Pakistan J. Zool., vol. 45(1), pp. 93-100, 2013.

Molecular Characterization of Multidrug-Resistant Isolates of *Mycobacterium tuberculosis* from Patients in Punjab, Pakistan

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Abstract.- Globally, Pakistan with population of around 180 million moved up two spots to 6th in the list of countries with the highest number of tuberculosis (TB) patients in the world according to WHO report (2011). Global TB control is further challenged by drug-resistant TB. For controlling the spread and better understanding of *Mycobacterium tuberculosis* (*M. tb*) isolates circulating in this region, it is important to explore the characteristics of these strains. In this study 71 isolates were tested for drug sensitivity and 41 out of 71 were found to be resistant. Out of these 41 isolates, 21 (51%) were resistant to isoniazid (INH) and rifampicin (RIF) and remaining have different drug resistance patterns. Genes associated with drug resistance like *ahpC, katG* and *inhA* for INH, *rpoB* for RIF, *rrs* and *rpsL* for streptomycin (STR) and *embB* for ethambutol (EMB) resistant isolates were checked for mutation using PCR amplification and sequencing. Out of 27 RIF resistant strains, 22 have mutation affecting four amino acid codons of *rpo*B; 531 (52%), 516 (15%), 526 (7.0%) and 512 (7.0%). In codon 512 two isolates showed insertion of GCC. These insertions were novel and observed for the first time in Pakistani isolates. The overall sensitivity of mutation detection in *rpoB* gene was 88.8% and specificity was 100%. Out of 24 INH resistant isolates, 19 (79 %) had S315T mutation in *katG* while 5 isolates had no mutation in this region. Thus, the overall sensitivity of mutation detection in *katG* gene was 79 % and specificity was 100%. These 5 isolates were further checked for mutation in promoter region of *inhA* and *ahpC*. In 2 isolates there was G to A transition at -88 of the promoter region of *ahpC* while 3 isolates had C to T transition at point -15 for *inhA* promoter region. Out of 14 STR resistant isolates, 7 isolates showed the mutation K88M, K88R and K43R in the gene *rpsL* while no mutation was found in the gene *rrs* in STR resistant strains. Six (67%) out of 9 EMB resistant isolates showed the mutation M306I while no mutation was found in 3 isolates. The mutations observed in this study are similar to the mutations commonly present worldwide. However, our results have shown one novel mutation in *rpoB gene* that can be considered for improving molecular methods used for the detection of multidrug-resistant strains. Furthermore wide range of screening is needed to characterize drug resistant strains prevalent in this region.

Key words: *Mycobacterium tuberculosis*, multidrug resistant strains, tuberculosis, sequencing, *rpoB* gene, acid fast bacilli.

INTRODUCTION

Tuberculosis (TB) the most hazardous bacterial infection worldwide is caused by the *Mycobacterium tuberculosis (M. tb)* complex and affects millions of people. TB can be transmitted by an individual with active pulmonary disease through droplet aerosol. However, patients with positive smears for acid fast bacilli (AFB) have high risk to transmit the disease (Tostmann *et al.*, 2008). Therefore public health is threatened throughout the world by TB disproportionately, especially affecting low-income nations. People living in area where TB is endemic, or in contact with pulmonary TB patients and those with weak immune system are at highest risk of suffering from TB (Sia and Wieland, 2011). This is the reason that TB is the eighth leading cause of death in the world. Major causes of failure to effective solutions are human immunodeficiency virus (HIV) infection, epidemics of TB and delay in diagnosis and proper treatment

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0030-9923/2013/0001-0093 $ 8.00/0

(Lönnroth *et al.*, 2010) and also emergence of MDR and XDR strains of *M. tuberculosis*. In 2010, the estimated incidence of TB was about 8.0 million, deaths from TB among HIV-negative patients were 1.0 million while deaths from HIV-positive TB were about 0.40 million (WHO, 2011).

Pakistan, with population of around 180 million, moved up two spots to the 6th in the list of countries with the highest number of TB patients in the world. TB kills around 48,000 Pakistanis every year and according to WHO estimates of 2008, the number of MDR TB cases varies between 1.6-22.3% in high TB burden countries including Pakistan (WHO, 2011).

Primarily TB treatment relies on two important antibiotics isoniazid (INH) and rifampicin (RIF). These two antibiotics are the first line of defense against TB and are given in combination with ethambutol (EMB) and streptomycin (STR) (WHO, 2010). *M. tb* strains resistant to isoniazid (INH) and rifampin (RIF) are called multi drug resistant (MDR). Drug-resistant TB may arise due to the failure in implementing proper TB control programs and properly managing TB cases. As a result, more than 450,000 MDR-TB cases are estimated to occur worldwide each year, of which about 1%-2% takes place in South Africa (Calver *et al.*, 2010).

