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Characterization of Oligonucleotides with LNA-Monomers for PCR Detection of Point Mutations in *Mycobacterium Tuberculosis* Genome

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Abstract—Point mutations associated with isoniazid resistance in*Mycobacterium tuberculosis* (MTB) have been analyzed in codon 315 of the katG gene by conventional polymerase chain reaction (PCR) using primers containing locked nucleic acid (LNA) modified nucleotides. Purity and structure of primers containing 5 LNA monomers of 17 nucleotides in length were characterized by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and a 17-mer duplex formed by two complementary oligonucleotides was characterized by the method of thermal denaturation. The duplex containing five LNA monomers per each strand was characterized by a higher melting temperature than it was expected using extrapolation of theoretical calculation for nucleotide modification of one strand of the duplex. Detection of any of six possible mutations in *katG* codon 315 (i.e. discrimination between sensitive and resistant MTB) requires just one PCR employing a set of two primers with one LNA-modified primer; this is an important advantage of oligonucleotides containing LNA over unmodified nucleotides: employment of multiplex PCR would require up to 12 primers. Problems of control of oligonucleotide modification by LNA monomers are discussed.

Keywords: DNA melting, point mutation, polymerase chain reaction, mycobacteria tuberculosis, locked nucleic acid, mass spectrometry with matrix-assisted laser desorption/ionization

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INTRODUCTION

The main solution of the problem of control of the spread of drug-resistance tuberculosis consists in its early diagnostics; this will allow to control the spread of particular strain of *Mycobacterium tuberculosis* (MTB) and correct schemes of chemotherapy in each case. Several traditional classic methods for detection of MTB resistance to anti-tuberculosis drugs have been approved by WHO. Determination of a spectrum of MTB drug resistance usually takes from 1 to 3 months; during this period primary patients are treated with standard first-line anti-tuberculosis drugs. Since resistance of clinical isolates of MTB to the prescribed drugs remains unknown certain evidence exists that such therapy will be ineffective. Rapid detection of resistance and especially multiple drug resistance of MTB represents a very important problem for selection of effective tools for anti-tuberculosis (anti-TB) therapy. This problem could be solved at the genotype level and this would shorten duration of corresponding analysis to 1–3 days.

Allele-specific PCR is a widely used technology for detection of point mutations: this approach requires construction of such primers that could be annealed at sites with point mutations. Ideally, use of a primer complementary to a specific sequence would amplify only one variant but in reality significant amplification of variants with primer annealing with targets containing non-complementary nucleotides occurs.

Allele-specific PCR with LNA-modified primers is based on the fact that melting temperature (and consequently, annealing temperature) of the complex primer-single stranded DNA is lower for the mutant type than for a perfect matched duplex. This means that in the presence of a mutation at the site containing LNA (locked nucleic acid) nucleotide the mis-

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matched complex primer-single stranded DNA template will have lower melting temperature (T_m) than the perfect duplex. Insertion of LNA nucleotides increases discrimination power of the primer. In LNA-modified primers some nucleotides are substituted by LNA monomers, nucleotide analogues containing the methylene bridge between 2'-O, 4'-C (Fig. 1), which limits conformational flexibility of the deoxyribose moiety and transforms the monomer into a rigid structure. This results in increased hybridization of the strand containing LNA monomers with the complementary strand of the duplex. Insertion of LNA nucleotides significantly increases thermal stability of the duplex compared with unmodified DNA (Fig. 1c). For example, in the case of a 13-mer oligonucleotide duplex insertion of 9 and 10 substituted LNA monomers increased T_m by 25°C and 28°C, respectively. Thus, for the 13-mer duplex contribution of one LNA monomer to the increase in T_m (ΔT_m) is about 3°C. This means that in the presence of point mutation at the site containing LNA nucleotide the complex primer-single stranded template will have lower melting temperature compared with the perfect matched duplex.

Streptomycin and isoniazid resistance make major contribution to anti-TB drug resistance; isoniazid resistance is mainly associated with mutations located in codon 315 of the *katG* gene encoding MTB catalase-peroxidase. Probability isoniazid resistance associated with mutations in codon 315 of the katG varies from 50 to more than 90% (in various geographic regions) [1, 2].

