516) to detect point mutations depicting the AGC/ACC mutation in the katG gene and TCG/TTG, CAC/TAC or GAC/GTC mutation in the *rpoB* gene (Figure 1 & 2). It was found that six out of eight samples harbored the AGC/ACC mutation for the *katG* gene. This mutation exhibits a S315T substitution on the respective translated proteins. The remaining 2 strains were found to be drug sensitive. Five out of the eight samples showed the TCG/TTG mutation for the *rpoB* gene, the remaining three were found to be drug sensitive. This mutation exhibits a S531L substitution on the respective translated proteins.

DISCUSSION

Anti-tuberculosis drug resistance poses a significant threat to human health, which usually develops due to the alteration of drug targets by mutations in *M. tuberculosis* chromosomal genes[10,18]. A large number of mutations in several genes that confer resistance to *M. tuberculosis* have been reported from different countries. However no study until now has managed to reveal the range of mutation in clinical samples from India, one of the countries with the highest TB prevalence. Hence, in the present study, we have attempted to identify the molecular basis of the drug resistance of *M. tuberculosis* in India specifically in and around Mumbai.

Multi-Drug Resistant Tuberculosis (MDR-TB) is based on the culture and Drug Sensitivity Testing (DST) for confirmation which is considered as the 'gold standard' test for identification of rifampicin and isoniazid resistance. But these phenotypic techniques require 6–8 weeks for a definitive diagnosis. Rifampicin, the most potent anti-tubercular drug, is bactericidal to the *M. tuberculosis* as it inhibits mycobacterial DNA-dependent RNA polymerase by binding with its b-subunit which is coded by the *rpoB* gene. Mutations in *rpoB* gene indicate resistance to rifampicin and are often associated with resistance to other drugs also, most notably isoniazid.

Rifampicin resistance as reported in previous studies is due to mutation in the Rifampicin Resistance Determining Region (RRDR) of *rpoB* gene. The most frequent mutations reported in RRDR of *rpoB* gene include codon 531 followed by codon 526 and codon 516[2,19]. In the present study, we have sequenced RRDR of *rpoB* gene in rifampicin resistant/sensitive samples (DST proven) and observed that codon 531 (TCG/TTG) is the most frequent site of mutation leading to rifampicin resistance. Five out of eight samples harbored the TCG/TTG mutation for the *rpoB* gene. This mutation exhibits a S531L substitution on the respective translated proteins.

Bostanabad *et al*.[20] in 2008 demonstrated mutations in the 'hot spot' region (RRDR) of *rpoB* gene leading to rifampicin resistance with predominant nucleotide changes in codon 510, 523, 526 and 531. Study by Deepa *et al*.[21] in 2005 reported dominant presence of mutations at codon 531 (TCG/TTG). Meera *et al*.[22] in 2003 and Mani *et al*.[23] in 2001 studied strains of rifampicin resistant *M. tuberculosis* by Line Probe Assay (LiPA) have reported codon 531 (TCG/TTG)and codon 526 (CAC/TAC) to be the most frequent site of mutation in RRDR of *rpoB* gene. Mechanisms such as a permeability barrier or membrane proteins acting as drug efflux pumps may also confer resistance to rifampicin. In our study, we characterized only the RRDR of *rpoB*. A second formal possibility to account for the inability of detecting *rpoB* mutations in these rifampicin- resistant cases is that changes might have occurred in one or more genes whose products participate in antibiotic permeability or metabolism[24] Bostanabad *et al.* (2008)[20] in their study reported that 5 rifampicin resistant isolates did not show any mutations in RRDR of *rpoB* gene. Mani *et al*.[23] in 2001 from Tuberculosis Research Centre (TRC), Chennai reported that 13.6% of mutations occur outside RRDR of *rpoB* gene.

