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Evaluation of Multiplex Polymerase Chain Reaction Assay for the Detection of *katG* (S315T) gene Mutation in *Mycobacterium tuberculosis* Isolates from Puducherry, South India

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To evaluate the Multiplex Allele Specific-Polymerase Chain Reaction (MAS-PCR) assay for the detection of *katG*S315T mutations in *Mycobacterium tuberculosis* (MTB) clinical isolates. Totally, 78 MTB clinical isolates were analyzed by conventional Lowenstein–Jensen (LJ), BACTEC MGIT-960™ liquid cultures, MAS-PCR, PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and DNA sequencing methods. Fifty one (65.4%) out of 78 isolates were drug resistant, and 27 isolates (34.6%) were drug susceptible. MAS-PCR assay identified *katG*315 mutations in 47 (60.2%) isolates, in which 41 (52.5%) isolates had mutation at codon S315T (AGC→ACC), 6 (7.7%) isolates had mutation at codon S315N (AGC→AAC), and none of the 27 susceptible isolates had mutation. Concordant result was obtained by PCR-RFLP and DNA sequencing method was used further to validate the results obtained by MAS-PCR and PCR-RFLP assays. Novel mutations were found at G305V (GGC→GTC) and Q295P (CAG→CCG) codons in combination with S315T codon in 5 (6.4%) and 4 (5.1%) drug resistant strains respectively. *katGS315T* was the frequently mutated codon of *katG gene* in *M. tuberculosis* isolates, which can be used as a marker of choice for direct detection of isoniazid resistance by MAS-PCR assay in regions where *katG*S315T codon mutation is predominant among drug resistant isolates.

Key words: *Mycobacterium tuberculosis*, Multi drug resistance, *katG,* multiplex-PCR, PCR-RFLP, Isoniazid resistance.

One-third of the world population carries an asymptomatic infection with *Mycobacterium tuberculosis*, (MTB) which results in 9.0 million people were infected with tuberculosis (TB) and 1.5 million deaths in 2013 [1,2]. Multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) are major public health problems, especially in developing countries[1]. Rapid diagnosis and appropriate chemotherapy become the first priority in controlling the growing epidemics of TB infection[1,2].

Isoniazid (INH), a mainstay of the first-line drug against TB and mutations in various genes namely, *katG, inhA*, *oxyR, kasA*, *aphC* and *ndh* are responsible for INH drug resistance[3]. Particularly, *katG* gene codes for catalase-peroxidase and *inhA* gene codes for enoyl-acyl carrier protein reductase enzymes are key factors in mycolic acid biosynthesis and presence of mutations in these

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genes show INH resistance (INH^R)[4-6]. Clinical isolates were identified with absence/complete deletion of *katG* gene has also exhibited INH^R phenotype[7]. Altogether, mutations occurring in *katG* and *inhA* genes account for up to 80% in resistance to INH drug[5,8]. Moreover, majority of mutations are associated with *katG* gene at codon 315 in 30–90% of INH^R isolates spans across the world[9,10].

Many conventional approaches were used to detect drug resistance in isolates but, they are mainly time depending. Hence, numerous genotypic assays have been developed in past decades to precisely target the mutated codon and to rapidly detect drug resistance among clinical isolates[5,6,11-13]. The genotypic methods described so far for the detection of mutations in *katG* gene at different codons which includes *katG315*. More specifically, the second base of the *katG* gene at 315 codon S315T (AGC→ACC) substitution is the target for MAS-PCR assay[11].

Hence, this study was attempted to evaluate the efficiency of MAS-PCR and PCR-RFLP techniques to rapidly detect *katG*315 mutations in MTB clinical isolates in this region.

MATERIALS AND METHODS

M. tuberculosis clinical isolates and susceptibility testing

Totally, 78 *M. tuberculosis* (MTB) isolates were isolated from hospitalised patients with newly diagnosed pulmonary TB infection and were admitted as advised by physician to the hospital in Government Hospital for Chest Diseases, Intermediate Reference Laboratory at Puducherry. Species identification was performed as described previously[2,14]. Conventional solid Lowenstein-Jensen (LJ) medium was used for the identification and culturing of isolates, and susceptibility testing was performed by the proportion method as followed previously[6,15].

