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Molecular Technologies Used in Detecting of Sensitive and Isoniazid-Resistant *Mycobacterium tuberculosis*

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Abstract—Two variants for the detection of single nucleotide polymorphisms in codon 315 of the *katG* gene of Mycobacterium tuberculosis (MTB) (mutations in this gene are associated with resistance to isoniazid, which is an antituberculosis drug of the first line) have been developed. 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), permitted the identification of the most frequent AGC ⟶ ACC and AGC ⟶ AGA point mutations in codon 315 of the *katG* gene. Conduction of PCR with a set of two primers, one of which contained five LNA monomers, permitted the detection of any of the six known mutations in codon 315 of the *katG* gene and, thereby, for the discrimination between isoniazid-sensitive and isoniazid-resistant MTB. The purity and structure of the 17 bp long primers containing LNA-modified nucleotides were characterized by time-of-flight MALDI mass spectrometry, and the 17 bp duplex formed by two LNA-containing complementary oligonucleotides was analyzed by thermal denaturation. The molecular genetic test systems created for differentiating between the wild-type MTB isolates and isoniazid-resistant MTB (an antituberculosis drug of the first line) can be used in clinical laboratories equipped with standard PCR devices; such systems permit the shortening of the time required for the detection of isoniazid resistance of MTB: from 1–3 months by the standard bacteriological methods to 1–3 days by PCR.

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INTRODUCTION

Infectious diseases are still very serious public health threats: every year, 51 million people die from infectious diseases, and 17 million people among them die from infectious and parasitic diseases [1]. Special attention is paid to tuberculosis (TB), which remains one of the actual public health care problems in the whole world. According to the data obtained by WHO in 2009 [2], notwithstanding the fact that the progression of the active form of this disease occurs in only a small proportion of infected people, the average annual death rate caused by tuberculosis reaches 2 million cases, and 9.27 million cases of this disease were registered in the whole world in 2007. At the same time, Sergiev et al. [1] emphasized that the number of TB patients was not counted correctly (indeed, the number of TB patients should be larger). According to expert estimations, the total number of TB patients reaches 1.3 billion.

In Ukraine, as well as in most countries of the Community of Independent States (Table 1), the indexes of morbidity and mortality demonstrate a multifold increase compared to the same indexes in Western Europe (the database of the WOH European Regional Committee, http://data.euro.who.int/hfadb/). Ukraine, similarly to some other countries, belongs to countries with high levels of TB morbidity (87.5 per 100000 population in 2006 and 89.7 per 100000 population in 2007) and mortality (21.4 per 100000 population in 2004) (http://data.euro.who.int/hfadb/).

Along with the increase in TB morbidity, the wide prevalence of multiresistant tuberculosis mycobacterial strains (MTB) is not less a global problem. The main solution to the problem of control of the prevalence of drug-resistant forms of TB is their timely detection in early stages of disease progression, which permits the controlling of the prevalence of specific strains and making adjustments in the chemotherapy design for each patient. There are several traditional classic methods (both direct and indirect) used for detecting resistance of mycobacteria to antituberculosis drugs; these methods were approved by the WHO. At that, the detection of a spectrum of medical resistance of mycobacteria of tuberculosis takes from one to three months, during which primary patients receive a therapeutic course with the use of standard antituberculosis drugs of the first line. As the resistance of MTB clinical isolates to prescribed drugs remains uncertain throughout this time, the ineffectiveness of taking drugs is probable. The fast determi-

Table 1. Number of estimated tuberculosis (TB) cases with multidrug resistance (N, MDR) in the states of the Community of Independent Countries in 2007, the number of TB cases with MDR (information regarding these cases (K) was published in 2007), and the predicted number of cases where a course of chemotherapy was expected (P) in 2008 and 2009

Country	Number of estimated cases in 2007			It was informed		Anticipated number of cases of chemotherapy treatment, P	
	%	N	SS+	K, 2007	SS+, 2007	2008	2009
Russia	21	42969	31397	5297	17	4 221	9 897
Ukraine*	19	9835	5568	–	–	–	–
Uzbekistan	24	9450	6936	484	7	334	720
Moldova	29	2231	1656	896	54	466	490
Belarus	16	1101	758	870	115	–	–
Tadzhikistan*	23	4688	3286	–	–	–	–
Kirgizstan	17	1290	813	322	40	–	–
Armenia	17	486	373	123	33	–	–

Note: SS+ designates the cases with positive results of sputum analysis; "–" shows that no data was provided. *Countries that did not provide data concerning TB cases with MDR and anticipated chemotherapy.

nation of resistance and, especially, multidrug resistance of MTB is very important for the selection of an effective method of antituberculosis therapy. Getting a solution to this problem looks possible at the genotype level; therefore, it will permits the shortening of the time required to perform an appropriate analysis to 1–3 days.

At the present time, many publications contain a number of molecular biological methods for the detection of resistant MTB strains [3–6]. The study of molecular mechanisms of resistance showed that the resistance of MTB to any drug was caused by single mutations in certain genes. To detect such point mutations or single nucleotide polymorphisms, the use of different variants of PCR is possible: using a primer with a nucleotide that is noncomplementary with the wild-type DNA template at the 3'-end [7]; using an additional primer with a blocked 3'-end [8]; using an LNA-modified primer (LNA locked nucleic acid) [9]; and, at last, using an additional competitive LNA oligonucleotide that assumes the utilization of the Stoffel fragment of *Taq* DNA polymerase (which lacks 5' to 3' exonuclease activity) [10] (Fig. 1).

It is known that isoniazid resistance significantly contributes to the development of antituberculosis drug resistance of MTB along with streptomycin resistance; isoniazid resistance is mainly caused by mutations localized in codon 315 of the *katG* gene encoding the synthesis of catalase and peroxidase. Isoniazid resistance is associated with mutations in codon 315 with a probability from 50 to over 90% [11, 12].

The modern strategy of detection and typing of human and animal pathogens lies in the development of technologies based on molecular genetic analysis with the use of PCR and its modifications; it is also based on the DNA polymorphism phenomenon. New methods of detection of point mutations (mainly, for real-time PCR) have appeared on the market of molecular genetic products. Each of these methods has both certain advantages and disadvantages.