MTB DNA molecules from wild type isolates represent perfect duplexes, while DNAs from isoniazid resistant isolates are characterized by the presence of mutations in the katG codone 315 and therefore by lower melting temperature. These mutations are mainly found at the second and third (but not the first) positions of codon 315 and so six mutation variants have been detected in codon 315 [3–5]. It should be mentioned that in the presence of single nucleotide polymorphism changes in T_m of the complex LNA primer—DNA template depend on both the number of LNA monomers and length of the primer. In the case of a 20-mer duplex insertion of an additional LNA monomer results in the T_m increase by about 1°C. Consequently, discriminating power of primer may be increased by high relative content of LNA monomers. On the other hand, the increase in primer length (required for increased specificity) results in a decrease of relative contribution of the LNA monomer (at their preserved quantity) and therefore to decreased discriminating potency of such primer.

In this study, using modern achievement in chemical synthesis of oligonucleotide primers containing LNA-modified nucleotides we have developed molecular genetic test systems for PCR detection of mutations in codon 315 of the *katG* MTB gene. The primers

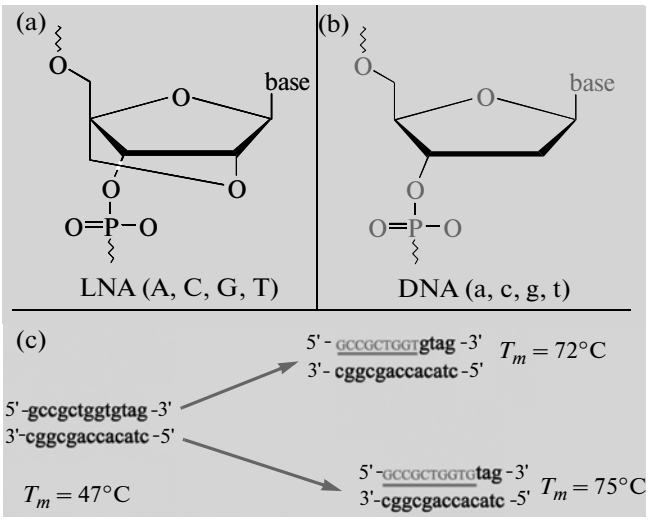

Fig. 1. Locked nucleic acid (LNA), a new analogue of a DNA monomer (a), which can replace nucleotides in DNA and RNA sequences (b). LNA follows Watson-Crick base pairing but is characterized by increased specificity and affinity during hybridization. Constructed mixed LNA/DNA oligonucleotides (c) facilitate control of melting of samples and primers and also optimize sequence discrimination at a current temperature. Insertion of an additional LNA-modified monomer for a 13-mer oligonucleotide containing 9 LNA-modified nucleotides (c) resulted in the increase of temperature of oligonucleotide melting by 3°C.

with LNA-modified monomers were characterized by matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) and their duplexes were characterized by the method of thermal denaturation. Using the developed kits containing LNA-modified primers it is possible to detect any of six possible mutations in codon 315 by means of just one reaction. Consequently, proposed test is applicable for discrimination of wild type and mutant MTB (carrying mutation in *katG* codon 315), i.e. MTB strains sensitive and resistant to the first-line anti-TB drug, isoniazid, respectively.

MATERIALS AND METHODS

UV Spectroscopy of Oligonucleotide Duplexes

Thermal denaturation with registration of optical density at 260 nm was used for characterization of oligonucleotide duplexes (each of which contained 5 complementary LNA-modified nucleotides). It should be noted that analysis of duplex melting at 270 nm is more correct because at this wavelength contribution of extinction coefficients of AT and GC pairs is the same. The UV studies were performed using a Specord M40 spectrophotometer (Carl Zeiss Jena, Germany) equipped with a temperature-con-

trolled cell holder. Optical density was registered at the temperature interval of 0.05°C and the heating rate of 0.5°C/min. Experiments on thermal denaturation were performed in microcuvettes with an optical path-length of 1 cm. Duplex melting was performed in 1, 10, and 50 mM sodium cacodylate buffer, pH 7.0, using DNA concentrations of 20–20 pmol/μl. Differential melting profiles were obtained by differentiation of the integral melting profile using the Origin 7.0 software (USA).

Polymerase Chain Reaction

The developed sets of primers for detection of wild type (WT) and isoniazid-resistant MTB isolates were tested using a DNA collection extracted from WT MTB isolates and also isoniazid-resistant MTB clinical isolates with mutations (MT) in *katG* codon 315. Testing of the sets of primers was performed using MTB DNA with the point mutations AGC ⟶ ACC, AGC ⟶ ACG, and AGC ⟶ AGA in codon 315; these samples were obtained from isolates characterized by the level of isoniazid resistance of 1–25 μg/μ.

