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Acta Microbiologica et Immunologica Hungarica, 58 (1), pp. 51–63 (2011)
DOI: 10.1556/AMicr.58.2011.1.6

PREVALENCE OF MUTATIONS AT CODON 463 OF *KATG* GENE IN MDR AND XDR CLINICAL ISOLATES OF *MYCOBACTERIUM TUBERCULOSIS* IN BELARUS AND APPLICATION OF THE METHOD IN RAPID DIAGNOSIS

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(Received: 11 January 2011; revised: 19 February 2011; accepted: 25 February 2011)

Isoniazid (INH) is a central component of drug regimens used worldwide to treat tuberculosis. In respect to high GC content of *Mycobacterium tuberculosis,* nonsynonymous mutations are dominant in this group. In this study a collection of 145 *M. tuberculosis* isolates was used to evaluate the conferring mutations in nucleotide 1388 of *katG* gene (KatG463) in resistance to isoniazid. A PCR-RFLP method was applied in comparison with DNA sequencing and anti-mycobacterial susceptibility testing. From all studied patients, 98 (67.6%) were men, 47 (32.4%) were women, 3% were <15 and 9% were >65 years old; male to female ratio was 1:2.4. PCR result of *katG* for a 620-bp amplicon was successful for all purified *M. tuberculosis* isolates and there was no positive *M. tuberculosis* culture with PCR negative results (100% specificity). Subsequent PCR RFLP of the *katG* identified mutation at KatG463 in 33.3%, 57.8% and 59.2% of our clinically susceptible, multidrug resistant TB (MDR) and extensively drug resistant (XDR) isolates, respectively. Strains of H37Rv and Academic had no any mutations in this codon. *M. bovis* was used as a positive control for mutation in KatG463. Automated DNA sequencing of the *katG* amplicon from randomly selected INH-susceptible and resistant isolates verified 100% sequence accuracy of

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the point mutations detected by PCR-RFLP. We concluded that codon 463 was a polymorphic site that is associated to INH resistance (a missense or "quiet" mutation). RFLP results of *katG* amplicons were identical to those of sequence method. Our PCR-RFLP method has a potential application for rapid diagnosis of *M. tuberculosis* with a high specificity.

Keywords: *mycobacterium tuberculosis,* KatG463, multidrug Resistant, extensively drug resistant

Introduction

The consequences of tuberculosis (TB) on society are immense. *Mycobacterium tuberculosis* responsible for more deaths than any other single infectious organism; there are more than 8 million new cases and 1.7 million deaths annually [1].

Worldwide, one person out of three is infected with *M. tuberculosis* – two billion people in total. TB accounts for 2.5% of the global burden of disease [2, 3].

Effective drugs to treat and cure the disease have been available for more than 50 years, yet every 15 seconds, someone in the world dies from TB. Even more a person is newly infected with *M. tuberculosis* second of every day. Aperson with active TB without treatment infect an average of 10 to 15 other people every year [4].

Ninety-five per cent of all cases and 99% of deaths occur in developing countries [1, 5].

Drug resistance is a matter of great concern for TB control programs since there is no cure for some multidrug-resistant TB (MDR-TB) strains of *M. tuberculosis* [1].

MDR-TB is defined as resistance to at least rifampicin (RIF) and isoniazid (INH), while extensively drug resistant TB (XDR-TB) shows resistance to at least RIF and INH, in addition to any fluoroquinolone, and to at least 1 of the 3 following injectable drugs used in anti-TB treatment: capreomycin, kanamycin and amikacin [1, 6].

There is a confirmed emergence of XDR-TB as a serious worldwide public health threat. From 19.9% of identified MDR-TB isolates, 9.9% met the criteria for XDR [7].

Countries of the former Soviet Union had a median prevalence of resistance to the four drugs of 30%, compared with 1.3% in all other settings [1].

In Belarus, number of all TB cases in 1990 was 3948 (incidence 38 in 100,000 persons) and in 2006 the number of cases increased to 5989 (incidence 61

in 100,000 persons) [8]. According to a WHO report, the number of XDR patients was 65 in this country in 2007 [9].

Genetic study of *katG* that codes for catalase-peroxidase enzyme confirmed that this gene takes part in acquisition of resistance to INH.

