

A comparative study of ARMS – PCR and RFLP – PCR as methods for rapid SNP identification

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GEORGIANA. DUTA-CORNESCU¹, ALEXANDRA. SIMON-GRUITA¹, NICOLETA. CONSTANTIN¹, F. STANCIU¹, MICHAELA DOBRE² DORINA. BANICA³, RODICA. TUDUCE⁴, P. CRISTEA⁴, VERONICA. STOIAN¹

¹Department of Genetics, Faculty of Biology, University of Bucharest, ² Faculty of Medicine and Pharmacy, Low Danube University of Galati, ³Institute of Pneumophthisiology “Marius Nasta”, Bucharest, ⁴Bio-Medical Engineering Center, “Politehnica” University of Bucharest

Abstract

Identification of single nucleotide polymorphisms (SNPs) is now possible by many techniques, but choosing one of these methods for a particular case represents quite a challenge, because the researcher must take into consideration many factors.

In this article the authors are trying to present a comparative study of two methods, used currently in our laboratory, for identification of SNPs polymorphisms: ARMS – PCR (amplification refractory mutation system) and RFLP – PCR (restriction fragment length polymorphism). The two SNPs on which we focused belong to human VDR gene (vitamin D receptor gene) and are ApaI (a G→T base change in intron 8) and TaqI (a silent T→C base change in codon 352), named after the restriction enzymes which recognize these variations.

Since the results obtained by both methods were confirmed by direct sequencing, we concluded that ARMS-PCR method is the most adequate for detecting the alternative genotypes determined by single base mutations. The simplicity of this method makes it suitable for the analysis of large number of samples, situation which is usually met in case-control and population genetic studies because this test is easy to use, cost – effective and have an accuracy of 99,9%

Keywords: ARMS – PCR, RFLP – PCR, sequencing, VDR gene, Apa I and Taq I polymorphisms

Introduction

Single nucleotide polymorphisms (SNPs) from human genome are important markers for understanding the fine structure, expression and functions of normal genes and also in presymptomatic and prenatal diagnostic and genetic concealing.

In present, a large number of methods for rapid detection of SNPs are described, therefore choosing one of them for a particular case represents quite a challenge, because the researcher must take into consideration many factors: the test must be easy to use and cost-effective; accuracy must be in 99.9% range and the method must have a day-to-day reliability (S. Lakhotia et al 2002, [1]).

The general strategy for detecting single-base mutations is to amplify the sequence of interest by PCR method, scan the PCR products for the presence of mutation by a rapid procedure and then sequence only those fragments positive for mutation in order to locate and identify the nature of the mutation.

In this article the authors are trying to present a comparative study of two methods, used currently in our laboratory, for identification of SNPs polymorphisms. The two SNPs on which we focused belong to human VDR gene (vitamin D receptor gene) and are ApaI (a G→T base change in intron 8) and TaqI (a silent T→C base change in codon 352), named after the restriction enzymes which recognize these variations (Bellamy R. et al. 2000

[9]). The two methods of choice are ARMS – PCR (**amplification refractory mutation system**), RFLP – PCR. Direct sequencing was used to confirm the correct assignment of the genotypes.

ARMS – PCR method was first described by Newton et al. (1989) as a general technique for the analysis of any point mutation or small deletion. A typical ARMS test, which can detect a known SNP polymorphism consists of two complementary reactions: one containing an ARMS primer specific for the normal DNA sequence and cannot amplify mutant DNA at a given locus and the other one containing a mutant-specific primer and does not amplify normal DNA. The genotype of an individual can be determined by analysis of the amplification products: for homozygote individual PCR, products will be obtained in only one reaction (either the one with the “wild type” primer, either the one with “mutant” primer) and for a heterozygote genotype PCR products will be obtained in both reactions. **RFLP – PCR method** for detection of these two SNPs is based on PCR amplification of a 740pb fragment located between intron 8 and exon 9 of *VDR* gene (9, 10) which contains both polymorphisms, followed by enzymatic restriction of the amplicon with the appropriate enzyme.

In order to certify the results obtained by these methods we have analyzed the two polymorphisms by direct sequencing analysis

Materials and methods

Blood samples collected on EDTA from 50 unrelated Romanians donors was used for DNA extraction, using Wizard® Genomics DNA Purification Kit (Promega Corporation, Madison, WI, USA).