In the majority of drug-resistant *M. tb* isolates, drug resistance is due to mutations in genes or promoters region of genes activating the drug or encoding the drug targets (Ong *et al.*, 2010). *M. tb* isolates become resistant to RIF, INH, EMB and STR due to mutations in several genes and genomic regions. Studies have pointed out that the *M. tb* becomes resistant to RIF due to the mutations in *rpoB*, INH due to *katG* and *ahpC* (Ramaswamy and Musser., 1998), EMB due to *embB* (Telenti *et al*., 1997b) and *pncA* (Scorpio and Zhang, 1996).

Rapid detection of RIF resistance is of particular importance because it is one of the important surrogate markers for MDR resistance (Heep *et al.*, 2001). The main target of RIF is *rpoB* gene encoding the beta subunit of RNA polymerase. Studies have shown that about 98% of RIF resistant isolates have mutations in *rpoB* gene. More than 90% of the mutations have been found in 81 bp (codons 507 to 533) core region of RNA polymerase beta subunit (*rpoB)* gene (Mokrousov *et al.*, 2002).

Resistance to isoniazid can be conferred by mutations in multiple genes (Zhang *et al.*, 2005). In *M. tb* isolates mutation in *katG* gene encoding catalase-peroxidase enzyme is most frequently associated with INH resistance as demonstrated by several studies. INH, one of the important first line drugs, is in inactive form and catalytic conversion is required to convert it into its active form. This function is accomplished by an enzyme catalase-peroxidase in *M. tb* isolates (Dobner *et al.*, 1997). In addition promoter mutation of the gene *inhA* was shown to confer low level resistance to INH.

The aim of the present study was to analyze the mutations responsible for antibiotic resistance in the target genes, in *M.tb* isolated from Pakistan. It will help to find the extent of drug-resistant TB and to track the routes of infection in combination with the molecular epidemiology data of these drug resistant strains. These types of studies are designed to find out polymorphisms in the drug target genes, and mutations found in this study might be considered as molecular markers for drug resistance identification in *M. tb* isolates in Pakistan and other settings.

MATERIALS AND METHODS

Collection of M. tb. cultures

A total of 71 *M. tb.* cultures were collected from three different centers of Punjab. Both susceptible and drug resistant strains were collected from Pakistan Medical Research Council (PMRC), TB Research Center, Mayo Hospital Lahore, National Institute of Health (NIH), Islamabad and National Reference Laboratory, National TB Control Program, Islamabad. Lowenstein–Jensen media was used for isolation and culturing of *M. tb* isolates from sputum and other specimens. The *M. tb* isolates were then tested for drug susceptibility against rifampicin (RIF), isoniazid (INH), ethambutol (EMB) and streptomycin (STR) on Lowenstein–Jensen media.

Sensitivities to the first-line drugs RIF, INH, STR and EMB of the *M. tb* isolates obtained from National Reference Laboratory (NRL) Islamabad, National Institute of Health (NIH), Islamabad and Pakistan Medical Research Council (PMRC),

Lahore were checked on Lowenstein Jensen medium by Proportion Method.

Extraction of DNA from mycobacterial culture

DNA was extracted from mycobacterial colonies by method described by Somerville *et al*. (2005). A loop full of colonies was collected and transferred to 400 µl Tris-EDTA buffer and heated for 20 min at 80°C to kill the mycobacterium. Then lysozyme (1 mg/ml) was added to each tube, and incubated for 2 hours at 37°C. Then proteinase K (0.2 mg/ml) and 10% SDS (1.1%) was added and tubes were vortexed and incubated at 65°C for 20 min. During this step a mixture of N-acetyl-N, N, N-trimethyl ammonium bromide (CTAB: final concentration, 40 mM) and NaCl (final concentration, 0.1 M) was added, followed immediately by the addition of NaCl alone (final concentration, 0.6 M). The tube were then vortexed until the suspension turned milky, and were incubated at 65°C for 10 min. A mixture (750 µl) of chloroform-isoamylalcohol (24:1) was then added, again vortexed and then centrifuged at 13,000 rpm in a microcentrifuge for 5 min at room temperature. The genomic DNA was then precipitated with 70% ethanol and resuspended in 30 µl TE buffer. DNA concentration was determined by spectrophotometer at optical density of 260 nm and 280 nm. The ratio of 260/280 nm was used to determine the quality of DNA. Most of DNA samples gave ratio between 1.5 and 2.0. The samples with lower ratio were retreated with chloform-isoamylalcohol to remove proteins.