The frequency of mutation in *kat*G gene is 100 fold higher than *rpoB* gene[25] and is regarded as first step in evolution of MDR-MTB. Also it creates more possibility for acquiring mutations in other genes[26]. The combination of INH resistance and maintained virulence might make it possible for some INH resistant strains, especially those with the *kat*G Ser315Thr mutation, to acquire extra drug resistance and become MDR-TB[27]. The molecular basis of resistance to isoniazid is more

complex and is caused by a variety of mutations in four different genes of MTB *i.e. kat*G encoding catalase peroxidase, *inhA* encoding the enoyl acyl carrier protein (ACP) reductase, *kas*A encoding â-ketoacyl ACP synthase and *ahp*C encoding alkyl-hydroperoxide reductase. Even then, nearly 5-10% of isoniazid-resistant *M. tuberculosis* isolates do not have an identifiable mutation[28]. This study was conducted to gain further insight into molecular basis of INHr MTB circulating in India specifically in and around Mumbai. We found that mutation in *kat*G gene (Ser315Thr substitution) was responsible for six of INH resistant strains. Out of the eight samples sequenced, two samples were found to be sensitive to isoniazid. We compared our result of mutation at *kat*G codon 315 associated with INH resistance and results of demographic findings with those of earlier studies done worldwide.

Several studies have revealed that mutation in *kat*G gene is responsible for 60-70% of INH resistant strains. A study by Marahatta S.S *et al.*[25] in 2006, India revealed *kat*G S315T mutation in 74.19% of strains of MTB from Delhi. In Nepal where incidence of all forms of TB is 173 cases per 1, 00,000 population[29] the Ser315Thr mutation found was not as high as those of north-western Russia (93.6%)[30] and Germany (86.41%). Similarly, Wang Y.C *et al.*[30] from China reported 68.6% of INHr strains associated with mutation in *kat*G due to S315T substitution. Our finding of mutation in the *kat*G Ser315Thr substitution (*i.e.* 75%) is similar to those reported in other parts of the world, which reflects a global pattern. Since all the INHr isolates were resistant to RIF as well so we concluded that all the strains under study are MDR-MTB isolates. Thus, chances that Mumbai may carry extensive transmission rate of MDR-TB with *rpoB* and *katG* gene mutation cannot be ignored.

The advantage of DNA sequencing method opted in the current study is the time between sampling and availability of the test results for clinical decision making. In general, Conventional methods of culture and DST take 6-8 weeks for definitive diagnosis. Consequently, clinical decisions cannot be taken until then. In comparison, the results of rifampicin and isoniazid susceptibility of *M .tuberculosis* based on genetic alteration in the RRDR of *rpoB* and *katG* gene are available within 3–5 days. This period is based on 1–2 days for DNA extraction from clinical sample followed by PCR and 2–3 days for amplification and sequencing of gene.

CONCLUSION

To conclude, early detection of MDR-pulmonary TB cases is of immense clinical importance. DNA sequencing is rapid, conclusive and more advantageous over conventional drug susceptibility testing (DST). It determines the exact site and frequency of mutation of *rpoB* and *katG* gene which lead to rifampicin and isonizid resistance in different geographical areas. This DNA sequencing serves two fold functions. First, it provides an accurate and rapid prediction of rifampicin and isoniazid resistant *M. tuberculosis* which is clinically useful. Secondly it can serve as useful data in developing a screening protocol for detection of MDR-TB in future. Early detection of the resistant strains will facilitate the modification of treatment regimen for patients and appropriate infection control measures can be taken in time to reduce the risk of further development and transmission of MDR-TB. An understanding at the molecular level of the mechanism of drug resistance in MTB will enable us to develop improved tools. It deserves further investigation to determine which mechanism may play the critical role in the epidemic of MDR-TB, since the implication behind it could be meaningful to evaluate the performance of local TB control as well as to determine the MDR-TB strategies.

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REFERENCES

1. Organization WH, Global tuberculosis report 2013; World Health Organization; 2013.
2. Nathanson, E., Nunn, P., Uplekar, M., Floyd, K., Jaramillo, E., Lönnroth, K., *et al*. MDR tuberculosis critical steps for prevention and control. New. England. *Journal. of. Medicine.,* 2010; **363**(11):1050-8.
3. Poudel., A., Nakajima, C., Fukushima, Y., Suzuki, H., Pandey, B.D., Maharjan, B., *et al.*

Molecular characterization of multidrug-resistant *Mycobacterium tuberculosis* isolated in Nepal. *Antimicrobial. agents and chemotherapy.,* 2012; **56**(6):2831-6.