The **double ARMS-PCR** reaction was performed in 25µl final volume, with 2.5µl Buffer, 0.5µl dNTP mix, 0.5µl MgCl₂, 0.5µl for each primer (A, a, T and t, for sequence see Table 1), 0.2µl Taq Polymerase, 1µl DNA and distilled water. Amplification parameters were: 96⁰C/1min; 5 cycle (95⁰C/25sec, 61⁰C/40sec, 72⁰C/42sec.); 21 cycle (96⁰C/25sec, 65⁰C/50sec, 61⁰C/45sec.); 4 cycle (96⁰C/25sec, 55⁰C/60sec, 72⁰C/120sec.); 72⁰C/5min.

For **RFLP analysis** of *Apa* I and *Taq* I polymorphisms the PCR reaction was carried on in a Gene AmpPCR System - 2000 (Perkin Elmer), in 25µl final volume: 2.5µl Buffer, 0.5µl dNTP mix, 0.7µl MgCl₂, 0.5µl for each primer (P1 and P2, for sequence see table1), 0.2µl Taq Polymerase, 1µl DNA and distilled water; the initial denaturation step was 94⁰C/4min, followed by 30 cycles (94⁰C/1min., 55⁰C/1min., 72⁰C/1min.), 72⁰C/5min. All primer sequences are summarized in Table 1. Enzymatic restriction for *Apa*I and *Taq*I polymorphism were performed according to manufacturer specifications.

Table 1. Nucleotide sequence of the primers used in this study

Primers sequence	Nucleotide sequence
ARMS-PCR assay	
Apa – A	5' - GTG GGA TTG AGC AGT GAG GT - 3'
Apa – a	5' - GTG GGA TTG AGC AGT GAG GG - 3'
Taq – T	5' - CGG TCC TGG ATG GCC TCA - 3'
Taq – t	5' - CGG TCC TGG ATG GCC TCG - 3'
RFLP-analysis	
Apa - Taq - Rw2 (P1)	5' GCA ACT CCT CAT GGC TGA GGT CTC - 3'
Apa - Taq - Fw2 (P2)	5' CAG AGC ATG GAC AGG GAG CAA - 3'

PCR products and restriction fragments have been visualized by electrophoresis in 2% agarose gel (Sigma).

The **direct sequencing** of the 740 bp fragment was carried out on a 3130 Genetic analyser (Applied Biosystems).

Results

All 50 individuals from our group were analyzed by both methods (ARMS – PCR and RFLP – PCR), eighth genotypes being identified (Table 2). From this only one individual for each possible genotype were subsequently sequenced, in order to confirm the results.

Table 2. Genotypes distribution in the studied group

Genotype	Male	Females	Total
TT AA	1	-	1
TT aa	5	5	10
TT Aa	4	2	6
Tt AA	2	1	3
Tt aa	-	-	-
Tt Aa	11	9	20
tt AA	5	3	8
tt aa	1	-	1
tt Aa	-	1	1
Total	29	21	50

The differentially amplification of the four haplotypes corresponding to *TaqI* and *ApaI* polymorphisms was performed by double ARMS-PCR method using a pair of allele-specific PCR primers: a forward primer (in our case, a specific primer for *ApaI* polymorphism) and a reverse one (for *TaqI* polymorphism). This way, four amplification reactions are initiated, but amplification can occur in maximum two of the four reactions, according to the chromosomal haplotype of each individual. In our study, by VDR gene genotyping with sequence-specific primers for *ApaI* and *TaqI* polymorphisms, a unique 117 bp amplification product was obtained, corresponding to the primer combination used in PCR amplification (**AT**, **At**, **aT** and **at**) (Figure 1).

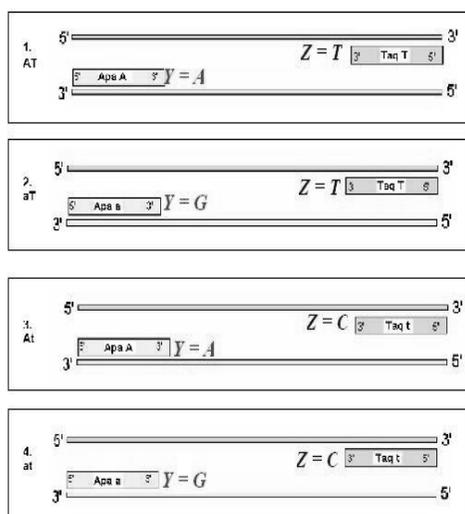


Figure 1. Schematic representation of the double ARMS-PCR reaction for *Apa I* and *Taq I* polymorphisms of the VDR gene. There are four possible reactions, for each possible haplotype: reaction 1-AT, reaction 2-At, reaction 3-aT and reaction 4-at. PCR reaction will generate amplicons according to the chromosomal haplotype of the individual. (i.e. for an “Aa tt” individual there will be amplicons only in the 3rd and 4th reactions)(Dalton D.L, 2005, [9])