PCR was performed using a dry amplification kit (Isogen, Russia) in 20–50 μl of the reaction mixture containing 4 mM NaCl, 50 mM KCl, 12 mM Tris HCl, pH 8.0,2.5 mM MgCl2,200 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 which were purchased from Exiqon (Denmark, http://www.exiqon.com/lna-technology).

Conventional PCR was performed using a thermocycler manufactured by the Scientific company Tochnost' (Tula, Russia) under the following reaction conditions: pre-denaturation at 95°C for 2 min followed by 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 two-step PCR was performed under the following reaction conditions: pre-denaturation at 95°C for 2 min followed by 45 cycles including denaturation at 95°C for 1 min, annealing and elongation at 71°C for 1 min. The annealing temperatures T_{an} were optimized in a series of reactions with stepwise increase of T_{an} (2–0.5°C per step) until the complete elimination of the nonspecific amplicon. The template contained 0.05 μg of genomic MTB DNA. For amplifon visualization PCR reaction products (10–20 μl) were resolved by electrophoresis in 1.5% agarose gel containing ethidium bromide at a field strength of 10 V/cm. Annealing temperature of the complex LNA-modified oligonucleotide—single stranded DNA was calculated by a free program available at http://lna-tm.com [6], thermodynamic analysis of primers and amplicons was performed using the MeltCalc program [7, 8].

Mass Spectrometry of Oligonucleotides

The structures of LNA-modified oligonucleotides were characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF). Aliquots of the oligonucleotides purified by PAAG electrophoresis (0.5 μl) were applied to matrix crystals obtained by drying of aqueous matrix template solution containing 3-hydroxypicolinic acid (50 mg/ml) and dibasic ammonium citrate (2 mg/ml) on a stainless steel target.

The target was dried at room temperature until complete evaporation of the solvents and placed in the source of an Autoflex II mass spectrometer (Bruker Daltonics Inc., Germany) equipped with a nitrogen laser (λ = 337 nm), which allowed pulsed ion extraction. Mass spectra of oligonucleotides 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 was 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 spectra.

RESULTS AND DISCUSSION

Studies of molecular mechanisms of drug resistance have shown that MTB resistance to particular drugs is determined by single mutations in certain genes. Earlier, for detection of point mutations in genomes of pathogens we used PCR with a primer containing non-complementary nucleotide at 3'-end to a DNA template of one of investigated species (in the case of gene typing) [9].

In this study for detection of point mutations or single nucleotide polymorphisms in the MTB *katG* gene codon 315 we have developed two sets of 17-mer primers, each of which contained one LNA-modified primer (Mykat25^{LNA} and L8^{LNA}). Each LNA-modified primer contain 5 LNA-monomers: three LNA monomers of each primer were complementary to *katG* codon 315 and two others flank this codon. The principal difference between these two sets of primers consists in localization of the LNA-modified primer: in one set (L7–L8^{LNA}) the LNA primer is complementary to the "+" strand of DNA (Fig. 2a), whereas in the other primer (Mykat24–Mykat25^{LNA}) the LNA primer is complementary to the "—" strand of DNA (Fig. 2b).

Analysis of amplification products at low T_{an} shows that both primer sets amplified corresponding *katG* fragments of expected length: 214 bp for the primer set L7—L8^{LNA} (Fig. 3, tracks *3*, *4*) and 110 bp (Fig. 3, tracks *5*, *6*) for the primer set Mykat24—Mykat25^{LNA}. Since band intensity for the 110 bp amplicon (Fig. 3, tracks *5*, *6*) was extremely low at T_{an} = 58°C, all subse-

