Tetra-Primer ARMS PCR and Its Applications in Genotyping Point Mutations and Single Nucleotide Polymorphisms

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ABSTRACT

Single nucleotide polymorphism is the most frequent type of polymorphism in the genome sequence, mostly used as the genetic marker for identification. Moreover, point mutations are diagnosable and detectable similar to single nucleotide polymorphisms which cause genetic diseases. Thus, it is important to suggest a technique to detect many mutations with high speed and accuracy and low cost. Tetra-primer ARMS PCR is able to provide the possibility of identifying different allele forms of a single nucleotide polymorphism in a PCR reaction. In this study, two common point mutations of IVSII-I (G-A) and IVSI-110(G-A) in beta thalassemia were detected and genotyped by tetra-primer ARMS technique. Finally, accuracy and precision of this techniques were analyzed and evaluated using sequencing results. As the results of genotyping by tetra-primer ARMS and sequencing were the same, it seems that the former is capable to turn into a routine test in diagnostic laboratories and clinics.

Keywords: Single nucleotide polymorphism, tetra-primer ARMS, point mutation, IVSII-1, IVSI-110

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Introduction

Studying the sequence of nucleic acid and the diversity of DNA sequences in genomes of different individuals plays an important role in molecular genetic studies. The importance of genomic variability is evident when it is used for population studies, variation among species, as well as diagnosis and genotyping of pathogenic mutations. Single nucleotide polymorphism (SNP) has more applications due to its higher frequency compared with other genetic variations[1]. It is estimated that approximately for every 250 - 1,000 base pairs in human genome sequence, one SNP is present. The high density of SNPs in the genome and the very small chance of variability against the mutagenic agents have made them very good candidates for DNA markers in population genetics studies and identifying effective genes involved in complex genetic diseases. Also, many genetic diseases occur as a result of point mutations that can be identified and tracked similar to different SNP types[2]. So, identifying genetic changes and diversity and introducing a technique which can identify a large number of point mutations or single nucleotide polymorphism on a large scale with high speed and precision and low cost is very important. Today, techniques used to identify SNPs are: restriction fragment length polymorphism (RFLP), allele specific oligonucleotide (ASO) hybridization, single-strand conformation polymorphism (SSCP), amplification refractory mutation system (ARMS), and sequencing. On a large scale, for identifying multiple mutations simultaneously, techniques such as real time PCR using special probes and microarray are used[3]. Each of these techniques has shortcomings that limit their use in determining genotype of SNPs. Identification and determination of genotype in most of the above techniques are done based on PCR reaction and its costly and time-consuming following steps.. In addition, this technique may have limitations. On the other hand, in mentioned routine techniques, the user does cannot simultaneously detect multiple mutations or different SNPs[2-6]. Although in the standard reaction of ARMS, genotype identification is carried out without additional steps following PCR, it is essential that different allelic forms of a SNP be detected in two separate PCR reactions in this test. Since the accuracy of detection depends on the type and specificity of primers for different allelic forms, lack of a control product in PCR reaction reduces the precision and accuracy of diagnosis[2]. Although sequencing seems to be the most accurate technique for genotyping mutation and SNP, determining the sequences is very time-consuming and if done endemically, special technology and expensive equipment are needed. Thus, it is not possible to use this technique as a routine test in diagnosis and research centers and it is mostly applied as a complementary and confirmatory test alongside other tests. None of routine tests is able to identify different mutations and polymorphisms simultaneously on a large scale, despite the fact that some genetic diseases are poly-genes and also, in the field of population genetics, it is necessary to identify several mutations or polymorphisms simultaneously. Although microarray technology and real time PCR using specific labeled probes and weighted spectroscopy can simultaneously identify multiple SNPs on a large scale, these technologies are very expensive and it is impossible to use them in all diagnostic and research centers[1].