A microbial suspension was prepared according to the McFarland turbidity standards (0.5, 1) and was diluted 1:10; then, 0.2 ml of the dilution was added to LJ medium slant with or without a drug. Drug resistance was considered as if bacterial growth was occurred at a concentration of 0.02µg/ml of INH and resistance proportion was calculated for each isolate[16,17]. The liquid method of drug susceptibility by BACTEC MGIT 960™ was performed as described previously by Balabanova *et al.*,[15]. Results of both solid and liquid culture systems were compared and final results were documented.

DNA extraction

Genomic DNA was isolated from all the clinical isolates by Phenol:Chloroform extraction method as described previously[18]. Isolated genomic DNA was stored at -20ºC for further PCR analysis.

PCR Amplification

PCR amplification of IS*6110* element in Mycobacterium genome was performed in an authorized thermal cycler (Eppendorf Gradient Cycler). The primers and PCR reaction conditions were used this assay is listed in table 1 (2,19). Totally 50 µl PCR mixture was used which consists, 10 pmol of each primer, 1.5 mM $MgCl_2$, 1U of Taq DNA polymerase (New England Biolabs), 200µM deoxynucleotide triphosphate. Amplified PCR product was run on a 2% agarose gel and bands were photographed using a Geldoc (Bio-Rad, Germany).

MAS-PCR Assay

MAS-PCR assay was performed by using two outer primers (Table 1)[11] to amplify a product of 435-bp (mutated allele) in the absence of the *katG*315 AGC codon, and one inner reverse primer, binds at *katG*315 AGC second base and amplifies a product of 292-bp, (wild-type allele) in an authorized thermal cycler (Eppendorf Gradient Cycler). Totally 30 µl PCR mixture was prepared which consists, 10 pmol of each primer, 1.5 mM $MgCl_2$, 1U of Taq DNA polymerase (New England Biolabs), 200µM deoxynucleotide triphosphate. The amplified fragments were electrophoresed in 2% agarose gels and photographed by Geldoc system (Bio-Rad, Germany).

PCR amplification for PCR-RFLP analysis

A primer pair, *katG*1F and 4R (Table 1), was used to amplify a 200-bp *katG* gene fragment, as described[11]. Totally 30 µl PCR mixture was carried out which consists, 10 pmol of each primer, 1.5 mM $MgCl_2$, 1U of Taq DNA polymerase (New England Biolabs), 200µM deoxynucleotide triphosphate. The amplified fragment was assessed by electrophoresis in a 2% agarose gel.

Restriction Enzyme digestion

In a PCR tube, 10 µl of the PCR amplified

Table 1. PCR primers and reaction conditions for amplifying and DNA sequencing of *M. tuberculosis* drug resistance genes

Gene	Primer (5'–3')	PCR product (bp)	PCR reaction condition	Reference
IS*6110*[a]	GTGAGGGCATCGAGGTGG CGTAGGCGTCGGTCACAAA	123	94°C 5min, 35x (94°C 1min, 57°C 1min, 74°C 1min) 74°C 10min	Helen et al. 2003
*mtp*40 gene[a]	GGTTCCCAACACCACGTTAG CCAACATCGACGCAGTACC	332	95°C 5min, 35x (94°C 1min, 60°C 1min, 72°C 1min), 72°C 5min	Alexis et al. 1996
katG[b]	GAAACAGCGGCGCTGATCGT GTTGTCCCATTTCGTCGGGG	206	94°C 5min, 30x (94°C 1min, 57°C 1min 74°C 1min) 74°C 10min	Somoskovi et al. 2006
*katG*1F[c] *katG*4R[c]	AGCTCGTATGGCACCGGAAC AACGGGTCCGGGATGGTG	200	96°C 3min, 20x (94°C 1min, 60°C 1min, 72°C 1min) 72°C 5min	Mokrousov et al. 2002a
katG 0F[d] *katG* 5R[d] *katG* 1F[d] *katG* 4R[d]	ATACGACCTCGATGCCGC GCAGATGGGGCTGATCTACG AGCTCGTATGGCACCGGAAC AACGGGTCCGGGATGGTG	292 (or) 435	96ºC 3min, 5x (95ºC 1min, 64ºC 1min, 72ºC 1min), 5x (95ºC 1min, 62ºC 1min, 72ºC 1min) 20x (94ºC 1min, 60ºC 1min, 72ºC 1min), 72ºC 5min	Mokrousov et al. 2002a

