ISSN 0026-8933, Molecular Biology, 2010, Vol. 44, No. 4, pp. 559–567. © Pleiades Publishing, Inc., 2010.
Original Russian Text © O.Yu. Limanskaya, T.N. Mukhina, V.N. Stepanshina, I.G. Shemyakin, X. Wud, J. Zhang, T.V. Fesenko, V.A. Pokrovskiy, A.P. Limanskii, 2010, published in Molekulyarnaya Biologiya, 2010, Vol. 44, No. 4, pp. 635–645.

GENOMICS AND TRANSCRIPTOMICS

UDC 616:579.873.21; 615.015.8

Identification of Wild-Type *Mycobacterium tuberculosis* Isolates and Point Mutations Associated with Isoniazid Resistance

O. Yu. Limanskaya[a, b], T. N. Mukhina[c], V. N. Stepanshina[c], I. G. Shemyakin[c], X. Wu[d], J. Zhang[d], T. V. Fesenko[e], V. A. Pokrovskiy[e], and A. P. Limanskii[a]

[a] *Mechnikov Institute of Microbiology and Immunology, Academy of Medical Sciences of Ukraine, Kharkov, 61057 Ukraine;*
e-mail: olga.limanskaya@mail.ru
[b] *Institute of Experimental and Clinical Veterinary Medicine, National Academy of Agrarian Sciences, Kharkov, 61023 Ukraine*
[c] *State Research Center for Applied Microbiology and Biotechnology, Obolensk, Moscow region, 142279 Russia*
[d] *Institute of Tuberculosis Research, Beijing, 100091 China*
[e] *Chuyko Institute for Surface Chemistry, National Academy of Sciences of Ukraine, Kiev, 03164 Ukraine*

Received December 25, 2009
Accepted for publication February 9, 2010

Abstract—Isoniazid resistance in *Mycobacterium tuberculosis* (MBT) is associated with point mutations in codon 315 of the *katG* gene. Two PCR technique were developed for detection of point mutations in codon 315. Most frequent point mutations (AGC ⟶ ACC and AGC ⟶ AGA) were identified in codon 315 by using two sets of primers, either of which included an additional competitive blocking primer with a 3'-terminal phosphate group in order to prevent nonspecific amplification. PCR with a set of two primers, one of which contained five locked nucleic acid monomers (LNA), permits one to detect any of six known mutations in codon 315 of *katG* and, thereby, discriminate between isoniazid-sensitive and resistant MBT isolates. The structure and purity of the 17-nt long LNA-containing oligonucleotides were characterized by MALDI-TOF mass spectrometry; and the 17 bp duplex formed by two LNA-containing complementary oligonucleotides was analyzed by thermal denaturation.

DOI: 10.1134/S0026893310040096

Key words: point mutation, PCR, *Mycobacterium tuberculosis*, isoniazid, drug resistance, LNA monomers, MALDI-TOF mass spectrometry

Infectious diseases represent a serious threat for all humanity. The annual death toll from infections and helminthic diseases constitutes nearly 17 million people of 51-million total annual mortality [1]. Tuberculosis is one of unsolved problem in public health service. According to WHO reports, annual tuberculosis mortality is approximately 2 million people [2]. The rates of tuberculosis morbidity and mortality in Russia and most CIS countries are several times as high as in Western Europe (http://data.euro.who.int/hfadb/). Approximately 120000 people contact tuberculosis in Russia annually. The total number of recorded cases is 2500000, and the expert judgment is approximately 7000000 [1]. Russian Government Decree 715 of December 1, 2004, assigned tuberculosis to socially significant diseases and enrolled it on the list of diseases hazardous for neighboring people (http://arhiv.inpravo.ru/index.htm).

The dissemination of *Mycobacterium tuberculosis* (MBT) strains resistant to drugs is no less significant. The main approach to its solution is timely detection of such strains at early stages of the disease. This would allow control of the dissemination of a particular strain and correction of the treatment regimen in each case. There are conventional methods of detection of mycobacterium resistance to antituberculous drugs approved by WHO. However, the determination of the range of drug resistance in MBT takes 1–3 months. In this period, newly admitted patients receive the standard drug treatment, which includes first-line pharmaceuticals. According to WHO recommendations, first-line antituberculous drugs include isoniazid, rifampicin, and ethambutol, which are used in a short tuberculosis treatment schedule together with pyrazinamide [2]. When data on the sensitivity/resistance of a particular MBT isolate to drugs used in the therapy are absent, the treatment can be inefficient. Rapid detection of

Abbreviations: LNA (Locked Nucleic Acid)—closed nucleic acid; MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization-Time Flight)—time-flight matrix activated laser desorption/ionization; MT (Mutant Type)—isolate of mutant type; T_{an}—annealing temperature; T_m—melting temperature; WT (Wild Type)—isolate of wild type; ISN—isoniazid; MBT—mycobacteria of tuberculosis (*M. tuberculosis*); MDS—multiple drug stability.

multiple drug resistance (MDR) in MBT is critical for the choice of efficient treatment. This problem can be solved by using modern molecular methods and data on MBT strain genotyping. If one is fortunate, the analysis can be completed within 24 h.

Numerous molecular methods have been developed for detecting resistant MBT strains [3–6]. Studies of the molecular pathways underlying resistance indicate that it is determined by mutations in certain genes. Such mutations can be detected by PCR-based methods with specific types of primers: (1) a primer with a 3'-terminal nucleotide noncomplementary to the wild-type MBT template [7], (2) an additional primer blocked at the 3' end [8], (3) an LNA-containing primer [9], and (4) an additional competitive LNA-modified oligonucleotide [10].