Tetra-primer ARMS (T-ARMS) is a varied form of ARMS classic technique which, in addition to overcoming shortcomings of other test, makes it possible to identify different mutations on a large scale with high speed and precision and low cost[1,7]. Genotyping in T-ARMS is based on PCR reactions, followed by electrophoresis on agarose gel. In this method, unlike standard ARMS, different allelic forms are identified by four primers. Primers are designed so that the

sizes of the amplified fragments of allelic forms differ and it is possible to separate them by electrophoresis on agarose gel[1, 2, 8, 9]. Using this method, 2-3 products are made in every PCR reaction based on homozygous and heterozygous genotypes (Fig. 1). Primers are designed to have a base un-pairing at the third position from the 3' end of primer. The created change increases the specificity of the T-ARMS primers. The type of base un-pairing, selection of which is done by a series of specific rules, greatly affects the accuracy of genotyping results. If the lack of base un-pairing at the 3' end of inner primer results from a strong allelic variation (G/A or C/T), the second base pairing must be weak (C/A and or G/T) and vice versa. If the base pairing at the 3' end is medium (A/A, C/C, G/G or T/T), the second base pairing will also be medium[1, 2, 5]. Some technical features of T-ARMS are forming control band by outer primers, mixed PCR reaction (four primers in a reaction simultaneously), and simultaneous identification of mutation alleles by a reaction. If some measures are taken in designing different mutations primers to make their performances in PCR technique similar, it will be possible to identify different mutations simultaneously.

According to the aforementioned features of T-ARMS, it seems that this method is more accurate and precise and also more cost-effective than other methods, and can be used as a routine test replacing other clinical tests. This study aims to analyze the accuracy and precision of T-ARMS by simultaneous identification of two mutations involved in

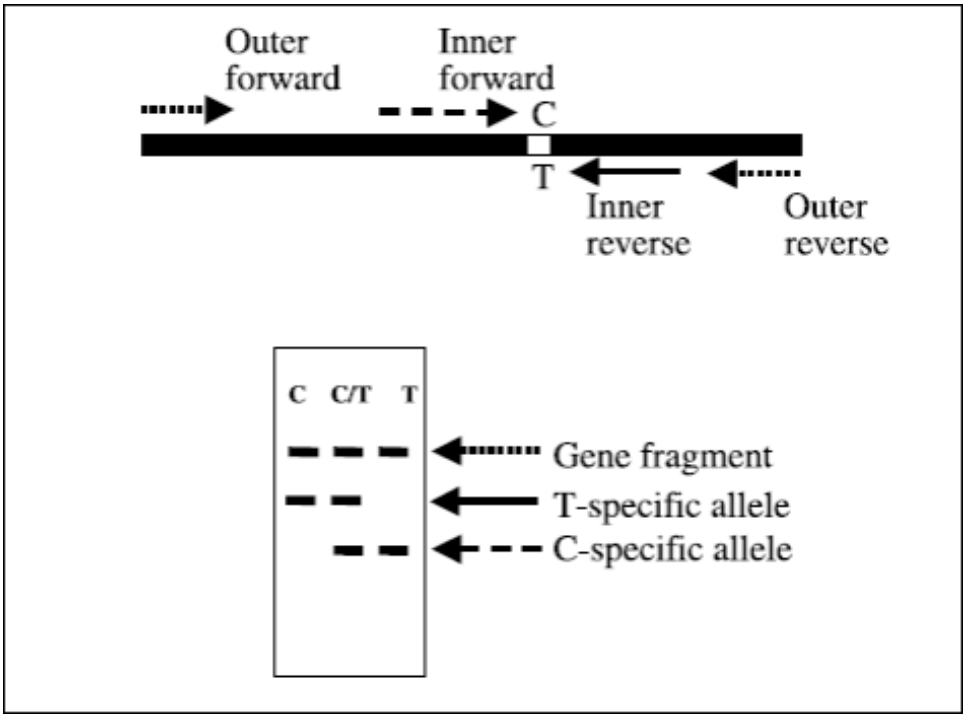


Fig. 1: A schematic view of the four primers of T-ARMS and how reaction is conducted

thalassemia, IVS II-I (G to A) and IVSI-110 (G to A), and comparing the results with the those of sequencing.