Taking into consideration the data from publications concerning various modifications of PCR [13], as well as the fact that the majority of clinical laboratories have standard PCR equipment, two variants of molecular genetic systems for the detection of mutations in codon 315 of the MTB *katG* gene were developed in this study. In the first variant, we used primers containing LNA-modified nucleotides to detect point mutations in the MTB *katG* gene. In the second variant, we conducted PCR with the use of blocking primer containing a 3'-terminal phosphate group that permits detecting of the point mutations AGC $\longrightarrow$ ACC and AGC $\longrightarrow$ AGA that frequently occur in codon 315 of the *katG* gene.

The primers containing LNA-modified monomers were characterized with the use of time-of-flight mass spectrometry with template-activated laser desorption/ionization, and the duplex formed by them was characterized by thermal denaturation. The molecular genetic kits developed by us permitted the determination of the presence of any of the six possible variants of mutations in codon 315 of the *katG* gene by using only one PCR reaction; therefore, it becomes possible to discriminate between MTB sensitive and MTB resistant to isoniazid (an antituberculosis drug of the first line), respectively.

MATERIALS AND METHODS

PCR analysis. The sequenced chromosomal DNA nucleotide sequences of the *katG* gene of 62 wild-type isolates and the isoniazid-resistant MTB taken from

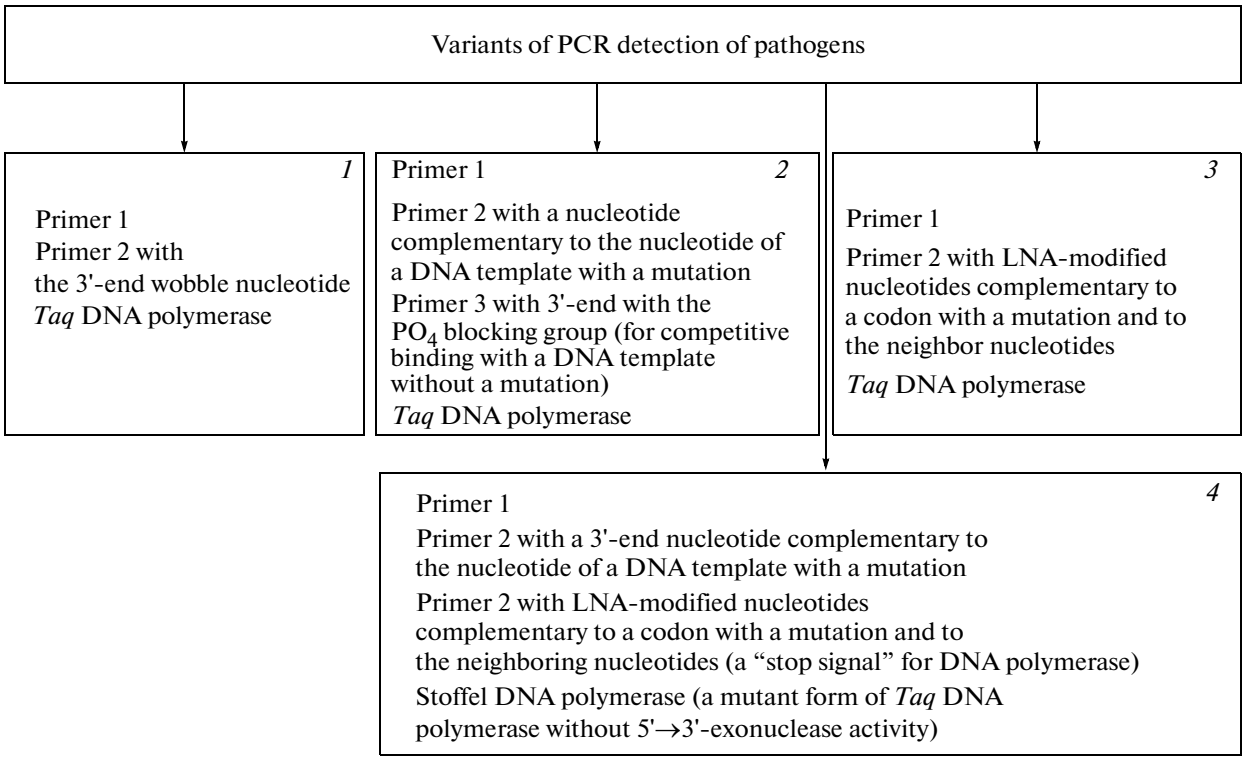

Fig. 1. Scheme of the possible modifications of PCR analysis for the detection of wild-type and mutant (resistant to isoniazid) *Mycobacterium tuberculosis*. Methods *1–3* were used in this study.

the GenBank were used for computer analysis. We carried out a multiple alignment (MSA) and further statistical analysis by the programs from the GeneBee software package [14].

To check the primer sets developed for the detection of wild-type and isoniazid-resistant MTB isolates, we utilized DNA extracts from 43 wild-type (WT) and mutant (MT) MTB isolates containing point mutations in codon 315 of the *katG* gene. The primer sets were tested on DNA from MTB isolates with the AGC ⟶ ACC, AGC ⟶ ACG, and AGC ⟶ AGA point mutations in codon 315 of the *katG* gene; the taken MTB isolates were characterized by the level of isoniazid resistance at 1–25 μg/μl. The MTB DNA collection was kindly provided by Dr. V.M. Stepanshina (Scientific National Research Center for Applied Microbiology and Biotechnology, Russian Federation). Genomic DNA was isolated by the method of phenol–chloroform extraction.

To check the sets of primers Mykat4–Mykat5–Mykat5P and Mykat4–Mykat9–Mykat9P (PCR with the use of these primers permitted the detection of the variants of point mutations in codon 315 of the MTB *katG* gene AGC ⟶ ACC and AGC ⟶ AGA, respectively), we used three DNA templates: DNA of *M. tuberculosis* strain HB385 that contains an AGC ⟶ AGA mutation in codon 315 of the *katG* gene; DNA of *M. tuberculosis* strain HB125 with an AGC ⟶ ACC mutation; and DNA of *M. tuberculosis* strain H37Rv that served as negative control of amplification (codon 315 contains the AGC sequence).