Selective pressure of INH widely used for TB treatment triggers high frequency of mutations in *katG*gene of *M. tuberculosis*strains circulating on the territory of the Republic of Belarus. The majority of mutations in *katG*gene of *M. tuberculosis*are observed in codon 315.

A missense mutation (a type of nonsynonymous mutation) is a point mutation in which a single nucleotide is changed, resulting in a codon that codes for a different amino acid. This can render the resulting protein nonfunctional. Not all missense mutations lead to appreciable protein changes. An amino acid may be replaced by an amino acid of very similar chemical properties, in which case, the protein may still function normally; this is termed a neutral, "quiet", or conservative mutation. In this study, we evaluated conferring missense mutations in codon 463 at *katG* gene (KatG463) responsible for phenotypic resistance to INH by using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method in comparison with DNA sequencing and anti-mycobacterial susceptibility testing.

Materials and Methods

Clinical isolates

Samples were collected from patients suffering from chest symptoms and/or chest radiographic infiltrates in the Belarusian National Research Institute of Phthisiopulmonology. Specimens were processed for direct smear followed by concentration for acid-fast bacilli (AFB) culture [10]. N-acetyl-L-cysteine was used as a digestant in the isolation and culture of mycobacteria from sputum.

Catalase activity was assayed using a mixture of hydrogen peroxide (15%) and Tween 80 (10%) according to the method of Nolte and Metchock [10]. M. tuberculosisH37Rv was included as a susceptible control strain in the drug-susceptibility testing and also as a positive control for the catalase assay. Conventional biochemical and antimicrobial susceptibility testing was performed using the proportional method and the BACTEC system.

DNA extraction for PCR

DNA purification from isolates was performed by using of modified Chelex 100 method [11]. In brief, 3–4 colonies of fresh culture of an isolate were solved in 270 ml TAE buffer (1×) and heated in 95°C for 45 min, followed by a 10 min centrifugation in 14000 rpm (3 times) for total removal of Chelex 100 that would interfere with the PCR reaction.

PCR-RFLP

Identification of isolates was confirmed by detection of *katG* gene by PCR. The INH-resistance mutation in KatG315 was identified by RFLP using *HpaII* digestion (restriction site C:CGG as described by Leung et al. [12].

Primers *katG904* (5'-AGCTCGTATGGCACCGGAAC-3', forward primer, positions 904–923) and *katG1523* (5'-TTGACCTCCCACCCGACTTG-3', reverse primer, positions 1523–1502) [12] were used to amplify a 620-bp fragment of *katG*. 50 µl of a reaction mixture containing 10 µl purified DNA, 5 µl 10x Taq Buffer (containing $(NH_4)_2SO_4$ and 20 mM $MgCl_2$, Fermentas B34), 1 µl of deoxynucleotide-triphosphates (dNTPs) mix, 10 mM each (Fermentas #R0192), 1U *Taq* polymerase and 25 pmoles of each set of primers Subsequent temperature cycling for 45 cycles started at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min and a final cycle at 72°C for 10 min to complete the elongation of the PCR intermediate products. A 10 µl aliquot of the PCR product was electrophoresed for 1 h through 1.5% agarose gel in 1× TAE buffer.

Samples positive for the 620-bp fragment of katG were identified as MTB. A 12 µl aliquot of the PCR product was digested using 5 U HpaII restriction endonuclease (Fermentas Restriction Enzymes) in a 20 µl reaction mixture containing 2 µl 10× buffer Tango and 5.5 µl distilled water (with 18.2 MΩ.cm) at 37°C for 3h, followed by a 20 min incubation in 65°C for stopping of enzyme activity. Electrophoresis was performed in a 2.5% agarose gel, the bands were visualized by ethidium bromide staining.

DNA sequencing

To verify the point mutations detected by PCR-RFLP, the sequencing method was used for some randomly selected samples. PCR with forward primer