Drug resistant genes analysis

For analysis of mutations in drug resistant genes PCR amplification of target genes and sequencing was done. The primers sequences along with other details used for different genes are listed in Table I.

PCR amplification of target genes

The target genes were amplified by preparing 25 µL PCR reaction mixture containing 2.5 µl of 10X PCR buffer, 0.5 µl of 10 mM d-NTPs, 0.75 µl of 25 mM magnesium chloride solution, 1.25 µl each of the 10 µM primers, 0.125 µl of 2 U AmpliTag Gold polymerase (Perkin Elmer, USA), 1.25 µl of DMSO, 2 µl of genomic DNA, and 15.3 µl of double distilled water. Amplification was done by using protocol of 3 min at 95°C for initial denaturation; 40 cycles each of denaturation at 95°C for 1 min, 1 min 55°C, 30 sec 72°C; and then final extension at 72°C for 5 min. The PCR amplified products were confirmed on 1.5 % agarose gel.

EXOSAP clean up of PCR products for sequencing

Five µl PCR products were mixed with 1µl exonuclease and 1µl alkaline phosphatase. The products were then placed in thermal cycler with hot lid off. The cycling parameters were for 30 min at 37°C and 15 min at 80°C.

DNA sequencing analysis

The purified products were sequenced with forward and reverse primers by using Bigdye-terminator kit and ABI Prism *3130xl* genetic analyzers (Perkin-Elmer, USA). The SeqScape version 2.6 (Applied Biosystems) was used for DNA sequence comparisons and mutations were detected in respective gene by comparing with the reference *Mycobacterium tuberculosis* strain H37Rv sequence.

RESULTS

Resistance patterns of first-line drugs

Out of 41 drug resistant isolates 26 isolates (63%) were at least resistant to RIF and INH, 6 (14%) were only resistant to RIF, 3 isolates (7%) to INH and the remaining had different resistance patterns (Table II).

Analysis of rpoB gene for mutations in rifampicin resistant isolates

The 157 bp region including the 81bp hyper-variable RIF resistance detection region (RRDR) of *rpoB* gene was amplified using TR8 and TR9 primers as described by Telenti *et al.* (1997a). Out of 27 RIF resistant strains, 22 have mutation affecting four amino acid codons of *rpoB*; 531 (52%), 516 (15%), 526 (7.0%) and 512 (7.0%). In codon 512 two isolates showed insertion of GCC. These insertions were novel and observed for the first time in Pakistani isolates. Five isolates having no mutation in RRDR were checked for mutation

Table I.- Primers used for various drug resistant genes amplification.

Genes	Primers	Sequences 5'→3'	Product size (bp)	Reference
rpoB	TR8	TGCACGTCGCGGACCTCCA	157	Talenti *et al*. (1997a)
	TR9	TCGCCGCGATCAAGGAGT		
rpoB	TB-176	CTTCTCCGGGTCGATGTCGTTG	365	Heep *et al.* (2001)
	TB-176	CGCGCTTGTCGACGTCAAACTC		
rpoB	TBB-1	ATCACACCGCAGACGTTG	749	Heep *et al.* (2001)
	TBB-2	TGCATCACAGTGATGTAGTCG		
katG	TB86,	AAACAGCGGCGCTGGATCGT	209	Talenti *et al*. (1997a)
	TB87	GTTGTCCCATTTCGTCGGGG		
inhA	TB92	CCTCGCTGCCCAGAAAGGGA	248	Talenti *et al*. (1997a)
	TB93	ATCCCCCGGTTTCCTCCGGT		
ahpC	TB90	CGATGAGAGCGGTGAGCTG	236	Talenti *et al*. (1997a)
	TB91	ACCACTGCTTTGCCGCCACC		
rrs	(+1202)	CTTATGTCCAGGGCTTCA	363	Self designed
	(+1565)	CAGTTGGGGCGTTTTC		
rpsL	(-203)	CGGCGGGTATTGTGGTTGCTCGTG	800	Self designed
	(597)	CCTCCAGGGCGGGTTTGACATTG		
embB	(725)	TGGACGGGCGGGGCTCAAT	333	Self designed
	(1058)	GGCAGGCGCATCCACAGACT		

Table II.- Drug resistance profiles of drug resistant *M. tb* isolates.