a, Species identification
b *katG* amplified products for DNA sequencing
c PCR-RFLP for *katG* gene (132bp for the mutated (315 AGC→ACC) allele, the INH^r strain, 153bp for the *katG* 315 wild-type or differently mutated alleles by *MspI* digestion)
d MAS-PCR for *katG* gene (292-bp in the absence of mutation at *katG* 315 and 435-bp in the presence of mutation at *katG* 315)

Table 2. Prevalence of nucleotide and amino acid change among drug resistant isolates with *katG* mutations

Codon(s)	Change of Nucleotide(s)	Change of amino acid(s)	No. of isolates
315	AGC→ACC	Ser→Thr	33
315	AGC→AAC	Ser→Asn	5
305	GGC→GTC	Gln→Val	1
295	CAG→CCG	Gln→Pro	1
com. 315, 305	AGC→ACC GGC→GTC	Ser→Thr Gln→Val	4
com. 315, 295	AGC→ACC CAG→CCG	Ser→Thr Gln→Pro	2
com. 315, 275	AGC→ACC GCA→CCA	Ser→Thr Gln→Pro	1
com. 315, 295	AGC→AAC CAG→CCG	Ser→Asn Gln→Pro	1
com. 315, 305, 295	AGC→ACC GGC→GTC CAG→CCG	Ser→Thr Gln→Val Gln→Pro	1

com-combination

product, 2U *MspI* restriction endonuclease (New England Biolabs), 2µl of 10X buffer4™, 18µl of nuclease free water were added and incubated at 37ºC for 1h in an authorized thermal cycler as described[11]. The restriction fragments obtained were electrophoresed in a 2% agarose gel and documented.

DNA Sequencing

PCR reaction of *katG* gene was performed in all MTB isolates, spanning codon 315 (206-bp fragment) with the primers listed in Table 1[6,20] and subjected to direct DNA sequencing. Obtained sequences were analysed using the BLASTn and BLASx bioinformatics tools[21] available at the National Centre for Biotechnology Information (NCBI) to determine the specificity of PCR amplification and to identify the nucleotide and amino acid changes in comparison with wild type *M. tuberculosis* (H37Rv). Moreover, MAS-PCR and PCR-RFLP assay results were further confirmed with DNA sequencing data.

Results

Drug susceptibility

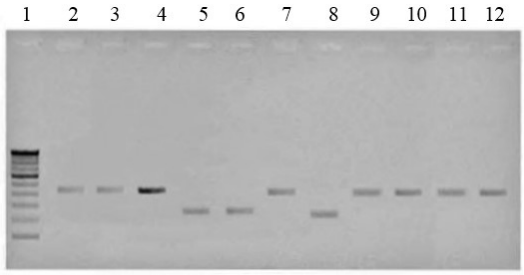
Totally, 27 (34.6%) isolates were susceptible and 51 (65.4%) isolates were isoniazid resistant. Out of 51 drug resistant, 17 (26%) were isoniazid mono-resistant and 34 (52%) were MDR-TB isolates. 36 out of 51 (70.5%) drug-resistant clinical isolates showed a high level of resistance against the INH drug (1µg-10µg/mL) had S315T mutations. However, strains having S315N and Q295P mutations were susceptible at 1µg/mL. Drug susceptible strains were used as controls in this study to avoid discrepancy of MAS-PCR and PCR-RFLP results.

