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# Tuberculosis Pathogenicity, Drug Resistance and Molecular Diagnostic Methods

**Zoha Tavakkoli***

*Department of Microbiology, University of Barcelona, Barcelona, Spain*

## Abstract

**Aim and objective:** Tuberculosis associated whit drug resistances is one of the most important infectious diseases in the world today. A High Resolution Melting Analysis (HRM) was expanded to detect Isoniazid (INH) and Rifampin (RMP) in Mycobacterium tuberculosis by targeting resistance related mutations in the *katG, rpoB, inhA genes* in this study we aimed nature and frequency of this *genes* mutations by HRM analysis.

**Materials and Methods:** 95 clinical Mycobacterium tuberculosis isolates was selected for evaluation by HRM. DNA extracting of isolates and HRM analysis in presence of a single dye was performed and PCR amplification were sequenced from the *katG, rpoB, inhA genes*.

**Results:** A set of 20 (21/05%) INH-resistence strains and 12 (12/63%) RMP-resistence strains were found to have a mutation in analysis katG gene or *inhA locus* and *rpoB* gene. Our detection shows that the most frequency mutation pattern was at 315 codon and codon 279. A nucleonic change was display which is related with INH resistance from 15$^{th}$ C to T in the locus in the rpoB gene, codons that had point mutation were 531 and 545 codons.

**Conclusion:** It is concluded that High Resolution Melting Analysis is a rapid and sensitive method when compared to other molecular methods for detection of drug resistance in Mycobacterium tuberculosis which could be used for screening individual isolates when drug resistence is doubtful.

**Keywords:** Contraceptives • Abortion • Morbidity • Mortality

## Introduction

At the beginning of the 20$^{th}$ century, infectious diseases were the leading cause of death in different societies. Tuberculosis is one infectious disease responsible for 3 million deaths worldwide, which is exacerbated by the prevalence of drug-resistant patients and the AIDS epidemic. Therefore, infection control is essential when drug resistant is likely to remain infectious for a long time, so the public health consequences of drug-resistant may be more serious than those that are drug-sensitive. When drugs are used alone, resistant floods rapidly appear and proliferate, so it is advisable to use a combination drug that has a recovery rate of over 95%. The two main drugs used in the treatment of tuberculosis are rifampicin and isoniazid, which are the most important drugs in the treatment of tuberculosis and resistance to these drugs has undesirable consequences. Tuberculosis resistance problem is growing worldwide. The rapid detection of drug resistance in TB patients allows us to use appropriate treatment to better control of disease.

Limiting the transmission of drug-resistant strains and reducing the time between effective diagnosis and treatment, requires rapid detection of resistance. Since the detection of MTB drug resistance takes 6 to 8 weeks by old culturing methods, molecular methods are important to detect this drug resistance [1-3].

The development of new methods to evaluate resistance to anti-tuberculosis has led to the control of TB. Nucleic acid proliferation assays can reduce detection time. According to molecular genetic methods, rifampicin and isoniazid resistant regions can be identified. We need fast, easy and cost-effective methods to detect these mutations that are also suitable for huge populations. The best technique for identifying MTBs drug-resistant is molecular methods, especially PCR-based molecular methods. The aim of this study was to introduce different methods for tuberculosis and also drug resistance tuberculosis patient's detection shortly after sample collection and a standard method that can detect mutated *genes* rapidly in clinical specimens. Mycobacteria are belonging to the *Mycobacteriaceae* family and to the class of actinomycetes.

***Address for Correspondence:** Zoha Tavakkoli, Department of Microbiology, University of Barcelona, Barcelona, Spain; E-mail: zohatavakoly@yahoo.com*



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Pathogenic species are belong to the mycobacterium tuberculosis complex and are the most common and the most important cause of the disease in humans [4-6].