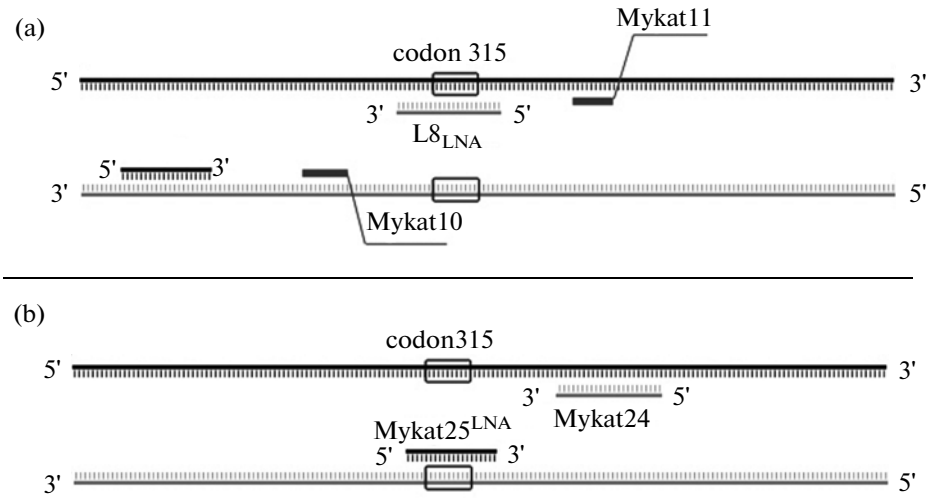

Fig. 2. Localization of primers on the *katG* gene of *Mycobacterium tuberculosis* DNA versus codon 315; mutations in this codon are linked with resistance to the antituberculosis drug isoniazid. Using sets of primers L7–L8^{LNA} and Mykat24–Mykat25^{LNA} it is possible to amplify the fragments of 214 and 110 base pairs of the *katG* gene containing codon 315. Each primer, L8^{LNA} and Mykat25^{LNA}, contains five LNA-modified nucleotides, two of which flank and three of which are complementary to the "+"strand (a) and the "—" strand (b) of the codon 315 region of the *katG* gene. The set of primers Mykat10–Mykat11 was used for amplification of the fragment of 151 bp in length containing codon 315 as a molecular mass marker.

quent experiments were performed using the primer set L7–L8^{LNA}.

Analysis of PCR amplification products showed that the primer L7–L8^{LNA} primer sets amplified the *katG* fragment of expected length of 214 bp at both annealing temperatures 66°C (Fig. 4a, track 3) and 71°C (Fig. 4b, tracks *3*, *4*). Using PCR with the primer set L7–L8^{LNA} it is possible to discriminate mutant (MT) and wild type (WT) variants of MTB *katG* gene (Fig. 4b, tracks *3*, *4*). In the case of WT *katG* a gene fragment of 214 bp was amplified, while in the case of a MT MTB isolate such amplicon was not detected despite the presence of a band of nonspecific amplicon suggesting that amplification occurred (appearance of this nonspecific band can be attributed to rather short length of the L8^{LNA} primer). The intensity of the nonspecific band decreased with the T_{an} increase from 66 to 71°C (in this case, two-step PCR was performed for reliable discrimination of MT and WT MTB isolates).

The L8^{LNA} and Mykat25^{LNA} primers from two different primer sets were designed so that they were complementary to each other and able to form a 17-mer double-stranded oligonucleotide. Theoretically calculated melting temperature (T_m) for the unmodified L8–Mykat25 duplex was 45°C at the ionic strength (I) of 1 mM Na^+. Modification of each strand with five LNA monomers sharply increased T_m of the L8^{LNA}–Mykat25^{LNA} duplex to 88°C (Fig. 5). One can see, that the contribution of one pair of LNA monomers to the T_m increase of the 17-mer duplex was 8.6°C and that of a single LNA monomer was 4.3°C. At the same time, for the 17-mer oligonucleotide duplex L8^{LNA}–Mykat25 (containing modified nucleotides only in one strand), the theoretically calculated ΔT_m value was just 2.8°C. Thus, LNA-modification of five nucleotides in each strand results in higher duplex stabilization, than it was predicted by theoretical calculations based on extrapolation of data on single stranded LNA modification to the case of LNA modification of both strands of the duplex. (It should be mentioned that theoretical calculation of T_m using the

