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Genotyping of Related Mutations to Drug Resistance in Isoniazid and Rifampin by Screening of *katG, inhA* and *rpoB* Genes in *Mycobacterium tuberculosis* by High Resolution Melting Method

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Abstract

Introduction: Tuberculosis is one of the infectious diseases and this is responsible for 3 million mortalities in the world which is increased by the rise of drug-resistant tuberculosis patients. Thus, it seems essential to detect the drug resistances in tuberculosis patients. Rifampin and isoniazid are two essential drugs for treatment of tuberculosis patients. The new High Resolution Melting method is simple, rapid and inexpensive for detection of these mutations responsible for these resistances compared to conventional methods in tuberculosis patients.

Materials and methods: 2500 sputum samples were collected from patients with suspected tuberculosis referred to Iran Remedial Center over a period of 2 years in which, 1650 samples had positive smear for *Mycobacterium tuberculosis*. After extraction of genomic DNA from samples, High resolution melting method was used for samples based on the color of SY to-9 and PCR product were sequenced to verify the mutation.

Results: Our findings showed that, 116 out of 1650 positive smear samples, were resistant to isoniazid due to mutations in *katG* and *inhA* genes, which this resistance is created by mutation of 20 samples in *inhA* gene and 96 samples in *katG* gene. Whist 65 samples had resistance to rifampin with mutation in *rpoB* gene.

Conclusion: High resolution melting method is quick, easy and affordable without wasting time for culturing and Post PCR processes for diagnosing these resistances in tuberculosis clinical samples.

Keywords: *Mycobacterium tuberculosis*; Isoniazid; Rifampin; High resolution melting

Introduction

At the beginning of the 20th century, infectious diseases were the main cause of mortality in different societies [1] that Tuberculosis is one of the most dangerous infectious diseases [2,3], which increased by prevalence of drug-resistant patients and rise of epidemic AIDS [4] patients. Therefore, it is necessary to control the infection since; drug-resistant tuberculosis patients [5] are likely to remain infectious for a long period, thus [6], the public health consequences of drug-resistant tuberculosis is more serious [7] than those who are sensitive to the drug [8]. Isoniazid and rifampin are two main drugs which are used to [9] treat tuberculosis. Isoniazid antibiotic activity depends on bacterial activation [10] by catalase peroxidase (*katG*) enzyme which creates active radicals and many regions in the *Mycobacterium tuberculosis* [11]. *inhA* is another gene which, mutation in this gene creates resistance to isoniazid [12]. Enol-Acp reductase is a protein involved in mycolic acid that interferes in the cell wall biosynthesis [13]. So, *katG* and *inhA* are the most popular mutations associated with resistance to Isoniazid and since the Isoniazid destroys the *tubercle bacillus* after starting therapy in a short period of time [14], rifampicin is another treatment drug for tuberculosis patients, which is associated with creating problems in RNA synthesis. Resistance to these drugs can be associated with incredible results [15]. Considering detection of these resistances with old methods such as culturing, require a long period of time and associated with pollutions [16], so it is better to use molecular methods based on PCR for rapid diagnosis of these resistances [17]. High resolution melting method is rapid, easy and economical for isolating genotypes without using specific probes [18] which are shown in HRM analysis of nucleic acid sequences with high accuracy based on the difference between the formed curves [19]. This study was conducted to evaluate Izoniazid and rifampin drugs resistance in patients referred to health and care centers in all tuberculosis centers in Iran and provide a quick and convenient method for the detection of mutations that cause resistance in strains *Mycobacterium tuberculosis*.

Materials and Methods

Sample collection

This study was conducted on 2500 patients with suspected tuberculosis as an infectious disease which referred to health and care centers in Iran in the past two years. Samples of suspected tuberculosis patients were studied in the case of having positive smear. First 2500 sputum samples collected, samples were homogenized and decontaminated using sodium and N-acetyl-L-cystein. Then, we examined with the help of Zihel Nelson strains for the presence of acid fast bacillus. Among them, 1650 patients had positive smear and their sputum samples were kept in physiology serum and -20°C freezers.