MAS-PCR results

A 292-bp PCR amplified product was observed in drug susceptible isolates indicating the absence of mutation in the target *katG*315 codon (*katG*315AGC) (Fig. 1, Lanes 5,6 and 8). A 435-bp amplified product was observed in drug-resistant isolates with the presence of *katG*315 AGC→ACC (Fig. 1, Lanes 2–4, 7, 9 and 10) and 292-bp amplified fragment was observed in isolates harboured *katG*315 AGC→AAC codons (Fig. 1, Lanes 11 and 12).

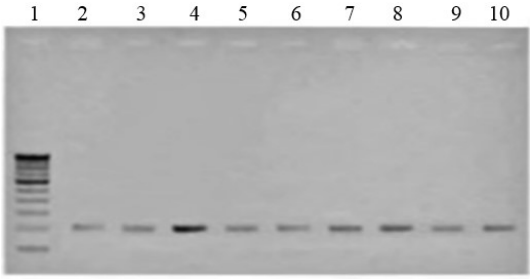
PCR-RFLP

PCR-RFLP reaction was performed and 200-bp amplified product was observed in all the strains (Fig. 2, Lane 2-7) Amplified PCR products were further digested by *MspI* enzyme. Totally, 41 (52.5%) isolates had ACC at codon 315 (S315T substitution) (Fig. 3, Lane 2, 3 and 5) and resulted in a 132-bp small fragment, INH^s isolates had wild type codon at 315 (AGC) produced band at 153-bp



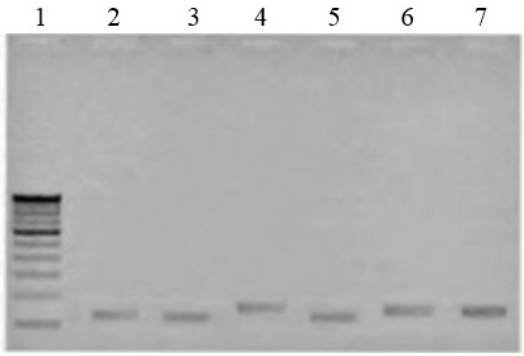
Lane 1: 100bp DNA ladder, Lane 2-4, 7 and 9-12, a 435-bp amplified product of strains having *katG315* AGC→ACC mutation; Lanes 5,6 and 8 a 292-bp amplified products of strains having *katG315* wild type allele.

Fig. 1. Examples of MAS-PCR amplification



Lane 1: 100-bp DNA ladder; Lane 2-10: 200-bp amplified product

Fig. 2. Examples of PCR-RFLP amplification



Lanes 1: 100-bp DNA ladder, Lanes 2,3 and 5: showed the 132-bp amplified products of strains harbouring *katGS315T* AGC→ACC mutation; Lane 4: 153-bp amplified product of strain harbouring *katG315* wild type allele; Lanes 6 and 7: a 153-bp amplified product of strains with *katGS315N* AGC→AAC mutation.

Fig. 3: Examples of Restriction digestion by *MspI* Enzyme

large fragment (Fig. 3, Lane 4) after enzyme digestion. Clinical isolates had S315N (AGC→AAC) and other mutations produced 153-bp large fragment (Fig. 3, Lane 6 and 7).

MAS-PCR amplification of either 292-bp or 435-bp fragment provided a PCR quality confirmation with results that were consistent with the results generated by PCR-RFLP analysis. This ruled out the eventual false-negative results obtained because of lack of amplification.