Total no. of isolates	*Phenotype of resistance to drugs*			
	INH	RIF	STR	EMB
4	R	R	R	R
4	R	R	R	
13	R	R		
3	R			
6		R		
6		R	R	
5	R	R		R

INH, Isoniazid; RIF, Rafampicin; STR, Streptomycin; EMB, Ethambutol.

outside of this region as described by Heep *et al.* (2001). The 365 bp region for V176F mutation and 749 bp region containing upstream region of RRDR were amplified and sequenced for mutation. No mutation of V176F type was found in any isolate but mutations in 2 isolates outside of RRDR region were found. The detail is given in Table 3. Thirty susceptible isolates were also checked for mutation in these regions to validate our results. No mutations were detected in 30 susceptible strains. Therefore the overall sensitivity of mutation analysis in *rpoB* gene was 88.8% and specificity was 100%.

Mutations detection in INH resistant isolates

In order to find the relative frequency of mutations in INH resistant isolates, three most commonly effected genes like *katG*, promoter region of *ahpC* and *inhA* were amplified and sequenced by using primers as described by Telenti *et al.* (1997a). Out of 24 INH resistant isolates, 19 (79 %) isolates had S315T mutation in *kat*G while 5 isolates had no mutation in this region. These 5 isolates were checked for mutation in promoter region of *inhA* and *ahpC*. In 2 isolates there was G to A transition at -88 of the promoter region of *ahpC* while 3 isolates had C to T transition at point -15 for *inhA* promoter region. The results are shown in Table III. Thirty susceptible strains were also sequenced for *katG* gene mutation to validate our results and no mutation was found in these isolates. Thus, the overall sensitivity of mutation detection in *katG* gene was 79 % and specificity was 100%.

Detection of mutations in EMB resistant isolates

In this study a total of 9 EMB resistant isolates were checked for mutation in *embB* gene at codon 306. Six (67%) out of 9 EMB resistant showed M306I mutation while no mutation was found in 3 isolates (Table III).

Table III.- Resistance conferring mutations to different drug resistant *M. tb* isolates.

Gene	Codon/nucleotide change	Amino acid change	No. of isolates
rpoB	TCG→TTG	S531L	12
	TCG→TGG	S531W	2
	GAC→TAC	D516Y	2
	GAC→GTC	D516V	1
	GAC→TTC	D516F	1
	CAC→TAC	H526Y	2
	ins512 +GCC	512	2
	ATC→TTC	I572F	1
	AAC→AAT	N582N	1
katG	AGC→ACC	S315T	19
	No mutation		5*
ahpC	G→A	-88	2
inhA	C→T	-15	3
embB	ATG→ATA	M306I	6
rpsL	AAG→ATG	K88M	1
	AAG→AGG	K88R	2
	AAG→AGG	K43R	4

* These 5 Isolates were further checked for *ahpC* and *inhA* mutations. Out of 5 isolates 2 have mutation in *ahpC* gene and 3 have mutation in *inhA* gene.

Detection of mutations in STR resistant isolates

The genes *rrs* and *rpsL* have been identified to be associated with mutations in STR resistant isolates. Therefore, 14 STR resistant isolates were analyzed for mutation in *rpsL* and *rrs* gene. Out of 14 STR resistant isolates, 7 (50 %) isolates showed mutation at codon K88M, K88R and K43R in *rpsL* gene as shown in Table III. No mutation was found in *rrs* gene in STR resistant isolates.

DISCUSSION

The genetic basis of antibiotic resistance in drug resistant *M. tb* isolates has been widely studied and is generally believed to be caused by point mutations in some important resistance genes like *katG, rpoB, embB* and *rpsL* etc (Ramaswamy and Musser, 1998). Since MDR is big threat to human healthcare therefore we designed this study to check various drug resistant isolates for mutations in these loci in Pakistani isolates.