DNA extraction from strains

After transferring the samples to the laboratory, High pure PCR Template preparation kit from Roch Company was used for extracting DNA. After lyses of the samples and denaturation of other proteins

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by suitable buffer, samples were incubated at 56°C. Lysed substances were added into the column and when other cellular components were removed from the column environment, DNA was bonded to the column. The samples were then rinsed to remove other components from environment. The obtained DNA was centrifuged with 95% cold ethanol for 10 min at the rotation of 14000 rpm. Supernatant was removed from the environment and the sample DNA was rinsed with 70% cold ethanol. DNA was centrifuged again and ethanol was removed. The DNA was air dried and dissolved in 70 µl buffer.

Primer design and PCR reaction conditions

Two pairs of Forward and Reverse primers were designed for areas of *katG* and *inhA* genes, also one pairs of forward and reserve primers were designed for *rpoB* gene which have the most probability for the presence of mutations that the sequence of Forward and Reverse primers are listed (Table 1).5 µL from genomic DNA with the mixture of 2.5 µl from 10X buffer, 1 µl from (50 µM) $MgCl_2$, 1 µl from each primer (10 pmol/µl) F and R, 1 µl dNTP (10 µM) and enzyme Taq 0/4 µl were added. The volume of reaction mixture is 25 µl. The PCR reaction conditions were the same for both genes which included 95°C for 10 min and 40 cycles of 95°C for 30 sec, 55°C for 30 sec and 72°C for 40 sec were performed.

High resolution melting analysis

HRM curve analysis was performed on Rotor Gene 6000 device. When PCR amplification was completed, melting curve data were analyzed using initial stage holding at 60°C for 30 sec. And temperature changes were examined with Rotor Gene 6000 (Corbet Life Science). Rotor Gene software examined nucleotide differences samples in three forms of normalized graph, difference graph and melting Curve (Table 2).

DNA sequencing

Samples were selected randomly from analyzed groups by Rotor Gen 6000. After PCR, purified DNA was used as the template for sequencing PCR in a BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA). The samples were heated at 96°C for 1 min and then run for 35 cycles of 96°C for 10 s, 50°C for 5 s, and 60°C for 4 min. The extension products were purified using the ethanol precipitation method described in the manufacturer's manual. The pellet was rehydrated in 15 µl of formamide, heat denatured at 95°C for 5 min in a thermal cycler, and immediately put on ice for 5 min. The samples sequenced with the BigDye Terminator kit were electrophoresed on an ABI Prism 3130 instrument (Applied Biosystems).

Name	5´→3´ sequence
Tb92-Forward Isoniazid: *inhA* (248 bp) Tb92-Reverse	5´-CCTCGCTGCCCAGAAAGGGA-3´
	3´-ATCCCCCGGTTTCCTCCGGT-5´
Tr9-Forward Rifampicin: *rpoB* (109 bp) Tr9-Reverse	5´-TCGCCGCGATCAAGGAGT-3´
	3´-GTGCACGTCGCGGACCTCCA - 5´
Tb86-Forward Isoniazid: *katG* (209 bp) Tb86-Reverse	5´-GAAACAGCGGCGCTGATCGT-3´
	3´-GTTGTCCCATTTCGTCGGGG-5´

Table 1: Primers designed to amplify genes resistant to Isoniazid and Rifampin

Cycle	Cycle Point
Hold @95°C, 7 min 0 sec	
Cyclying (40 Repeats)	Step 1 @96°C, hold 30 secs
	Step 2 @62°C, hold 30 secs, acquiring to Cycling A (Green) [1] Step 3 @68°C, hold 45 secs
Hold 2 @68°C, 7 min 0 sec Hold 3 @50°C, 5 min 0 sec HRM (75°C-95°C) hold 2 secs on the 1st step, hold 2 secs on next steps, Melt A (HRM)	