A brand new approach to MDR detection is provided by microarrays. Their high sensitivity allows simultaneous identification of the causative agent and analysis of its resistance to several drugs. The new test kit TB-Biochip identifies a total of 50 mutations in the *rpoB, katG, oxyR*, and *inhA* genes of MBT. The MDR range can be determined within 48 h.

Resistance to isoniazid (INH) is one of the most widespread resistances in MBT. It is provided mainly by mutations in codon 315 of the *katG* gene, which encodes catalase–peroxidase [12, 13]. The generally used strategy of point mutation detection involves allele-specific PCR. One or both primers are designed so that they are annealed to sites containing target point mutations. Ideally, a primer complementary to a certain sequence supports amplification of only one fragment. Actually, sequences containing noncomplementary nucleotides can also be amplified. Modern approaches to the detection and genotyping of human and animal pathogens include the development of methods based on DNA polymorphism and molecular analysis of this polymorphism by various PCR modification. Assignment of an isolate to the wild (WT) or mutant (MT) type with the amplification refractory mutation system (ARMS PCR) demands analysis of six mutation variants in codon 315 of *katG* with seven primer pairs [14]. Primer design is much simpler in common PCR with LNA-modified primers. In this variant, one primer pair is sufficient. One member of this pair contains three to five LNA monomers complementary to (or complementary to and flanking) the codon of interest.

Taking into consideration the above reasoning, data from the literature on various PCR modification [15], and the fact that common equipment for PCR is available in most hospital laboratories, we developed two protocols for identification of mutations in codon 315 of *katG*. In the first protocol, LNA-modified primers are used for detection of point mutations. The designed sets of such primers allow identification of any of the six mutations that can occur in codon 315 in one reaction. In this way, WT and MT isolates can be discriminated. In the second protocol, PCR is conducted with an additional competitive blocking primer with 3' phosphate group. This variant allows detection of two most frequent mutations in codon 315: AGC ⟶ ACC and AGC ⟶ AGA.

EXPERIMENTAL

Polymerase chain reaction. Softwares GeneBee [16] and MeltCalc [17, 18] were applied for analysis of nucleotide sequences of *katG* gene from 62 MBT isolates from GenBank database. The melting temperature of the LNA-containing oligonucleotide–ssDNA duplex was calculated with Exiqon T_m prediction software, available at http://lna-tm.com [19]. Primer sets were tested with a collection of DNA samples obtained from clinical MBT isolates of known phenotypes: WT or MT. Isolates of the latter, INH-resistant, type are characterized by mutations in *katG*. DNA of MT isolates resistant to 1–25 μg/ml INH carried point mutations in codon 315: AGC ⟶ ACC, AGC ⟶ ACG, and AGC ⟶ AGA.

The primer sets Mykat4–Mykat5–Mykat5P and Mykat4–Mykat9–Mykat9P designed for identification of point mutations AGC ⟶ ACC and AGC ⟶ AGA, respectively, were tested with DNA templates from three strains: HB385, carrying AGC ⟶ AGA; HB125, carrying AGC ⟶ ACC, and H37Rv with the wild-type codon 315 (negative control for amplification).

The reaction mixture (20–50 μl) was prepared from a dry amplification kit (Isogen, Russia). Its composition was: 4 mM NaCl, 50 mM KCl, 12 mM Tris HCl pH 8.0, 2.5 mM $MgCl_2$, 200 μM dNTP, 0.1–0.5 μM each primer and 1 U of antibody-inhibited *Taq* DNA polymerase for hot-start PCR. Electrophoretically pure primers were purchased from Syntol (Russia). LNA-modified primers contained five LNA monomers each, purchased from Exiqon, Denmark. Amplification was conducted in a thermocycler manufactured by Scientific company Tochnost' (Russia). The amplification program was as follows: predenaturation at 95°C for 2 min and 38–45 cycles including denaturation at 95°C for 1 min, annealing at 58–71°C for 1 min, and elongation at 70–74°C for 1 min. The annealing temperatures T_{an} were experimentally optimized in a series of reactions with T_{an} increased at intervals first 2°C and then 0.5°C until the absence of the nonspecific band. The template contained 0.05 μg of MBT genomic DNA. The reaction products were resolved by electrophoresis in 1.5% agarose gel with ethidium bromide at the field strength 10 V/cm.

Mass-spectrometrical analysis of oligonucleotides. The structures of LNA-modified oligonucleotides were verified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). The oligonucleotides were purified by electrophoresis in 20% polyacrylamide gel with 8 M urea. Oligonucleotide samples (0.5 μl of 10 μg/ml solution)

1	NCBI007 (H37Rv)	TGGCACCGGAACCGGTAAGGACGCGATCACCAGCCCGTACGAGGTCGTATGGACGTTGTGG
2	DQ056356 – Chin	. . . . . AAC . . . . .
3	AF314112 – Ital	. . . . . ATC . . . . .
4	41309 – Finlan	. . . . . ACA . . . . .
5	AY155353 – Taiw	. . . . . ACC . . . . .
6	HB385 – Chin	. . . . . AGA . . . . .
7	DNA780 – Rus	. . . . . ACG . . . . .

Fig. 1. Fragment of the *katG* gene. Mutations in its codon 315 (SNPs are underlined) are associated with isoniazid resistance in MBT. Accession numbers in GenBank and country of sequencing are shown: Ital, Taiw, Chin, Finlan, and Rus designate Italy, Taiwan, China, Finland, and Russia, respectively. H37Rv is a laboratory *M. tuberculosis* strain.

were applied to matrix crystals obtained by drying of aqueous matrix template solution (50 mg/ml 3-hydroxypicolinic acid and 2 mg/ml dibasic ammonium citrate) on a stainless steel target plate.

