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Detection of Mutations in *Mycobacterium tuberculosis pncA* Gene by Modified High-Resolution Melting Curve Analysis of PCR Products

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We developed a protocol for detection of mutations in the *pncA* gene associated with *M. tuberculosis* resistance to pyrazinamide by analyzing melting curves of 7 overlapping amplicons with artificial heteroduplex formation (H-HRM) formed by co-amplification of wild-type DNA and test DNA and compared its efficiency and robustness with those of classical HRM analysis. Using HRM and H-HRM, we analyzed 35 PZA^R DNA isolates carrying mutations in the *pncA* gene, 3 PZA^R isolates without mutations in the *pncA* gene, and 20 PZA^S isolates without mutations in the *pncA* gene were analyzed. The sensitivity and specificity of HRM for detection of mutations in the *pncA* gene were moderate: 88.57% (CI 73.26%-96.80%) and 82.61% (CI 61.22%-95.05%), respectively. The sensitivity of the H-HRM test was 97.14% (CI 85.08%-99.93%) and specificity was 95.65% (CI 78.05%-99.89%), with a significant improvement in accuracy — 96.55% *vs.* 93.85% for HRM. In general, despite addition stage of equalizing the concentrations of the test and control mycobacterial DNA, H-HRM showed greater stability and reproducibility at standard settings of the melting curve analysis software.

Key Words: *Mycobacterium tuberculosis; drug resistance; mutations; pyrazinamide; pncA gene*

Pyrazinamide (PZA), a structural analog of nicotinamide, is the first-line drug in therapy for tuberculosis, used since 1952. Due to PZA the treatment period can be shortened from 9 to 6 months; this fact explains its frequent use in the protocols for the treatment of drug-resistant tuberculosis (MDR-TB) [13]. Pyrazinamide is a prodrug that is activated in cells upon conversion into toxic for mycobacteria form, pyrazinoic acid, under the effect of pyrazinamidase (bacterial enzyme). Drug resistance often correlates with the absence of pyrazinamidase activity caused by mutations in the *pncA* gene [13]. Mutations (deletions, insertions, or single nucleotide substitutions) in PZA-resistant strains (PZA^R) are in 72-97% cases located in the *pncA* gene and its promoter part [9,12]. An important characteristic is the absence of frequent mutations. A great variety of mutations in this gene leading to inactivation or attenuation of specific activity of the corresponding enzyme can be explained by small size of pncA protein, consisting from just 186 amino acid residues. In fact, any kind of amino acid substitution negatively affects the function of this protein [9]. Analysis of correlations between mutations and minimum inhibitory concentration of PZA for some *Mycobacterium tuberculosis* (MBT) strains demonstrated high PZA resistance of isolates with mutations in codons 63, 138, and 14 of *pncA* gene (MIC>500 μg/ml) [7]. The absence of mutations in *pncA* gene in an appreciable fraction of PZA^R isolates indicates the

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existence of some other genes associated with PZA resistance (*e.g.*, 28%, according to some data [14]).

Evaluation of the resistance of MBT by classical bacteriological methods takes 1-2 months "lost" for the patient. Moreover, testing of the sensitivity to PZA involves some modifications of the standard cultural methods, as the drug is active *in vitro* only at low pH. Some attempts were made at using agar 7H10 (pH 5.5) and drug concentrations 25-50 μg/ml [10]; however, many MBT isolates did not grow at all or grew poorly at pH 5.5. At present, the use of BACTEC MGIT 960 fluorometric system (Becton Dickinson) is regarded as the reference method for testing the sensitivity to PZA; the results can be obtained within 14 days [10]. However, this method is very expensive and requires special equipment.

Numerous attempts at using molecular genetic methods for rapid detection of mutations associated with drug resistance of MBT have been undertaken [4]. Molecular testing has certain advantages over traditional methods. This is primarily little time needed for the analysis due to the absence of culture re-inoculation in media with the test antibiotic. High specificity of the methods based on amplification of nucleic acids allows using clinical material without initial culturing of MBT, which accelerates drug resistance testing: the results can be obtained within just 1-2 days. For these reasons, the molecular approaches can become an alternative to the culturing methods. The molecular genetic method best fit for testing of PZA resistance is sequencing, due to the absence of frequent mutations in the *pncA* gene and its promoter sequence. However, this method involves the use of expensive equipment and can be realized only by highly qualified specialists.