Table 2: Temperature conditions of HRM reaction

Results

The results of resistance to isoniazid and rifampin and evaluation of *katG*, *inhA* and *rpoB*

HRM analysis was used for 2500 positive smear samples to evaluate fragment 209 of *katG* gene, fragment 248 of *inhA* and *rpoB* genes, samples placed in different groups for analysis, based on differences in melting point and shape of curves. Graph differences were probably due to nucleotide differences in the samples. In order to verify the results and to ensure the nucleotide differences in samples and identifying the wild and mutated groups considered to our interested regions, the samples from each group were sequenced. On the analysis of 1650 samples, samples were classified in three different groups for examining sector 209 from *katG* gene, sector 248 from *inhA* and *rpoB* gene based on difference in melting point of these samples. Below we represent some samples in 3 different graphs.

HRM results for *katG* gene: HRM analysis was performed for *katG* gene and samples were divided in three different groups based on HRM and melting curve (Figure 1). The samples that had the same melting point were perched on same group. According to thermal shift of 70°C-90°C and CT 90% we can observe normalized graph and different graph below.

Normalized graph is a basic display that shows different mutation based on change shape of curve. In fact the samples which have the similar curve shape represented in a same group and divided into A, B and C groups (Figure 2).

Difference graph is a graph in which one sample (red line) was to be considered as base line for positive control and other samples were compared with that positive control. Generally in difference graph melting profile of each sample compared with selection sample that is horizontal line and samples because of having slope near X axis (± 4), stand on 3 different groups that analyzed indication of sequence changes of amplicon. 2D sample indicated as positive control (Figure 3).

Melting curve each sample denatured and gives pick in different temperature according to the length of the Goanin and Cytosine plots. If the samples show different pick, nucleotide difference will be observed (Figure 4) difference between samples picks show difference in samples sequences which are divided in 3 different A, B and C groups. That they confirmed by sequencing analysis (Tables 3 and 4).

HRM result for *inhA* gene: Difference graph, normalized graph and melting curve were performed for this gene as other genes (Figures 5-7), according to this analysis samples seated on 3 different A, B and C groups (Tables 5 and 6).

HRM results for *rpoB* gene: Difference graph, normalized graph and melting curve were performed for this gene as other genes (Figures 8-10), according to this analysis samples seated on 3 different A, B and C groups (Tables 7 and 8).