The target was dried at room temperature until complete evaporation of the solvents and placed to an Autoflex II mass spectrometer (Bruker Daltonics Inc., Germany) equipped with a nitrogen laser, which allowed pulsed ionization. Mass spectra were obtained in the positive ion mode. The samples were ionized with a UV laser of 337 nm wavelength in the pulse mode: pulse length 3 ns, frequency 20 Hz, maximum energy 65 μJ, ion extraction delay 30 ns. Positive ions were detected in the reflectron mode. The accelerating voltage was 20 kV. The resulting mass spectra were obtained from no less than 100 individual ones.

UV spectrometry of oligonucleotide duplexes. The formation of oligonucleotide duplexes containing five complementary LNA monomers each was confirmed by thermal denaturation with optical density measurement at 260 nm. Optical density was recorded at temperature intervals 0.05°C and heating rate 0.5°C/min with a Specord M40 spectrometer (Carl Zeiss Jena, Germany) equipped with a temperature-controlled cell holder. Denaturation experiments were conducted in cells with optical path length 1 cm. The duplexes were melted in sodium cacodylate buffer pH 7.0, concentrations 10^{-3}, 10^{-2}, and 5×10^{-2} M, at the DNA concentration 20–30 pmol/μl. Differential melting profiles were deduced from integral ones with Origin 7.0 software (United States).

RESULTS

Our developed methods for detection of point mutations in codon 315 of the *katG* gene in MBT are based on two PCR variants: allele-specific with a competitive blocking primer and common PCR with LNA-modified primers. The first step of primer design included analysis of sequences of the complete *katG* gene and its fragments from MBT isolates accessible in GenBank. The results of computer analysis of *katG* with various point mutations conferring INH resistance to MBT isolates show that mutations in codon 315 can be used for detecting both MT and WT MBT isolates (Fig. 1).

Allele-Specific PCR with a Competitive Blocking Primer

This PCR variant demands a blocking primer in addition to a set of allele-specific primers. In the protocol for discrimination between WT and MT, a pair of conventional primers is supplemented by a competitive oligonucleotide. Its sequence is complementary to the DNA strand of a WT MBT isolate and contains the target mutation site, i.e., codon 315 of *katG* (Fig. 2). The competitive oligonucleotide is blocked at the 3' end to prevent further replication of the nonmutant strand by DNA polymerase. This favors selective amplification of mutant DNA. The blocking primer for our PCR protocol for detection of INH-resistant MBT isolates had a phosphate group at its 3' end, which terminated cDNA synthesis.

The system for detection of INH-resistant MBT strains was tested with a collection of DNA samples of clinical MBT isolates varying in INH resistance and bearing various point mutations in codon 315 of *katG*.

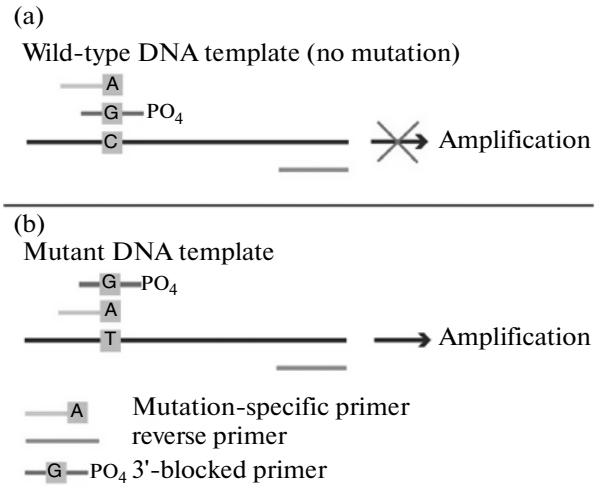

Fig. 2. Allele-specific PCR with the blocking competitive primer. This primer contains a phosphate group at its 3' end and suppresses amplification of the wild-type allele.

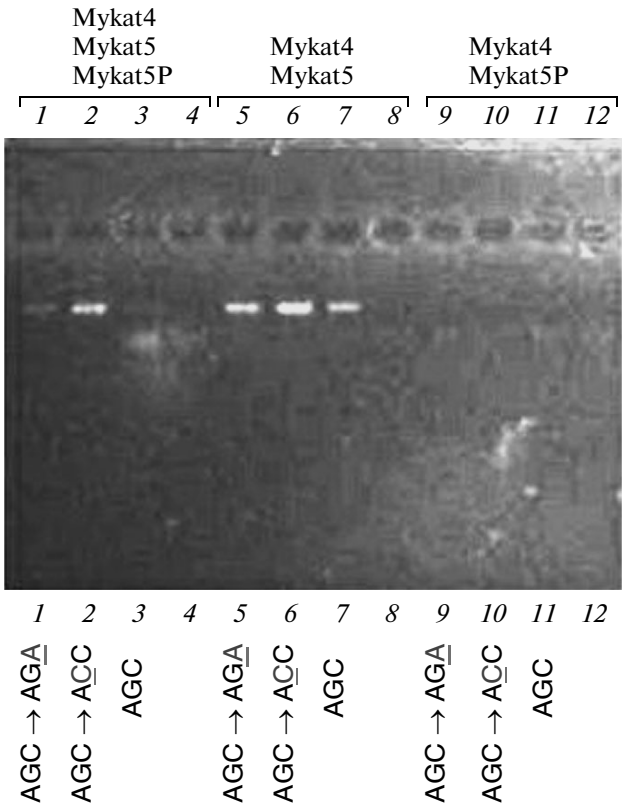

Fig. 3. Detection of the AGC ⟶ ACC SNP in *katG*. PCB is performed with three primer sets: Mykat4–Mykat5–Mykat5P, Mykat4–Mykat5, and Mykat4– Mykat5P. Amplification products are resolved by electrophoresis in 2% agarose gel: Lanes: *1, 5, 9*, DNA of *M. tuberculosis* strain HB385, possessing the AGC ⟶ AGA SNP in codon 315; *2, 6, 10*, DNA of *M. tuberculosis* strain HB125, possessing the AGC ⟶ ACC SNP; *3, 7, 11*, DNA of *M. tuberculosis* strain H37Rv, negative control (wild-type AGC codon); *4, 8, 12*, water, negative control. At the T_{an} used in the experiment, the Mykat4–Mykat5–Mykat5P set discriminates the AGC ⟶ ACC SNP (Lane *2*) from AGC ⟶ AGA (Lane *1*) and the WT sequence AGC (Lane *3*). Point mutation positions are underlined.