In 1997 a new method for detecting mutations has been proposed; it is based on the use of intercalation fluorescent dyes and analysis of PCR product melting curves, recorded with the use of the RT-PCR equipment [1]. Mutations within the analyzed PCR-fragment of DNA lead to slight changes in the melting temperature shifting the curve along the temperature axis or in some cases modifying its shape. This approach is called high resolution melting curve analysis (HRM, HRMA, and HRMCA) [1].

The HRM analysis for detection of mutations in the *pncA* gene of MBT isolates was proposed in 2011 [2]. By the present time, the use of HRM for detection of mutations causing MBT resistance to rifampicin, isoniazid, streptomycin, and ethambutol was reported [5,11]. Later, the use of HRM with 7 overlapping primer pairs for detection of mutations in the *pncA* gene was proposed for evaluation of PZA resistance [6]. However, this method showed insufficient accuracy (94%). We previously suggested a modified HRM method for detection of mutations in *rpoB* gene [15]. Its special feature consisted in the formation of "artificial" heteroduplexes of amplified DNA fragments due to simultaneous co-amplification of wild-type DNA (DNA-W) and mutant DNA (DNA-M). The heteroduplexes are characterized by lower melting temperature, which facilitates their detection and making this process more reliable. The base of real time PCR, analysis of PCR products in a closed tube, is strictly adhered to, which excludes the possibility of contamination with amplicons.

Our aim was to develop protocols and to compare the efficiency of detecting mutations in *pncA* gene by means of "artificial" formation of heteroduplex in the course of co-amplification of wild type DNA and test DNA and by standard HRM method.

MATERIALS AND METHODS

Clinical strains of MBT were isolated at the Microbiological laboratory of the Novosibirsk Research Institute of Tuberculosis from patients, residents of Novosibirsk and the Novosibirsk region. The study was approved by the Local Medical Ethics Committee. PZA resistance of the isolates was evaluated in a BACTEC MGIT 960 fluorometric system (Becton Dickinson). Strains growing in the presence of PZA in a concentration of 100 μg/ml were assumed to be PZA-resistant. Genomic DNA of MBT was isolated as described previously [1].

Amplification of a 760 bp *pncA* fragment was carried out using the following primers: pncA-7: 5'-GCCGCCAACAGTTCATCC-3' and pncA-8: 5'-GATTTGTCGCTCACATCACC-3'. The reaction mixture (25 μl) contained 65 mM Tris-HCl (pH 8.9), 16 mM $(NH_4)_2SO_4$, 3 mM $MgCl_2$, 0.05% Tween-20, 0.2 mM dNTP, 1 U *Taq*-polymerase (Fermentas), 0.3 μM each primer, and 1-10 ng genomic DNA of MBT. Amplification was carried out in a PTC-200 amplifier (Bio-Rad) by the following protocol: 3 min at 96°C, after which 38 cycles as follows: 10 sec at 95°C, 10 sec at 64°C, and 20 sec at 72°C, and final elongation at 72°C — 3 min. The presence of amplification product of the target size was verified by 6% PAAG electrophoresis with visualization of DNA fragments stained with ethidium bromide in UV light. The sequences of PCR products were determined using Big Dye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) and ABI 3130xL Genetic Analyzer for capillary electrophoresis (Life Technologies) at Genomika Common Use Center, Institute of Chemical Biology and Fundamental Medicine. The mutations were detected by comparing the resultant nucleotide sequences with MBT gene *pncA* (H37Rv) using Unipro UGENE software (version 1.11.3) (http://ugene.unipro.ru/).

