I am Capt. Aung Aung. I have commissioned and graduated from Defense Services Medical Academy, Myanmar. I obtained M.B., B.S and Dip in Med.Sc (Molecular Biotechnology) from DSMA at 2008 and 2017 respectively. I am serving as medical officer for 11 years at the Medical Battalions and Military Hospitals. Now, I am serving at Department of Biochemistry, Defense Services Medical Academy.
Comparison between Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) Method and Sequence Specific Primer Polymerase Chain Reaction (PCR-SSP) Method for ApoE-gene Genotyping

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ABSTRACT

There are number of polymerase chain reaction based genotyping methods to find out the single nucleotides polymorphism (SNPs) in genetic study in relation with detection of risk associated genotypes linked to human disease. To develop a convenient and accurate method with flexible difference throughput genotyping of SNPs, the study selected the target gene as ApoE gene polymorphism genotyping linked to coronary heart disease (CHD) and aimed to compare the sensitivity and specificity of PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) and Sequence Specific PCR (PCR-SSP) techniques for genotyping of ApoE gene polymorphism. The forty-eight DNA samples of CHD patients were selected. In PCR-RFLP analysis, DNA amplification was done by using thermal cycler and treated with HhaI restriction enzyme which cleaves at the GCGC sequence that encodes Arg112 and Arg158 to detect three different isoforms (ε2, ε3 and ε4). In PCR-SSP analysis, allele-specific primers were used with three PCR reactions to determine three main isoforms. Two forward primers were designed with variations in their 3' nucleotides specific for one of the two variants (T/C) in the 2059 locus and two reverse primers for the nucleotide variants (C/T) in the 2197 locus. These primers were then combined in three haplotype-detecting reaction mixtures "Primer Mix E2, E3 and E4". Control primers must be amplified to verify PCR efficiency in each PCR reaction. PCR cycling conditions were optimized only for the perfectly matched primers was able to hybridize correctly that can detect presence or absence of SSP amplicon which was important for genotyping. The ApoE allele frequency for ε2, ε3 and ε4 was 8.3%, 79.2%, 12.5%, respectively by two methods with 100% concordance results. In comparison, PCR-RFLP method had various steps, more time consuming and labour intensive than PCR-SSP method. However, the sensitivity and specificity for genotyping by these two methods were the same. Therefore, PCR-SSP method for ApoE genotyping was relative simplicity, rapid, precise and cost effective with the potential for high-throughput application in assessing the risk for a variety of vascular and neurodegenerative diseases.
**Keywords:** PCR RFLP, Sequence specific Primer PCR, *ApoE* gene genotyping

**Introduction**

With the development of biotechnology, SNPs were becoming favored genetic markers that are used in the detection of risk-associated alleles linked to human diseases. Several different PCR based genotyping methods had been developed. Among them, the PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis was a conventional method applied to genotyping. PCR-RFLP analysis was amplification of a fragment containing the variation that followed by treatment of the amplified fragment with an appropriate restriction enzyme [1]. Since the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes, allele identification could be done by electrophoretic resolution of the fragments [2].

PCR-SSP method was based on the principle that *Taq* DNA polymerase was more specific for the oligonucleotide primers that completely match the target gene [3]. If a primer that completely matches one genotype of the allele was designed and the PCR process was strictly controlled, then the matching primer would be amplified (positive results), whereas the mismatched primer would not (negative results). Thermal profile must be adjusted to denature the DNA template and in order to bind the primers to 3'end completely. Considering that numerous factors affect a PCR procedure, an internal reference must be used for each reaction.

The aim of this study was to compare and evaluate the difference between PCR-RFLP and PCR-SSP method. To develop a convenient and accurate method with flexible difference throughput genotyping of SNPs, the study selected the target gene as *ApoE* gene polymorphism genotyping linked to coronary heart disease (CHD).