All three regions including the V176F codon, RRDR and upstream region of RRDR of *rpoB* gene in RIF resistant isolates were checked. Out of 27 RIF resistant strains, 22 (81%) have mutation in RRDR affecting four amino acid codons of *rpoB*; 531 (52%), 516 (15%), 526 (7.0%) and 512 (7.0%) while in codon 512 two isolates showed insertion of GCC. This insertion is novel and observed for the first time only in Pakistani isolates which may be considered as an addition to series of known mutations. The findings of this study are quite similar with respect to the mutations in the RRDR of *rpoB* gene and those reported from other parts of the world (Taniguchi *et al.*, 1996; Sun *et al.*, 2008). Mutations at codons 531, 516 and 526 of the *rpoB* gene in RIF resistant isolates are also consistent with the data from Pakistan and India (Ali *et al.*, 2009; Ajbani *et al.*, 2011). Nineteen percent of isolates in this study did not show any mutation in RRDR and this result differs from the data showing that only 4% of the RIF resistant isolates lack RRDR changes (Yue *et al.*, 2003). This difference may be due to the difference in genotypes prevailing in different parts of the world. However in a study carried out by Heep *et al.* (2000) revealed that a mutation associated with RIF resistance can also be located outside the RRDR, although this does not occur frequently. Therefore those isolates having no mutation in RRDR region were also checked for mutation in V176 codon and upstream region of RRDR of *rpoB* gene. Mutations in codons 572 and 582 upstream region of RRDR were found in 2 isolates which is similar to those mutations found by Heep *et al.* (2001). Therefore, overall three (11%) out of twenty seven RIF resistant isolates did not show any mutation in any of the three regions studied. Absence of known mutations in these resistant strains might be an indication to the presence of other rare rpoB mutations heteroresistance (a mixed infection of susceptible and resistant subpopulations), or less likely another mechanism of resistance to rifampicin (Bártfai *et al.*, 2001; Heep *et al.*, 2001). In this study mutations were found in 24 isolates out of 27 RIF resistant isolates and this study supports the hypothesis of Hasnain *et al.* (1998) which considered MDR TB rifampin resistance as an excellent surrogate marker.

Similarly all INH resistant isolates were checked for mutations not only in *katG* gene, but also in regulatory region of *inhA* and *ahp*C. Frequency of *katG* mutation among INH resistant *M. tb* isolates was 79% which is comparable with

the recent study carried out by Campbell *et al.* (2011) having frequency rate of 80.5% whereas the frequency of *katG* S315T mutation in this study was higher than that previously reported rate for patients diagnosed in Netherlands and Kuwait with 55% and 65% respectively. On the other hand the frequency was lower when compared to Russia *i.e.*, 95% (Ahmad and Mokaddas, 2004; Van Doorn *et al.*, 2006; Lipin *et al.*, 2007). The different frequencies of S315T mutation in *katG* may be due to different geographical distribution of these isolates. Five isolates having no mutation in S315T in *katG* were checked for mutation in promoter region of *inhA* and *ahpC*. In 2 isolates there was G to A transition at -88 of the promoter region of *ahpC* and frequency of *ahp*C mutations was 8%, which fall in the range of mutation frequencies *i.e.*, 4.8-24.2% described in various other studies (Kelley *et al.*, 1997; Lee *et al.*, 2001; Cardoso *et al.*, 2004). Similarly 3 isolates had C to T transition at point -15 for *inhA* promoter region reported to be associated with INH resistance as described by Lavender *et al.* (2005) and mutation frequency of 12% in *inhA* is also comparable with other studies showing frequencies varying from 10% to 34.0% (Kiepiela *et al.*, 2000; Kim *et al.*, 2003).

Six (67%) out of 9 EMB resistant isolates checked for mutation in *embB* gene at codon 306 showed M306I mutation while no mutation was found in 3 isolates in this codon. Studies carried out by Sreevatsan *et al.* (1997) and Telenti *et al.* (1997b) showed that most of the mutations in EMB resistant *M. tb* isolates are present in *emb*B codons 306, 497 and 406 and *ini*A codon 501. However, the most prevalent mutations are in *emb*B codon 306, accounting for more than 50% of EMB-resistant strains in some studies. Although, our results are quite consistent with these studies, but three isolates showing no mutations in this study may have mutation at either *mb*B406 or *emb*B497 as confirmed by recent study of Shi *et al.* (2011), who have shown importance of mutations at *emb*B497 and *emb*B406 as hotspots in addition to *emb*B306 for evaluating ethambutol resistance.

Out of 14 STR resistant isolates, 7 (50%) isolates showed mutations at codon K88M, K88R and K43R in *rpsL* gene. In STR resistant isolates mutations in codons 43 and 88 of the *rpsL* gene are considered as major cause of resistance development. The results of this study are consistent with one of the previous studies conducted by Sreevatsan *et al.* (1996) in which it was pointed out that substitution of amino acid 43 or 88 of ribosomal protein S12 is common in STR resistant *M. tb* isolates. These mutations were identified in isolates from widespread geographic localities. For example, 4 out of 8 resistant isolates from Japan had *rpsL* changes, including codon 43 mutations (K43R) and codon 88 substitutions (K88Q) and also one isolate each from Yemen and the other from Peru had a mutation at codon 43.