Mycobacteria are aerobic bacilli with little or no curvature of 0.2 to 0.6 in 1 microns to 10 microns. The cell wall of this bacterium is composed of a multilayer structure containing large amounts of lipid complex. Some of these building blocks are exclusively present in this genus of bacteria and can have profound biological effects on the host. In terms of chemical composition, the cell wall of Mycobacterium differs from gram-negative or gram-positive organisms. Mycobacterium tuberculosis is a compulsive aerobic organism that does not grow in lack of oxygen and even a slight decrease in oxygen pressure, significantly slows down the growth of the organism. A complex medium containing potato-egg or serum-agar should be used for primary isolation (from infected specimens). Organisms grow slowly even in the most ideal conditions; therefore, for growth observation, cultures are kept at 37°C for 10 days to 20 days. In solid media, the colonies produce small, dry, scaly-shaped colonies, but in liquid media, due to their hydrophobic properties, they grow on the surface of the medium. The addition of the Tween 80 detergent prevents the bacteria from accumulating and releases them into the form, thus providing a uniform suspension of the bacilli in the liquid medium, which can be measured by turbidity measurement [7-9].

## Materials and Methods

### Pathogenicity

Among the mycobacterial species, Mycobacterium tuberculosis is one of the most important human pathogens. The bacterium is easily dispersed in the community through the respiratory secretions of the tuberculosis and can cause disease in susceptible individuals at low concentrations. Mycobacteria are clinically divided into three categories:

- **Forced pathogens:** Mycobacterium tuberculosis and *Mycobacterium leprae*.
- **Opportunistic pathogens:** Live freely in the environment and cause opportunistic infections in humans.
- **Saprophytes:** Rarely causes disease in humans [10-13].

### Pathogenicity of tuberculosis in human can divided in four different steps

**Primary infection:** Phagocytosis of bacteria in the lung; circulation in the lymph nodes and circulation; establishment and proliferation in the lungs, kidneys, skeletal vessels and lymph nodes; stimulation of the cellular immune response and increased tuberculin sensitivity hand control of the immune system [14-17].

**Secondary infection:** Silent foci of primary infection; faster phagocytosis of bacilli due to cellular acquired immunity, localized lesions, tuberculosis formation, delayed hypersensitivity reaction that accelerates cheese-making [18-21].

**Pulmonary TB-85% of cases:** Main symptoms; gan complex; distinguished by primary pulmonary calculus and lymph node symptoms; nonspecific symptoms; fever, weakness, fatigue, anorexia, night sweat and slimming; specific symptoms; cough and tremor and chest pain [22-24].

**Extra-pulmonary tuberculosis:** Inadequate immune response, Tuberculosis, small foci throughout the body; most bones, genitourinary system, meninges, lymph nodes and peritoneum; often happen in children with inadequate immune response against primary infection [25-27].

**Tuberculosis and HIV:** Studies have shown that tuberculosis is an opportunistic disease in AIDS patients. The immune system is resistant to TB by macrophages and lymphocytes. In macrophages, bacterial cell division is restricted by CD4 cells and T lymphocytes. HIV, on the other hand, decreases the function of lymphocytes and decreases the function of macrophages by decreasing phagocytic capacity and by decreasing cytokine production and antigen delivery capacity. Therefore, in HIV-positive patients, the risk of Mycobacterium tuberculosis infection is increased towards tuberculosis. The risk of tuberculosis in a normal person infected with this bacterium is 5%-10% but in an infected person with AIDS it is around 50%. Therefore, the risk of HIV infection in people with HIV is 10 times higher than that of ordinary people [28-31].

**Tuberculosis patient's treatment:** Koch recovered the bacterium from the patient's sputum and lesions and for the first time, it was cultured on serum from cattle and sheep. In addition, by inoculating the susceptible animal, it caused disease in the animal and acquired the primary bacterium from the animal. This process is known today as Koch's principles. By the beginning of the 19$^{th}$ century, physicians had two important tools for diagnosing tuberculosis; microscopy method and x-ray of the chest, which are the basic tools for diagnosis of tuberculosis [32-35].