as (5'-TTCGGCCGGGTCGACCAGT-3') and reverse primer as (5'-CGGAATT CCAGGGTGCGAATGACCT-3') was performed by annealing temperature of 62°C for 30 seconds. The 975 bp bands in the PCR products were detected by 1.5% agarose-ethidium bromide gel electrophoresis and extracted from agarose gel by DNA extraction kit (Fermentas, K0513) according to the manufacturer's instructions with some modification. Salts and impurities were removed from the glass beads containing deposited DNA. The DNA-containing solution was placed on the gel. A piece of gel containing the DNA band was removed. Approximate volume of the piece was determined (1 g is approximately equal 1 ml) and was put in the plastic tube. Three volumes of interaction solution were added to one gel volume. The mixture was incubated at 35ºC for agarose dilution. Five ml of silicon powder suspension per 2.5 mg of DNA was added, and incubated for 5 minutes at 55ºC. Then the solution was mixed by vortexing the probes every minute. The silicon powder/DNA mixture was centrifuged for 5 seconds to obtain the glass beads, then the supernatant was removed, 500 ml of ice washing buffer was added, the mixture was vortexed and centrifuged for 5 seconds again, supernatant was removed. This procedure was repeated three times. DNA was eluted in water or TE buffer, the beads were diluted in the proportional volume of water, and the tubes were incubated at 55°? for 5 minutes. Then the tubes were centrifuged, supernatant was transferred to a new tube without touching the bead; elution was repeated with different volume of water or TE buffer to remove silicon powder particles. The tubes were centrifuged once again for 30 seconds and supernatant was transferred to another tube. DNA quantity was determined and compared with the control one, followed by a centrifugation for 1 minute at 14000 rpm.

Extracted DNA concentration s were measured by Nucleic Acid analyzer (DU 730, Life Science UV/Vis spectrophotometer).

Extracted fragments 571–1408 of *katG* were amplified in a Rotor-Gene (RG-3000, Corbett Research Inc.) by Thermo-Sequenase Cy5 Dye Terminator Sequencing Kit (GE Healthcare 27-2682-01) using the oligonucleotide primer that were designed as (5'-TGCGGTCGAAACTAGCTGTGA -3') from the *M. tuberculosis* H37Rv genome sequence, by the help of server programs (Integrated DNA Technologies, DNA Services Facility, Primer Design, etc.) and DNAMAN (Quebec, Canada). Sequence annealing temperature for amplification was 56°C for 60 seconds and was sequenced directly using an automatic DNA sequencer (Amersham auto sequencer). The analysis of the results was accomplished by using the ALFwin Sequence Analyser module V2, Mega, NCBI-BLAST and BLASTP, DNAMAN (Quebec, Canada) and BioEdit programs.

Results

The 145 clinical isolates were identified as *M. tuberculosis*. Drug susceptibility testing revealed that 109 isolates were MDR, 27 were XDR and 9 isolates were susceptible *M. tuberculosis* strains. Two standard strains, H37Rv and Academic, and an isolate of *M. bovis* were used as controls. From all 145 isolates, 98 (67.6%) were obtained from men and 47 (32.4%) were obtained from women. Three% were <15 and 9% were >65 years old; male to female ratio was 1:2.4. differences were seen mainly in age group of 25–65years.

The *katG* PCR for a 620-bp amplicon was successful for all purified M. tuberculosis isolates, including standards, susceptible, MDR and XDR isolates (specificity 100%) *(Figure 1)*. There was no PCR negative sample observed.

The *katG* amplicons were analysed by restriction endonuclease analysis of RFLP using *HpaII* digestion.

Four distinct RFLP patterns were generated. Pattern A represented no mutations in codons KatG315 and KatG463. Pattern B indicated mutated KatG463 but no mutations in KatG315, in contrast to pattern C, demonstrating mutations in KatG315 but not in KatG463. Pattern D indicated mutations in both codons (*Table I* and *Figures 1* and *2*).

Table I

Four patterns distinguishable by restriction endonuclease analysis of the samples

Patterns	A		B		C		D	
Codon numbers	315	463	315	463	315	463	315	463
Nucleotides numbers	944	1388	944	1388	944	1388	944	1388
Status of mutations	–	–	–	+	+	–	+	+

We identified mutation at KatG463 in 33.3%, 57.8% and 59.2% of our clinically susceptible, MDR and XDR isolates, respectively *(Table II).*

Table II

RFLP results of KatG463 in clinical isolates as number (%)

Isolates	Susceptible isolates	MDR isolates	XDR isolates	Total isolates
Not mutated KatG463	6 (66.7%)	46 (42.2%)	11 (40.7%)	63 (43.5%)
Mutated KatG463	3 (33.3%)	63 (57.8%)	16(59.2%)	82 (56.5%)
Total	9 (100%)	109 (100%)	27 (100%)	145 (100%)