Sequencing results for *katG* gene

Samples of three different groups were sequenced for sector 209

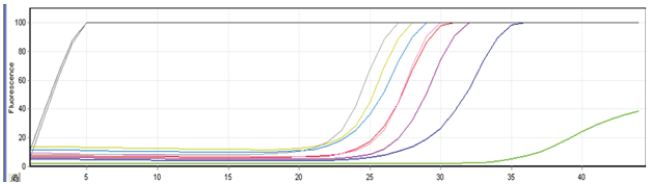

Figure 1: Unequipped data chart

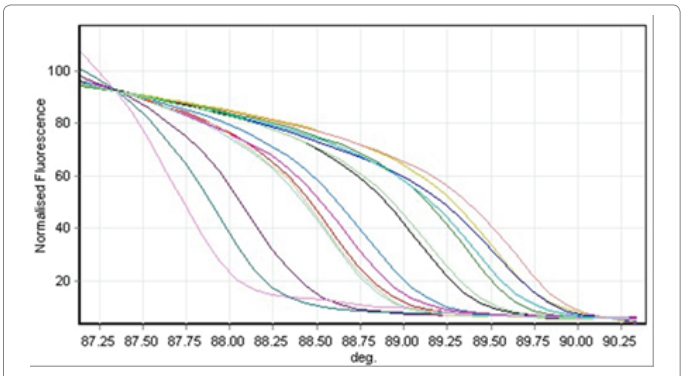

Figure 2: Normalized graph according to curve shape change

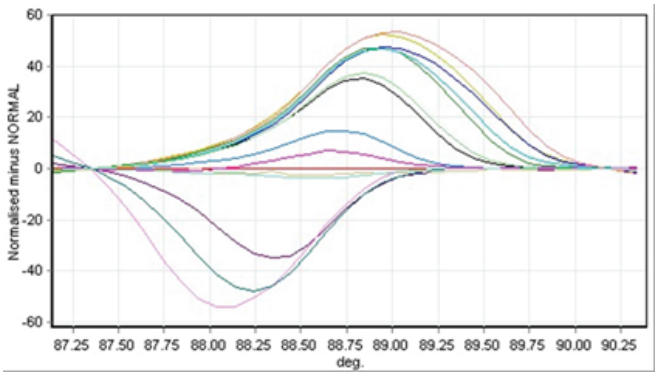

Figure 3: Difference graph according to control sample

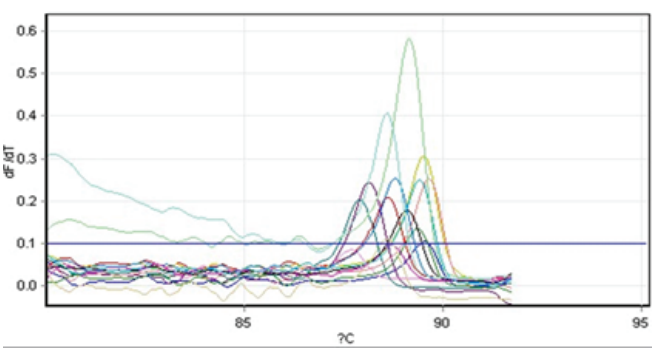

Figure 4: Melting curve graph

from *katG* gene based on HRM analysis. According to sequencing results, two groups of samples had mutated sequence and one group had normal sequence. According to sequencing process, 86 samples were placed in two mutated groups and 1564 samples were placed in normal sequence group. Among 86 mutated samples, 66 samples (71/25%) were mutated in 315 codons from *katG* gene and 11 samples (19%) were placed in 279 codon of the same gene. The following mutations were

1		1-D	100.00	**A**
2		2-D	89.00	**B**
3		3-D	96.45	**B**
4		4-D	88.07	**C**
5		5-D	89.32	**C**
6		6-D	29.83	**A**
7		7-D	100.00	**C**
8		8-D	97.34	**B**
9		9-D	95.70	**B**
10		10-D	78.97	**A**
11		11-D	88.05	**B**
12		12-D	66.72	**B**
13		13-D	93.04	**A**
14		14-D	98.01	**B**
15		15-D	91.38	**A**

Table 3: Color differences in difference graph for each sample

in 66 codon samples 5/3 from *katG* gene. 51 samples (41/25%) (ser → Thr) AGC → ACC, 10 samples (11/25%) (ser → Ile) AGC → ATC, 3 samples (2/25%) (ser → Asn) ACC → AAC, 2 samples (1/5%) (ser → Thr) AGC → ACA.

Following changes were carried out in 11 samples which had mutation in Codon 279 from *katG* gene: 7 samples (2/8%) (GLY → Thr) GGC → ACC, 3 samples (0/8%) (GLY → Arg) GGC → CGC, 1 sample (0/4%) (GLY → Ile) GGC → ATC.

Sequencing results for *inhA* gene

Samples from three different groups were sequenced for sector 248 from *inhA* gene based on HRM analysis. The sequencing results for this gene showed that two groups of investigated samples had normal sequence and one group had mutated sequence. From 1650 positive samples, only 20 samples had mutations. All mutated samples responsible for resistance to Isoniazid in *katG* and *inhA* genes are given (Table 2). Among 1650 investigated samples, 86 samples with mutation in *katG* gene and 20 samples in *inhA* gene had resistance to Isoniazid.

