

High Resolution Melting Analysis in Leishmania Resistance: The Role of Multi Drug Resistance 1 Gene

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Research note

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Abstract

Objective Pentavalent antimonial compounds are currently used to treat Leishmaniasis. Resistance to these drugs is a serious problem. The purpose of this study was to evaluate the mutations in the multi-drug resistance 1 (MDR1) gene in biopsy specimens of *Leishmania Tropic*a with high resolution melting (HRM) method.

Results In this experimental study, genomic DNA was extracted from 130 patients with skin Leishmaniasis. Then, nucleotide changes were investigated throughout the gene length using HRM and sequencing methods. The results of the nucleotide changes showed that 61% of the samples that were unresponsive to drug had mutations in the MDR1 gene in different groups, which were confirmed by the sequencing method. These mutations can be one of the factors responsible for non-response to the treatment of the disease. HRM method can be used to diagnose drug resistance in Leishmaniasis. It is also recommended that further studies be done regarding the importance of drug resistance in the affected patients.

Introduction

Cutaneous Leishmaniasis is one of the major diseases in tropical and subtropical areas (1). It is caused by parasites of *Leishmania Tropic*a and *Leishmania major* (2). It is a health problem in 88 countries, and because of the importance of controlling and preventing its spread, it is one of the top priorities of the international community, including the World Health Organization (3).

Currently, pentavalent antimony compounds, including glucantime, are used to treat Leishmaniasis. It has been shown that pentavalent antimony affects the amastigote form and inhibits the activity of enzymes in the oxidative pathway of fatty acids. While its active form acts as trivalent antimony against the forms of amastigote and promastigote *Leishmania* (4). On the other hand, pentavalent antimony including sodium stibogluconate has been shown to stimulate macrophages to produce *Leishmania* lethal molecules such as nitric oxide (NO) and reactive oxygen species (ROS) (5). In an animal model, the five-degree antimony mechanism depends on a number of factors, including T cells and cytokines. Therefore, the results indicate that sodium stibogluconate (SSG) may eliminate this parasite by both direct and indirect mechanisms. Studies show that trivalent antimony by creating the flow of intracellular thiol leads to disturbances in the oxidation potential and decreases the parasite thiol and by inhibiting trypanosome reductase leads to the parasite death by oxidative stress (6). Antibody destroys the parasite by a process that allows DNA fragmentation and phosphatidylserine output at the outer surface of the membrane (7). High levels of trypanoiton are involved in resistant strains to the synthesis of GSH and polyamines (8). Various mechanisms of drug resistance are known for Leishmaniasis. Studies on the failure of a pentavalent antimony treatment have recently been reported in several countries (4). Uncovering the molecular mechanisms involved in drug resistance will help to treat patients. In order to identify gene sequence variations, regarding the accuracy, cost, and time spent, the HRM method seems to be a suitable and powerful tool. HRM is a new and homogeneous method after polymerase chain reaction

(PCR) amplification performed in a sealed tube(9). This method of genetic variation analysis (SNPs, mutations, and methylation) measures PCR products based on the amount of fluorescence reduction during the process of thermal melting slope of the DNA due to color exhaustion and HRM products can be sequenced without any additional replication and purification (10–12).

Because of the reports of drug resistance caused by glucantime in patients with cutaneous Leishmaniasis, this study was conducted to determine and diagnose mutations in the multi-drug resistance 1 (MDR1) gene in paraffin tissue samples of Leishmaniasis.

Material And Methods

130 samples of parasitic tissues of Leishmaniasis and two standard parasite samples, including *Leishmania Tropica* of sensitive (MHOM/IR/10/175) and resistant (MHOM/IR/10/827) strains were obtained from Dermatology Department of Afzalipour Hospital in Kerman.

DNA extraction

Genomic DNA was isolated by using QIAamp DNA Mini Kit (QIAGEN Germany). We used the manufacturer's protocol for extraction. DNA concentration was determined by Nano Drop[®] at a wavelength of 260 nm, and the absorption ratio of a pure sample DNA at a wavelength of 260 nm to 280 nm was calculated (A_{260}/A_{280}).

HRM Analysis

Specific primers were designed to amplify the MDR1 encoded region of the MDR gene. The designed primers have the following sequence and were synthesized by TibMolbiol (Berlin, Germany):

HRM.F: 5'-ATTGTCGCTTCTGGGGTTG-3'

HRM.R: 5'-ATCGTGTCGCTTGTGTAC-3'

HRM real-time PCR was performed using 10 μ L of Type-It Master Mix (QIAGEN, Germany), 2 μ L of DNA, 0.7 μ L of each of forward and reverse primers (10 pmol) and 6.6 μ L of nuclease-free water in a total volume of 20 μ L. Thermal cycling conditions included an initial activation step at 95°C for 5 min followed by 40 cycles including a denaturation step at 94°C for 20 s and a combined annealing/elongation step at 60°C for 30 s.