The sequences of primers with appropriate positions in the *katG* gene (a full sequence of this gene is 2223 bp) are the following:

L7 (739–762)	5'-gacattcgcgagacgtttcggcgc-3';
$L8^{LNA}$ (952–936)	5'-cgatgcCGCTGgtgatc-3' (LNA monomers are highlighted);
Mykat4 (739–758)	5'-gacattcgcgagacgtttcg-3';
Mykat5 (962–944)	5'-catacgacctcgatgccgg-3';
Mykat5P (962–944)	5'-catacgacctcgatgccgc-3'-PO_4;
Mykat9 (962–943)	5'-tccatacgacctcgatgcct-3';
Mykat9P (962–943)	5'-tccatacgacctcgatgccg-3'-PO_4;
Mykat10 (895–918)	5'-ggctggttcagctcgtatggcacc-3';
Mykat11 (1045–1023)	5'-cagcagggctcttcgtcagctcc-3'.

The PCR was carried out by a dry kit of reagents for DNA amplification (Isogen, Russian Federation) in 20–50 μl of reaction mixture containing 4 mM NaCl, 50 mM KCl, 12 mM Tris HCl (pH 8.0), 2.5 mM $MgCl_2$, 200 μM dNTPs, 0.1–0.5 μM of each primer, and 1 unit of *Taq* DNA polymerase inhibited with antibodies that enable hot start PCR. The primers

used in the study (received from Syntol, Russian Federation) were purified by PAGE. LNA-modified primers (Synthol, Russian Federation), containing five LNA nucleotides, were each received from Exiqon, Denmark (http://www.exiqon.com/lna-technology).

We used a Tochnost amplifier (Tula, Russian Federation); the PCR conditions were as follows: initial incubation at 95°C for 4 min; denaturation at 95°C for 40 sec; annealing at 58–71°C for 60 sec; synthesis at 70–74°C for 60 sec; and 38–45 cycles. The annealing temperature (T_{an}) was optimized by conduction of reactions with an increase in the temperature by 2–0.5°C until the complete elimination of the nonspecific amplicon band. For amplification, 0.05 μg of *M. tuberculosis* genomic DNA were used. To visualize the amplicons, 10–20 μl of PCR products were separated by electrophoresis in 1.5–2% agarose gel that contained ethidium bromide (field strength 10 V/cm).

The melting temperature of the complex of oligonucleotide containing LNA monomers, single-stranded DNA was calculated by the program that has free access and can be found at http://lna-tm.com; the thermodynamic analysis of primers and amplicons was performed by the MeltCalc program [15].

MALDI Mass Spectrometry of Oligonucleotides

The characterization of LNA-modified oligonucleotides was performed by time-of-flight mass spectrometry with matrix-assisted laser desorption and ionization (MALDI). This method is based on the ionization of bioorganic molecules, during which a short powerful laser impulse causes the evaporation and ionization of the molecules of the laser matrix; such molecules capture the molecules examined in the experiment and transfer the electric charge to them for their future acceleration in the electric field. In time-of-flight MALDI mass spectrometry, time-of-flight detectors are used; their operation is based on the fixation of time differences between ions with various masses in the process of reaching a sensor.

Aliquots of nucleotides (0.5 μl) purified by PAGE were placed on the matrix crystals, which had been obtained through drying a water matrix solution that contained 3-hydroxypicolinic acid (50 mg/ml) and ammonium citrate (2 mg/ml) on targets made from stainless steel. A target was dried under room temperature until the complete evaporation of the solvent and placed in the source of an Autoflex II time-of-flight mass spectrometer (Bruker Daltonics Inc., Germany) equipped with a nitrogen laser, which allows for the impulse ion extraction. The mass spectra of oligonucleotides were obtained in the regime of positive ions registration. The ionization of the specimens was conducted by a UV-laser at a wavelength of 337 nm in the impulse regime; the impulse duration was 3 ns, and the frequency was 20 Hz. We used a 30-ns delay time of the ion extraction. Positively charged ions were detected in the reflectron operation regime. To obtain total mass spectrum, we summed up and averaged 100 individual spectra.

UV spectroscopy of oligonucleotide duplexes. To characterize the oligonucleotide duplexes, each of which contained five complementary LNA-modified nucleotides, we used thermal denaturation with registration of the optical density at a 260-nm wavelength. UV spectroscopic investigations were conducted using a Specord M40 spectrophotometer (Carl Zeiss Jena, Germany) with temperature-controlled cell. The optical density was registered in the increment of 0.05°C and at a heating rate of 0.5°C/min. The melting was conducted in a 10^{-3} M, 10^{-2} M, and 5×10^{-2} M sodium–cacodylate buffer (pH 7.0) at a DNA concentration of 20–30 pmol/μl. Differential melting profiles were obtained through a differentiation of a melting integral profile using the Origin program (version 7.0, United States).

RESULTS AND DISCUSSION

Two technologies for the detection of point mutations were used in the study: (1) allele-specific PCR with a competitive blocking primer and (2) conventional PCR with the use of LNA-modified primers for the differentiation between sensitive and isoniazid-resistant mycobacteria tuberculosis. To perform allele-specific PCR, one or both primers are designed in the following manner: annealing occurs at sites containing point mutations. Ideally, a site complementary to a specific sequence results in the amplification of only one variant, but, practically, a significant amplification that involves annealing of primers with targets containing uncomplementary nucleotides occurs.

At the first development stage of primer sets, we carried out an analysis of the complete *katG* gene and its fragments from MTB isolates taken from the GenBank database. A computer analysis of the *katG* gene of chromosomal DNA from isoniazid-resistant MTB isolates with variants of typical point mutations showed that a mutation in codon 315 could be used for the detection of both isoniazid-resistant mutant bacteria and wild-type MTB isolates.