Sequencing results for *rpoB* gene

For this gene such as other two genes samples putted in three groups .all groups were sequenced for fragment 109 bp from *rpoB* gene based on HRM analysis. According to sequence results one group had normal sequence and two groups had mutation sequence. From 1650 samples 65 samples were resistance to rifampin. Finding mutations in this gene were shown below. 44 samples from 66 rifampin resistance samples had mutation in codon 531 which changed (ser → leu) TCG → TTG and 22 samples had mutation in codon 545 that this mutation changed leucine to methionine (LEU → MET) (CTG → ATG). All mutations in *rpoB*

No.	Colour	Name	Group	Peak 1	Peak 2	Peak 3	Peak 4	Peak 5	Peak 6	Peak 7	Peak 8	Peak 9
1		1-D	A	88.62								
2		2-D	B	89.53								
3		3-D	B	89.55								
4		4-D	C	88.15								
5		5-D	C									
6		6-D	A	88.80								
7		7-D	C	87.93								
8		8-D	B	89.65								
9		9-D	B	89.37								
10		10-D	A									
11		11-D	B	89.10								
12		12-D	B	89.42								
13		13-D	A	88.60								
14		14-D	B	80.63	82.42	83.73	84.63	85.27	85.58	86.38	89.15	
15		15-D	A	80.20	83.25	83.92	84.42	85.20	85.27	86.03	86.33	88.60

Table 4: Melting curve peak results

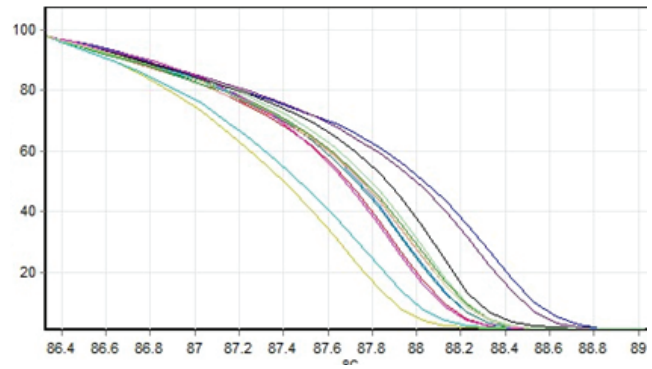

Figure 5: Normalized graph

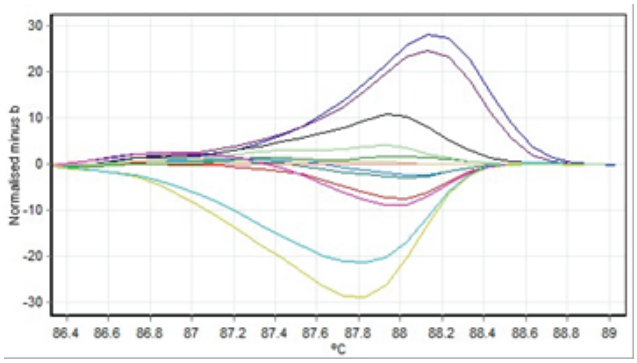

Figure 6: Difference graph according to base line

were represented in Table 9.

Discussion

According to national TB directions [20], there are many different factors that caused drug-resistant tuberculosis [21], including: being uninformed of free treatment for the disease, ignorance of patients about the disease, factors related to the drug such as unsuitable quality and dosage of medication, failing to educate patients and their families. In 2005, about 260,000 cases of total MDR were reported and in some areas up to 10% prevalence of extensive drug resistance (XDR) were reported. Providing methods for determining the amount of sensitivity seems necessary after the correct diagnosis of bacterial infections [22]. Understanding the genetic basis of drug resistance will help to develop effective methods for rapid determination of drug-resistant *Mycobacterium tuberculosis* [22]. The aim of our study was to investigate *katG* and *inhA* genes in tuberculosis patients that are resistant to Isoniazid whilst detection of mutation in *rpoB* gene that it is caused resistance to rifampin antibiotic and also to identify the most common mutations in these genes with the evaluation of high resolution melting method which is a simple, rapid and standard method without the need for post-PCR procedures, specific probes and long period of time for culturing [21]. These resistances set suitable treatment for these patients and prevent transmission of the disease to others. In contrast to previous studies carried out by researchers, we directly used sputum samples to purify DNA without the use of culturing techniques. Also we examined common mutations in