The reaction took place in Applied Biosystem Step One (ABI). For HRM analysis, the PCR products were melted by warming up the temperature from 40°C to 95°C at a ramp rate of 0.007°Cs⁻¹ by 20 fluorescence acquisition every degree of temperature. Dissociation of fluorescent dye from double-stranded DNA occurred with an increase in temperature(9). The normalized graph and the normalized temperature-shifted difference graph (difference graph) from the gene scanning analysis were used to analyze the data. HRM data were analyzed using Applied Biosystem Step One (ABI) software.

Sequencing

To compare the difference between the samples that were found in HRM analysis some of the samples were amplified by MDR1-R and MDR1-F primers, bilateral sequencing was accomplished by Bioneer Company in South Korea. The results of sequencing were studied by the chromas pro.v.2.1 software, and sequence alignment was carried out for two-sided sequences with each other. Alignment diagram was drawn by the use of software Vector NTI advance 11.

Results

The amplification by primers was confirmed by simple PCR and then it was shown by gel electrophoresis that all samples have 160 bp amplification products (figure 1).

Real time PCR and HRM Results

Primer pairs of HRM amplified a 100 bp product. In HRM analysis, all samples had melting peaks. This shows a difference between genome, which caused changes in melting peaks. To further differentiate, a high-resolution difference plot of the HRM assay was plotted by subtracting the melting curve of each sample from the baseline (or reference) curve. Based on the patterns of the difference plots, the assay was able to discriminate the group that has same sequence. One sample from each group was sequenced and compared with each other by alignment to find nucleotide difference one by one (figure 2).

Alignment

To compare the amplified products, some of them were sent for sequencing. The nucleotide sequence was subjected to alignment with other sample nucleotides and plotted in figure 3. In this method every nucleotide was compared with all sequences of other samples and any similarities and differences between the sequences were shown. Every category in HRM curve was compared with each other and the differences between them were shown in alignment.

Discussion

Analyzing the drug resistance mutations is useful in achieving an effective drug for treating the disease. Currently, chemotherapy especially the use of pentavalent-antimony compounds, such as glucantime, is used more than other methods in the treatment of leishmaniasis. The use of glucantime in patients with cutaneous Leishmaniasis has some side effect on vital organ such as kidney, liver, heart, and blood (13). Studies conducted in different parts of the world show drug resistance to glucantime in some people with Leishmaniasis (14). Various factors contribute to glucantime resistance, including: 1- Gene factors, 2- Protein factors, 3- Enzyme factors, 4- Carrier passing medicine, 5- Change in drug accumulation, 6- Inadequate doses of drug, 7- Thiol attached to glutathione, 8- Apoptosis, 9- Cell's skeleton changes, and 10- Host's safety system (4, 15).

A family of transmembrane proteins is ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp), which is encoded by the MDR1 gene. MDR1 gene is 37% similar to human MDR gene and is located on chromosome 34. P-gp is an ATP-dependent drug efflux pump, which can reduce the drug's accumulation in resistant cells and often increase drug resistance (16). According to the importance of drug resistance in patients, modern molecular techniques can provide an opportunity for disease control and treatment for rapid identification of drug resistance. In this study we used HRM method to scan the MDR1 as a molecule that involves in resistance in *Leishmania* spp. Alizadeh and colleagues collected the samples of cutaneous Leishmaniasis in some endemic areas of Iran including Yazd, Mashhad, and Kashan province and by RFLP-PCR method showed that 11% of the patients had mutations in MDR1 gene (17). Our results showed that 61% of the specimens had mutations in the region of MDR1 gene. These changes may cause differences in response to treatment. The HRM method is an easy way to scan sequence and it is an economic, rapid, and closed tube method that has advantages in diagnosis. This study showed new way to diagnose mutations of MDR1 gene in a wider range than RFLP-PCR method. This method can be used to diagnose *Leishmania* drug resistance. It is also suggested that studies be carried out to determine the prevalence of genetic factors affecting the resistance of antimony compounds in endemic areas of cutaneous Leishmaniasis along with the determination of species based on precise molecular methods. Also, studies are needed to replace appropriate drugs for treatment in case of drug resistance to antimony compounds.

Limitations

Finding the samples and also the standard parasite samples, including *Leishmania Tropica* of sensitive (MHOM/IR/10/175) and resistant (MHOM/IR/10/827) strains is the most time consuming part of the research.

Abbreviations

multi-drug resistance 1 (MDR1)

high resolution melting (HRM)

ATP-binding cassette (ABC)

P-glycoprotein (P-gp)

Declarations

Ethics approval and consent to participate

The study approved in Ethical Committee of Kerman University of Medical Sciences. The Ethical Approval Code is IR.KMU.REC.1397.267.