Two sets of primers were chosen: Mykat4–Mykat5–Mykat5P and Mykat4–Mykat9–Mykat9P. They allowed detection of point mutations AGC ⟶ ACC and AGC ⟶ AGA, respectively.

The ability of Mykat5P to block DNA polymerase was confirmed by the absence of the amplicon in PCR with the primer pair Mykat5P–Mykat4 (Fig. 3) and its specificity was confirmed by the absence of selectivity without this primer (Fig. 3, Lanes *5*, *7*) with either WT or MTs with various mutations in codon 315. The reaction with the blocking primer amplifies only DNA with the AGC ⟶ ACC mutation (Fig. 3, Lane *2*).

Similar results were obtained in experiments on AGC ⟶ AGA detection with the Mykat4–Mykat9–Mykat9P primer set (data not shown).

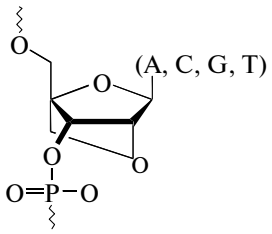

Fig. 4. A locked nucleic acid (LNA) monomer able to replace nucleotides in DNA and RNA sequences.

PCR with LNA-Containing Primers

LNA monomers are analogs of nucleotides differing in that they contain an additional methylene bridge between 2' oxygen and 4' carbon atoms in the furanose ring. This bridge locks the 3' endo conformation in the sugar (Fig. 4).

Two sets of LNA-containing primers were designed in this work for detection of point mutations, or single nucleotide polymorphisms (SNPs), in codon 315 of the MBT *katG* gene: L7–$L8^{LNA}$ and Mykat24–$Mykat25^{LNA}$. Each set included one LNA-modified primer. Each of the 17-nt LNA-modified primers, $Mykat25^{LNA}$ and $L8^{LNA}$, had five LNA monomers. Three LNA monomers of each primer were complementary to codon 315 of *katG*, and two other primers flanked the codon. These sets differed in the orientation of the LNA-modified primer. The LNA primer in the L7–$L8^{LNA}$ set was complementary to the plus strand of the DNA (Fig. 5a), and in Mykat24–$Mykat25^{LNA}$ it was complementary to the minus strand (Fig. 5b).

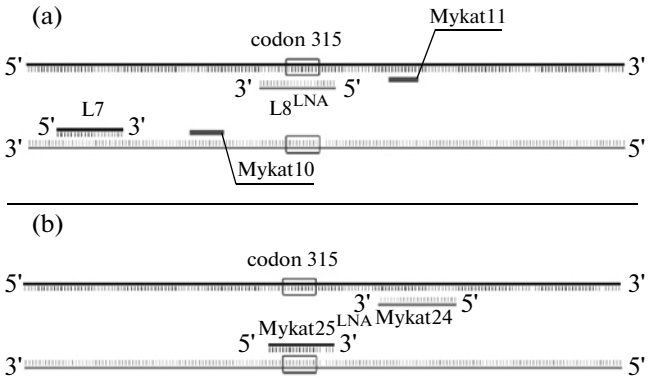

Fig. 5. Location of primers on the MBT *katG* sequence with reference to codon 315. The L7–$L8^{LNA}$ and Mykat24–$Mykat25^{LNA}$ primer sets allow amplification of 214 and 110-bp amplicons in *katG*. The $L8^{LNA}$ and $Mykat25^{LNA}$ primers have five LNA monomers each. Two of them are flanking and three are complementary to (a) plus strand and (b) minus strand at codon 315. The Mykat10–Mykat11 primer set amplifies a 151-bp long fragment containing codon 315 and serving as a molecular weight marker.

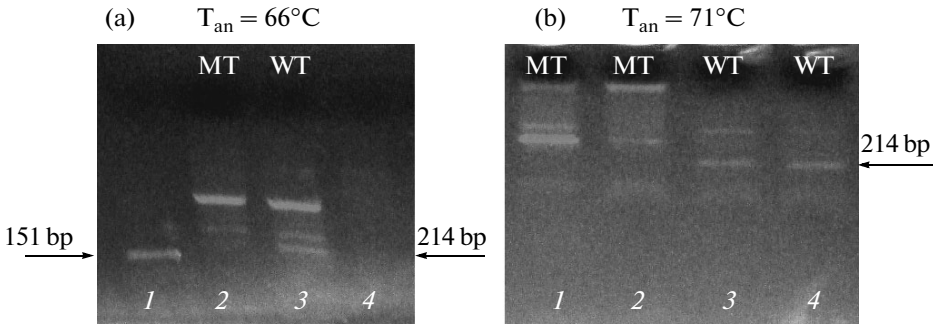

Fig. 6. Detection of amplification products for fragment *katG* gene of the MTB wild (WT) and mutant (MT) types by electrophoresis in 1.5% agarose gel. Panel (a). Annealing temperature 66°C. Lane *1*. Amplificate of the 151-bp *katG* fragment containing codon 315 with Mykat10–Mykat11 primers, serving as a length marker. Lane *2*. Amplification of DNA from MT isolate with L7–$L8^{LNA}$ primers. Lane *3*. Amplification of a WT isolate. With the presence of a mutation in codon 315, the 214-bp band is absent. Lane *4*. Negative control. Panel (b). Annealing temperature 71°C. Lanes *1, 2*. Amplificons of the *katG* gene from two clinical MT MBT isolates: *1*, AGC ⟶ AGA and *2*, AGC ⟶ ACC. The amplificate of the expected length is absent. Only bands corresponding to longer nonspecific amplicons are seen. Lanes *3, 4*. Two different WT isolates. The characteristic 214-bp band is seen in both lanes.