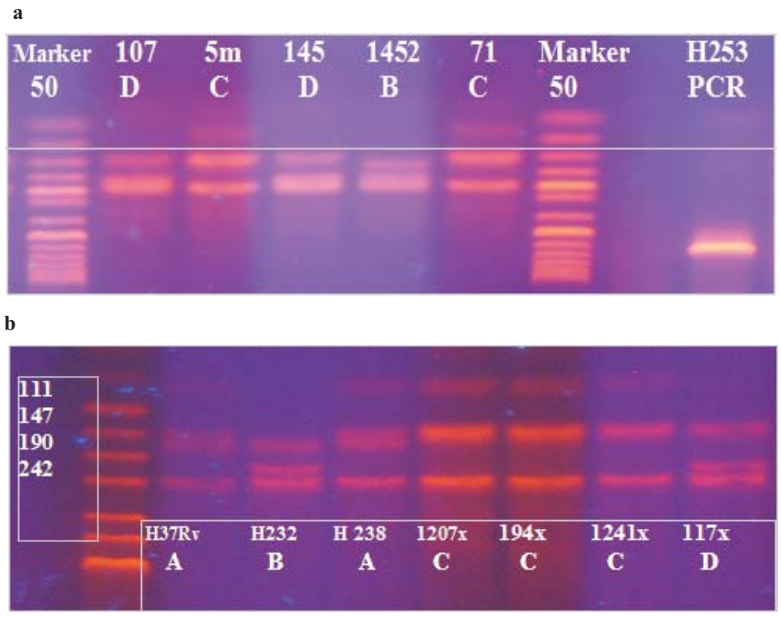


Figure 1. Results of PCR-RFLP. Different patterns of A ,C (non-mutated KatG463) and B, D (mutated KatG463)

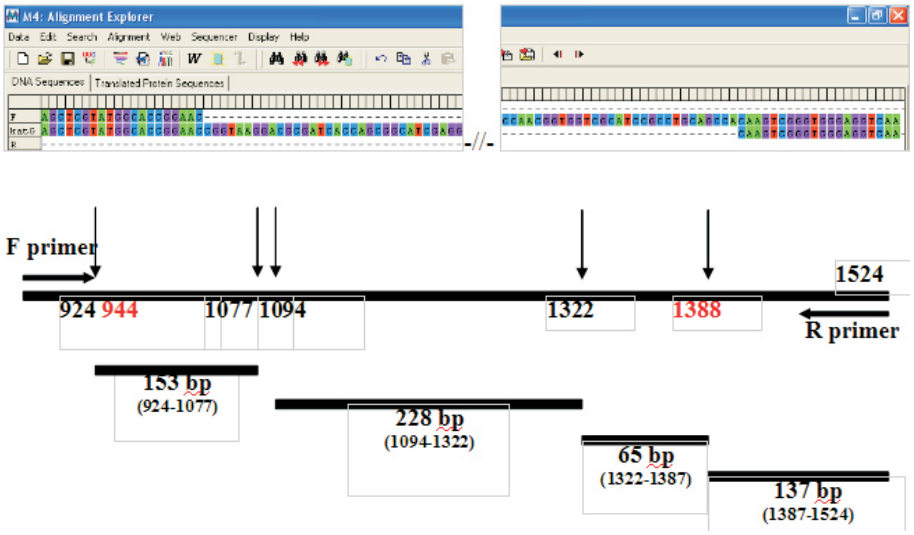
a: MDR isolates of three different patterns and a PCR product of 620 bp of *katG* fragment (before treatment by *HpaII* endonuclease)
b: Standard H37Rv strain, susceptible (H232, H238) and four XDR isolates that show mutated and non-mutated forms of KatG463

Standard strains of H37Rv and Academic had no mutations in this codon. *M. bovis* has a B pattern that indicates mutation in KatG463.

Totally, of the 145 isolates (in addition to the standard strains and *M. bovis*) 82 isolates (56.5%) had mutation in KatG463. On the other hand, 6 (66.7%) of susceptible isolates did not have any mutation in this codon, and 3 (33.3%) had mutated codon.

DNA sequencing

Automated DNA sequencing of the *katG* amplicon from randomly selected INH-susceptible and resistant isolates verified 100% sequence accuracy of the point mutations detected by PCR-RFLP.