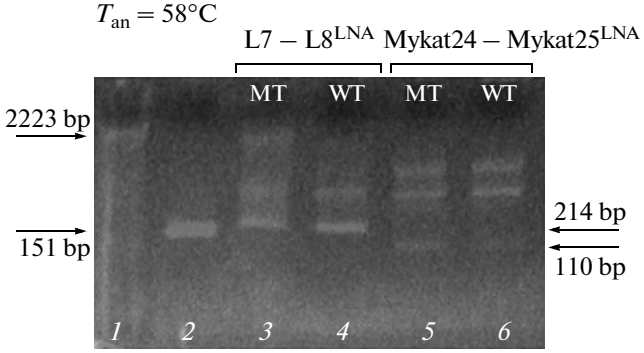

Fig. 3. Detection of amplification products of the *katG* gene fragment of wild type (WT) and isoniazid-resistant (carrying mutation in *katG* codon 315; MT) *Mycobacterium tuberculosis* strains after PCR with a set of primers L7—L8^{LNA} and electrophoresis in 1.5% agarose gel. The annealing temperature (T_{an}) was 58°C. *1*–amplification of full length *katG* (2223 bp); *2*–a fragment of 151 bp containing condon 315; *3*, *4*–a fragment of 214 bp after DNA amplification of isoniazid-resistant (*3*) and wild type (*4*) MTB with the set of primers L7—L8^{LNA}; *5*, *6*–a fragment of 110 bp after DNA amplification of isoniazid-resistant (*5*) and wild type (*6*) MTB with the set of primers Mykat24—Mykat25^{LNA}. L8^{LNA} and Mykat25^{LNA} are modified primers containing LNA nucleotides.

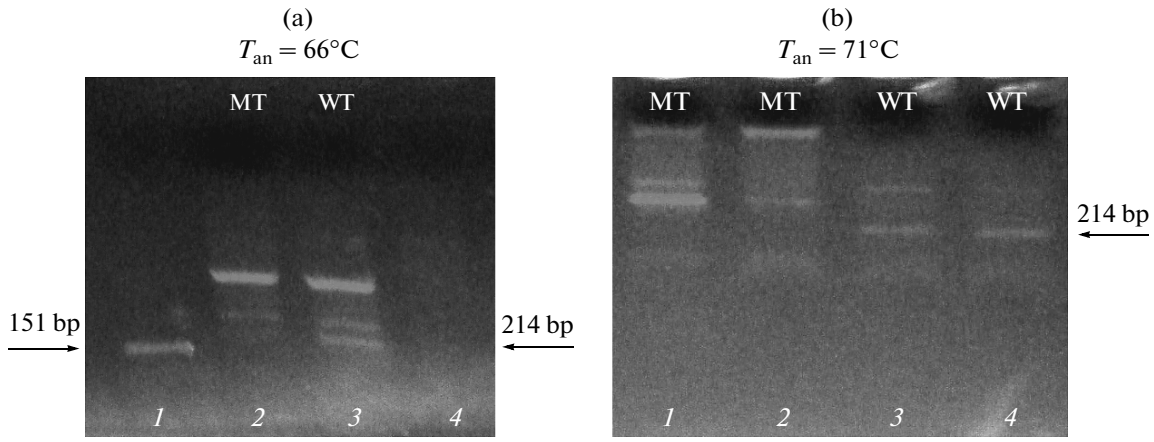

Fig. 4. Detection of amplification products of the *katG* gene fragment of wild type (WT) and isoniazid-resistant (carrying mutation in *katG* codon 315; MT) *Mycobacterium tuberculosis* strains after PCR with a set of primers L7–L8^{LNA} and electrophoresis in 1.5% agarose gel. L8^{LNA} is a modified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

program [7, 8] is only possible for the case of LNA modification of one strand).

Experiments on melting of the L8^{LNA}–Mykat25^{LNA} duplex at different ionic strength (I = 5 mM Na^+ and I = 50 mM Na^+) revealed only an initial melting interval due to high T_m of the duplex (data not shown). This may be explained by the fact that the increase in melting temperature of the duplex is proportional to the logarithm of cation concentration [10].

MALDI-TOF mass spectrometry is one of the most effective methods of analysis of macromolecules. For nucleic acids MALDI mass spectra ranged from 600 Da to 90 kDa (about 300 nucleotides in length) were obtained; interpretation of results below 600 kDa is complicated due to the absence of matrix ions. Methods used for preparation of nucleic acid sample are very important for registration of high quality mass spectra. The latter is influenced by many factors: purity of samples, solvent composition, matrix composition and surface properties of a substrate.

Table 1. Theoretically calculated melting temperature T_m (°C) of the unmodified duplex (L8—Mykat25) and the duplex with 5 LNA monomers in one strand (L8^{LNA}–Mykat25) at various ionic strength (I) and total concentration of primers 0.5 μM

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

In this study MALDI-TOF mass spectrometry was used for control of quality of chemical synthesized single stranded oligonucleotides containing LNA monomers. Registered MALDI mass spectra of L8^{LNA} and Mykat25^{LNA} (Figs. 6, 7) were used for control of removal of protecting groups from nucleotide bases (i) and also for detection of contaminant oligonucleotides lacking some monomers (ii). Mass spectra of LNA-modified oligonucleotides (Fig. 6) were characterized by the presence of peaks with the mass/charge ratio (m/z) of 5373.8 (L8^{LNA}) and 5347.3 (Mykat25^{LNA}); these peaks confirm the presence of LNA monomers in these primers (Table 2). The peaks with these masses were assigned to the cluster ions $[L8^{LNA} + Na]^+$ and $[Mykat25_{LNA} + Na]^+$.