The method of allele-specific PCR based on the utilization of allele-specific primers and blocking oligonucleotide assumes the presence of both a pair of regular primers and an additional nucleotide, the sequence of which is complementary to wild-type DNA and contains a potential mutation site (Fig. 2a). A competitive nucleotide is blocked at the 3'-end in such a way that the further elongation of a nonmutant strand by DNA-polymerase becomes impossible, and only selective amplification of mutant DNA is observed. Using this variant of PCR for the detection of isoniazid-resistant *Mycobacterium tuberculosis* and blocking DNA polymerase synthesis were conducted with a primer containing a phosphate group at the 3'-end; this group

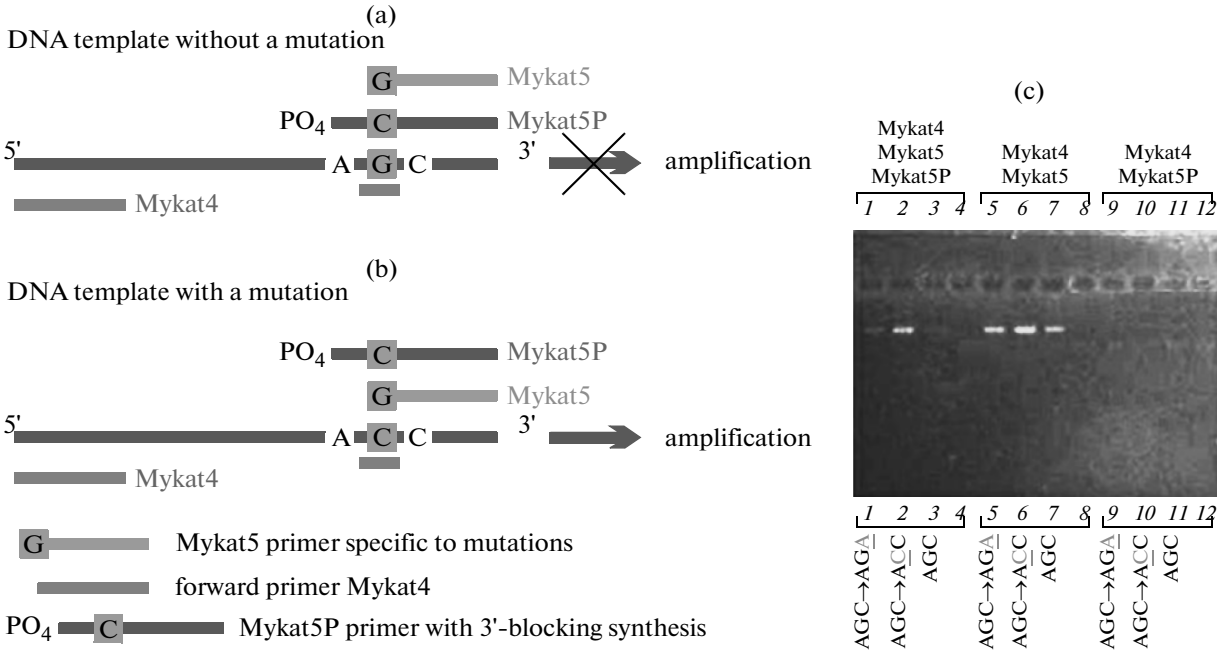

Fig. 2. Scheme of allele-specific PCR for the detection of the AGC ⟶ ACC point mutation in codon 315 of the *katG* gene with an additional blocking competitive reverse primer with the 3'-end phosphate group (for the elimination of the amplification of wild-type allele DNA). The 3'-end nucleotide of the Mykat5 primer specific to the mutation is complementary (a) and noncomplementary (b) to the nucleotide localized at the second position of codon 315; (c) detection of the AGC ⟶ ACC point mutation in codon 315 of the *katG* gene of *M. tuberculosis* DNA associated with isoniazid resistance after PCR with the set of primers Mykat4–Mykat5–Mykat5P and electrophoresis in 2% agarose gel; *1*, *5*, *9*—DNA of *M. tuberculosis*, strain HB385, containing mutation AGC ⟶ AGA; *2*, *6*, *10*—DNA of *M. tuberculosis*, strain HB125, containing mutation AGC ⟶ ACC; *3*, *7*, *11*—DNA of *M. tuberculosis*, strain H37Rv, negative control of amplification (codon 315–AGC); and *4*, *8*, *12*—water, negative control of amplification. In case of selection of an optimal annealing temperature, the set of primers Mykat4–Mykat5–Mykat5P allows for the differentiation between the mutations AGC ⟶ ACC (lane *2*) and AGC ⟶ AGA (lane *1*), as well as between the AGC sequence in codon 315 of the *katG2* gene of mycobacterial DNA (lane *3*). The positions of point mutations are underlined.

resulted in the termination of the synthesis of DNA polymerase.

Two sets of primers were designed in this study (Mykat4 –Mykat5–Mykat5P and Mykat4–Mykat9–Mykat9P) using a nucleotide with attached PO_4 group at the 3'-end. PCR with such primers permitted the detection of the following types of point mutations: AGC ⟶ ACC (Fig. 2c) and AGC ⟶ ACA, AGC ⟶ AGA, respectively.

The absence of an amplicon as a result of PCR with Mykat5 (and also with Mykat4) confirms that the competitive Mykat5 primer really blocks the synthesis of DNA polymerase (Fig. 2c). The absence of the blocking Mykat5P primer resulted in nonselective amplification of DNA fragments of *M. tuberculosis* of both wild type and mycobacteria containing different possible mutations in codon 315 of the *katG* gene (Fig. 2c, lanes *5*, *7*). The incorporation of a blocking primer only resulted in the synthesis of an amplicon that contained an AGC ⟶ ACC mutation (Fig. 2c, lane *2*) and to the impossibility of amplification of nonspecific fragments. Similar results were obtained for the set of primers Mykat4–Mykat9–Mykat9P, which permits the detection of the AGC ⟶ AGA mutation in codon 315 of the *katG* gene (the data are not shown).

Allele-specific PCR with LNA-modified primers is based on the following observation: the primer–single-stranded DNA template complex melting temperature (and, thus, the annealing temperature) for the mutant type is lower compared to the perfect duplex melting temperature. This means that in the presence of a point mutation in a site containing LNA nucleotide, the primer–single-stranded DNA template complex will have a lower melting temperature (T_m) compared to the perfect duplex. Concerning *M. tuberculosis*, the DNA molecules from the wild-type isolates are perfect duplexes, whereas the DNA molecules from the isoniazid-resistant isolates are characterized by the presence of mutations in codon 315 of the *katG* gene and, therefore, have a lower melting temperature. It should be noted that the value of a change in the LNA primer–DNA template complex T_m in the presence of oligonucleotide polymorphism depends on both the number of LNA monomers and the length of the primer.