In order to equalize the concentrations of the test and control DNA (DNA H37Rv), quantitative PCR analysis was carried out and calibration curves were plotted on the basis of amplification results of serial 4-fold dilutions of H37Rv DNA in a concentration of 20 ng/μl. The reaction mixture (20 μl) contained 10 mM Tris-HCl (pH 8.9), 50 mM KCl, 0.05% Tween-20, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 μM SYTO82, 0.3 μM oligonucleotide primers, 1 U act. *Taq*-polymerase, and 1-10 ng MBT DNA. Amplification was carried out in a CFX 96 amplifier (Bio-Rad) according to the following protocol: 3 min at 96°C, followed by 41 cycles: 6 sec at 95°C, 6 sec at 65°C, and 10 sec at 72°C. Fluorescence was recorded at 87°C for 5 sec. The efficiency of PCR was 90-94% (r^2>99).

Seven overlapping pairs of primers covering 561 bp coding region of *pncA* gene and 40 bp promoter sequence were designed. The structures of oligonucleotide primers for HRM are presented in Table 1.

PCR was carried out as described above with a minor modification: introduction to the amplification protocol of the heteroduplex formation stage (3 min at 97°C and 2 min at 50°C) and fluorescent detection of melting with increasing the temperature from 85 to 96°C (0.2°C at each step). PCR was carried out in 3 repetitions for each pair of primers with formation of heteroduplexes (H-HRM) and without it (HRM).

MBT H37Rv DNA was used in each analysis as DNA-W. The resultant melting curves were analyzed using available commercial Precision Melting Analysis Software (Bio-Rad) with standard setups.

RESULTS

Nucleotide sequences of *pncA* gene were identified for 38 PZA^R and 20 PZA^S isolates of MBT. Mutations leading to modification of the amino acid sequence of the gene were identified in 33 PZA^R isolates, two of these contained single-nucleotide A→G substitutions in position -11 of the gene promoter. The spectrum of *pncA* mutations in the studied sample of MBT isolates and the results of HRM and H-HRM analyses are presented in Table 2.

The classical HRM and modified HRM (H-HRM) were used for the analysis of DNA in 35 PZA^R strains carrying mutations in the *pncA* gene, 3 PZA^R strains without mutations in *pncA*, and 20 PZA^S isolates without mutations in *pncA*. Amplification product melting curves were plotted using 7 overlapping primer pairs for DNA-W, test isolates (DNA-M) and mixture of DNA-W and DNA-M (DNA-H). Typical profiles of normalized (appropriate option of the software) melting curves are presented in fragment *a* of Figure 1. The mutation detectable at the appropriate level of significance (>95%; the percent confidence in the Precision Melting Analysis Software) only by H-HRM analysis was analyzed (Fig. 1, *a*). As a rule, HRM was less effective for A↔T and G↔C substitutions that only slightly shift PCR fragment melting temperature in certain nucleotide contexts. In these cases, H-HRM exhibited obvious advantages. Substitution of A for G with the formation of a local short G/C-rich area (Fig. 1, *b*) significantly elevated melting temperature of the PCR fragment. These types of single-nucleotide

TABLE 1. Structures of Oligonucleotide Primers for HRM of MBT *pncA* Gene

Segment	Primer	Fragment size, bp	Nucleotide sequence	Position	Temperature of amplified fragment, °C
1	Pnc-prom	157	5'-GCGTCGGTAGGCAAACTG-3'	(-50)-(-32)	94.20
	Primer 1		5'-GCCAGGTAGTCGCTGATG-3'	90-107	
2	pnc13U	108	5'-TGCGAGGGTGGCTCGCTG-3'	40-57	93.95
	pnc14R		5'-GTCCTTGGTTGCCACGACG-3'	129-147	
3	Primer 6	108	5'-ACCATCACGTCGTGGCAAC-3'	122-140	90.14
	pnc16R		5'-GAGTACCGCTGACGCAATG-3'	211-229	
4	pnc17U	123	5'-GGACTATTCCTCGTCGTGG-3'	186-204	91.32
	pnc18R		5'-TACGCTCCGGTGTAGGCAC-3'	290-308	
5	pnc19U	185	5'-GAGGCGGTGTTCTACAAGG-3'	271-289	92.72
	Primer 9		5'-GCCAAGCCATTGCGTACCG-3'	437-455	
6	Pnc21U	155	5'-CACGCCACTGCTGAATTGG-3'	339-357	92.93
	pnc25R		5'-CCGACACACCCGCTGTCAG-3'	475-493	
7	Pnc_14U	137	5'-CAGGGTGCTGGTGGACCT-3'	459-476	93.62
	pnca7R		5'-GCCGCCAACAGTTCATCC-3'	578-595	