MALDI mass spectra of higher resolution (Fig. 7) revealed the presence of nucleotides with protecting groups. (It is known that for prevention of oligonucleotide strand branching amino groups of nitrogenous bases are blocked in dG, dA, and dC phosphoramidites [11]). In the case of deblocked protecting groups, besides main (expected) band the oligonucleotide mass spectrum is characterized by the presence of an additional peak of higher mass. For example, isobutyryl (for dG) and benzoyl (dA and dC) used for amino group protection increased oligonucleotide mass by 70 and 104 Da, respectively [12].

In addition to the main peak (Fig. 5a) with predicted m/z of 5373.8, the mass spectrum of L8^{LNA} also contained a peak of lower m/z (4755.7), which may correspond to a shorter oligonucleotide of 15 nucleotides in length. Detection of such sorter oligonucleotide in the MALDI mass spectrum explains high

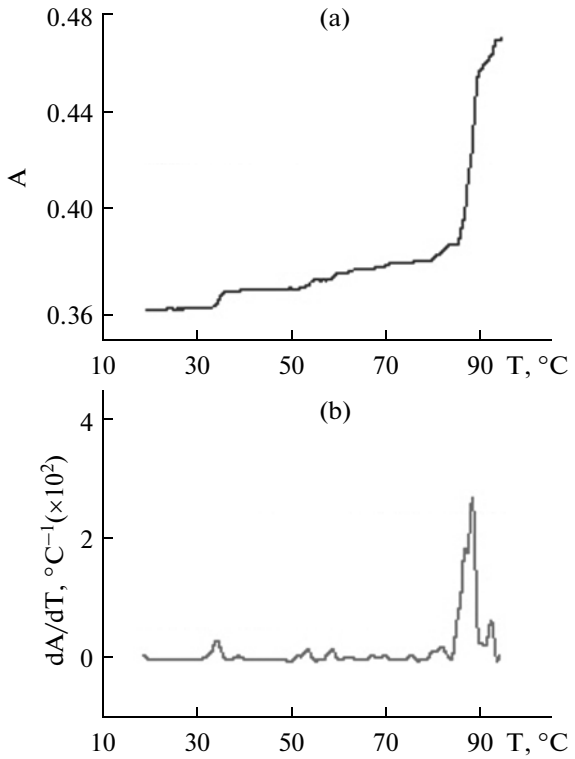

Fig. 5. Integral (a) and differential (b) melting profiles for a duplex formed by 17-mer oligonucleotides ($L8^{LNA}$–$Mykat25^{LNA}$), each of which contains 5 LNA-modified nucleotides. Melting was performed in 1 mM sodium cacodylate (pH 7.0).

intensity of the nonspecific amplicon formed during PCR with the L7–$L8^{LNA}$ primers (Fig. 4a, tracks *2*, *3*; Fig. 4b, tracks *1*, *2*). The ratio of areas under the peaks with predicted (*m/z*) 5373.8) and lower (*m/z*) 4755.7) masses shows that the 15-mer nucleotide represents about 25% of the total amount of $L8^{LNA}$; this values is sufficient for PCR (concentration of the $L8^{LNA}$ primer used for PCR was 0.5–1 μM).

Existence of several peaks (Figs. 6, 7) instead of one peak with predicted mass (Table 2) expected in the case of correct synthesis [13] is an essential drawback of the synthesized oligonucleotides with LNA monomers. It is possible that most these additional peaks originate from incomplete desalination of the sample containing this oligonucleotide.