Wild Type (without any mutations) (A):	153 bp, 228 bp, 65 bp, 137 bp
Mutation in nucleotide 1388 (KatG463) (B):	153 bp, 228 bp, 202 bp (65 + 137)
Mutation in nucleotide 944 (KatG315) (C):	132 bp, 228 bp, 65 bp, 137 bp
Mutations in nucleotides 1388 and 944 (D):	132 bp, 228 bp, 202 bp (65 + 137)

Figure 2. Resulting bands due to the action of restriction enzyme HpaII on the 620 bp amplicon of *katG* gene. The vertical arrows indicate pointes cutting places of HpaII

DNA sequencing also revealed that there was no mutation other than Arg/Leu463 within the 837-bp *katG* amplicons and 100% of all sequenced mutated codon were as Leu463.

Discussion

The 145 isolates were confirmed by PCR as *M. tuberculosis* (without any negative PCR). Leung et al. [12] observed negative PCR results of some purified cultures. Our results, however, indicate that the method of *katG*-based PCR may be used for confirmation of the diagnosis of *M. tuberculosis* infection.

There are no mutations in the standard strains of H37Rv and Academic, indicating specificity of the used method for detection of KatG463. In addition, *M. bovis* was used in this study as a positive control, because of a mutation at KatG463 in this strain.

Implementation of the method of PCR-RFLP indicated that a proportion of the resistant isolates harbored mutation in KatG463. On the other hand, this muta-

tion was also observed in susceptible isolates, suggesting that codon 463 is a polymorphic site that does not contribute to INH resistance. Our finding is concordant with the results of van Doorn [14], Leung et al. [12], and Zhang et al. [15].

The RFLP method detects a loss in an HpaII restriction site which occurs when the R463L is present. Restriction enzyme HpaII (C'CGG) cuts the wild-type amplicon at three positions but cuts an amplicon with the Arg463Leu (CGG→CTG) mutation at only two positions *(Fig. 1)*. Yielding 4 bands from wild type comprise a 153 bp, a 228 bp, a 65 bp and a 137 bp fragments. In mutant types with mutation in nucleotide 1388, HpaII can not recognize the mutated site and the yielding bands will comprise a 153 bp, a 228 bp and a 202 bp (65 bp band + 137 bp band) fragments. In the wild type, *HpaII* does not cut the *katG944* (as GCGG), and the produced band is a 153 bp fragment. In the KatG315 mutant (G944C), however, the enzyme cuts mutated site (CCGG) and the produced band is a 137 bp fragment (153 bp – 17 bp = 137). The action of *HpaII* on the 620 bp amplicon of *katG* gene is shown in *Fig. 2*.

Standard strains (without any mutations) formed four bands of 153 bp, 228 bp, 65 bp and 137 bp fragments (A). Some isolates with mutation in nucleotide 1388 (KatG463) yielded three bands of 153 bp, 228 bp and 202 bp (B). Isolates harboring mutation in nucleotide 944 (KatG315) have four bands as: 132 bp, 228 bp, 65 bp, and 137 bp (C) and some of the isolates showed three bands of 132 bp, 228 bp and 202 bp, that have mutations in both nucleotides 1388 and 944 (D) (*Table I, Figures 1* and *2*).

Based on Leung's et al. theory [12], KatG463 mutation associates with region and geography. These authors suggested that the Arg463 wild-type MTB isolates predominant in the Western world were replaced by isolates carrying Leu463 in the South China region. Our findings are in disagreement with this suggestion. KatG463 mutations were found in Europe and USA and other regions of the world. Prevalence of mutation in KatG463 in USA was 16–44%, in China 40–80%, in The Netherlands 28% and in Korea up to 78%. We concluded that the prevalence is not associated with regional conditions *(Table III).*

On the other hand, prevalence of KatG463 was different in the different studies carried out in the same country, e.g. in Korea it varied from 22% to 78%, or in Japan from 3.2% to 71.2%. Some authors presented the prevalence in both of the resistant and the susceptible isolates. When this percent was low in resistant forms, it was also low in susceptible strains, and so on in contrast. It is interesting that Zhang et al [15] did not detect any mutation at codon 463 of *katG* among the 50 INH-susceptible isolates, and in contrast, none of the 67 INH-resistant isolates

Table III

Prevalence of mutation in nucleotide 1388 (KatG463) in resistant and susceptible isolates in some studies