Consent for publication

All authors consent for publication in **BMC Research Notes** journal.

Availability of data and material

Please contact corresponding author (S.D) for data requests.

Competing interests

Not applicable

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Authors' contributions

S.D. proposed the original concept and designed the experiment and supervised all aspects of the work. MA.F., A.M., R.V., and I.R. equally participated in the data acquisition and analysis. All authors contributed to writing the manuscript. S.D. provided critical reviews in order to promote the manuscript.

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Figures

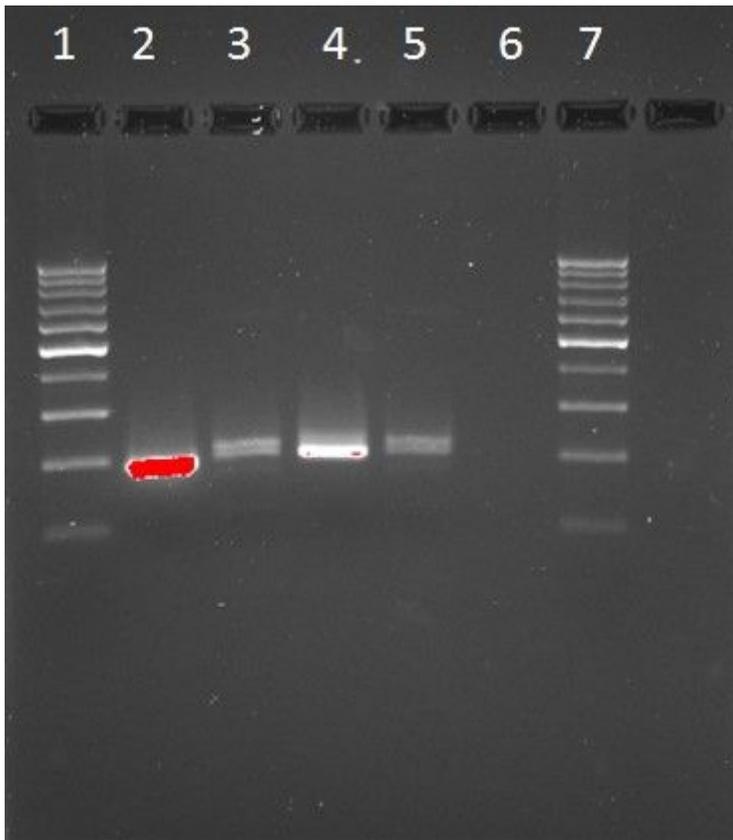


Figure 1

Electrophoresis of MDR1 showed gene PCR product in the samples of Leishmania. Tropica on the 2% agarose gel; from left to right, column1: molecular marker100bp; columns2,3,4,5: fragment length 160bp; column6: contamination control; column7: molecular marker 100bp