Methods

Sampling and genomic DNA extraction

After obtaining consent from healthy individuals and patients with major thalassemia who had referred to Iran Blood Transfusion Organization and Omid Hospital in Isfahan, Iran, 5 mL blood from 10 healthy individuals and 100 patients with major thalassemia were collected in EDTA-containing tubes. Genomic DNA of blood samples was extracted from Leukocyte cells using the standard salting out method[11] and was kept at -20 °C after determining its concentration.

Design of the primers

Primer design in T-ARMS method is as follows: two outer primers amplify the control gene fragment containing SNP and the greatest product of PCR, and every inner primer and one of outer primers amplify smaller fragments that determine different forms of SNP alleles. Size of fragments should be designed in order to allow the separation of bands on electrophoresis gel. In order to design primers, first the sequence of related mutations' surroundings in beta-globin gene was obtained by referring to the database on http://www.ncbi.nlm.nih.gov and appropriate primers were designed using Primer 3 website (http://bioinfo.ut.ee/cgi-bin/primer3-0.4.0/cat_humrep_and_simple.cgi). Next, the final analysis was conducted by Oligo 7 and BLAST available at http://ncbi.nlm.nih.gov/blast and then the best primers were selected. Table 1 shows the primers' sequence, their individual bonding temperature, and the length of PCR products for each allele.

Optimizing PCR reaction

Since PCR reactions in T-ARMS are multiple, it is expected that at least two different products (control band and allele-band) be produced in each reaction. Since there is some interference between primers' performances, and their functions are competitive, it is essential to optimize reaction conditions to produce main products of PCR for genotyping. To optimize PCR reaction numerous factors such as bonding temperature of primers, inner and outer primers' and reagents' concentrations were probed. The temperature gradient and the gradient of the various inner and outer primers' ratios were used in order to optimize multiplex conditions. Because of production of various products in a PCR reaction, it is expected that, in addition to consumed primers' concentrations, the concentration of other reagents such as ion, dNTP, and polymerase enzymes affect the quality and quantity of the PCR reaction products.

Polymerase chain reaction and electrophoresis gel

PCR reaction was conducted in a final volume of 25 µl, containing100 ng of genomic DNA, 0.5 mM dNTP, 6 mM MgCl2, 2.5 µl PCR buffer, and a proper concentration of each primer including 5 and 15 pM of inner and outer primers, respectively. The optimized thermal-time schedule of polymerase chain reaction was completed in the following steps: the initial melting step of 94 °C for 5 min, then 30 cycles of thermal repetition with denaturation at 94 °C for 30 s, bonding temperature optimized for each specific primer of every mutation listed in Table 2 for 30s, followed by initial replication at 72 °C for 30s, and then a final elongation at 72 °C for 5 min. After PCR reaction, the products were electrophoresed on 2% agarose gel with a 100 bp marker and stained with ethidium bromide, and then the bands of interest were investigated by UV Gel Doc.

Validation of genotyping

To determine the accuracy of genotyping results, genomic DNA of 16 patients were randomly selected

Table 1: Nucleotide sequence of designed primers, their bonding temperatures, and the length of amplified products for identifying the genotype of IVS II-I and IVI-110 mutations