Countries	Prevalence of mutation in KatG463	Numbers of INH-resistant (MIC > or = 1 mcl/ml)	Percent of KatG463 mutant (in INH-susceptible)	Year of study	Author (reference)
USA (Maryland)	34%	26	–	1995	Rouse [16]
USA	44%	9	–	1995	Cockerill [17]
USA	16%	81	–	1997	Temesgen & Cockerill [18]
Korea	22%	32	39% (18)	1996	Shim [19]
Korea	78%	32	61% (18)	1997	Shim [20]
China	80%	102	–	2003	Leung [12]
China	57.4%	101	53.5% (43)	2005	Chen [21]
China	40.2%	87	0% (50)	2005	Zhang [15]
Japan	71.2%	66	–	2008	Abe [22]
Japan	3.2%	68	–	2007	Sekiguchi [23]
Poland	30%	24	–	2004	Wojtyczka [24]
Mexico	0%	67	–	2003	Viader-Salvadó [25]
South Africa	7%	87	–	1997	Haas [26]
The Netherlands	28%	225	32% (100)	2001	van Doorn [27]
Finland	7%	13	–	1996	Marttila [13]

tested by Viader-Salvadó et al. [25], showed mutation at codon 463. These results indicate that this mutation is only a polymorphism.

However, in previous studies, the mutation at codon 463 was found in 0 to 61% of INH-susceptible *M. tuberculosis* isolates [5, 6, 13]. Also, it was found that the activity of catalase, a *katG*-encoded enzyme, did not differ among isolates having either Arg or Leu at codon 463 [21, 22]. Furthermore, complementation of *katG*-negative INH-resistant *M. tuberculosis* strains with *katG* having the CGGàCTG (Arg463Leu) mutation fully restored the virulence and catalase activity of these strains [12, 14]. Hence, there was no biochemical support for the observation that CGGàCTG (Arg463Leu) was associated with resistance to INH [12]. The results of our study support this observation. Our results on sequencing of the randomly selected INH-resistant isolates that had a mutation in this recognition site confirmed the presence of the CGG→CTG (Arg463Leu) mutation, and the results of our PCR_RFLP show that the mutation in KatG315 of *M. tuberculosis* does not confer resistance to INH.

In 2002, site-directed mutagenesis of *katG* codon 463 revealed that the codon 463 Arg to Leu substitution does not significantly alter the level of expression of *katG* or the peroxidase activity of the *M. tuberculosis* catalase-peroxidase [28].

Results of Cockerill et al. in 1995 indicated that the mutation of arginine to leucine in codon 463 of the catalase-peroxidase gene occurs in a significant fraction (44.2%) of *M. tuberculosis* strains with INH MICs > or = 1.0 microgram/ml. But none of the strains with an INH MIC<1.0 microgram/ml had mutations affecting codon 463 [17].

We detected mutation in KatG463 in a clinical isolate of *M. bovis*. This observation is in agreement with the results of Musser et al. [29]. Since all 16 strains of *M. bovis* and *M. microti* studied by these authors had Leu463 rather than Arg463 in KatG, their observation was consistent with the hypothesis that Leu463 is the ancestral condition in *M. tuberculosis* [29]. Furthermore, Doorn in 2001 [14] mentioned that this mutation is also present in *M. intracellulare, M. bovis, M. bovis* BCG, *M. africanum,* and *M. microti* isolates. These mycobacterial species are in general less susceptible to INH [9, 10]. We used *M. bovis* as a positive control in experiments [30].

Conclusion

We conclude that because of its high specificity, application of our method for fast detection of *M. tuberculosis* may be useful. Furthermore, because of its occurrence in all sensitive and resistant forms, codon 463 is a polymorphic site that does not contribute to INH resistance. Results were compared with anti-mycobacterial susceptibility testing and DNA sequencing.

Acknowledgements

We sincerely thank our colleagues at the National Reference Laboratory of the Research Institute for Pulmonology and Pathophysiology that they collected the samples and performed the drug susceptibility tests.