The L7–$L8^{LNA}$ set allows discrimination between the wild and mutant types of MBT *katG* (Fig. 6). With WT, the system yields a fragment of the predicted length 214 bp in the T_{an} range from 66 to 71°C, but this amplificate is absent in the case of MT, although amplification occurs, as indicated by the presence of a nonspecific amplificate. The intensity of the nonspecific band decreases with T_{an} increase from 66 to 71°C. In this case, two-step PCR was conducted for reliable discrimination.

The Mykat10–Mykat11 primer set served as a positive control. The reaction with these primers yielded the 151-bp fragment of *katG*, which covered codon 315 (Fig. 6, Lane *1*). Further sequencing of this short amplificate will allow identification of the mutation in codon 315 of MBT strains circulating in the region in question. Analyses with this set of primers and sequencing of the resulting amplificates for identification of polymorphisms in codon 315 are scheduled for the future.

Characterization of Primers with LNA Monomers

MALDI-TOF mass spectrometry for testing structure and composition of synthesized single-stranded LNA-containing oligonucleotides with LNA monomers $L8^{LNA}$ and $Mykat25^{LNA}$ (Figs. 7, 8). The check included completeness of protecting group removal from nucleobases and presence or absence of shorter oligonucleotides, lacking one or several monomers. The mass spectra of the oligonucleotides analyzed contained peaks with m/z ratios 5373.8 for $L8^{LNA}$ and 5347.3 for $Mykat25^{LNA}$, thereby confirming the presence of LNA monomers (Table 1). These peaks were assigned to cluster ions $[L8^{LNA} + Na]^+$ and $[Mykat^{LNA} + Na]^+$.

Table 1. Oligonucleotides containing LNA-modified monomers

Designation	Sequence*	Formula/calculated molecular weight, Da	
		unmodified	LNA-modified
$Mykat25^{LNA}$	5`-GATCAC**<u>CAGCG</u>**GCATCG-3`	$C_{164}H_{207}N_{67}O_{98}P_{16}$ 5180.4	$C_{169}H_{207}N_{67}O_{103}P_{16}$ 5320.4
$L8^{LNA}$	5`-CGATGC**<u>CGCTG</u>**GTGATC-3`	$C_{165}H_{209}N_{63}O_{102}P_{16}$ 5202	$C_{170}H_{209}N_{63}O_{107}P_{16}$ 5342

* LNA-modified nucleotides are indicated in bold and underlined.

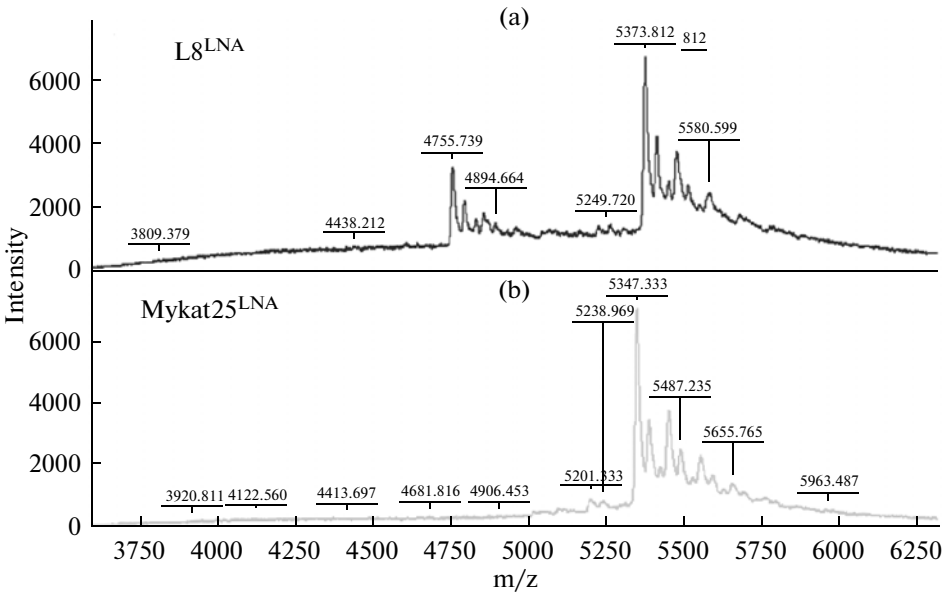

Fig. 7. MALDI-TOF spectra of 17-mer oligonucleotides having five LNA monomers: (a) $L8^{LNA}$; (b) $Mykat25^{LNA}$.