Amplicon size	Bonding temperature	Primer sequence	Mutation
172 bp (A allele)	55	Forward inner primer (IVS II-I IF) (A allel): 5'-CGTGGATCCTGAGAACTTCATGA-3'	IVS II-I (G-A)
268 bp (G allele)	55	Reverse inner primer (IVS II-I IR) (G allel): 5'-AAACATCAAGCGTCCCATAGACTAAC-3'	
391 bp (control)	55	Forward outer primer (IVS II-I OF): 5'-TCTATTTTCCCACCCTTAGGCTG-3'	
	55	Reverse outer primer (IVS II-I OR): 5'-CTAAAACGATCCTGAGACTTCCACA-3'	
127 bp (A allel)	61	Forward inner primer (IVI-110 IF) (A allel): 5'- GATAGGCACTGACTCTCTCTGCCTAGTA -3'	IVI-110 (G-A)
323 bp (G allel)	61	Reverse inner primer (IVI-110 IR) (G allel): 5'- AGCCTAAGGGTGGGAAAATAGTCC -3'	
395 bp (form two outer primers)	61	Forward outer primer (IVI-110 OF): 5'- CCAGGGCTGGGCATAAAAG -3'	
	61	Reverse outer primer (IVI-110 OR): 5'- CATAACAGCATCAGGAGTGGACAG -3'	

and sequenced. Sequencing specific primers were designed so that they would include the most frequent mutations of beta thalassemia in Iran as identified in literature. Sequence of sequencing primers and the length of amplified fragment are shown in Table 2.

PCR sequencing reaction occurred in a total volume of 25 µl containing 100 ng genomic DNA, 2.5 µl 10X buffer, 3 mM magnesium ion, 2.0 mM dNTP, and 0.5 unit Taq polymerase enzyme for 5 min at 94 °C, 30 cycles at 94 °C for 30s, at 55 ° C for 30s, at 72 ° C for 30s, and the final polymerization was carried out for 5 min at 72 °C. After completing the PCR reaction, about 5 µl PCR product was electrophoresed on 2% agarose gel. After viewing the band of interest with a length of 645 bp, the band was purified by a gel purification kit manufactured by BioNeer and then was sent for sequencing to Sina Clone Company (Fig. 2).

Result

Genotyping the mutations of IVS II-I (G to A) and IVSI-110 (G to A) using T-ARMS and designed primers was successfully completed. Primers' concentrations, denaturation temperature, and concentration of magnesium ions had key roles in optimizing reaction conditions. The ratio of outer primers' concentration to that of inner primers affects the

Table 2: A list of the nucleotide sequence and order of designed primers for sequencing beta thalassemia gene		
Amplicon size	Primer sequence	Sequencing primers
645 bp	5'- AGGGCAGAGCCATCTATTGCTTAC-3'	Forward
	5'- CACACTGATGCAATCATTCGTCTG -3'	Reverse

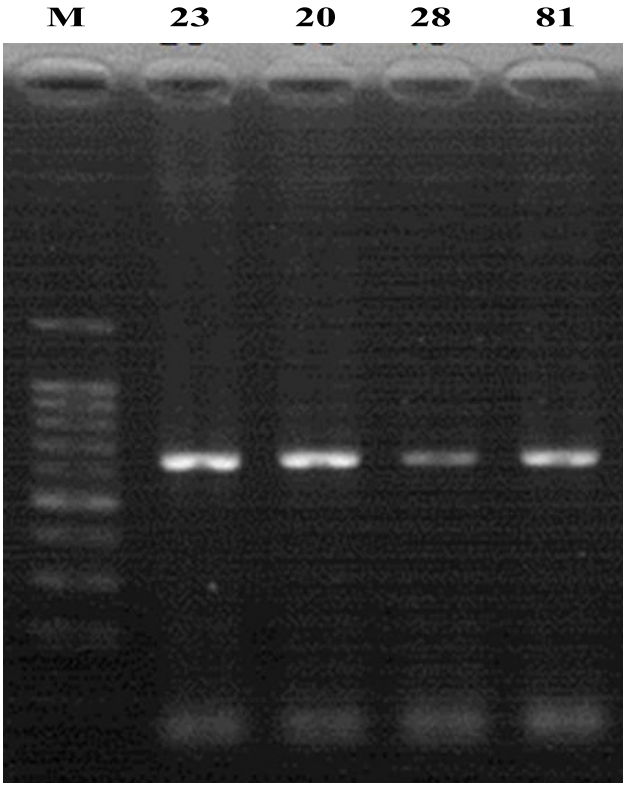


Fig. 2: PCR product with the length of 645 bp of four beta thalassemia patients for sequencing with 100 bp marker

production rate of PCR reaction products. Results show that increased concentrations of inner primers compared with outer primers provide the possibility of producing different allelic products. Results of the study showed that the 1:3 ratio of outer to inner primers is the most desirable condition, and increases the production rate of smaller products (Fig. 3).