References

1. Nalin, R., Christophe, S.: Molecular Evolution of the *Mycobacterium tuberculosis* Complex (Chapter 2); Montoro, E., Rodriguez, R.: Global Burden of Tuberculosis (Chapter 7); Martin, A., Portaels, F.: Drug Resistance and Drug Resistance Detection (Chapter 19). In Palomino, J. C., Cardoso Leão, S., Ritacco, V. (eds): Tuberculosis 2007. From basic science to

patient care. www.tuberculosistextbook.com (http://www.tuberculosistextbook.com/tuberculosis2007.pdf).
2. Dye, C., Dolin, P., Pathania, V., Raviglione, M. C., Scheele, S.: Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA **282,** 677–86 (1999).
3. Smith, I.: What is the health, social, and economic burden of tuberculosis. In Frieden, T. (ed.): Toman's Tuberculosis Case Detection, Treatment, and Monitoring: Questions and Answers. 2nd ed. Geneva, WHO, WHO/HTM/TB/2004.334 (2004).
4. Dye, C., Watt, C. J., Bleed, D. M., Mehran Hosseini, S., Raviglione, M. C.: Evolution of tuberculosis control and prospects for reducing tuberculosis incidence, prevalence, and deaths globally. JAMA **293,** 2767–2775 (2005).
5. Dye, C.: Global epidemiology of tuberculosis. Lancet **367,** 938–940 (2006).
6. CDC, Centers for Disease Control and Prevention; Extensively Drug-Resistant Tuberculosis (XDR TB), www.cdc.gov/tb, pp. 1–3, July (2007).
7. Shah, S. S., Wright, A., Bai, G. et al.: Worldwide emergence of extensively drug resistant tuberculosis. Emerg. Infect. Dis. **13,** 380–387 (2007).
8. World Health Organization. TB burden (2006 estimates), TB country profile, Belarus. www.who.int/GlobalAtlas/predefinedReports/TB/PDF_Files/blr.pdf.
9. World Health Organization. The Global MDR-TB & XDR-TB Response Plan 2007, WHO/HTM/TB/2007.387 (2008).
10. McGowan, J. E. Jr, Metchock, B., Nolte, F. S.: Laboratory diagnosis of tuberculosis: Past, present, and future. J. Med. Assoc. Ga **84,** 215–220 (1995).
11. Walsh, P. S., Metzger, D. A., Higuchi, R.: Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques **10,** 506–513 (1991).
12. Leung, E. T. Y., Kai-Man, K., Agatha, C., Pak-Leung, H., Wing-Hong, S., Kwok-Yung, Y., Wing-Cheong, Y.: Detection of KatG Ser315Thr substitution in respiratory specimens from patients with isoniazid-resistant *Mycobacterium tuberculosis* using PCR-RFLP. J. Med. Microbiol. **52,** 999–1003 (2003).
13. Marttila, H. J., Soini, H., Eerola, E., Vyshnevskaya, E., Vyshnevskiy, B. I., Otten, T. F., Vasilyef, A. V., Viljanen, M. K.: A Ser315Thr substitution in KatG is predominant in genetically heterogeneous multidrug-resistant *Mycobacterium tuberculosis* isolates originating from the St. Petersburg area in Russia. Antimicrob. Agents Chemother. **42,** 2443–2445 (1998).
14. van Doorn, H. R.: The Susceptibility of *Mycobacterium tuberculosis* to Isoniazid and the Arg3Leu Mutation at Codon 463 of *katG* Are Not Associated. J. Clin. Microbiol. **39,** 1591–1594 (2001).
15. Zhang, M., Yue, J., Yang, Y. P., Zhang, H. M., Lei, J. Q., Jin, R. L., Zhang, X. L., Wang, H. H.: Detection of mutations associated with isoniazid resistance in *Mycobacterium tuberculosis* isolates from China. J. Clin. Microbiol. **43,** 5477–5482 (2005).
16. Rouse, D., Li, Z., Bai, G., Morris, S. L.: Characterization of the katG and inhA genes of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. Antimicrob. Agents Chemother. **39,** 2472–2477(1995).
17. Cockerill, F. R., 3rd, Uhl, J. R., Temesgen, Z., Zhang, Y., Stockman, L., Roberts, G. D., Williams, D. L., Kline, B. C.: Rapid identification of a point mutation of the *Mycobacterium tuberculosis* catalase-peroxidase (katG) gene associated with isoniazid resistance. J. Infect. Dis. **171,** 240–245 (1995).
18. Cockerill, F. R., 3rd, Williams, D. E., Eisenach, K. D., Kline, B. C., Miller, L. K., Stockman, L., Voyles, J., Caron, G. M., Bundy, S. K., Roberts, G. D., Wilson, W. R., Whelen, A. C.,