*ApoE* gene was one of the most studied genes which was responsible for stabilizing and solubilizing circulating lipoproteins in our body and also responsible for the development of CAD. *ApoE* gene located on the chromosome at position 19q13.2 has been known to be polymorphic. [4,5] SNPs at positions 112 (rs 429358) and 158 (rs 7412) determine three major alleles: ε2 (T to C substitution at position 158), ε3, and ε4 (C to T substitution at position 112); 3 isoforms: ApoE2 (Cys112, 158Cys), ApoE3 (Cys112, 158Arg), and ApoE4 (Arg112, 158Arg); [6] and 6 genotypes having 3 homozygous: E2/E2, E3/E3, E4/E4, and 3
heterozygous: E2/E3, E2/E4, E3/E4 [7]. ApoE2: exhibits reduced affinity for the LDLR, with reduced clearance of ApoE-containing remnant lipoproteins. Individuals homozygous for Apo E2/E2 have higher plasma ApoE levels, often develop hyperlipidemia due to accumulation of remnant lipoproteins, and are at risk for premature atherosclerotic disease. ApoE4: has increased LDLR affinity with more rapid clearance of ApoE-containing remnant lipoproteins. Carriers of apoE4 have lower plasma ApoE levels, but increased LDL cholesterol levels and increased cardiovascular risk [8]. The increasing requests for the evaluation of Apo E genotype in several clinical settings warrant the development of fast, accurate and as much as possible, automated methodologies.

**Materials and Methods**

**Isolation of genomic DNA**

Genomic DNA was purified from 3 ml of human whole blood using Phenol Chloroform method from 48 subjects of coronary heart disease patients.

**ApoE Genotyping by PCR RFLP method**

The DNA amplification by PCR using Thermal Cycler (T professional, Analytik Jena) and the endonuclease restriction HhaI (New England Biolabs, USA) were performed as the following approach. The PCR, which amplifies a 271-bp fragment, was carried out using the primers: Forward 5’- GCA CGG CTG TCC AAG GAGC TGC AGGC - 3’ and Reverse 5’- GGC GCT CGC GGA TGG CGC TGAG -3’. For each sample containing 12.5 µL of nuclease free water, 5µL of 10 X PCR buffer, 2.5µL of DMSO, 1.25µ L of MgCL2, 0.5µL of each deoxynucleotide triphosphate, 0.5µLof each primer, 0.25µL of Taq polymerase and 2µl of genomic DNA in a final volume of 25µl. The PCR cycling conditions were initial denaturation for 5 cycles of 1 min of 95°C and 3 mins of 72°C, followed by 30 cycles of 1 min for 95°C,1 mins for 65°C,1 mins for 72°C and final extension 5 mins for 72°C. PCR products were digested with 10U of restriction enzyme (HhaI) and then digested products were incubated at 37°C for overnight digestion. The next morning, the digested products were analyzed by 8% polyacrylamide gel electrophoresis.

**ApoE Genotyping by PCR SSP method**
In PCR-SSP analysis, allele-specific primers were used with three PCR reactions to determine three main isoforms (ε2, ε3 and ε4). Two forward primers were designed with variations in their 3' nucleotides specific for one of the two variants (T/C) in the 2059 locus and two reverse primers for the nucleotide variants (C/T) in the 2197 locus (Table 1).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5' 3'</th>
<th>Primer identifies</th>
<th>Forwards or reverse orientation</th>
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<tr>
<td>Primer 8</td>
<td>TGC CAA GTG GAG CAC CCA A</td>
<td>(control) HLA-DRB 1 region</td>
<td>Forward</td>
</tr>
<tr>
<td>Primer 9</td>
<td>GCA TCT TGC TCT GTG CAG AT</td>
<td>(control) HLA-DRB 1 region</td>
<td>Reverse</td>
</tr>
<tr>
<td>Primer 1</td>
<td>CGG ACA TGG AGG ACG TGT</td>
<td>APOE -112 Cys</td>
<td>Forward</td>
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<tr>
<td>Primer 2</td>
<td>CGG ACA TGG AGG ACG TGC</td>
<td>APOE -112 Arg</td>
<td>Forward</td>
</tr>
<tr>
<td>Primer 3</td>
<td>CTG GTA CAC TGC CAG GCA</td>
<td>APOE -158 Cys</td>
<td>Reverse</td>
</tr>
<tr>
<td>Primer 4</td>
<td>CTG GTA CAC TGC CAG GCG</td>
<td>APOE – 158 Arg</td>
<td>Reverse</td>
</tr>
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</table>