The results of investigating different concentrations of the PCR reagents including ions, dNTP, and polymerase enzyme indicated that except for magnesium ion, the concentrations of other materials

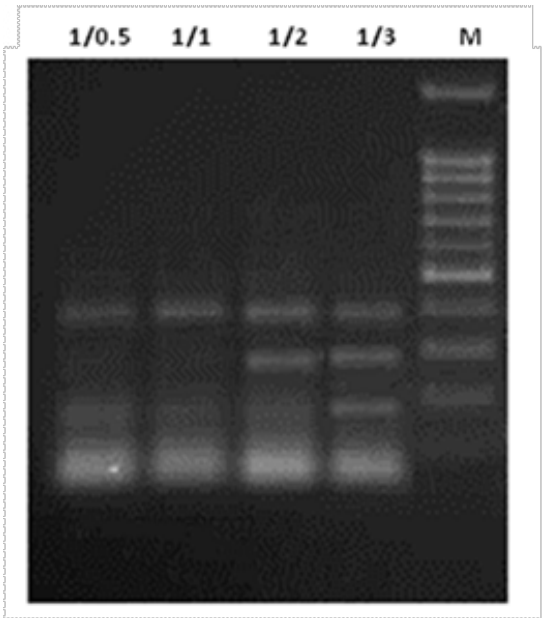


Fig. 3: Optimizing conditions of multiplex PCR for IVII-1 primers using different ratios of outer to inner primers' concentrations, M marker of 100 bp

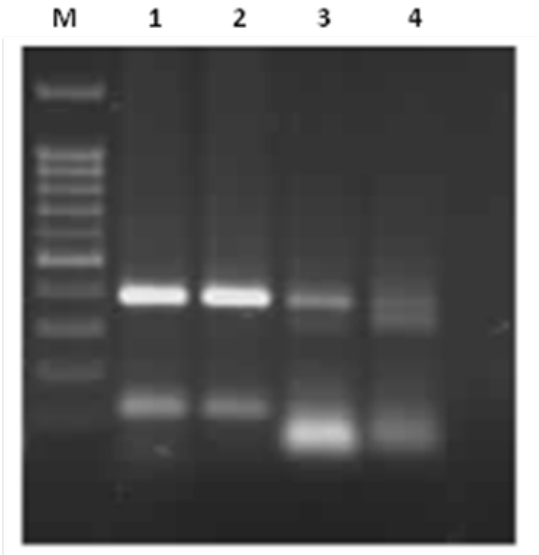
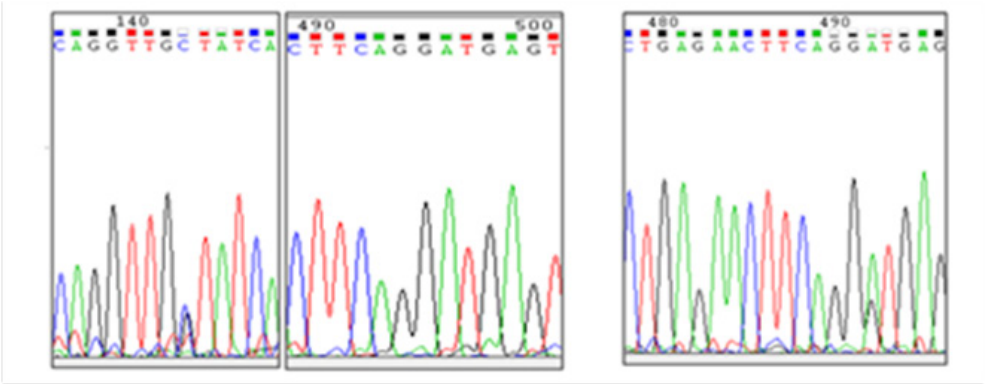


Fig. 4: Optimizing reaction conditions of multiplex PCR for IVI-110 mutation. Samples 1 and 2 homozygous mutant of a sick person; samples 3 and 4 a healthy homozygote of a control participant. The used concentration of magnesium ions in the samples 1-4 were 6, 5, 4, and 3 M, respectively.