The incorporation of LNA nucleotides increases the discrimination power of primers. In the LNA-modified primers, some nucleotides were replaced with LNA monomers; LNA monomers are the analogs of nucleotides containing the 2'-O,4'-C methylene

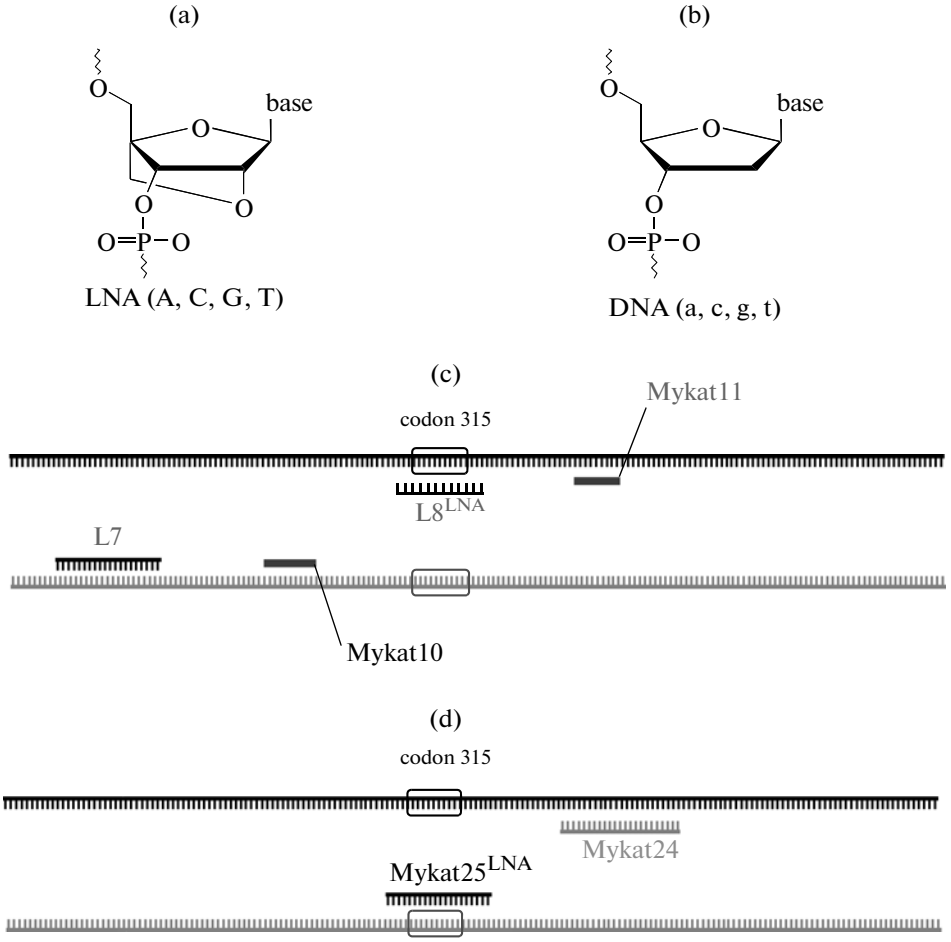

Fig. 3. Locked nucleic acid is a chemical analogue of DNA monomer (a), which can replace nucleotides in DNA and RNA sequences (b). Primers localization on the *katG* gene of mycobacteria tuberculosis DNA relatively to codon 315; the mutations in this codon are linked with resistance to the first-line antituberculosis drug isoniazid. The sets of primers L7–L8^{LNA} and Mykat24–Mykat25^{LNA} permit the amplification of 214-bp and 110-bp fragments of the *katG* gene, which contains codon 315. Each of the L8^{LNA} and Mykat25^{LNA} primers contains five LNA-modified nucleotides: two of them are flanking sequences, and three are complementary to the "+" (c) and "–" (d) strands in and around of the region of codon 315 of the *katG* gene. The set of primers Mykat10–Mykat11 was used as a marker of molecular mass for the amplification of a 151-bp fragment, which contains codon 315.

bridge (Fig. 3), which limits the conformational flexibility of the deoxyribose ring and transforms a monomer into a rigid structure. This results in the strengthening of the hybridization of a strand, which contains LNA monomers, with a complementary strand of the duplex. The incorporation of LNA nucleotides significantly increases the duplex thermal stability compared to the nonmodified DNA thermal stability. For a 20 bp duplex, the incorporation of one additional LNA monomer results in an increase in T_m by approximately 1°C. Therefore, to increase the discrimination power of primers, from one side, the content of LNA monomers should be elevated, and, from the other side, increasing the length of a primer (to enhance the specificity) results in a decrease in the relative content of LNA monomers (the number of LNA monomers remains unchanged); therefore, this results in a lower primer discrimination power.

In this study, to detect point mutations or single nucleotide polymorphisms in codon 315 of the MTB *katG* gene, two sets of primers (L7–L8^{LNA} and Mykat24–Mykat25^{LNA}) were developed; each of these sets contained one LNA-modified primer. Each of 17-nucleotide LNA-modified primers, i.e., Mykat25^{LNA} and L8^{LNA}, contains five LNA monomers; moreover, three LNA monomers from each primer are complementary to codon 315 of the *katG* gene, and two other primers flank this codon. The principal feature of these sets of primers is the different localization of the LNA-modified primer: in the L7– L8^{LNA} set, the LNA primer is complementary to "+" DNA strand, and in another set (Mykat24– Mykat25^{LNA}), it is complementary to "–" DNA strand (Fig. 3d).