CONCLUSIONS

Using LNA-modified primers it is possible to detect point mutations in genomes of drug-resistant MTB by allele-specific PCR; this does not require additional amplicon sequencing needed for differenti-

Table 2. Parameters of oligonucleotides containing LNA-modified monomers, which were characterized by MALDI-TOF mass spectrometry. LNA-modified nucleotides are shown in capital letters. Formulas and molecular masses of expected compounds are shown in bold

Oligonucleotide	Sequence	Formula/molecular mass	
		Unmodified oligonucleotide	Oligonucleotide with **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**

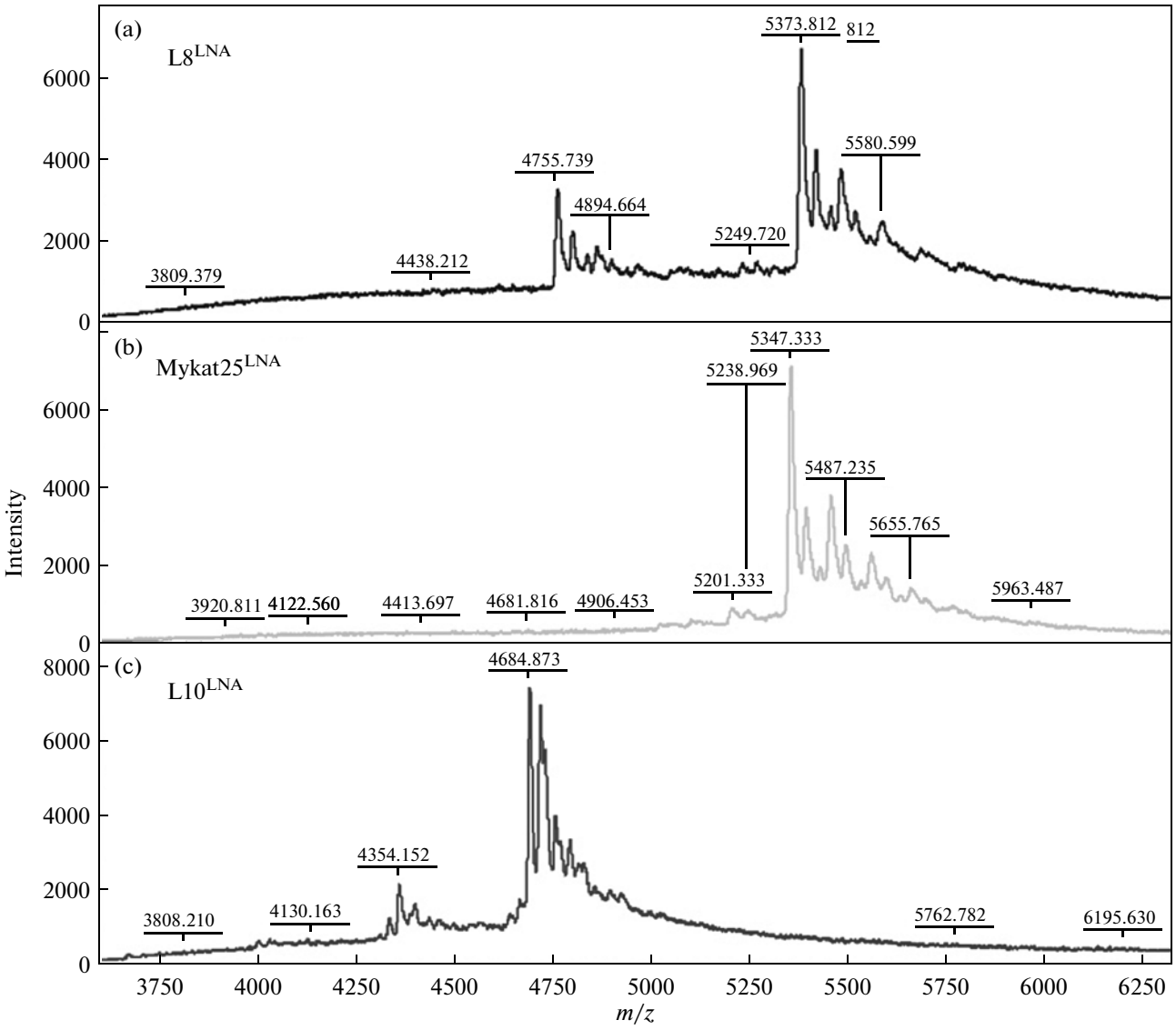

Fig. 6. Mass spectrometry analysis of the 17-mer oligonucleotides $L8^{LNA}$ (a) and $Mykat25^{LNA}$ (b) containing 5 LNA monomers.

ation of WT and MT MTB (in *katG* codon 315), sensitive and resistant to the first-line antitubercular drug isoniazid, respectively. This suggests reasonable purity of primers used for PCR. However, use of primers purified by HPLC will definitely increase effectiveness of the PCR analysis.