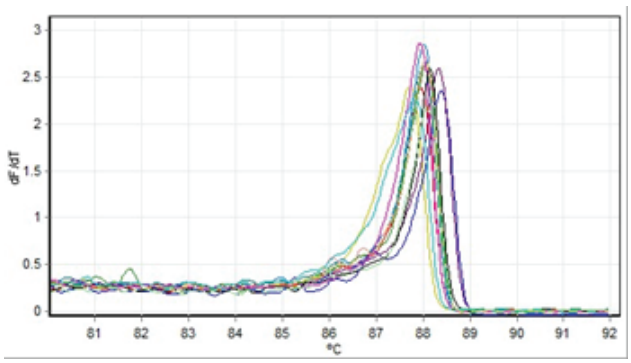

Figure 7: Melting curve according to difference melting temperature

No.	Colour	Name	Genotype	Confidence
7		8a	B	95.93
8		9a	B	100.00
9		5a	B	97.79
10		3s	B	66.72
11		8s	B	53.56
12		10a	A	72.70
13		4a	B	99.43
14		6a	B	89.94

Table 5: HRM result for *inhA*

No.	Colour	Name	Genotype	Peak 1
1		1s	B	87.95
2		6s	A	87.72
3		12s	C	88.4
4		14a	C	88.35
6		5s	B	88.02
7		8a	B	88.02
8		9a	B	88.05
9		5a	B	88.07
10		3s	B	87.95
11		8s	B	88.15
12		10a	A	87.85
13		4a	B	88.05
14		6a	B	88.1