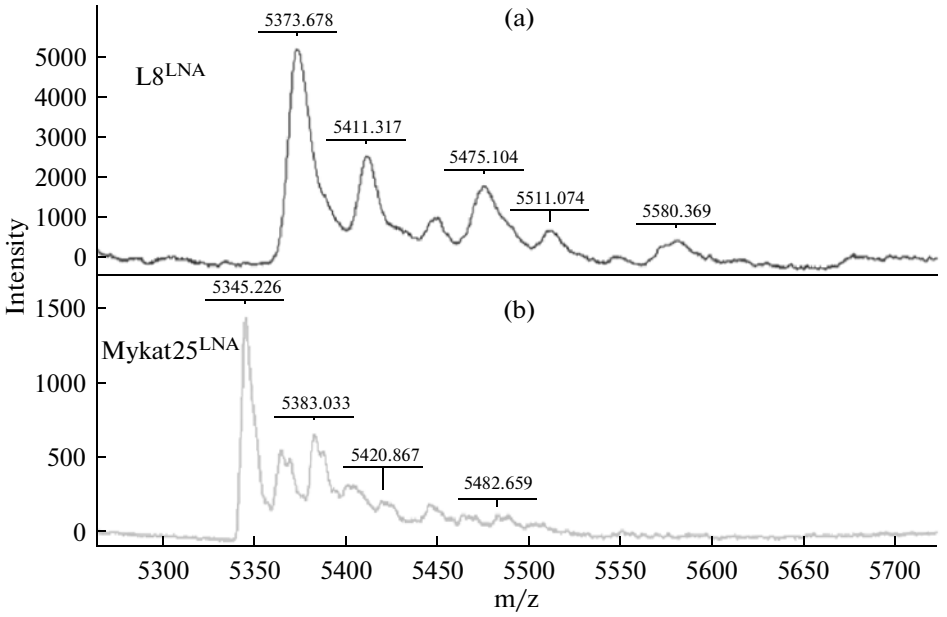

Fig. 8. High-resolution mass spectra of LNA-containing oligonucleotides: (a) $L8^{LNA}$; (b) $Mykat25^{LNA}$.

The $L8^{LNA}$ and $Mykat25^{LNA}$ primers were designed so that they were complementary to each other and able to form a 17-bp long double-stranded oligonucleotide. The predicted melting temperature (T_m) of the unmodified L8–$Mykat25^{LNA}$ duplex at the ionic strength 1 mM Na^+ is 45°C. The presence of five LNA monomers in each strand of the $L8^{LNA}$–$Mykat25^{LNA}$ duplex dramatically increases T_m to 88°C (Fig. 9). The contribution of one pair of LNA monomers to T_m increase is 8.6°C and that of a single LNA monomer is 4.3°C. The ΔT_m value predicted for the 17-bp $L8^{LNA}$–Mykat25 duplex with only one modified strand is 2.8°C (Table 2). Thus, the LNA modification of five monomers in each strand stabilizes the duplex much more than expected if the data on LNA modification of one strand are extrapolated to the modification of

both strands. It should be mentioned that T_m can be predicted with the program [4, 5] only in the case of LNA modification of one strand.

Experiments on the effect of ionic strength (5 and 50 mM Na^+) on T_m of the $L8^{LNA}$–$Mykat25^{LNA}$ duplex provided little information, as only an initial portion of the melting profile was recorded because of the highT_m of the duplex (data not shown). The melting temperature of the duplex is in the logarithmic relationship with cation concentration [7].

A general approach to the analysis of clinical samples is proposed on the basis of the developed methods, which allow identification of WT and MT isolates and genotyping of SNPs associated with INH resistance (Fig. 10). A specific feature of this approach is that WT isolates can be rapidly detected, and newly admitted patients can be immediately given a short course of drug therapy, including INH.

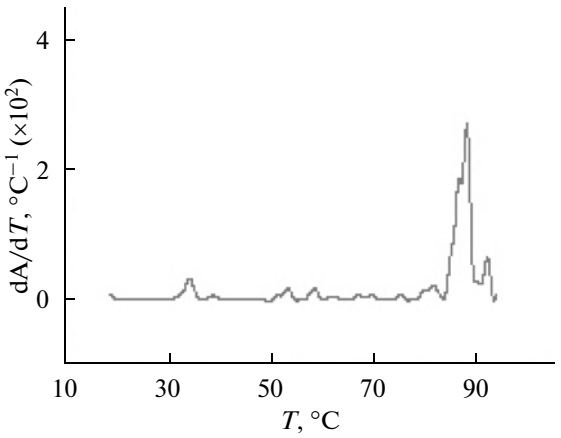

Fig. 9. Differential melting curve of the duplex formed by two 17-bp oligonucleotides ($L8^{LNA}$–$Mykat25^{LNA}$), each having five LNA monomers. The sample was melted in 1 mM sodium cacodylate pH 7.0.

DISCUSSION

LNA monomers obey Watson–Crick base pairing rules, but their hybridization is more specific and affine [20]. Duplexes with LNA monomers are extremely thermostable [21]. Introduction of one LNA monomer to one strand of a 13-bp duplex increases its T_m by 3–4°C. Nine LNA monomers increase it by 25°C, and ten increase it by 28°C. Thus, the contribution of one LNA monomer to the increase in T_m is 3°C. Generally, the temperature shift (ΔT_m) depends on duplex length.

Identification of genetic markers of INH resistance by multiplex PCR requires six primer pairs: one pair per one SNP allele in codon 315 of MBT *katG* gene. The same goal can be achieved by common PCR with one set of primers, including a primer with three to five LNA monomers complementary to the codon in question or to the codon and flanking regions.