The basis of anti-tuberculosis treatment is drug therapy. Drug treatment should be initiated as soon as the test results show that the smear is positive or if the patient is in very bad shape and suspected of having tuberculosis. The first antibiotics used to treat tuberculosis were streptomycin and para-aminosalicylic acid, which were prescribed in the 1940's.

These drugs were successfully administered separately, but after a short time, resistance to the two drugs was seen. In the late 1950's, isoniazid was added to the TB treatment regimen. Atambutol was first introduced and then in the early 1970s, rifampicin was introduced. After three decades, drugs were added to the TB drug regimen used in phase I and II clinical trials. Second-line drugs for the treatment of tuberculosis include fluoroquinolones, amikacin, kanamycin, caproomycin, ethionamide, para-aminosalicylic acid, cycloserine, thiactazone. All second-line drugs are expensive, have low efficacy and specificity and are highly toxic [36-39].

**Drug resistance in Mycobacterium tuberculosis:** Drug resistance in tuberculosis arises from spontaneous chromosomal mutations at low abundance. Clinical drug resistance of TB occurs due to genetic changes in an unpredictable genetic source; undesirable physician diagnosis and patient poverty. MTB strains that were resistant to streptomycin appeared after the introduction of drugs for the treatment of tuberculosis in 1994.

Genetic resistance to an anti-tuberculosis drug depends on spontaneous chromosomal mutations at a frequency of 6-10 to 8-10 in mycobacterial homologs. Isoniazid resistance is more common to other anti-TB drug resistance, in isolation or in combination with other drugs.

Standard drug therapy is successful when all 4 drugs isoniazid, rifampin, pyrazinamide and ethambutol are used concurrently and can be completed in 6 months. When 4 drugs to rifampicin and isoniazid are decreased after 2 months, the rate of return increases after 6 months of treatment by 10%. There may be little chance of resistance to rifampin [40].

Rifampicin TB resistance has ominous predictions. The result of the standard Short Course of Chemotherapy (SCC) for disease is poor condition at the 6-month stopping treatment and relapse. It is recommended that disease be treated with isoniazid, pyrazin amide and ethambutol for 18 hours to 24 hours. Some feel that during the course of treatment they can be reduced to 12 months by adding one fluoroquinolone to these three drugs. Furthermore, single rifampicin resistance in MTB is rare except occasionally in HIV patients and rifampicin resistance is generally used as an alternative marker for two species resistance to rifampin and isoniazid. This proxy seems particularly valid for patients who have had previous treatment. SCG can treat less than 60% of patients with refractory TB (MDR.TB) and a high recurrence rate of approximately 28% between these apparent are successes. A second line of treatment is needed to manage the condition. Increased resistance progression with slowing for ethambutol and pyrazinamide has been observed when WHO. treatment occurs despite treatment failure. Diagnosis of resistance to Pyrazine Amide (PZA) and Ethambutol (EMB) in the diagnosis of MDR-TB, for example; resistance plus dual resistance to isoniazid and rifampin generally indicates an undesirable diagnosis, especially when patients receive only the second line of anti-TB treatment. They often compare the two drugs in addition to Fluoroquinolone (FQ) and aminoglycoside and Capromycin (CPM) for the treatment of MDR-TB. The high prevalence of resistance to PZA or EMB as well as impaired SCC, such as PZA, plays a unique role in sterilizing TB lesions to prevent recurrence. FQS is generally considered to have a critical position in the treatment of MDR-TB. *In vitro* resistance to FQS has been demonstrated in the treatment of TB MDR. Most of the resistance to FQS in MTB is associated with the inappropriate use of these drugs in the control of TB, especially MDR-TB. MDR, rifampin and isoniazid resistance differentiation ranged from 0 to 22.3% in new cases. Understanding the mechanisms of mycobacterial resistance to anti-tuberculosis drugs not only advances further molecular diagnostic tests and embodies the concept of designing new anti-tuberculosis drugs, it also helps to prevent the development of drug resistance (Table 1) [41].