Table 6: Melting curve result

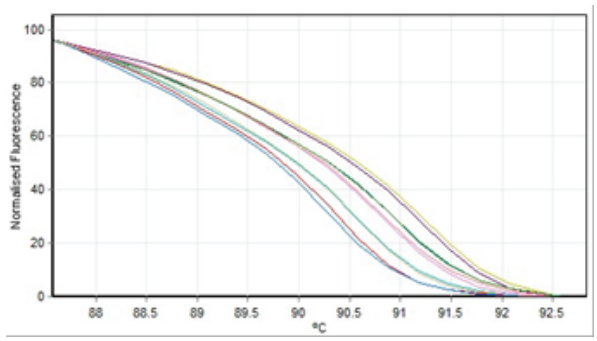

Figure 8: Normalized graph

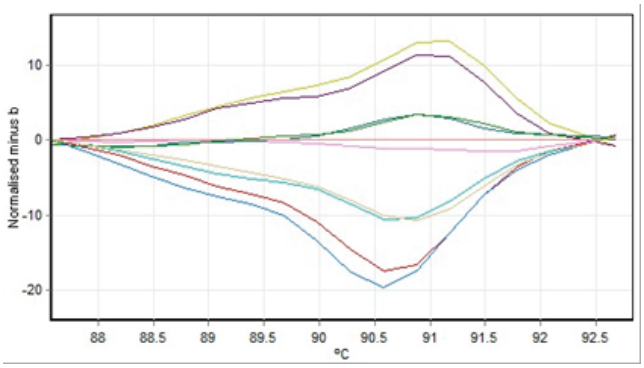

Figure 9: Difference graph according to normal control sample

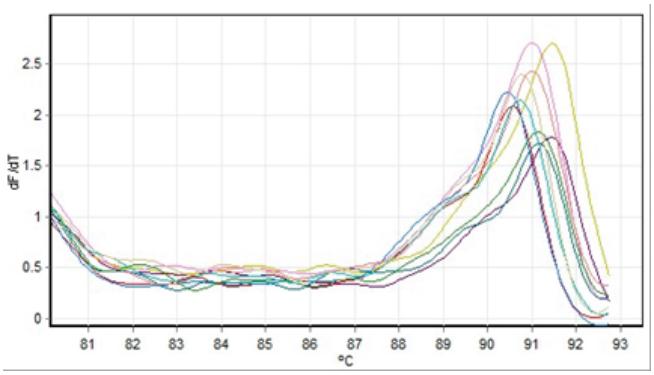

Figure 10: Melting curve for *rpoB* gene

the sequence of TB86, TB92 and *rpoB* and also showed that HRM techniques have this ability to identify all of the mutations without the use of culturing. PCR method have post-PCR section which has a lot of pelotons; also real time PCR methods need specific probe that spend more time whilst more currency. While, in the previous studies culturing technique was used to evaluate known resistant and sensitive samples. According to this study, among 1650 examined samples, 116 samples were resistant to Isoniazid in which 86 samples with mutations in codon 315 and 279 in *katG* gene and 20 samples with mutations in the C → T 15th area in *inhA* gene had resistance to Isoniazid. In a study by Gonzalez and colleagues for examination of MTB drug-resistance by Fluor-genetic probes, mutations in codon 315 of *katG* gene (AGC → AGC) in the C → T 15th area had *inhA* gene and among 1650 samples 65 samples had resistance to rifampin with mutation in codon 531 and 545 *rpoB* whit changing in TCG → TTG (SER → LEU) and CTG → ATG (LEU → MET). In a study conducted by Gulhon and colleagues,

No.	Colour	Name	Genotype	Confidence
1		1s	A	100.00
2		3s	C	97.14
4		5a	C	100.00
5		5s	B	98.83
6		6s	A	96.17
7		8a	B	95.80
8		8s	B	100.00
9		9a	B	95.54
12		12s	A	82.07
13		14a	A	80.24

Table 7: HRM result

NO.	Colour	Name	Genotype	Peak1
1		1s	C	90.56
2		3s	B	91.46
4		5a	B	91.4
5		5s	A	91.01
6		6s	C	90.44
7		8a	A	91.16
8		8s	A	91.01
9		9a	A	91.16
12		12s	C	90.74
13		14a	C	90.8

Table 8: Melting curve color differences

Analyzed Gene Region	Location of mutation	Nucleotide Change	Amino Acid Change	No (%) of strains
katG	315	AGC→ACC AGC→ATC AGC→AAC AGC→ACA	SER→Thr SER→Ile SER→Asn SER→Thr	55 15 3 2
inhA	279	GGC→ACC GBC→CGC GGC→ATC	GLY→Thr GLY→Arg GLY→Ily	14 4 2
	15^{th} Locus	C→T	C→T	20
rpoB	531	TCG→TTG	SER→LEU	44
	545	CTG→ATG	LEU→MET	22

Table 9: Mutations found in the *katG* and *inhA* genes by Silver sequence

HRM method was used for detecting MTB resistance to Isoniazid and Rifampin. This study has been carried out to identify specific mutations using resistant and sensitive cultured samples that mutation in S315T and D310A areas in *katG* gene, in the C → T 15^{th} area was shown to evaluating the resistance to Isoniazid [21]. Thus, all previous studies used PCR techniques, culturing, RFLP and Real time PCR for examining HRM technique, known resistant and sensitive samples. Likewise HRM method is a very convenient and sensitive molecular method for screening pointed mutations in clinical samples with sensitivity 98/6% and specificity 100% [18] because this technique is fast, inexpensive and amenable to a large extent. So there is no need for further processing of samples and it can be suggested to drug-resistant TB patients as the easiest way to check and detect pointed mutations [12]. This procedure is also performed in the same tube without any post-PCR processing, which is useful for routine diagnostic testing. In the present study the SYTO dyes were used which is useful for HRM application against other colors. Also, understudy samples showed the most common mutations associated with drug-resistance to Isoniazid, that were in agreement with previous studies. The study suggests that a broader and more samples of the types of mutations associated with drug-resistance should be discussed and examined.

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