These primers were then combined in three haplotype-detecting reaction mixtures "Primer Mix E2" (Primer 1 and 3), "Primer Mix E3" (Primer 1 and 4) and "Primer Mix E4" (Primer 2 and 4). Because this genotyping system is based on the presence or absence of PCR amplification by allele-specific primers, it is imperative to ensure PCR amplification for those reactions that do not produce haplotype-specific amplicons. For this reason, each specific Primer Mix also contained a pair of “control primers” (primers 8 and 9), which amplify two regions of chromosome 6 in the HLA-DR locus, to verify PCR amplification in the absence of haplotype-specific amplification in each PCR reaction. Control primers must be amplified to verify PCR efficiency in each PCR reaction. PCR cycling conditions were optimized only for the perfectly matched primers was able to hybridize correctly that can detect presence or absence of SSP amplicon which was important for genotyping. There were three PCR reaction tubes (E2, E3, E4) for one sample was prepared. For each sample containing 13.2 µL of nuclease free water, 5 µL of 10 X PCR buffer, 1.25 µL of MgCl₂, 0.5 µL of each deoxynucleotide triphosphate, 2 µL of specific primer, 0.25 µL of Taq polymerase, 0.8 µL of control primer, 2 µL of DMSO and 0.5 µL of genomic DNA in a final volume of 25 µl. The PCR cycling condition were as follows: initial denaturation for 1 min at 96°C; followed by 5 cycles of 20 s at 96°C, 45 s at 70°C, and 25 s at
72°C; 21 cycles of 25 s at 96°C, 50 s at 65°C, and 30 s at 72°C; 4 cycles of 30 s at 96°C, 60 s at 55 °C, and 120 s at 72 °C. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized under ultraviolet illumination.

**Results**

**Figure 1** 8% Polyacrylamide Gel electrophoresis ApoE genotyping. PCR-RFLP using HhaI on ApoE DNA fragment amplified by PCR. The digestion patterns of the genotypes that detected in this study. Lane 1–7: seven different samples. Their genotypes are: lane 1 E3/E4; lane 2 E3/E3; lane 3 E2/E3; lane 4 E2/E4; lane 5 E2/E3. lane 6 E3/E3; lane 7 E3/E4. M: molecular marker (25 bp ladder).

Figure (1) showed the digestion patterns of all genotypes that were obtained by PCR RFLP method in this study. It was the 8% polyacrylamide gel electrophoresis of the digested Apo E amplification products. The 25 bp ladder was used for molecular maker. The four genotypes were obtained. Lane 1 has 4 fragments (91bp, 72 bp, 48 bp, 35 bp), so it is E3/E4 genotype. Lane 2 has 3 fragments (91 bp, 48bp, 35 bp), it is E3/E3 genotype. Lane 3 has 4 fragments (91bp, 83 bp, 48 bp, 35 bp), it means E2/E3. Lane 4 has (91bp, 83 bp, 72bp, 48 bp, 35 bp), it is E2/E4 genotype. Lane 5 has (91 bp, 83 bp, 48 bp, 35 bp), it is E2/E3. Lane 6 has 3 fragments (91,48,35) it means E3/E3 genotype. Lane 7 has 4 fragments (91 bp, 72 bp, 48 bp,35 bp). it means E3/E4.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of cases (%) PCR RFLP</th>
<th>No of case (%) PCR SSP</th>
<th>Accuracy</th>
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</table>
In figure (2) showed the all genotype that were obtained by PCR-SSP method in this study. Figure (2) (a) show the agarose gel electrophoresis of 173 bp amplification products. The first sample had the presence of E2 and E3 amplicons. The E4 amplicon was absence, but all control band (785bp) are present. So, this sample was counted as E2/E3 genotype. The next sample was E3/E4 genotype. The another band (1598 bp) in this sample was also control band that was variable with samples. In Fig 2 (b) showed the amplification products of genotype of E3/E3 and E2/E4 respectively.