The electrophoretic separation of this 117bp fragment allowed us to identify the haplotype combination for each tested individual (Figure 2). In the studied group we have identified all possible genotypes except **ttAA**: homozygote for both polymorphisms: complete dominate **AATT** (Figure 2A), complete recessive (**ttaa**) and dominant/recessive (**TTaa** - data not showed). In Figure 2 the other genotypes which we have identified are also presented: homozygous for only one polymorphism: **aaTt** (Figure 2B), with the recessive “a” allele at *Apa* I locus, **AATt** (Figure 2E), with the dominant “A” allele at *Apa* I locus, **AaTT** (Figure 2D) with the dominant “T” allele at *Taq* I locus, and **Aatt** (data not showed) with the recessive “t” allele at *Taq* I locus. We were also able to identify the compound heterozygote genotype **AaTt** (Figure 2C).

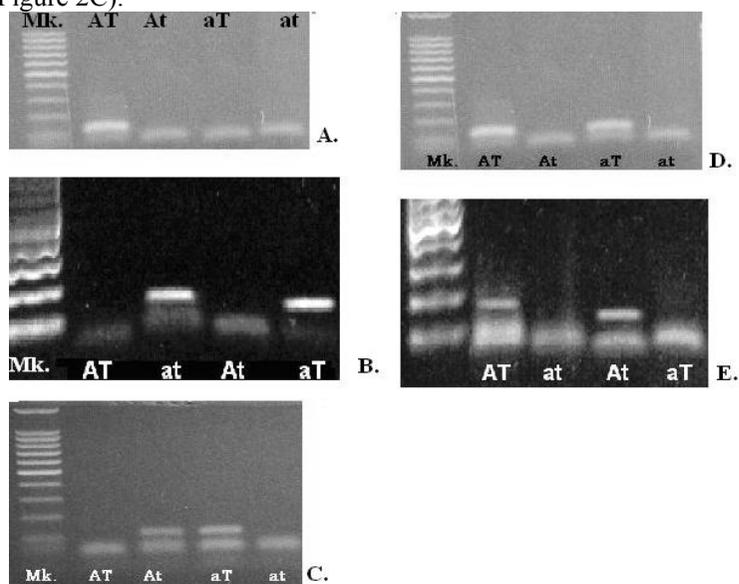


Figure 2 ARMS - PCR of the *Apa* I and *Taq* polymorphisms at different individuals. **A.**- a homozygote AATT individuals - according PCR product obtained only in the reaction where primers for “A” and “T” alleles were used; **B.**- genotype aaTt - PCR products are visible in two reaction; **C.**- genotype AaTt; **D.**- genotype AaTT; **E.**- genotype AATt;

The other method for *Apa*I and *Taq*I polymorphisms identification used was RFLP – PCR. For this purpose, we amplified a 740bp fragment, localized between intron 8 and exon 9 of VDR gene. In order to determine the possible presence of *Apa* I and *Taq* I recognition sites, the obtained amplicon was digested with the two restriction enzymes. After the digestion with *Apa*I enzyme all three possible genotypes were identified: **AA** - which corresponds to an unique, undigested fragment of 740 bp (Figure 3A); **aa** - represented by two fragments of 515 bp and 225 bp (Figure 3B lane 2) and **Aa** heterozygote genotype, made up of three fragments of 740 bp, 515 bp and 225 bp (Figure 3B - lane 1).

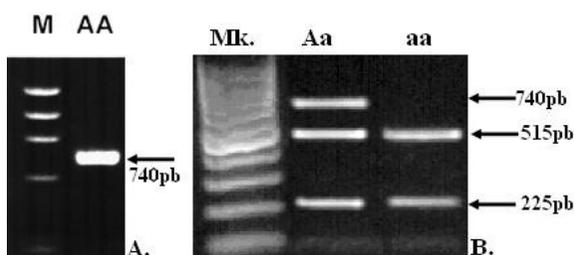


Figure 3. Electrophoretic separation of *Apa I* enzymatic restriction products. **A.** The homozygote genotype “AA” is represented by a single, undigested 740pb fragment, meaning that in the wild type the *Apa I* recognition site is absent; **B.** The heterozygote wild - mutant genotype “Aa” presents beside the 740bp fragment (wild type) two more fragments (515pb and 225pb) generated by a G→T transversion (which inserted an *Apa I* recognition site); the “aa” genotype is represented by only two fragments, the 515pb fragment and 225pb one.