TABLE 2. Spectrum of *pncA* Gene Mutations in MBT Isolates and Results of HRM and H-HRM Analyses

No.	Isolate	Nucleotide substitution	Gene	Amino acid substitution	PZA	Segment	HRM	H-HRM	Sum
1	5328	A→G	-11		R	1	1	1	1
2	6354	A→G	-11		R	1	1	1	1
3	5103	TTG→TGG	11	4 Leu→Trp	R	1	1	1	1
4	273	GTC→GGC	20	7 Val→Gly	R	1	1	1	1
5	10	CAG→CCG	29	10 Gln→Pro	R	1	1	1	1
6	276	GAC→CAC	34	12 Asp→His	R	1	0	1	1
7	312	TGC→TGA	42	13 Cys→STOP	R	1, 2	1	1	1
8	6514	insG	50		R	2	0	0	0
9	78	GCC→GAC	83	Ala→Asp	R	2	1	1	1
10	5494	ATC→AGC	92	31Ile→Ser	R	2	1	1	1
11	6190	ATC→AGC	92	31Ile→Ser	R	2	1	1	1
12	287	ATC→AGC	92	31Ile→Ser	R	2	1	1	1
13	300	ATC→AGC	92	31Ile→Ser	R	2	1	1	1
14	5339	GTG→ATG	133	45 Val→Met	R	2	1	1	1
15	153	CAC→CGC	170	57 His→Arg	R	3	1	1	1
16	133	TTC→TTA	174	58 Phe→Leu	R	3	0	1	1
17	262	CAT→GAT	211	71 His→Asp	R	3, 4	0	1	1
18	151	TGC→CGC	214	72 Cys→Arg	R	3, 4	1	1	1
19	57	ACT→CCT	226	76 Thr→Pro	R	3, 4	1	1	1
20	175	TTC→TCC	281	94 Phe→Ser	R	4	1	1	1
21	149	TAC→TAG	309	103 Tyr→STOP	R	5	1	1	1
22	288	AGC→AGA	312	104 Ser→Arg	R	5	1	1	1
23	5225	GGA→AGA	322	107 Gly→Arg	R	5	1	1	1
24	111	GTG→GGG	389	130 Val→Gly	R	5, 6	1	1	1
25	184	GGT→CGT	394	132 Gly→Arg	R	5, 6	1	1	1
26	5348	GGT→GCT	395	132 Gly→Ala	R	5,6	1	1	1
27	191	ATT→ACT	398	133 Ile→Thr	R	5, 6	1	1	1
28	138	ACC→CCC	403	135 Thr→Pro	R	5, 6	1	1	1
29	182	TGT→TGG	414	138 Cys→Thr	R	5, 6	1	1	1
30	173	GTG→GCG	416	139 Val→Ala	R	5, 6	1	1	1
31	194	CAG→CCG	422	141 Gln→Pro	R	5, 6	1	1	1
32	237	GTG→GCG	464	155 Val→Ala	R	6,7	1	1	1
33	200	ACA→CCA	478	160 Thr→Pro	R	7	1	1	1
34	286	GCG→GAG	512	171 Ala→Glu	R	7	1	1	1
35	103	TTG→TCG	545	182 Leu→Ser	R	7	1	1	1
36	4851	DNA-W			R		0	0	0
37	4978	DNA-W			R		0	0	0
38	4998	DNA-W			R		0	0	0
40-59		DNA-W			S		3	1	1

substitutions were effectively detected by the classical HRM. The modified method (H-HRM) identified these types of mutations with a sufficiently high level of significance, though the differences in the melting curves were visually not so obvious. In addition, for substitutions C→A or C→T types (Fig. 1, *c*) the mutations could be detected by both, HRM and H-HRM (differences in the position and shapes of melting curves).

None of the approaches allowed detection of G insertion in position 50 of the gene inducing reading frame shift and enzyme inactivation with admissible level of statistical significance. The false-negative results of HRM were mainly associated with G↔C substitutions (34 GAC→CAC, 211 CAT→GAT), though the 174TTC→TTA substitution was not detected either, presumably, because of the nucleotide composition of the fragment.