Sequencing results

A clear band at 206-bp region was observed in 2% agarose gel and confirmed the amplification of the *katG* gene. Sequencing results of the amplified *katG* fragment showed common mutations were displayed in Table 2. *katG* S315T (AGC→ACC) mutation alone was found majority in 33 strains (42.3%) followed by *katG* S315N (AGC→AAC) mutation alone was found in 5 strains (6.4%). One strain (1.3%) had G305V (GGC→GTC) mutation; one strain (1.3%) had Q295P (CAG→CCG) mutation. Combination of mutations were observed as follows, 4 strains (6.4%) had mutations at S315T and G305V codons, 2 strains (2.5%) had mutations at S315T and Q295P codons, one strain (1.3%) had mutations at S315T and Q275P codons, one strain (1.3%) had mutations at S315N and Q295P, and one strain (1.3%) had triple mutations at S315T, G305V and Q295P codons. Two INH^R strains (2.5%) showed resistance in LJ and BACTEC MGIT culture methods, but no mutations was identified by MAS-PCR, PCR-RFLP and DNA sequencing methods.

DISCUSSION

Molecular methods for the detection of drug resistance more quickly, could be of great value in aiding the timely administration of appropriate anti-TB drugs. One concern with the development of new tools is that they may utilize expensive technologies that could be beyond the reach of developing countries like India affected with dual TB/HIV epidemics, where a simpler PCR-based method might have greater applicability.

Therefore, this study was undertaken to gain further insight into the prompt and rapid detection of *katG*S315T mutation responsible for INH^R in clinical isolates by performing a simple MAS-PCR assay. Majority of the mutations were occurred at *katG* codon 315 (Thr'→Ser) of *M. tuberculosis*, than other codons[6,22] (Table 2). Three types of mutations were observed in *katG315* codon AGC→ACC, AAC and AGG which constituted 75% of all isolates[23] but in our case we found two of these (AGC→ACC and AGC→AAC) mutations. A high level of INH drug resistance was observed in strains, which is attributable to the presence of mutation at S315T codon of *katG* gene[17,23].

In this study, *katG*S315T mutation was found in totally 47 INH^R strains and *katG*315 wild-type allele (AGC) was identified in all INH^S strains. Of the 47 out of 78 isolates, 41 isolates had S315T, 6 had S315N, 1 had G305V, and one had G295P mutations. In two strains, mutation was not identified by any of these methods used in this study, but those strains showed INH drug resistance in LJ and BACTEC methods. Therefore, this result is supportive to the hypothesis that the presence of mutations in other gene namely, *inhA*, *aphC*, *oxyR* are also responsible for INH drug resistant among drug resistant isolates[9,17].

In accordance with previous reports, almost 92.1% (47 out of 51 MDR-TB) of the multidrug resistant strains had S315T substitution. Hence, the data suggests that, the presence of a similar mutation at codon 315 of the *katG* gene was found in drug-resistant MTB isolates in South India during 2011-2012[24]. Although a point mutation (AGC→ACC) resulting in 315 (Ser→Thr) substitutions is the most common, other mutations involving at codon 315 in the *katG* gene also occur in INH^R strains[25]. In this regard, *katG* gene harbouring S315N (AGC→AAC) mutation was found as less frequent event in this region. 34 (66.7%) of the 51 drug-resistant strains with the *katG315* substitution showed resistance to other first-line drugs such as rifampicin, ethambutol and streptomycin. In this respect it is then supporting the concept of strains already have the S315T mutation have more possibility of acquiring mutations in other genes too[16].

27 INH^S strains were produced a single MAS-PCR product at 292-bp band, as anticipated, implying that no change was found in *katG*315 second base. On contrary, MAS-PCR amplified product at 435-bp was amplified in 47 INH^R strains, that directly proportional to the presence/change in the AGC (AGC→ACC or AAC) codon at 315.

The result of this study was highly corroborated with recent Indian data[17]. The specificity of the proposed method was 92.1% (47 out of 51 isolates), since all the results agreed with the conventional drug susceptibility test and with the sequence analysis performed in the isolates under investigation. Strains harbouring *katG* S315N (AGC→AAC) mutation and other mutations such as, 305, 295 which produced discrepancy results with *MspI* digestion which, required further attention like digestion by *MspA1I* would be of interest.