4. Bártfai, Z., Somoskövi, Á., Ködmön, C., Szabó, N., Puskás, E., Kosztolányi, L., *et al*. Molecular characterization of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Hungary by DNA sequencing and the line probe assay. *Journal. of. Clinical. Microbiology.,* 2001; **39**(10):3736-9.
5. Veluchamy, M., Madhavan, R., Narayanan, S., Rajesh, L. KatG Gene as a Surrogate Molecular Marker Leading to Cause Drug Resistance in *Mycobacterium tuberculosis* Isolates. *Nature.,* 2013; **1**(5):86-91.
6. Rodrigues, C., Shenai, S., Sadani, M., Thakkar, P., Sodha, A., Udwadia, Z.F., *et al*. Multidrug-resistant tuberculosis in Mumbai: it's only getting worse. *Int. J. Tuberc. Lung. Dis.,* 2006; **10**(12):1421-2.
7. Therese, K., Gayathri, R., Madhavan, H. Molecular biological techniques for detection of multidrug resistant tuberculosis (MDR) and extremely drug resistant tuberculosis (XDR) in clinical isolates of *Mycobacterium tuberculosis*. Understanding tuberculosis global experiences and innovative approaches to the diagnosis. In. Tech. Rijeka. *Croatia.,* 2012; 455-70.
8. Hillemann, D., Weizenegger, M., Kubica, T., Richter, E., Niemann, S. Use of the genotype MTBDR assay for rapid detection of rifampin and isoniazid resistance in *Mycobacterium tuberculosis* complex isolates. *Journal. of. Clinical. microbiology.,* 2005; **43**(8):3699-703.
9. Ramaswamy, S., Musser, J. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis* 1998 update. Tubercle. *Lung. disease.,* 1998; **79**(1):3-29.
10. Sajduda, A., Brzostek, A., Pop³awska, M., Augustynowicz-Kopeæ, E., Zwolska, Z., Niemann, S., *et al*. Molecular characterization of rifampin-and isoniazid-resistant *Mycobacterium tuberculosis* strains isolated in Poland. *Journal. of. Clinical. microbiology.,* 2004; **42**(6):2425-31.
11. Telenti, A., Imboden, P., Marchesi, F., Matter, L., Schopfer, K., Bodmer, T., *et al*. Detection of rifampicin- resistance mutations in *Mycobacterium tuberculosis*. *The. Lancet.,* 1993; **341**(8846):647-51.
12. Hazbón, M.H., Brimacombe, M., del Valle, M.B., Cavatore, M., Guerrero, M.I., Varma-Basil, M., *et al*. Population genetics study of isoniazid resistance mutations and evolution of multidrug-resistant *Mycobacterium tuberculosis*. *Antimicrobial. agents and chemotherapy.,* 2006; **50**(8):2640-9.
13. Kiepiela, P., Bishop, K., Smith, A., Roux, L., York, D., Genomic mutations in the katG , inhA and aphC genes are useful for the prediction of isoniazid resistance in *Mycobacterium tuberculosis* isolates from Kwazulu Natal, South Africa. *Tubercle. Lung. Disease.,* 2000; **80**(1):47-56.
14. Palomino, J.C., Leão, S.C., Ritacco, V. Tuberculosis 2007 from basic science to patient care ., 2007.
15. QIAamp D mini kit and QIAamp DNA blood mini kit handbook. US Patent., 2001;4.
16. Kim, M. H., Yang, H. Y., Suh, J.T., Lee, H.J. Comparison of in-house PCR with conventional techniques and Cobas Amplicor M. tuberculosis™ kit for detection of *Mycobacterium tuberculosis*. *Yonsei. Medical. journal.,* 2008; **49**(4):537-44.
17. Torres, M.J., Criado, A., Palomares, J.C., Aznar, J. Use of real-time PCR and fluorimetry for rapid detection of rifampin and isoniazid resistance-associated mutations in *Mycobacterium tuberculosis*. *Journal. of. Clinical. microbiology.,* 2000; **9**:3194-9.
18. Ramaswamy, S.V., Amin, A.G., Göksel, S., Stager, C.E., Dou, S.J., El Sahly, H., *et al*. Molecular Genetic Analysis of Nucleotide Polymorphisms Associated with Ethambutol Resistance in Human Isolates of *Mycobacterium tuberculosis*. *Antimicrobial. agents and chemotherapy.,* 2000; **44**(2):326-36.
19. Mahmoudi, A., Iseman, M.D. Pitfalls in the care of patients with tuberculosis: common errors and their association with the acquisition of drug resistance. *Jama.,* 1993; **270**(1):65-8.
20. Bostanabad, S.Z., Noghanian, M., Graviss, A.E., Bahrmand, A.R., Titov, P.L., Nojoumi, S.A. Multiple mutations in the rpoB gene of *Mycobacterium tuberculosis* isolates correlate with high level of resistance to rifampicin in patients with active pulmonary tuberculosis in Afghanistan border of Iran. *African. Journal. of. Microbiology. Research.,* 2008; **2**(4):95-102.
21. Deepa, P., Therese, K., Madhavan, H. Detection and characterization of mutations in rifampicin resistant *Mycobacterium tuberculosis* clinical isolates by DNA sequencing., 2005.
22. Katoch, V. Newer diagnostic techniques for tuberculosis. Indian. *Journal. of. Medical. Research.,* 2004; **120**:418-28.
23. Mani, C., Selvakumar, N., Narayanan, S., Narayanan, P. Mutations in the rpoB gene of