Allele-specific PCR with LNA-modified primers is based on the fact that the melting temperature (and, as a consequence, annealing temperature) of a primer–ssDNA template complex for a mutant is lower than T_m of the perfect duplex. Hence, T_m of a primer–ssDNA template complex with a point mutation at the position corresponding to an LNA monomer is lower than that of the perfect duplex. In experiments with MBT isolates, DNA of a WT isolate forms a perfect duplex with the LNA-modified primer, whereas the duplex of DNA of an INH-resistant isolate, bearing a mutation in codon 315 of *katG*, with the same primer has a lower T_m. It should be mentioned that T_m of an LNA primer–ssDNA template complex with an SNP depends on both the number of LNA monomers and primer length. Introduction of one additional LNA monomer to a 20-bp duplex increases T_m by approximately 1°C. The choice of primer length was based on that fact the discriminating ability of a primer can be enhanced by increasing the proportion of LNA monomers. The increase in the length of the primer itself, which is commonly used to increase specificity, will decrease the contribution of the same number of LNA monomers and, as a result, the discriminating ability of the primer.

The analysis of amplification products at low T_{an} values showed that both primer sets gave rise to *katG* fragments of predicted lengths: 214 bp for L7–$L8^{LNA}$ (Fig. 6b, Lanes 3, 4) and 110 bp for Mykat24–$Mykat25^{LNA}$ (data not shown). The 110-bp band obtained at T_{an} = 58°C was faint. At T_{an} values within 66–71°C, PCR with L7–$L8^{LNA}$ allows discrimination between MT and WT MBT isolates of the *katG* gene, whereas no amplification occurred with Mykat24–$Mykat25^{LNA}$. For this reason, most experiments were performed only with L7–$L8^{LNA}$. In our opinion, the low intensity of the 110-bp amplification band is related to the relatively large contribution of five LNA monomers to its T_m and melting of only part of ampl-

Table 2. The predicted melting temperatures (°C) of the unmodified L8–Mykat25 duplex and the $L8^{LNA}$–Mykat25 duplex with five LNA monomers in one of the strands at various ionic strengths. Primer concentration 0.5 μM

Primer	Ionic strength, mM Na^+		
	1	10	50
L8–Mykat25	41	51	58
$L8^{LNA}$–Mykat25	55	65	72

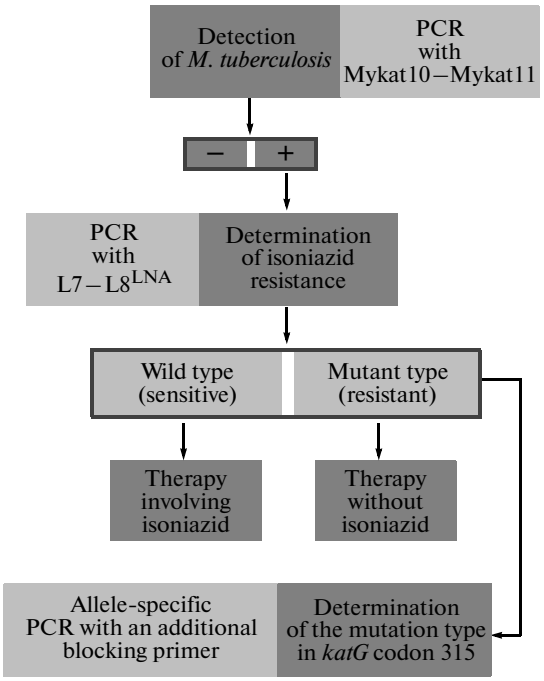

Fig. 10. Analysis of a clinical sample for (1) presence of *M. tuberculosis*, (2) isoniazid sensitivity/resistance of the MBT isolate, and (3) type of the point mutation in *katG* codon 315 associated with isoniazid resistance.

icon molecules at the 95°C denaturation temperature in each PCR cycle. An indirect piece of evidence is provided by the melting of the duplex formed by the complementary oligonucleotides $L8^{LNA}$ and $Mykat25^{LNA}$ at temperatures above 95°C and the low ionic strength typical of PCR mixtures (data not shown). The proportion of LNA monomers in the amplicon flanked by $L7–L8^{LNA}$ (214 bp) and their contribution to T_m are half as large as in the amplicon flanked by Mykat24–$Mykat25^{LNA}$. Thus, only part of Mykat24–$Mykat25^{LNA}$ amplicon molecules are denatured at 95°C, because the half-width of the melting range for the 110-bp duplex is 5–8°C. As a result, the number of the amplicon molecules is insufficient for DNA visualization by staining with ethidium bromide and UV illumination.

Note that the fluorescence intensity of the amplification band formed by PCR with $L7–L8^{LNA}$ (Fig. 6a, *3*) is much less than the intensity of the Mykat10–Mykat11 band (Fig. 6a, *1*). We suppose that the low intensity of the $L7–L8^{LNA}$ band is determined by the relatively large contribution of five LNA monomers to the T_m of this amplicon, as the introduction of LNA monomers to a duplex increases its T_m at the same ionic strength. Thus, the amplicon melts incompletely during denaturation at 95°C in each PCR cycle. Only part of the molecules produced by $L7–L8^{LNA}$ are denatured at 95°C, and the amount of the amplificate is less than with the Mykat10–Mykat11 primers, having no LNA monomers.

We suggested that T_m of a duplex with LNA monomers could be decreased by reducing the ionic strength of the reaction mixture. However, experiments on the effect of ionic strength of amplification showed that the fluorescence of the amplificate did not change when the ionic strength was decreased by a factor of 2.5: from 50 to 20 mM Na^+. Hence, the only way to increase the amount of the amplicon was to increase the reaction volume for PCR with LNA-modified primers from 20 to 50 μl.

To sum up, we have developed two protocols for detection of SNPs in codon 315 of the MBT *katG* gene. Both are based on optimization of PCR conditions with specific primer sets. In one protocol, the discriminating ability of the primer set was increased by using an additional blocking primer with a 3'-terminal phosphate at the position corresponding to the target codon. Two most common point mutations in codon 315, AGC ⟶ ACC and AGC ⟶ AGA, associated with INH resistance in MBT, can be detected with two primer sets: Mykat4–Mykat5–Mykat5P and Mykat4–Mykat9–Mykat9P, respectively. The second protocol is based on PCR with a set of two primers, one of which contains LNA monomers complementary to codon 315. In this method, WT and MT MBT isolates can be rapidly differentiated.