In conclusion, the frequencies and occurrence of mutations at various target loci in drug-resistant isolates of *M. tb* from Pakistan were analyzed. The profile of mutations in various loci is similar to that of the majority of isolates worldwide. However one novel mutation in *rpoB* has been found in this study. Various target loci exhibiting diversity of the polymorphisms by these drug-resistant strains indicates the prevalence of a large numbers of drug resistant strains in this region. More information about these mutations would be helpful in the development of novel molecular diagnostic methods such as DNA microarray and the DNA line probe, which may be implemented in Pakistan.

ACKNOWLEDGMENTS

This work was supported by the Higher Education Commission under project entitled “5000 HEC Indigenious Fellowship” and IRSIP fellowship for six months. This investigation also received financial support from EMRO-COMSTECH funded project “Mutator *M. tuberculosis* in Tehran and Rawalpindi; A Comparative Study on Fingerprinting, Mutator Genes, and Antibiotic Resistance Genes of the Isolates". Thanks to Dr. Rizwan Iqbal, PMRC TB research Centre, Mayo Hospital Lahore for providing cultures of *M. tuberculosis* isolates. Especial thanks to Mr. Matthias Merker, Dr. Andreas Routzer Dr. Silke Feuerriegel and Dr. Homolka, Molecular Mycobacteriology, Research Center Borstel, Germany, for providing the guidance, time and encouragement.

REFERENCES

AHMAD, S. AND MOKADDAS, E., 2004. Contribution of AGC to ACC and other mutations at codon 315 of the katG gene in isoniazid-resistant *Mycobacterium tuberculosis* isolates from the Middle East. *Int. J. Antimicrob. Agents*, **23**: 473-479.

AJBANI, K., RODRIGUES, C. SHENAI, S. AND MEHTA, A., 2011. Mutation detection and accurate diagnosis of extensively drug-resistant tuberculosis: report from a tertiary care center in India. [Research Support, Non-U.S. Gov't]. *J. clin. Microbiol.*, **49**: 1588-1590.

ALI, A., HASAN, Z., MOATTER, T., TANVEER, M. AND HASAN, R., 2009. *M. tuberculosis* Central Asian Strain 1 MDR isolates have more mutations in rpoB and katG genes compared with other genotypes. *Scand. J. Infect. Dis.*, **41**: 37-44.

AZIZ, M. A., WRIGHT, A., LASZLO, A., DE MUYNCK, A., PORTAELS, F., VAN DEUN, A.., WELLS, C., NUNN, P., BLANC, L. AND RAVIGLIONE, M., 2006. Epidemiology of antituberculosis drug resistance (the Global Project on Anti-tuberculosis Drug Resistance Surveillance): an updated analysis. *Lancet*, **368**: 2142-54.

BÁRTFAI, Z., SOMOSKÖVI, Á., KÖDMÖN, C., SZABÓ, N., PUSKÁS, E., KOSZTOLÁNYI, L., FARAGÓ, E., MESTER, J., PARSONS, L. M. AND SALFINGER, M., 2001. Molecular characterization of rifampin-resistant isolates of *Mycobacterium tuberculosis* from Hungary by DNA sequencing and the line probe assay. *J. clin. Microbiol*., **39**: 3736-3739.

CALVER, A. D., FALMER, A. A., MURRAY, M., STRAUSS, O. J., STREICHER, E. M., HANEKOM, M., LIVERSAGE, T., MASIBI, M., VAN HELDEN, P. D., WARREN, R. M. AND VICTOR, T. C., 2010. Emergence of increased resistance and extensively drug-resistant tuberculosis despite treatment adherence, South Africa. *Emerg. Infect. Dis.*, **16**: 264-271.

CAMPBELL, P. J., MORLOCK, G. P., SIKES, R. D., DALTON, T. L., METCHOCK, B., STARKS, A. M., HOOKS, D. P., COWAN, L. S., PLIKAYTIS, B. B. AND POSEY, J.E., 2011. Molecular detection of mutations associated with first-and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother*., **5**5: 2032-2041.

CARDOSO, R. F., COOKSEY, R. C., MORLOCK, G. P., BARCO, P., CECON, L., FORESTIERO, F., LEITE, C.Q.F., SATO, D.N., SHIKAMA, M.D. AND MAMIZUKA, E.M., 2004. Screening and characterization of mutations in isoniazid-resistant *Mycobacterium tuberculosis* isolates obtained in Brazil. *Antimicrob. Agents Chemother*., **48**: 3373-3381.