CONCLUSIONS

In conclusion, the *katG*S315T mutation can be serve as a reliable marker for the detection of INH resistance in *M. tuberculosis* clinical isolates among drug-resistant strains. The MAS-PCR and PCR-RFLP assays that are described herein are rapid, making easy for the detection of resistance to INH in clinical isolates where this mutation is prevalent.

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Conflicts of interest

The authors declare that they have no conflict of interest.

REFERENCES

1. World Health Organization. Global tuberculosis report 2014. WHO, Geneva, (http://www.who.int/tb/publications/global_report/en/2014 .
2. Kathirvel, M., Kommoju, V., Brammacharry, U., Thirumurugan, T., Ravishankar, N., Radhakrishnan, B., Muthaiah, M. Clinical evaluation of *mtp40* polymerase chain reaction for the diagnosis of extra pulmonary tuberculosis. *World. J. Microbiol. Biotechnol*., 2014; **30**: 1485–1490.
3. Johnson, R., Streicher, E.M., Louw, G.E., Warren, R.M., vanHelden, P.D., Victor, T.C. Drug Resistance in *Mycobacterium tuberculosis. Curr. Issues Mol. Biol.*, 2009; **8**: 97–112.
4. Zhang, Y., Heym, B., Allen, B., Young, D., Cole, S. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature*, 1992; **358**: 591–3.
5. Musser, J.M., Kapur, V., Williams, D.L., Kreiswirth, B.N., van Soolingen, D., van Embden, J.D. Characterization of the catalase-peroxidase gene *katG* and *inhA* locus in isoniazid-resistant and susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing: restricted array of mutations associated with drug resistance. *J. Infect. Dis*., 1996; **173**: 196–202.
6. Ravibalan, T., Maruthai, K., Samrot, A.V., Muthaiah, M. Characterization of *katG* and *rpoB* gene mutations in Multi Drug Resistant *Mycobacterium tuberculosis* clinical isolates. *Int. J. Curr. Microbiol. Appl. Sci*., 2014; **3**: 1072–1080.
7. Heym, B., Alzari, P.M., Honore, N., Cole, S.T. Missense mutations in the catalase-peroxidase gene, *katG,* are associated with isoniazid resistance in *Mycobacterium tuberculosis*. *Mol. Microbiol*., 1995; **15**: 235–245.
8. Morris, S., Bai, G.H., Suffys, P., Portillo-Gomez, L., Fairchok, M., Rouse, D. Molecular mechanisms of multiple drug resistance in clinical isolates of *Mycobacterium tuberculosis. J. Infect. Dis*., 1995; **171**: 954–960.
9. Kiepiela, P., Bishop, K.S., Smith, A.N., Roux, L., York, D.F. Genomic mutations in the *katG*, *inhA* and *ahpC* genes are useful for the prediction of isoniazid resistance in *Mycobacterium tuberculosis* isolates from Kwazulu Natal, South Africa. *Tuber. Lung Dis*., 2000; **80**: 47–56.
10. Mokrousov, I., Narvskaya, O., Otten, T., Limeschenko, E., Steklova, L., Vyshnevskiy, B. High prevalence of KatG Ser315Thr substitution among isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates from Northwestern Russia, 1996 to 2001. *Antimicrob. Agents Chemother*., 2002; **46**: 1417–1424.
11. Mokrousov, I., Tatiana, O., Maxim, F., Anna, V., Eugeny, C., Elena, L., Lidia, S., Boris, V., Olga, N. Detection of isoniazid resistant *Mycobacterium tuberculosis* strains by a Multiplex Allele-Specific PCR assay targeting *katG* codon 315 variation. *J. Clin. Microbiol*., 2002a; **40**: 2509-2512.
12. Herrera, L.L., Molina, T., Saiz, P., Antonio, J., Jimenez, S.N., Jimenez, M.S. New multiplex PCR for rapid detection of isoniazid-resistant *Mycobacterium tuberculosis* clinical isolates. *Antimicrob. Agents. Chemother*., 2004; **49**: 144–7.
13. Yadav, R., Sethi, S., Mewara, A., Dhatwalia, S.K., Gupta, D., Sharma, M. Rapid detection of rifampicin, isoniazid and streptomycin

resistance in *Mycobacterium tuberculosis* clinical isolates by high-resolution melting curve analysis. *J. Appl. Microbial*., 2012; **113**: 856–862.