**Table 1**. Genomic techniques based on PCR for detecting of tuberculosis antibiotic resistance patients.

| Genomic technique | Mode of action | Activity | Drug resistance |
|---|---|---|---|
| D90d-d59 | Prevents myolic acid biosynthesis and other affects | Changing function of drug | d-d0/1 |
| d59 | Prevents of start dediacted transcription | drug point | d0/09d-d2 |
| d91d-d59 | dEnergy discharge | drug point | d21d-d90 |
| d79d-d19 | Prevent of galatine disynthesis arabinose | Changing function of drug | d2d-d9 |
| d91d-d95 | Prevent protein disynthesis | drug point | d1d-d8 |
| d91 | Prevent protein disynthesis | drug point | d1d-d7 |

**Real time PCR:** This technique is a proliferation-based technology with flow meter that can detect point mutations in *genes*. This method involves a fluorescent amplification reaction monitor to quantify or characterize PCR products without post-PCR analysis (such as digestion with restriction enzymes, electrophoresis, etc.). In this technique, mutations are identified by differences in the melting point of oligonucleotide probes labeled with fluorescent materials when hybridized to different amplified alleles. In this method, which is also known as kinetic analysis (PQ-PCR), fluorescent signals which are detectable during or after each PCR cycle and the results are obtained in a short time. It is currently a powerful tool that measures the products using fluorescent in each cycle. The measured fluorescent is designed to be proportional to the number of cycles. The number of cycles represents the reproduction. The dye used in PCR has ability to bind to the small groove of double-stranded DNA and increase the fluorescence emission power after binding. As the number of PCR cycles increases, the number of PCR products also increases [42].

**DNA line probe assays:** Several Line Probe Assays (LPAs) have been developed, most of them focus only on the hotspot regions of drug-resistance and different assays target different *genes*. Line Probe Assays (LPAs) are the method based on DND-DNA hybridization which is performed by multiple probes in order to contemporary detection of different mutations. In this method, DNA is extracted and the target is amplified by PCR and specific oligonucleotide probes are used to amplicon hybridization in the target sequences and are immobilized on the surface of a strip. Non-specific banding should remove by hybridization washes, the band on the strip get colored when the amplicon-probe hybrids are provided. There are three models to detect first-line and second-line anti-TB drug resistance by LPAs. Targets for model 1 are *rpoB*, *katG* and the *inhA* promoter which are the isoniazid and rifampicin resistance gene targets, rpsL and rrs that are for detecting aminoglycoside resistance are supported by model 2 and model 3 covered gyrA and embB in order to detection of FQ and EMB resistance. The lower sensitivity estimated for EMB resistance (72.9%) in 3 models of LPAs, in the second line agents' resistance sensitivity is (between 90% and 100%) and also high sensitivity and specificity is belonged to the detection of RIF, INH, STR, FQs.

**Sequencing:** One of the best technologies for genotype organism rapidly analyzing is targeted gene sequencing which provide species identification, screening of all known and new mutations including synonymous and non-synonymous mutations, insertions and deletions mutation in a sample, drug resistance detection and evolution

of predicting the organism. In sequencing technology robust software and database tools need to be developed for the full exploitation, requirement of specialized personnel and bioinformatics facilities for data acquisition, experiments and data analysis; the high cost of next generation sequencing platforms; determination of the region of new mutations confer anti-TB drug resistance; and high amounts of high quality DNA are disadvantages of sequencing. However, some reports presented false-positive results. Nevertheless, increasing the cost of sequencing is already lower than that of phenotypic testing for first and second-line drug resistance in most settings. Several studies have reported that sequencing method can be performed directly by sputum samples. And the procedure is progressively becoming simpler, affordable and rapid.