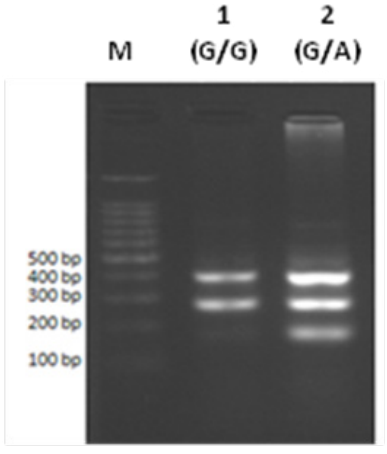
do not affect the quality and quantity of resulting products. The best concentration of magnesium ion for multiplex PCR reaction and for IVI-110 6 M mutation was calculated as 6 M (Fig. 4).

Determining the reliability of T-ARMS

Demographic studies of frequency of beta thalassemia mutations show that the most frequent mutation in the population of the Middle East and the Mediterranean is IVII-1 (G to A)[12-14]. Therefore, the majority of these patients are expected to be the carriers of that mutation. Studying 100 patients with T-ARMS technique showed that 52 patients with IVII-1 (G to A) mutation are homozygous or heterozygous. These results were consistent with determined genotypes by sequencing: from 16 samples submitted for sequencing, 6 samples contained IVII-1 (G to A) mutation, two of which were heterozygous. Tests conducted by T-ARMS for the samples no. 28 and 23 indicated that Sample 28 is homozygous mutation



5-1 5-2 5-3



5-4

Fig. 5. Comparison of the genotyping results by the T-ARMS and sequencing

Figures 5-1 and 5-2 show the results of sequencing for a beta thalassemia patient with compound heterozygous genotype. The patient carries IVI-5 (TTGG has turned into TTGC) and IVII-1 (GGG has converted to GGA) mutations. Figure 5-3 shows the result of sequencing homozygous sample of IVII-1 mutation. Figure 5-4 shows the results of genotyping by T-ARMS. Sample 1 shows the results of identifying homozygous mutant of the individual's genotype whose sequencing results are shown in Figure 3-5. Sample 2 shows the result of identifying heterozygous genotype of the individual whose results are shown in Figure 5-2.

homozygous genotype (Fig. 6).

Figure 6-1: Results of genotyping by T-ARMS: Sample 1, heterozygote; and Sample 2, patient's homozygote.

Figure 6-2: Results of sequencing Sample 2 (patient's homozygote).

Discussion

Progress in various fields of molecular biology has facilitated easier detection of many mutations and polymorphisms which cause genetic diseases[3]. But the important point about clinical techniques used in mutation detection

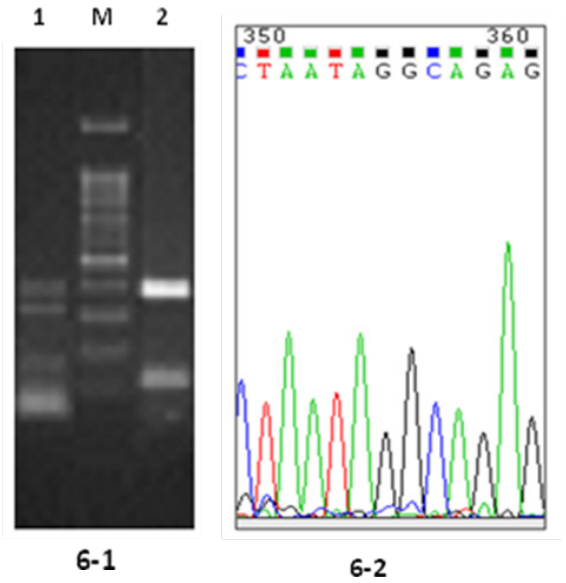


Fig. 6. Comparing the results of identifying genotype of IVI-110 mutation by T-ARMS and sequencing.