An analysis of PCR amplification products has shown that the set of primers L7–L8^{LNA} permits the

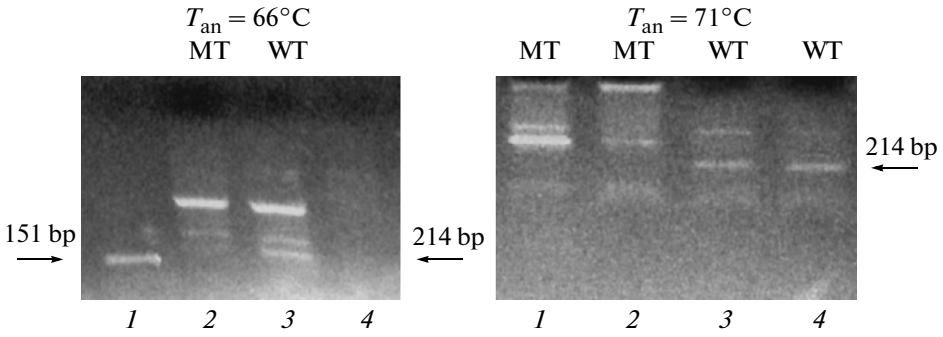

Fig. 4. Detection of the amplification products of a fragment containing the *katG* gene of mycobacteria tuberculosis' DNA of the wild type (WT) and isoniazid-resistant one (containing a mutation in codon 315 of the *katG* gene) (MT) after PCR with the set of primers L7–L8^{LNA} and electrophoresis in 1.5% agarose gel. L8^{LNA}-modified primer containing LNA nucleotides: (a) annealing temperature T_{an} is 66°C; *1* is the molecular mass marker is a 151 bp fragment (which contains codon 315) after amplification with the set of primers Mykat10–Mykat11; *2*, *3* are amplification with the set of primers L7–L8^{LNA} of mycobacteria tuberculosis DNA, which is mutant by codon 315 of the *katG* gene (*2*) and mycobacteria of wild-type tuberculosis (*3*); and *4* is negative control of amplification. The presence of a mutation in codon 315 results in the absence of the 214 bp amplicon band (*2*), which characterizes wild-type isolates (*3*); (b) annealing temperature T_{an} is 71°C. Amplification of the 214 bp fragment with the set of primers L7–L8^{LNA} for mycobacteria tuberculosis resistant to isoniazid (*1*, *2*) and for DNA of wild-type isolates (*3*, *4*).

amplification of the 214 bp fragment of the *katG* gene at annealing temperatures of 66°C (Fig. 4a, lane *3*) and 71°C (Fig. 4b, lanes *3*, *4*) (a 214 bp fragment length was expected). An analysis of PCR products of MTB DNA amplification at a low annealing temperature has shown that both primer sets permit the amplification of a fragment of the *katG* gene; the length of a 214 bp fragment was expected for the primer set L7–L8^{LNA} and the length of a 110 bp fragment was expected for the primer set Mykat24–Mykat25^{LNA} (the data are not shown). The intensity of an amplicon band was extremely low at T_{an} = 58°C. Moreover, at T_{an} = 66–71°C, the amplification of the *katG* gene with the Mykat24–Mykat25^{LNA} primer set did not occur; under these annealing temperatures (66–71°C), the differentiation of the mutant and wild-type MTB isolates by the *katG* gene was possible by PCR with the set of primers L7–L8^{LNA}. Therefore, most of the experiments were only carried out with the primer set L7–L8^{LNA}.

PCR with the use of the set of primers L7–L8^{LNA} permits the differentiation between mutant and wild types of the MTB *katG* gene (Fig. 4, lanes *3*, *4*): for the wild type of the *katG* gene, a 214 bp fragment is amplified; for the mutant MTB isolate, the amplicon is absent (although, amplification occurs, which is confirmed by the presence of a nonspecific amplicon band, formed as a result of the involvement of the short-length L8^{LNA} primer) in the reaction. The intensity of a nonspecific amplicon band decreases when T_{an} increases from 66 to 71°C (in this case, we conducted two-step PCR, which permitted accurate differentiation between mutant and wild-type MTB isolates).

The Mykat10–Mykat11 primer set served as control. PCR with this set permits the amplification of a 151 bp fragment of the *katG* gene; this fragment contains a locus with codon 315 (Fig. 4a, lane *1*). The following sequentcing procedure of this short PCR product permits the determination of its sequence and identification of the mutation type in codon 315 of the *katG* gene for *M. tuberculosis* clinical isolates circulating in the region.

For the purpose of quality control of the chemical synthesis of single-stranded oligonucleotides with LNA monomers, we used time-of-flight MALDI mass spectrometry. Recorded MALDI mass spectra of the oligonucleotides L8^{LNA} and Mykat25^{LNA} (Figs. 5a–5d) registered during the experiments were used for control of removing protection from reactive groups of nucleotide bases and also for detection of fraction of oligonucleotides with shorter lengths.

In the mass spectra of LNA-modified oligonucleotides (Figs. 5a, 5b), we registered peaks with mass/charge ratios (m/ch) of 5373.8 (L8^{LNA}) and 5347.3 (Mykat25^{LNA}), which confirms the presence of LNA monomers in the primers (Table 2).

The use of MALDI mass spectra with a high resolution (Figs. 5c, 5d) revealed the presence of nonremoved protecting groups on reactive nucleotide groups. It is known that to prevent the oligonucleotide strand branching during the elongation amino groups in phosphoramidites, the dG, dA, and dC nucleotides are blocked [16]. In the presence of nonremoved protecting groups in the nucleotide mass spectrum, additional peaks of large mass, along with a expected main band, are observed. For example, isobutyryl (for dG) and benzoyl (for dA and dC) used for the protection of amino groups increase the oligonucleotide mass by 70 and 104 Da, respectively [17].

The mass spectrum of L8^{LNA} (Fig. 5a) also contained a peak of a lower m/ch ratio of 4755.7 besides the main peak of the predicted m/z ratio of 5373.8; a lower mass peak can correspond to a shorter oligonucleotide of 15 nucleotides. The detection of such a