## Results

### High Resolution Melting (HRM)

High Resolution Melting (HRM) is a new, homogeneous, method after PCR amplification performed in a closed tube. This method enables genomic investigations to analyze the genetic alterations of SNPs, mutations and methylations in PCR products (HRM 71), distinguish nucleic acid samples by sequence, length, volume of GC, even easily detecting single open variants such as Single Nucleotide Polymorphisms (SNPs). We know that different base substitutions cause slight differences in melting behavior and the resolution of these melting differences requires an appropriate intercalation color. The new generation of intercalation colors such as LC Green, LCGreen PLUS and SYTO 9 are used to identify mutations. We used SYTO 9 which is very suitable for HRM application. SYTO 9 dye has several important properties that are suitable for high performance HRM; low fluorescence, high double stranded DNA fluorescence, minimal heat transfer from DNA melting due to dye binding, thermal stability with stand PCR cycles and inhibition of activity (Figure 1) [43].

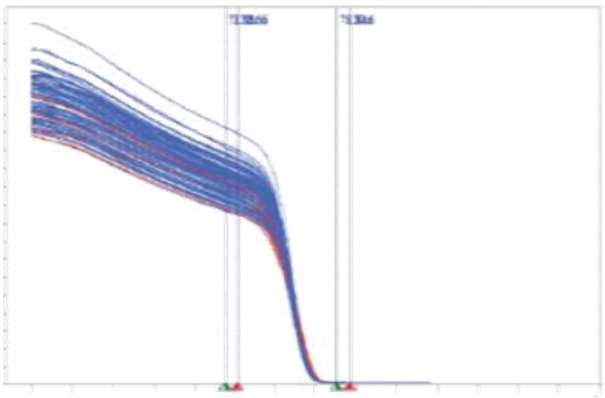

**Figure 1.** Raw melt curves.

The polymerase is highly toxic to the PCR for high performance of PCR and also has less toxicity in the replication reaction and is therefore used at higher concentrations to saturate double-stranded DNA samples. The presence of saturated pigments such as LC Green and SYTO-9 increases the sensitivity of DNA Melting analysis. Unsaturated pigment like SYBR Green because it jumps along the amplicon, causes less precision than the saturated colors. When saturated pigments bind to double-stranded DNA, the pigment is prevented from jumping during the melt-in and allowed to identify sequence changes (Figure 2).

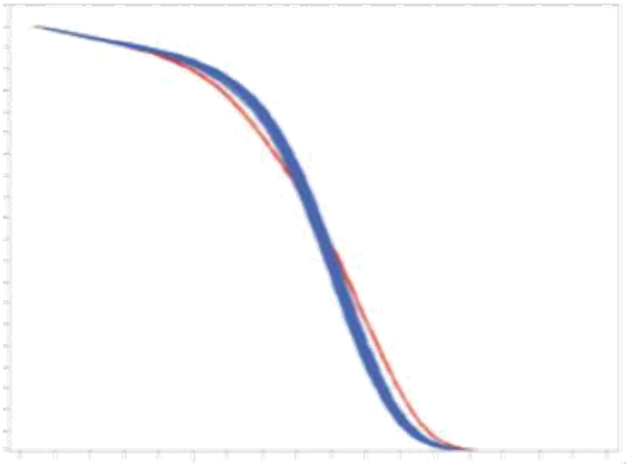

**Figure 2.** Aligned melt curves.

Fluorescent saturated dyes such as LC Green, LC Green Plus and Eva Green and SYTO-9 are more successful for HRM analysis. A very important application of HRM is gene screening, which investigates the presence of unknown variants in PCR primers before sequencing.

The mutations in the PCR products are detectable by the HRM method by the mutations alters in the shape of the melting curve. In fact, by the introduction of dyes attached to the luminous DNA as well as Real-time PCR tools that collect the fluorescent data at lower thermal resolution and transfer directly to software High Resolution Melting Analysis (HRM) is the method of choice for selecting genetic variants in a large number of samples.