14. Alexis, W., Pilkaytis, B.B., Butler, W.R., Woodley, C.L., Shinnick, T.M. The *mtp40* Gene is not present in all strains of *Mycobacterium tuberculosis*. *J. Clin. Microbiol*., 1996; **34**: 2309–11.
15. Balabanova, Y., Drobniewski, F., Nikolayevskyy, V., Kruuner, A., Malomanova, N., Simak, T., Ilyina, N., Zakharova, S., Lebedeva, N., Alexander, H.L., O'Brien, R., Sohn, H., Shakhmistova, A., Fedori, I. An integrated approach to rapid diagnosis of tuberculosis and multidrug resistance using liquid culture and molecular methods in Russia. *PLoS ONE*., 2009; **4**: e7129.
16. van Soolingen, D., de Haas, P.E.W., van Doorn, H.R., Kuijper, E., Rinder, H., Borgdorff, M.W. Mutations at amino acid position 315 of the *katG* gene are associated with high-level resistance to isoniazid, other drug resistance, and successful transmission of *Mycobacterium tuberculosis* in the Netherlands. *J. Infect. Dis*., 2000; **182**: 1788–90.
17. Unissa, A.N., Selvakumar, N., Narayanan, S., Narayanan, P.R. Molecular analysis of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis* from India. *Int. J. Antimicrob. Agents*, 2008; **31**: 71–5.
18. Mani, C., Selvakumar, N., Kumar, V., Narayanan, S., Narayanan, P.R. Comparison of DNA sequencing, PCR-SSCP and PhaB assays with indirect sensitivity testing for detection of rifampicin resistance in *Mycobacterium tuberculosis*. *Int. J. Tuber. Lung. Dis*., 2003; **7**: 625–59.
19. Helen, F.A., Donoghue, H.D., Taylor, G.M., Adri, G.M., Van der Zanden, A.G.M., Spigelman, M. Molecular analysis of *Mycobacterium tuberculosis* DNA from a family of 18th century Hungarians. *Microbiol*., 2003; **149**: 143–151.
20. Somoskovi, A., Dormandy, J., Mitsani, D., Rivenburg, J., Salfinger, M. Use of Smear-Positive samples to assess the PCR-based genotype MTBDR assay for rapid, direct detection of the *Mycobacterium tuberculosis* complex as well as its resistance to isoniazid and rifampin. *J. Clin. Microbiol*., 2006; **44**: 4459–4463.
21. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *J. Nucleic. Acids Res*., 1997; **25**: 3389–3402.
22. Zaker, S.B., Titov, L.P., Bahrmand, A.R., Taghikhani, M. Genetic mechanism of *Mycobacterium tuberculosis* resistance to antituberculosis drugs, isoniazid and rifampicine. *J. Dakladi. Hational. Akademia. Sci. Belarus*, 2006; **50**: 92–8.
23. Zaker, S.B., Titov, L.P., Bahrmand, A.R. Frequency and molecular characterization of isoniazid resistance in *katG* region of MDR isolates from tuberculosis patients in southern endemic border of Iran. *Infect. Genet. Evol*., 2008; **8**: 15-19.
24. Deepa, D., Achanta, S., Jaju, J., Rao, K., Samyukta, R. et al. The impact of Isoniazid resistance on the treatment outcomes of smear positive re-treatment Tuberculosis patients in the state of Andhra Pradesh, India. *PLoS ONE*, 2013; **8**: 8e76189.
25. Haas, W.H., Schilke, K., Brand, J., Amthor, B., Weyer, K., Fourie, P.B. et al. Molecular analysis of *katG* gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. *Antimicrob. Agents Chemother*., 1997; **41**: 1601-3.