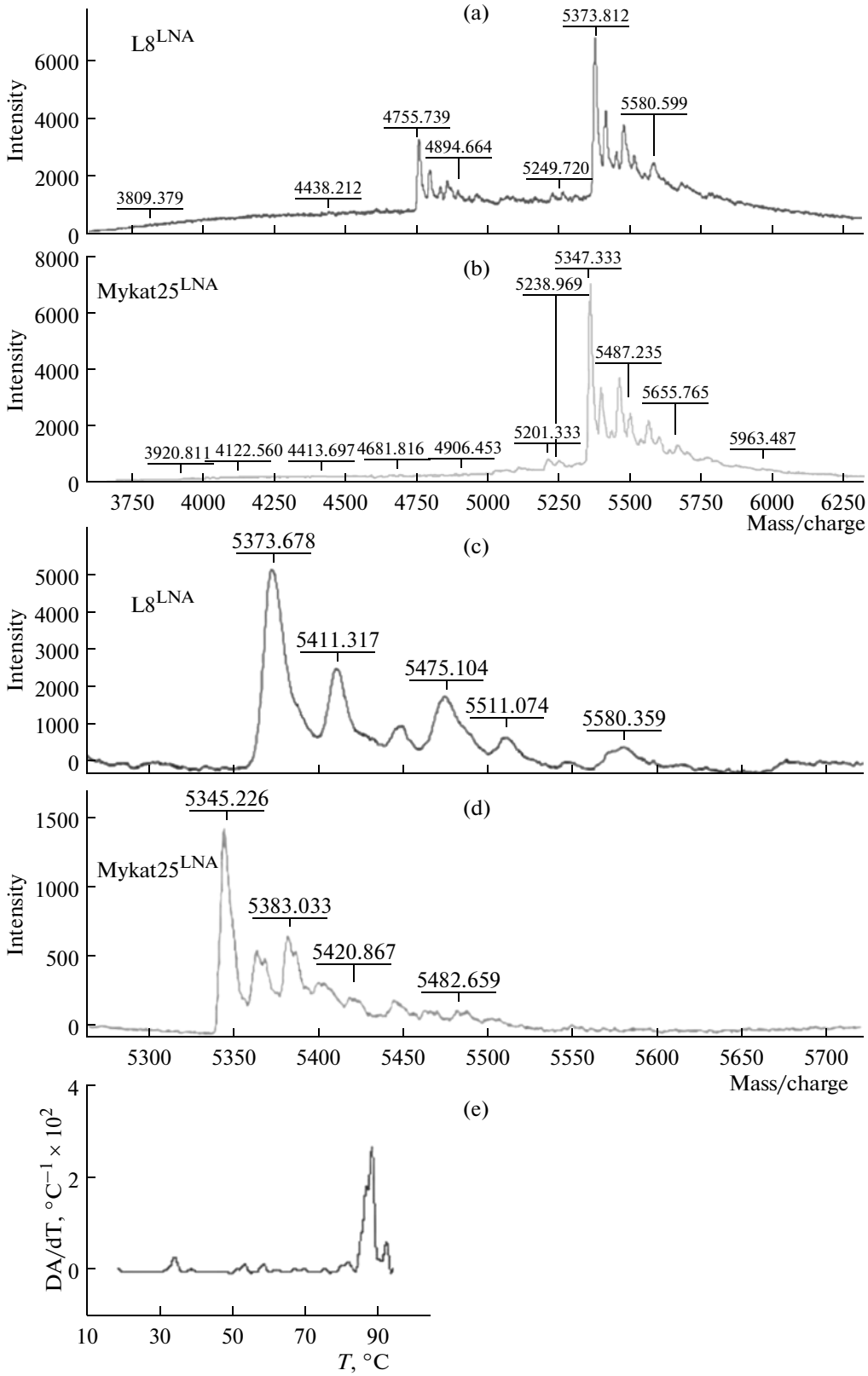

Fig. 5. Mass spectra of time-of-flight matrix-activated laser desorption/ionization (MALDI) of 17 bp oligonucleotides $L8^{LNA}$ (a) and $Mykat25^{LNA}$ (b) containing five LNA monomers. The MALDI mass spectra of the $L8^{LNA}$ primer (c) and $Mykat25^{LNA}$ primer (d) with a high resolution. The diferential melting profile of duplex which was formed by the $L8^{LNA}$–$Mykat25^{LNA}$ oligonucleotides. The melting was performed in a 1-mM solution of sodium cacodylate (pH 7.0) at a heating rate of 0.5°C/min (e).

Table 2. Parameters of oligonucleotides that contain LNA-modified monomers, which were characterized by time-of-flight MALDI mass spectrometry

Oligonucleotide	Sequence	Formula/molecular mass	
		unmodified oligonucleotide	oligonucleotide containing LNA monomers
Mykat25^{LNA}	5'-gatcacCAGCGgcatcg-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'-cgatgcCGCTGgtgatc-3'	$C_{165}H_{209}N_{63}O_{102}P_{16}$ 5202	$C_{170}H_{209}N_{63}O_{107}P_{16}$ 5342*

Note: LNA-modified nucleotides are capitalized and underlined. *Compounds, expected formulas, and the molecular mass.

shorter nucleotide in the MALDI mass spectrum explains the high intensity of the nonspecific amplicon, which is formed during PCR with the L7–L8^{LNA} set of primers. The square ratio under the predicted mass with m/ch of 5373.8 and a lower mass with m/ch of 4755.7 shows that the 15 bp oligonucleotide accounts for 25% of the total amount of L8^{LNA}; this value is sufficient for PCR (the concentration of the L8^{LNA} primer used for PCR was 0.5–1 μM).

The presence of a group of peaks instead of the presence of one peak of predicted mass (as it should be in the case of correct synthesis) is a substantial disadvantage of synthesized oligonucleotides containing LNA monomers (Table 2) [18]. It is possible that the appearance of most peaks could be a result of the incomplete desalinization of the specimens containing oligonucleotide.

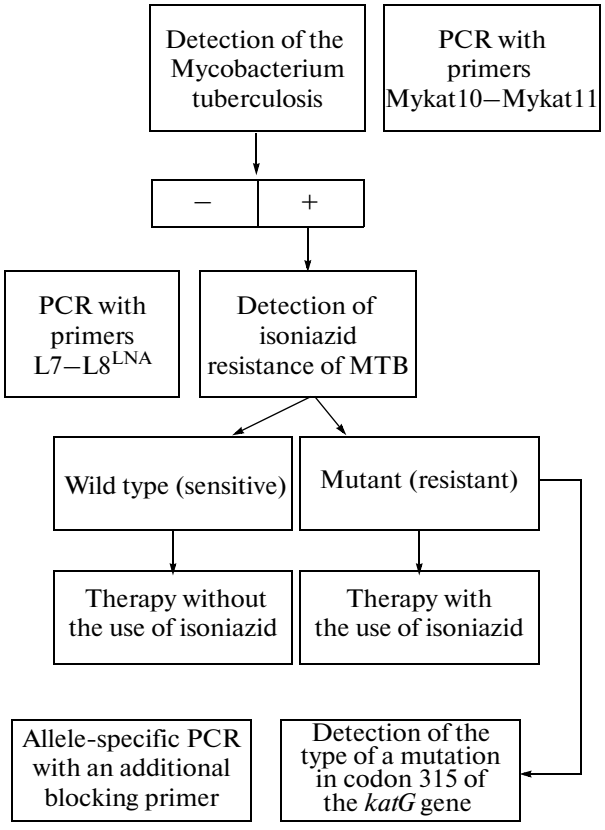

Fig. 6. Scheme of an analysis of a clinical specimen on the presence of mycobacteria tuberculosis, detection of resistance/sensitivity of an MTB isolate to isoniazid and the type of a point mutation in codon 315 of the *katG* gene associated with isoniazid resistance.