The procedure involves amplifying the gene in question in a double-stranded DNA binding dye. After binding, the reactive products are gradually denatured (thawed) resulted in a decrease of fluorescence. This is because that the single-stranded DNA is measured in real-time. Compared to other technologies for mutation selection, such as HPLC denaturing and Denaturing Gradient Gel Electrophoresis (DGGE), the HRM method is much easier, cheaper and less time consuming and consumes very low reactivity.

Rapid new genetic modifications are preferred and are more reproducible than dHPLC and DGGE, also require less researcher optimization and interpretation and provide more accuracy and sensitivity. In addition, HRM analysis is a closed-tube method that greatly reduces the risk of contamination of PCR products (Figure 3) [44].

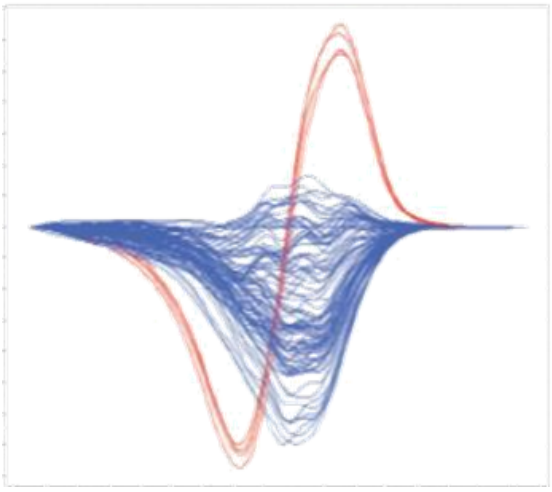

**Figure 3.** Difference plot.

For 95 samples with appropriate bacterial load, three *inhA*, *katG*, *rpoB genes* were analysis to evaluate the performance of high resolution melting method. Samples were placed into different groups by examining each gene of the samples based on differences in melting curve and their HRM. The differences in the graphs were probably due to nucleotide differences in the samples.

### HRM results for the *katG, inhA, rpoB genes*

HRM analysis was performed for all 3 *genes* and the samples were divided into three groups based on HRM and melting curve. Samples that had close melting temperatures were grouped together.

***Normalized graph for the katG, inhA, rpoB genes:*** Normalized Graph provides a basic representation of the different mutations based on the curve deformation in which the samples having the same curve shape are grouped into 3 groups, A, B and C, respectively.

**Difference graph for the *katG, inhA, rpoB genes*:** In the difference graph one sample (red line) is considered a positive control baseline and the other samples are compared. In fact, in the difference graph, the melting profiles of each sample are compared with the sample selected as horizontal lines and the specimens were subdivided into 3 groups because of their inclination near the x-axis, indicating intra-amplicon sequence changes. Sample D2 is positive control sample.

**Melting curve:** Each specimen will melt and peak at different temperatures based on the length of the amplified fragment and the amount of guanine-cytosine (denatured). If the samples have different peaks, there is a difference in their nucleotide sequence, as you can see in the figure, the difference in the peak curves implies the sequence changes in the samples in question, based on the difference of the samples into 3 different groups.

In the study of *katG* gene, two samples had a single nucleotide change at the mRNA level at codon 315, during which guanine was converted to cytosine, resulting in the conversion of the AGC to ACC coding and finally changing the amino acid. As a result, of the 6 sequenced samples, 3 were isoniazid-resistant and 2 were isoniazid-sensitive and since we had different grouping based on melting high resolution analysis, the samples shared this group, they had mutations also.