The digestion with *TaqI* allowed the identification of the dominant homozygote genotype **TT**, represented by two fragments of 490 bp and 245 bp (Figure 4 - lane 1) and the heterozygote haplotype **Tt**, represented by four fragments of 490 bp, 290 bp, 245 bp and 205 bp (Figure 4 - lane 2-4).

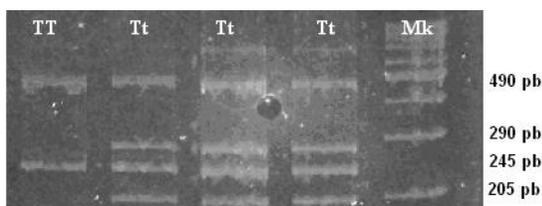


Figure 4. Electrophoretic separation of *Taq I* enzymatic restriction products. The wild type allele, “T”, of *TaqI* polymorphisms contains only one recognition site for *TaqI* enzyme and so, the homozygote “TT” genotype will generate two restriction fragments: a 490pb and a 245pb one. A T→C transition inserts a new recognition site for this enzyme, which, in a heterozygote individual will generate a four bands pattern.

Direct sequencing of the fragment of interest confirmed the genotypes obtained by ARMS-PCR and RFLP-PCR. The *Apa I* polymorphism is represented by a G→T transversion in intron 8 of the VDR gene and the *Taq I* polymorphism by a T→C transition in codon 352 of exon 9 of the gene. The homozygote individuals, either with dominant or recessive alleles presented only one peak (Figure 5 A and C) while the heterozygote individuals presents 2 peaks at the interest locus (Figure 5 B and D).

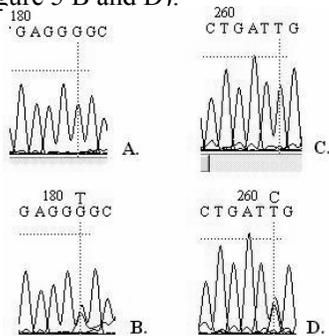


Figure 5. Chromatograms of corresponding sequence for *ApaI* and *TaqI* polymorphisms visualization with BioEdit Sequencer program: A - AA genotype; B - Aa genotype; C - TT genotype; D - Tt genotype

Discussions and conclusions

Current SNP genotyping techniques almost exclusively rely on amplification of the target DNA sequence by PCR, but differ in the means of discriminating between the different alleles, some involving significant post-PCR manipulation which increase the final costs of the analysis.

The ARMS system has several advantages over other PCR-based analysis systems: the method is rapid, reliable, and non-isotopic, and results can be easily obtained in one working day. The use of two reactions with internal controls ensures that false negative results are not obtained. In principle, ARMS tests can be developed for any mutation, and the technique has already been applied to the detection of several genetic polymorphisms. These include antitrypsin deficiency (Newton et al. 1989), sickle cell anemia (Wu et al. 1989), phenylketonuria (Sommer et al. 1989), cystic fibrosis (CF) (Ballabio et al. 1990; Wagner et al. 1990), thalassaemia (Old et al. 1990) and internal variation within the D1S80 locus (Jeffreys et al. 1991).

Our sequence of interest was: 5'GTGGGATTGAGCAGTGAG
GYGCCAGCTGAGAGCTCCTGTGCCTTCTTCTATCCCCGTGCCACAGATCGTC
CTGGGGTGCAGGACGCCGCGCTGATZGAGGCCATCCAGGACCG3', where **Y** can be either T, or G (and represents the *Apa*I polymorphisms) and **Z** can be either T or C (and represents the *Taq* I polymorphisms) and so, we have design the primers in such a way that mutant and wild type primers differed by a single base at the 3' end. This allows the differential amplification of the two polymorphisms, based on the particular genotypes of individuals.

By ARMS-PCR method the identification of particular genotypes was accomplished in a single step PCR, excluding other expensive and/or challenging post-PCR manipulation. Instead, RFLP-PCR method was more laborious, needing more optimization steps, reagents and human handling, which increase the cost-effective ratio.

The results obtained by both methods being confirmed by direct sequencing, we conclude that ARMS-PCR method is very adequate for detecting the alternative genotypes determined by single base mutations. The simplicity of this method makes it suitable for the analysis of large number of samples, situation which is usually met in case-control and population genetic studies.

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