The L8^{LNA} and Mykat25^{LNA} primers were designed from two different sets of primers in the following way: they are complementary to one another and can form a 17 bp duplex. The theoretically calculated value of melting temperature (T_m) of the L8–Mykat25 unmodified duplex is 45°C at an ionic strength of 1 mM Na^+ (I). The replacement of five monomers in each strand with LNA monomers results in an increase in the melting temperature (by 88°C) of L8^{LNA}–Mykat25^{LNA} (Fig. 5e). This spectrum shows that the contribution of one pair of LNA-modified nucleotides to the increase in the melting temperature of the 17 bp duplex is 8.6°C, and that for one LNA nucleotide is 4.3°C. At the same time, for the 17 bp oligonucleotide duplex L8^{LNQ}–Mykat25, the modification of one strand results in an increase in the melting temperature by 2.8°C (it was determined using the theoretically calculated value of melting temperature ΔT_m; the data are not shown). Thus, the LNA modification of five nucleotides in two strands of the duplex results in significantly stronger duplex stabilization than it was possible to expect proceeding from theoretical calculations and extrapolation of data on single-stranded DNA modification to the case of double-stranded LNA modification of the duplex. It should be noted that the theoretical determination of T_m using software [15] is only possible in case of LNA modification of one strand of the duplex.

The experiments on melting of the oligonucleotide duplex L8^{LNA}–Mykat25^{LNA} at the ionic strength I = 5 mM Na^+ and I = 50 mM Na^+ carried out in this study permitted only the registration of the beginning of the melting interval due to high T_m of the duplex (the results are not shown). This is explained by the increase in the ionic strength of the solution approximately in proportion to the logarithm of the cationic concentration [19].

Based on the technologies developed for the detection of wild-type and mutant MTB isolates associated with isoniazid resistance, we proposed a scheme of the analysis of MTB presence in clinical specimens; a

detection method of isoniazid resistance, and, in the case of presence, updating the type of a point mutation (Fig. 6). From our point of view, the peculiar feature of this design is the possibility of fast detection of wild-type MTB (i.e., sensitive to isoniazid), which permits an immediate beginning of chemical therapy, including the use of isoniazid. For the purpose of detection of whether a clinical isolate of *M. tuberculosis* belongs to the wild type or to a mutant strain, it is necessary to carry out the detection of six variants of point mutations in codon 315 of the MTB *katG* gene, using multiplex PCR with six pairs of primers. At the same time, the use of conventional PCR with LNA-modified primers permits a significant simplification of the design of a set of primers. In this case, it is sufficient to use only one pair of primers if one of them contains 3–5 LNA nucleotides complementary (or complementary and flanking) to the codon containing the mutations examined during the test.

The designed primers enhanced by LNA monomers are directed toward the standard variant of clinical laboratory equipment without using real-time PCR. However, taking into account the impressive tempo of development of medical biotechnology, in general, and the fast propagation of the real-time PCR technology, especially, we can expect the modernization of diagnostic laboratories through the placement and installation of devices for real-time PCR. To obtain optimal real-time PCR results, it is necessary to get a high level of purification of primers and probes to minimize the effects of background fluorescence.

Taking into account the statement mentioned above, PAGE oligonucleotide purification (based on their molecular mass) permits the removing of a major part of shortened oligomers, although it has a tendency to decrease the total yield of oligonucleotide. The use of double purification (by HPLC after conducting PAGE) permits the reaching of the highest level of purification; however, the yield is minimal. At the same time, using the reverse phase HPLC results in a high level of purification and yield of the product.

The appearance of the first high-tech systems with a closed-circuit cycle, such as the GeneXpert System (Cepheid Inc., United States) [20], which are based on real-time PCR, shortened the genotyping time or analysis of specimens on the presence of an infectious factor to 30–45 min, and the detection of rifampicin and isoniazid resistance of MTB isolates was shortened to 2 h. Such systems can serve as an alternative to highly expensive reference laboratories, because they do not need specialized staff and laboratory space. It is important that clinical material (for example, a drop of blood) is transferred into a disposable cartridge, which contains lyophilized beads with components for PCR (DNA polymerase, nucleotides, primers, scorpion probes, and primers for wild-type and mutant DNA).

Thus, two methods of detection of point mutations in codon 315 of the *katG* gene of *Mycobacterium tuberculosis* were elaborated. In one of them, the application of the molecular technology (based on conventional PCR utilizing the additional blocking primer with the phosphate group at the 3'-end) for the detection of variants of point mutations in codon 315 of the *katG* gene of *Mycobacterium tuberculosis*, which are associated with resistance to isoniazid (an antituberculosis drug of the first line), facilitate the increase in the discrimination power of primers. Two sets of primers were designed in the study: Mykat4–Mykat5–Mykat5P and Mykat4–Mykat9–Mykat9P. PCR with these primers permits the detection of point mutations, such as AGC $\longrightarrow$ ACC and AGC $\longrightarrow$ ACA, AGC $\longrightarrow$ AGA, respectively. The other method utilizing PCR with a set of primers, one of which contains LNA monomers complementary to the sequence of codon 315 of the *katG* gene of MTB, is helpful in differentiating between wild-type and mutant *Mycobacterium tuberculosis*.

The created molecular genetic test systems applied for the detection of wild-type MTB isolates resistant to isoniazid (an antituberculosis drug of the first line) may be used in clinical laboratories with conventional PCR equipment; in our opinion, such test systems will permit for the shortening of the time required for the detection of isoniazid resistance of MTB from 1–3 months in the case of using traditional bacteriological methods to 1–3 days in the case of using PCR.

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