In the high resolution melting study of inh A gene, the samples were divided into 3 groups and 2 samples from each group were sequenced. One sample from a group with mutation of 4 samples determined normal sequence. As a result, four isolates were susceptible to isoniazid and one isolate resistant to isoniazid and because of the grouping of these isolates, they were probably mutated in a common group of the 95 patients studied, only 3 were in the joint group with the mutation-containing sample, so a total of 3 (3.15%) had a melting temperature close to the sample that was resistant to isoniazid and 92% (96/84) Melting temperature had approximately 2 normal groups which showed sensitivity to isoniazid.

After examining the samples with high resolution melting for the rpoB gene, the samples were divided into 3 different groups based on their differences in melting point. Two samples from each group were sequenced, from them, 3 had mutation sequences and 2 had normal sequences.

In another example, a mutation was found in reductase 545 that converted cytosine to adenine, resulting in the alteration of the CTG-ATG codon, which eventually became the amino acid (leucine-to-mitonin) codon (Leu-Met). As a result of the sequenced samples, 3 samples had mutations that caused resistance to rifampin and 2 samples had normal sequences that caused the sample to be sensitive to rifampin. According to the probability of the samples in the common group having mutations, 5 samples (4.75%) had a melting temperature close to the sample with 531 mutation and 3 samples (3.15%) in the sample group with mutation. Those with mutations in the 545 had no mutation so that of the 95 samples tested, 8 (7.6%) were resistant to rifampin and 87 (82.65%) were susceptible to rifampin and had a melting point close to the sample. Which had a normal sequence with the development of these resistant strains, the implementation of drug susceptibility tests is more important than ever. Understanding the genetic basis of drug resistance will help to develop effective methods for the rapid determination of drug resistance of Mycobacterium tuberculosis strains [45].

## Discussion

Furthermore, the culture method is the most common method of TB diagnosis and antibiotic testing to detect drug resistance, takes about 6 to 8 weeks to develop and the disease may increase during this time. Therefore, molecular methods that have developed rapid methods for detecting the disease and drug resistance are techniques such as RFLP, spoligotyping, PCR, time-PCR-Real and so on.

RFLP and spoligotyping techniques require a great deal of time and energy in the laboratory and the PCR method requires a post-PCR step that may lead to contamination. In the time-PCR-Real method, we need dedicated probes that require a long time to design and are not economically viable. Resistant and also employs the standard, fast, inexpensive and cost-effective high resolution melting method without the need for post-PCR, dedicated probes and high time for culture to identify these mutations.

In addition to special devices, High Resolution Melting (HRM) uses special saturating dyes that only have fluorescence in the presence of double-stranded DNA. These dyes are embedded in amplified PCR products. When the sample is heated to high temperature, the DNA is denatured and the color of fluorescence

disappears so that the double stranded DNA cleaves and melts curves because different genetic sequences melt at slightly different rates. They can be viewed, identified and compared using these curves. Even a single base change will make a difference in the melting curve. Therefore, the HRM process can be used to compare sequence characteristics between two DNA samples and to select any sequence change between the two primers. High resolution DNA melting has become very popular because of its simplicity and accuracy and can be used to search for changes in many *genes* that in many cases require much reduced or eliminated sequencing.

## Conclusion

Therefore, High Resolution Melting (HRM) Analysis is a very suitable and sensitive molecular method for the screening of point mutations in clinical and diagnostic studies and since it is a fast, inexpensive, large-scale, responsive method, no further processing on the sample is required. A method for the detection and diagnosis of point mutations in patients with TB can be suggested. The advantage of this method is that PCR amplification and melting curve analysis are performed within the same tube or plate without any post-PCR processing. This feature is particularly important for routine diagnostic tests. HRM has many advantages over other techniques including that this technique is fast and powerful so that it can detect point mutations in a large number of samples and in less time. This is a simple technique. Mutations can be checked by measuring good quality HRM and accessing Real-time PCR. The present study showed the most common mutations associated with drug resistance to rifampicin and isoniazid and also showed the highest resistance to isoniazid, which is consistent with other studies.

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