The sensitivity and specificity of HRM for detection of mutations in *pncA* gene were moderate: 88.57 (confidence interval 73.26-96.80%) and 82.61 (61.22-95.05%), respectively. In turn, the sensitivity of H-HRM was 97.14% (85.08-99.93%) and sensitivity — 95.65% (78.05-99.89%), while the accuracy of the test was significantly higher: 96.55 *vs.* 93.85% (HRM).

For heteroduplex formation, DNA-W and DNA-M were added in equimolar proportion. However, this was practically not always possible because of errors in evaluation of the test isolate DNA concentration, and hence, we studied the possibility of modifying the proportion of these DNA concentrations. A series of experiments was carried out with ascending concentrations of DNA-W and unchanged DNA-M and *vice versa*. Primer pair pnc6/pnc16R was used in this experiment. H-HRM detected the mutation with a high level of statistical significance at DNA-W:DNA-M proportions 1:1, 1:3, and 3:1 (data not presented).

Mutations in the *pncA* gene are the main molecular mechanism of MBT resistance to PZA. Changes in the nucleotide sequence of the gene include missense mutations leading to amino acid residue substitution in the protein, insertions and deletions of different lengths, and mutations in the gene promoter essential for the level of its expression. The available data on dispersed location of mutations in the *pncA* gene sequence indicate that direct sequencing is the most adequate method for analysis of the genetic changes in it. With creation of high precision (for maintenance of preset temperature) equipment for RT-PCR, HRM analysis has become an alternative to sequencing at the screening stage 1 [8]. The aim of our study was to improve detection of various types of mutations in the *pncA* gene by HRM due to artificial formation of heteroduplexes with lower melting temperature at the expense of formation of unpaired base at the site of mutation.

Previously, HRM was used for detecting mutations in MBT *pncA* gene. A total of 98 clinical strains of MBT (41 PZA^S, 55 PZA^R, and 2 isolates with undefined status) were analyzed [6]. The gene sequences