Hunt, J. M., Jr, Persing, D. H.: Prospective evaluation of the utility of molecular techniques for diagnosing nosocomial transmission of multidrug-resistant tuberculosis. Mayo Clin. Proc. **71,** 221–229 (1996).
19. Shim, T. S., Yoo, C. G., Han, S. K., Shim, Y. S., Kim, Y. W.: Isoniazid resistance and the point mutation of codon 463 of katG gene of Mycobacterium tuberculosis. J. Korean Med. Sci. **12,** 92–98 (1997).
20. Shim, T. S., Yoo, C. G., Han, S. K., Shim, Y. S., Kim, Y. W.: Isoniazid resistance and the point mutation of codon 463 of katG gene of Mycobacterium tuberculosis. J. Korean Med. Sci. **12,** 92–98 (1997).
21. Chen, X., Ma, Y., Jin, Q., Jiang, G. L., Li, C. Y., Wang, Q.: Characterization of the katG, inhA, ahpC, kasA, and oxyR gene mutations in isoniazid-resistant and susceptible strain of *Mycobacterium tuberculosis* by automated DNA sequencing. Zhonghua Jie He He Hu Xi Za Zhi **28,** 250–253 (2005).
22. Abe, C., Kobayashi, I., Mitarai, S., Wada, M., Kawabe, Y. et al.: Biological and molecular characteristics of *Mycobacterium tuberculosis* clinical isolates with low-level resistance to isoniazid in Japan. J. Clin. Microbiol. **46,** 2263–2268 (2008).
23. Sekiguchi, J., Miyoshi-Akiyama, T., Augustynowicz-Kopec, E., Zwolska, Z., Kirrikae, F. et al.: Detection of multidrug-resistance in *Mycobacterium tuberculosis*. J. Clin. Microbiol. **45,** 179–192 (2007).
24. Wojtyczka, R. D., Dworniczak, S., Pacha, J., Idzik, D., Kepa, M., Wydmuch, Z., Glab, S., Bajorek, M., Oklek, K., Kozielski, J.: PCR-RFLP analysis of a point mutation in codons 315 and 463 of the *katG* gene of *Mycobacterium tuberculosis* isolated from patients in Silesia, Poland. Pol. J. Microbiol. **53,** 89–93 (2004).
25. Viader-Salvadó, J. M., Luna-Aguirre, C. M., Reyes-Ruiz, J. M., Valdez-Leal, R., del Bosque-Moncayo, M. de L., Tijerina-Menchaca, R., Guerrero-Olazarán, M.: Frequency of mutations in *rpoB* and codons 315 and 463 of *katG* in Rif^r and/or Iso^r isoniazid-resistant *Mycobacterium tuberculosis* isolates from Northeast Mexico. Drug Resist. **9,** 33–38 (2003).
26. Haas, W. H., Schilke, K., Brand, J., Amthor, B., Weyer, K., Fourie, P. B., Bretzel, G., Sticht-Groh, V., Bremer, H. J.: Molecular analysis of *katG* gene mutations in strains of *Mycobacterium tuberculosis* complex from Africa. Antimicrob. Agents Chemother. **41,** 1601–1603 (1997).
27. van Doorn, H. R., Kuijper, E. J., van der Ende, A., Welten, A. G. A., van Soolingen, D., de Haas, P. E. W., Dankert, J.: The susceptibility of *Mycobacterium tuberculosis* to isoniazid and the Arg3Leu mutation at codon 463 of *katG* are not associated. J. Clin. Microbiol. **39,** 1591–1594 (2001).
28. Tracevska, T., Jansone, I., Broka, L., Marga, O., Baumanis, V.: Mutations in the *rpoB* and *katG* genes leading to drug resistance in *Mycobacterium tuberculosis* in Latvia. J. Clin. Microbiol. **40,** 3789–3792 (2002).
29. 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. **173,** 196–202 (1996).
30. Setareh, M., Titov, L. P., Surkova, L. K.: High level association of mutation in KatG315 with MDR and XDR clinical isolates of *Mycobacterium tuberculosis* Belarus. Microbiol. Immunol. Hung. **56,**313–325 (2009).