Studies on IVI-110 (G to A) mutation by T-ARMS showed that among 100 studied patients, 11 patients were carriers of the mutation, among whom 2 patients were homozygous. Results of 16 samples sent for sequencing were consistent with the results of T-ARMS. For example, genotyping by T-ARMS and sequencing of Sample 81 showed the

or SNPs is the speed, accuracy, and low cost. Today, most techniques used for genotyping polymorphisms and mutations are based on amplification of DNA sequences by PCR, including the modified area, and then distinguishing between different alleles by steps following PCR. A similar process occurs in RFLP or in

hybridization of oligonucleotide fragments (ASO) where PCR products are subjected to the restricting enzymes or oligonucleotide probes. Although it seems that RFLP is highly accurate, due to some limitations such as the distance between detection area and mutation area, lack of access to the enzyme, or its high price, it is almost obsolete now. Instead, the classic ARMS is often used. However, this method has its own problems such as low accuracy, because detection of mutant and normal states is only based on an un-pairing at the 3' end. Moreover, in this method, primers are placed in separate vials and therefore the control band indicating accuracy and precision of PCR reaction cannot be achieved[5, 6]. Among these, sequencing seems to be a reliable method; however, this method is not free from error and must be accompanied by a secondary confirmatory method. In addition, sequencing requires advanced technology and expensive devices. Thus, there is no possibility of making it a routine technique in clinical laboratories or even in research centers[9, 15, 16]. Although newer techniques based on the use of labeled probes, weight spectrometry, and DHPLC with high sensitivity and accuracy to detect mutations and polymorphisms are present, they are very expensive and are not possible to be used for routine testing in most laboratories[2, 10].

T-ARMS is a simple and cost-effective method for genotyping single nucleotide variations. It involves a PCR reaction in a vial with two pairs of primers followed by electrophoresis on agarose gel. In this method, unlike ARMS, while designing internal primers, a second un-pairing is placed in the third position at the 3' end of the primer to increase specificity and accuracy of bonding, so that other mutant primers would not bond to normal sequences or vice versa[2].

Changes applied to this technique provide the following capabilities:

1. High speed, so that different genotypes in a PCR reaction can be detected without the steps following
2. High-precision: specific design for the four primers and placement of the base un-pairing in the vicinity of 3' in inner primers end increase the accuracy of detecting genotypes.
3. Ability to perform reactions in a vial and identifying homozygous and heterozygous genotypes based on the number of observed bands on electrophoresis gel.
4. Cost-effectiveness: T-ARMS does not need specific technology or steps following PCR, and it is possible to perform it with a simple PCR and ordinary reactants available at all diagnostic clinics and research centers.
5. Possibility of viewing the control band for controlling reaction performance in all conditions.
6. Since all reaction steps are performed in a vial and additional steps are not required, this method is the most economical method in terms of time and cost[10].

However, T-ARMS has some limitations too, the most important of which is the optimization of PCR conditions for the proper functioning of inner and outer primers at the same time. It seems that factors such as characteristics of the primers and their concentrations, bonding temperatures, duration of each step of PCR, and ion concentration of PCR reaction affect the quality of products. Results of this study on detecting IVI-110 (GA), IVI-5, and IVII-I (GA) mutations by T-ARMS show that the factors affecting the quality of T-ARMS PCR multiplex reaction are magnesium ion concentration and the concentration ratio of inner to outer primers. Overall, it seems that regarding the capabilities of T-ARMS in detecting mutations and single nucleotide polymorphism and their genotyping, it can be used as an alternative test in research laboratories and diagnostic clinics.

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