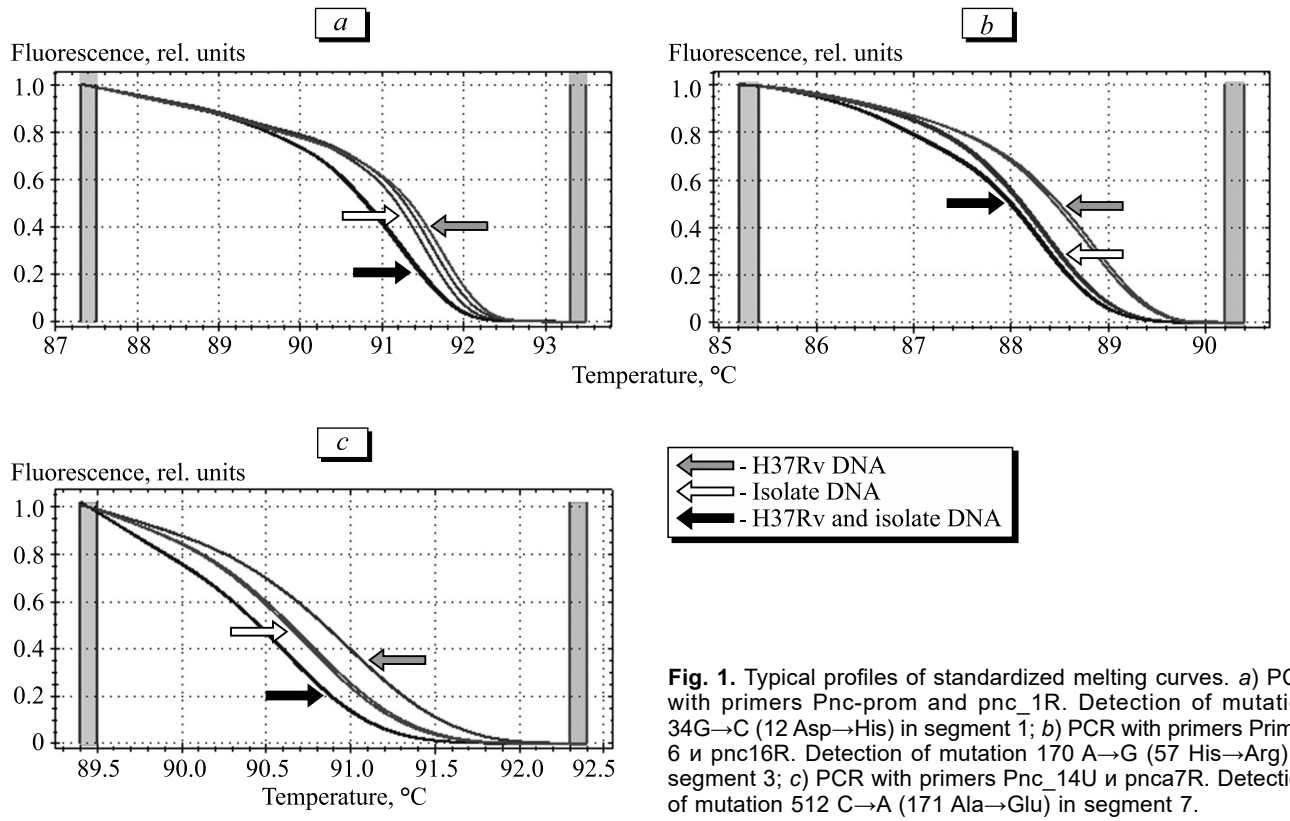

Fig. 1. Typical profiles of standardized melting curves. *a*) PCR with primers Pnc-prom and pnc_1R. Detection of mutation 34G→C (12 Asp→His) in segment 1; *b*) PCR with primers Primer 6 и pnc16R. Detection of mutation 170 A→G (57 His→Arg) in segment 3; *c*) PCR with primers Pnc_14U и pnca7R. Detection of mutation 512 C→A (171 Ala→Glu) in segment 7.

were subdivided into 7 overlapping amplicons, but this scheme was characterized by a lesser size of overlapping amplicon regions. The accuracy of the test was 94%, mainly because of worse detection of transversions, which was in line with our previous results with the "classical" HRM variant. In the next publication [3], these authors presented the results of searching for mutations responsible for MBT drug resistance in 10 genes using TaqMan array card (including a combination of hybridization hydrolyzed samples and HRM analysis). Despite rather high accuracy of the test in general (95%), for *pncA* gene it was only 81% (sensitivity 85% and specificity 79%), which was significantly lower than in our study.

As we mentioned, neither HRM, nor H-HRM could detect insertion G in position 50 of the gene with an admissible level of significance in the present study. Insertion of an additional nucleotide G leads to the formation of a short poly-G site. In a previous analysis of mutations in human *BRCA1* gene, we also observed lower efficiency of H-HRM in detection of single-nucleotide deletions and insertions, particularly in poly-G sites (data not presented).

In order to improve the formation of heteroduplexes, we added DNA-W to the isolated strain DNA-M. The proportion of DNA-W to DNA-M could vary at least 3-fold without loss of the resolving capacity of the method, this creating not very stringent requirements to equilibration of concentrations for testing.

It should be noted that our approach aimed at improving the efficiency of HRM analysis of mutations can be used in studies with all kinds of biological objects.

The limitations and flaws of the method are the need in a preliminary measurement of the concentration of analyzed MBT DNA by qPCR in order to choose the correct proportion of the control and test DNA in the reaction. In addition, similarly as all other tests based on the use of intercalation dyes without specific hybridization tests, this method detects silent or uninformative mutations, such as, *e.g.*, synonymous Ser65Ser (TCC→TCT) or His57Asp substitution (CAC→GAC), characteristic of MBT.

Hence, we have developed a highly effective method for detection of mutations in MBT *pncA* gene based on the analysis of melting curves of 7 overlapping amplicons with artificial formation of heteroduplexes due to co-amplification of wild type DNA and test DNA. The accuracy of the method is 96.55%, which is admissible for screening studies. The method can be easily adapted to studies of the structural polymorphisms of other bacterial species.

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