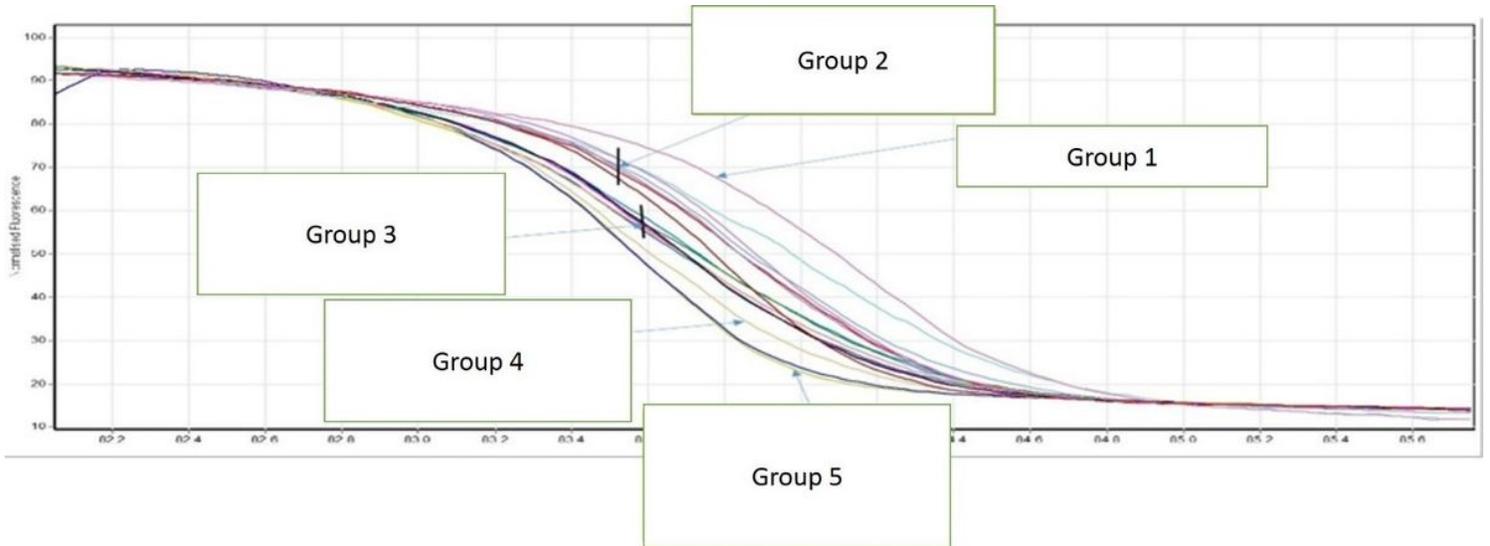


Figure 2

The HRM curve in which every sample was categorized in a group

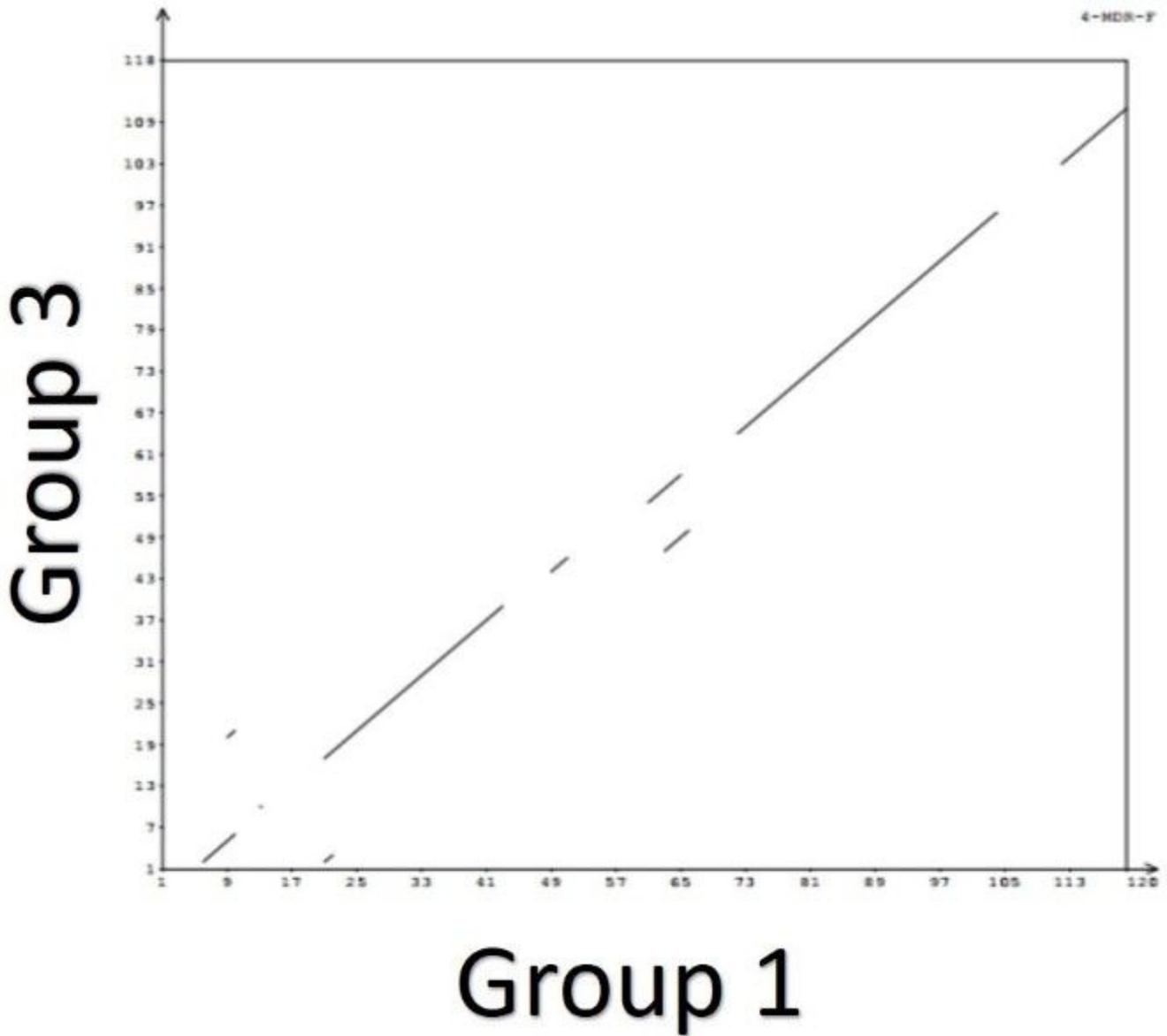


Figure 3

The alignment of two groups (1 and 3 in HRM curve) showed differences in each group (line brake showed this compared group has different nucleotide in that region).