The molecular test systems developed in our study allow detection of isoniazid-resistant MBT isolates within 1–3 days. The analysis can be performed in laboratories possessing common PCR equipment.

ACKNOWLEDGMENTS

This work was supported by grant AMS 72/2007 from Academy of Medical Sciences of Ukraine, and the *Mass-spectrometrical Complex with Laser Desorption-ionization Autoflex II* Multiple Access Center, National Academy of Sciences of Ukraine.

REFERENCES

1. Sergiev V.P., Filatov N.N. 2006. *Infektsionnye bolezni na rubezhe vekov* (Infectious Diseases at the Turn of the Centuries). Moscow: Nauka.
2. *Global Tuberculosis Control. Epidemiology, Strategy, Financing. WHO Report 2009*. Geneva: World Health Organization, 2009. http://www.who.int/tb.
3. Kolchinsky A., Mirzabekov A. 2002. Analysis of SNPs and other genomic variations using gel-based chips. *Hum. Mutat*. **19**, 343–360.
4. Mikhailovich V., Lapa S., Gryadunov D., Sobolev A., Strizhkov B., Chernyh N., Skotnikova O., Irtuganova O., Moroz A., Litvinov V., Vladimirskii M., Perelman M., Chernousova L., Erokhin V., Zasedatelev A., Mirzabekov A. 2001. Identification of rifampin-resistant *Mycobacterium tuberculosis* strains by hybridization,

PCR, and ligase detection reaction on oligonucleotide microchips. *J. Clin. Microbiol.* **39**, 2531–2540.

5. Wu X., Zhang J., Liang J., Lu Y., Li H., Li C., Yue J., Zhang L., Liu Z. 2007. Comparison of three methods for rapid identification of mycobacterial clinical isolates to the species level. *J. Clin. Microbiol.* **45**, 1898–1903.
6. Wu X., Zhang J., Chao L., Liang J., Lu Y., Li H., Yang Y., Liang Y., Li C. 2009. Identification of rifampin-resistant genotypes in *Mycobacterium tuberculosis* by PCR-reverse dot blot hybridization. *Mol. Biotechnol.* **41**, 1–7.
7. Limanskaya O.Yu. 2009. Species-specific detection of mycobacteria of the tuberculosis complex. *Tuberk. Bolezni Legkikh*. **10**, 49–56.
8. Orum H. 2000. PCR clamping. *Curr. Issues Mol. Biol.* **2**, 27–30.
9. Strand H., Ingebretsen O., Nilssen O. 2008. Real-time detection and quantification of mitochondrial mutations with oligonucleotide primers containing locked nucleic acid. *Clin. Chim. Acta*. **390**, 126–133.
10. Dominguez P., Kolodney M. 2005.Wild-type blocking polymerase chain reaction for detection of single nucleotide minority mutations from clinical specimens. *Oncogene*. **24**, 6830-6834.
11. Zasedatelev A.S., Sobolev A.Yu., Gryadunov D.A., Lapa S.A., KMikhailovich V.M., Mirzabekov A.D. 2005. RF Patent no. 2005140679, C12 Q 1/68, C12 R 1/32/, priority date: Dec. 26, 2005. *Byull. Izobret.* no. 19.
12. Nizova A.V., Stepanshina V.N., Maiskaya N.V., Bogun A.G., Maiorova A.A., Shemyakin I.G. 2007. Analysis of resistance to first-and second-line drugs in *Mycobacterium tuberculosis* clinical strains. *Epidemiol. Infekts. Bolezni*. **4**, 7–10.
13. Lipin M., Stepanshina V., Shemyakin I., 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.
14. Johnson R., Jordaan A., Pretorius L., Engelke E., van der Spuy G., Kewley C., Bosman M., van Helden P., Warren R., Victor T. 2006. Ethambutol resistance testing by mutation detection. *Int. J. Tuberc. Lung Dis.* **10**, 68–73.
15. Josefsen M., Lofstrom C., Sommer H., Hoorfar J. 2009. Diagnostic PCR: Comparative sensitivity of four probe chemistries. *Mol. Cell Probes*. **23**, 201–203.
16. Brodskii L., Drachev A., Tatuzov R., Chumakov K. 1991. GeneBee: Applied program package for analysis fobiopolymer sequences. *Biopolim. Kletka*. **7**, 10–14.
17. Schütz E., von Ahsen N. 1999. Spreadsheet software for thermodynamic melting point prediction of oligonucleotide hybridization with and without mismatches. *Biotechniques*. **27**, 1218–1224.
18. von Ahsen N., Wittwer C., Schütz E. 2001. Oligonucleotide melting temperatures under PCR conditions: Nearest-neighbor corrections for Mg^{2+}, deoxynucleotide triphosphate, and dimethyl sulfoxide concentrations with comparison to alternative empirical formulas. *Clin. Chem*. **47**, 1956–1961.
19. SantaLucia J. 1998. A unified view of polymer, dumbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl. Acad. Sci. USA*. **95**, 1460–1465.
20. Orum H., Jakobsen M., Koch T., Vuust J., Borre M. 1999. Detection of the factor V Leiden mutation by direct allele-specific hybridization of PCR amplicons to photoimmobilized locked nucleic acids. *Clin. Chem*. **45**, 1898–1905.
21. Latorra D., Arar K., Hurley J. 2003. Design considerations and effects of LNA in PCR primers. *Mol. Cell. Probes*. **17**, 253–259.