DOBNER, P., RUSCH-GERDES, S., BRETZEL, G., FELDMANN, K., RIFAI, M., LOSCHER, T. AND RINDER, H., 1997. Usefulness of *Mycobacterium tuberculosis* genomic mutations in the genes katG and inhA for the prediction of isoniazid resistance. *Int. J Tuberc. Lung Dis.*, **1**: 365-369.

HASNAIN, S. E., AMIN, A., SIDDIQI, N., SHAMIM, M., JAIN, N. K., RATTAN, A., KATOCH, V. M. AND SHARMA, S. K., 1998. Molecular genetics of multiple drug resistance (MDR) in Mycobacterium tuberculosis. In: *Drug resistance: mechanism and management* (eds. R. L. Singhal and O. P. Sood). Proceedings of the Fourth Annual Ranbaxy Science Foundation Symposium. Ranbaxy Science Foundation, New Delhi, India, pp. 35-40.

HEEP, M., BRANDSTÄTTER, B., RIEGER, U., LEHN, N., RICHTER, E., RÜSCH-GERDES, S. AND NIEMANN, S., 2001. Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical Mycobacterium tuberculosis isolates. *J. Clin. Microbiol.*, **39**: 107-110.

HEEP, M., RIEGER, U., BECK, D. AND LEHN, N., 2000. Mutations in the beginning of the rpoB gene can induce resistance to Rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother*., **44**: 1075-1077.

KELLEY, C.L., ROUSE, D.A. AND MORRIS, S.L., 1997. Analysis of ahpC gene mutations in isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother*., **41**: 2057-2058.

KIEPIELA, P., BISHOP, K., SMITH, A., ROUX, L. AND YORK, D., 2000. 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 Dis.*, **80**: 47-56.

KIM, S. Y., PARK, Y. J., KIM, W. I., LEE, S. H., LUDGERUS, C., KANG, S. J. AND KANG, C. S., 2003. Molecular analysis of isoniazid resistance in *Mycobacterium tuberculosis* isolates recovered from South Korea. *Diagn. Micr. Infect. Dis.*, **47**: 497-502.

LAVENDER, C., GLOBAN, M., SIEVERS, A., BILLMAN-JACOBE, H. AND FYFE, J., 2005. Molecular characterization of isoniazid-resistant *Mycobacterium tuberculosis* isolates collected in Australia. *Antimicrob. Agents Chemother*., **49**: 4068-4074.

LEE, A.S.G., TEO, A.S.M. AND WONG, S.Y., 2001. Novel mutations in ndh in Isoniazid-resistant *Mycobacterium tuberculosisIsolates*. *Antimicrob. Agents Chemother*., **45**: 2157-2159.

LIPIN, M., STEPANSHINA, V., SHEMYAKIN, I. AND SHINNICK, T., 2007. Association of specific mutations in katG, rpoB, rpsL and rrs genes with spoligotypes of multidrug-resistant Mycobacterium tuberculosis isolates in Russia. *Clin. Microbiol. Infect.*, **13**: 620-626.

LÖNNROTH, K., CASTRO, K. G., CHAKAYA, J. M., CHAUHAN, L. S., FLOYD, K., GLAZIOU, P. AND RAVIGLIONE, M. C., 2010. Tuberculosis control and elimination 2010–50: cure, care, and social

development. *Lancet*, **375**: 1814-1829.

MOKROUSOV, I., FILLIOL, I., LEGRAND, E., SOLA, C., OTTEN, T., VYSHNEVSKAYA, E., LIMESCHENKO, E., VYSHNEVSKIY, B., NARVSKAYA, O. AND RASTOGI, N., 2002. Molecular characterization of multiple-drug-resistant Mycobacterium tuberculosis isolates from northwestern Russia and analysis of rifampin resistance using RNA/RNA mismatch analysis as compared to the line probe assay and sequencing of the rpoB gene. *Res. Microbiol.*, **153**: 213-219.

ONG, D. C., YAM, W.C., SIU, G. K. AND LEE, A. S., 2010. Rapid detection of rifampicin- and isoniazid-resistant *Mycobacterium tuberculosis* by high-resolution melting analysis. [Comparative Study Evaluation Studies Research Support, Non-U.S. Gov't]. *J. clin. Microbiol.*, **48**: 1047-1054.

RAMASWAMI, S. AND MUSSER, J. M., 1998. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*. *Update. Tuberc. Lung Dis.*, **79**: 3-29.

SCORPIO, A. AND ZHANG, Y., 1996. Mutations in pncA, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat. Med.*, **2**: 662-667.