Using the method of thermal denaturation we have demonstrated that T_m of the oligonucleotide 17-mer duplex containing 5 complementary LNA monomers in each strand sharply increased (by about 47°C) compared with the unmodified duplex; this is significantly higher than it has been expected. Analysis of MALDI mass spectra of single stranded oligonucleotides forming the duplex has shown that despite electrophoretic purification in PAAG, the oligonucleotide containing LNA monomers were contaminated with oligonucleotides lacking some monomers and also with oligonucleotides with deblocked protecting groups.

The developed molecular genetic test systems for detection of WT and isoniazid-resistant MTB isolates may be used in diagnostic laboratories equipped standard PCR instruments. Their use can significantly decrease time required for determination of MTB drug resistance from 1–3 months needed for such analysis by traditional bacteriological methods employed in many anti-tubercular dispensaries to just 1–3 days using the PCR method.

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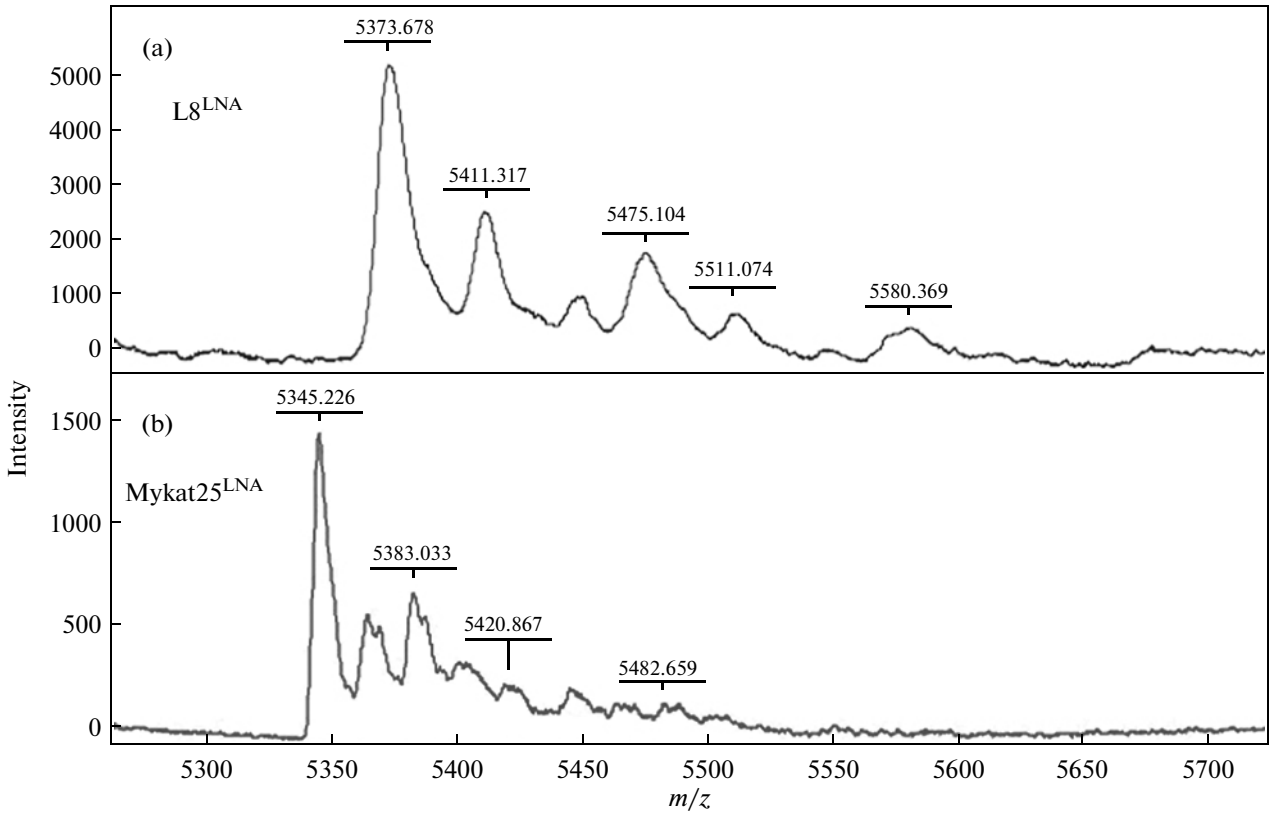

Fig. 7. High resolution MALDI-TOF mass spectra of $L8^{LNA}$ (a) and $Mykat25^{LNA}$ (b).

collective use of the device "Mass spectrometry complex with laser desorption and ionization Autoflex II" of National Academy of Sciences of Ukraine.

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