multidrug-resistant *Mycobacterium tuberculosis* clinical isolates from India. *Journal. of. Clinical. microbiology.,* 2001; **39**(8):2987-90.

24. Kapur, V., Li, L.L., Iordanescu, S., Hamrick, M.R., Wanger, A., Kreiswirth, B.N., *et al*. Characterization by automated DNA sequencing of mutations in the gene (rpoB) encoding the RNA polymerase beta subunit in rifampin-resistant *Mycobacterium tuberculosis* strains from New York City and Texas. *Journal. of. Clinical. microbiology.,* 1994; **32**(4):1095-8.
25. Marahatta, S., Gautam, S., Dhital, S., Pote, N., Jha, A., Mahoto, R., *et al*. katG (SER 315 THR) Gene Mutation in isoniazid resistant *Mycobacterium tuberculosis*. Kathmandu. University. *Medical. Journal.,* 2012; **9**(1):19-23.
26. Herrera, L., Jiménez, S., Valverde, A., Garcýµa-Aranda, M.A., Sáez-Nieto, J.A. Molecular analysis of rifampicin-resistant *Mycobacterium tuberculosis* isolated in Spain (1996–2001). Description of new mutations in the rpo B gene and review of the literature. *International. journal. of. Antimicrobial. agents.,* 2003; **21**(5):403-8.
27. Hu, Y., Hoffner, S., Jiang, W., Wang, W., Xu, B. Extensive transmission of isoniazid resistant M. tuberculosis and its association with increased multidrug-resistant TB in two rural counties of eastern China: a molecular epidemiological study. B.M.C. *infectious. diseases.,* 2010; **10**(1):43.
28. Santha, T., Nazareth, O., Krishnamurthy, M., Balasubramanian, R., Vijayan, V., Janardhanam, B., *et al*. Treatment of pulmonary tuberculosis with short course chemotherapy in south India—5-year follow up. *Tubercle.,* 1989; **70**(4):229-34.
29. Basnet, R., Hinderaker, S.G., Enarson, D., Malla, P., Mørkve, O. Delay in the diagnosis of tuberculosis in Nepal. B.M.C. Public. Health ., 2009; **9**(1):236.
30. Wang, Y.C., Zhu, R.Y., Xu, Y.Y., Zhao, M.Q., Liu, Y.H., Li, B., *et al*. Molecular characterization of drug resistant *Mycobacterium tuberculosis* isolates in Guangdong, *China. J. Infect. dis.,* 2009; **62**:270-4.