SHI, D., LI, L., ZHAO, Y., JIA, Q., LI, H., COULTER, C., JIN, Q. AND ZHU, G., 2011. Characteristics of embB mutations in multidrug-resistant *Mycobacterium tuberculosis* isolates in Henan, China. *J Antimicrob. Chemother.*, **66**: 2240-2247.

SIA, I. G. AND WIELAND, M. L., 2011. Current concepts in the management of tuberculosis. *Mayo Clin. Proc.*, **86**: 348-361.

SOMERVILLE, W., THIBERT, L., SCHWARTZMAN, K. AND BEHR, M.A., 2005. Extraction of Mycobacterium tuberculosis DNA: a question of containment. *J. clin Microbiol.*, **43**:2996-2997

SREEVATSAN, S., STOCKBAUER, K.E., PAN, X., KREISWIRTH, B.N., MOGHAZEH, S.L.W., JACOBS JR, R., TELENTI, A. AND MUSSER, J.M., 1997. Ethambutol resistance in *Mycobacterium tuberculosis*: critical role of embB mutations. *Antimicrob. Agents Chemother.*, **41**: 1677-1681.

SREEVATSAN, S., PAN, X., STOCKBAUER, K.E., WILLIAMS, D. L., KREISWIRTH, B. N. AND MUSSER, J. M., 1996. Characterization of rpsL and rrs mutations in streptomycin-resistant *Mycobacterium tuberculosis* isolates from diverse geographic localities. *Antimicrob. Agents Chemother.*, **40**: 1024-1026.

SUN, Z., CHAO, Y., ZHANG, X., ZHANG, J., LI, Y., QIU, Y., LIU, Y., NIE, L., GUO, A. AND LI, C., 2008. Characterization of extensively drug-resistant *Mycobacterium tuberculosis* clinical isolates in China. *J. clin. Microbi*ol., **4**6: 4075-4077.

TANIGUCHI, H., ARAMAKI, H., NIKAIDO, Y., MIZUGUCHI, Y., NAKAMURA, M., KOGA, T. AND YOSHIDA, S., 1996. Rifampicin resistance and mutation of the rpoB gene in *Mycobacterium tuberculosis*. *FEMS Microbiol. Lett.*, **144**: 103-108.

TELENTI, A., HONORE, N., BERNASCONI, C., MARCH, J., ORTEGA, A., HEYM, B., TAKIFF, H.E. AND COLE, S.T., 1997a. Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. *J. clin. Microbiol.*, **35**: 719-723.

TELENTI, A., PHILIPP, W. J., SREEVATSAN, S., BERNASCONI, C., STOCKBAUER, K. E., WIELES, B., MUSSER, J. M. AND JACOBS, W.R., 1997b. The emb operon, a gene cluster of *Mycobacterium tuberculosis* involved in resistance to ethambutol. *Nat. Med.*, **3**: 567-570.

TOSTMANN, A., KIK, S. V., KALISVAART, N. A., SEBEK, M. M., VERVER, S., BOEREE, M. J. AND VAN SOOLINGEN, D., 2008. Tuberculosis transmission by patients with smear-negative pulmonary tuberculosis in a large cohort in the Netherlands. *Clin. Infect. Dis.*, **47**: 1135.

VAN DOORN, H. R., DE HAAS, P. E. W., KREMER, K., VANDENBROUCKE-GRAULS, C. M. J. E., BORGDORFF, M. W. AND VAN SOOLINGEN, D., 2006. Public health impact of isoniazid-resistant *Mycobacterium tuberculosis* strains with a mutation at amino-acid position 315 of katG: a decade of experience in The Netherlands. *Clin. Microbiol. Infect.*, **12**: 769-775.

WHO, 2010. Treatemt of tuberculosis; guidelines for national programmes. 4th Ed. WHO Press

WHO, 2011. *Global tuberculosis control*. WHO report 2011, World health Organization (WHO).

YUE, J., SHI, W., XIE, J., LI, Y., ZENG, E. AND WANG, H., 2003. Mutations in the rpoB gene of multidrug-resistant *Mycobacterium tuberculosis* isolates from China. *J. clin. Microbiol.*, **41**: 2209-2212.

ZHANG, M., YUE, J., YANG, Y., ZHANG, H., LEI, J., JIN, R., ZHANG, X. AND WANG, H., 2005. Detection of mutations associated with isoniazid resistance in *Mycobacterium tuberculosis* Isolates from China. *J. clin. Microbiol.*, **43**: 5477-5482.

(Received 25 July 2012, revised 24 October 2012)