![Figure 2](image)

**Figure 2** 2% Gel electrophoresis; Amplification products of the Apo E genotypes by PCR-SSP (M = 100 bp ladder)

Table (2) showed the genotypes frequencies of ApoE gene polymorphism in study population by PCR-RFLP method and PCR-SSP method. There are six genotypes in ApoE polymorphism. But, the four genotypes were detected. In this study the homozygote of allele ε2 (E2/E2 genotype) and the homozygote of allele ε4

<table>
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<tr>
<th>Genotype</th>
<th>Frequency</th>
<th>Total</th>
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</thead>
<tbody>
<tr>
<td>E2/E2</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>E2/E3</td>
<td>7(14.58)</td>
<td>7(14.58)</td>
</tr>
<tr>
<td>E2/E4</td>
<td>1(2.08)</td>
<td>1(2.08)</td>
</tr>
<tr>
<td>E3/E3</td>
<td>29(60.41)</td>
<td>29(60.41)</td>
</tr>
<tr>
<td>E3/E4</td>
<td>11(22.9)</td>
<td>11(22.9)</td>
</tr>
<tr>
<td>E4/E4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>48(100)</td>
<td>48(100)</td>
</tr>
</tbody>
</table>

100%
(E4/E4) were not found in both methods. But, the heterozygote of allele ε2 (E2/E3, E2/E4) was obtained. The heterozygote of allele ε4 (E3/E4, E2/E4) was also detected.

Table 3 Genotype frequency distribution of ApoE gene polymorphism in this study

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Observe genotype frequency (%)</th>
<th>Expected genotype frequency (%)</th>
<th>$\chi^2$</th>
<th>df</th>
<th>HWE$_p$</th>
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<tbody>
<tr>
<td>E2/E2</td>
<td>0</td>
<td>0.7</td>
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<tr>
<td>E2/E3</td>
<td>14.58</td>
<td>13.1</td>
<td></td>
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<tr>
<td>E2/E4</td>
<td>2.08</td>
<td>2.1</td>
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<tr>
<td>E3/E3</td>
<td>60.41</td>
<td>62.7</td>
<td></td>
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<tr>
<td>E3/E4</td>
<td>22.9</td>
<td>19.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E4/E4</td>
<td>0</td>
<td>1.6</td>
<td>3.822</td>
<td>3</td>
<td>0.575</td>
</tr>
</tbody>
</table>

HWE = Hardy Weinberg Equilibrium, $p > 0.05$ = not significant, $\chi^2$ = Chi square test, df = degree of freedom

The result of number of cases and frequency percentage by two methods were shown in Table (2). The obtained ApoE genotypes by PCR-SSP method were 100% concordance with the results of PCR RFLP method. Table (3) shows the genotype frequency distribution of ApoE gene polymorphism. In this study population, the allele frequencies of ε2, ε3 and ε4 allele were 8.3%, 79.2%, 12.5% respectively. Genotype frequency were consistent with Hardy Weinberg Equilibrium in this study population ($\chi^2 = 3.822$, $p = 0.575$)

**Discussion**

The aim of this study is to compare and evaluate the difference between PCR RFLP and PCR-SSP methods for Apo E genotyping. ApoE gene is one of the most studied genes which responsible for stabilizing and solubilizing circulating lipoproteins in our body and also responsible for the development of CAD. The increasing requests for the evaluation of Apo E genotype in several clinical settings warrant the development of fast, accurate and as much as possible, automated methodologies. One of the mostly used methods to determine Apo E genotype was performed through a PCR-Restriction Fragment Length Polymorphism (PCR-RFLP) method that was time consuming and difficult to automate because it requires many different steps and procedures after polymerase chain reaction, such as restriction enzyme digestion ($HhaI$, the recognition site is 5' GCGY C 3') and electrophoresis on acrylamide gels.
Although the methodology requires only a single PCR reaction per sample, the restriction enzyme digestion step increases the post-PCR processing time by 2 hours. Furthermore, because incomplete restriction enzyme digestion can lead to false genotyping data, many researchers prefer to perform overnight digestions, thus increasing the assay time even further. The small size fragments produced after restriction enzyme digestion are usually resolved by acrylamide gels electrophoresis, thus limiting the number of samples that can be examined at one time.

PCR-RFLP is a traditional and conventional method which uses a pair of primer for SNPs site determination. In this type of PCR reaction only one reaction tube is needed for one sample of DNA. So it can reduce the consumption of reagents, comparing to PCR-SSP. Optimizing thermal profile is not much complex and need one annealing temperature is to be setup.

There are various steps in this method such as, reagent preparation step, PCR step, preparation of 2% agarose gel for PCR product check, digestion with restriction enzyme, preparation of 8% polyacrylamide gel, detection under UV light. This method required time consuming for about two days. The length differences between restriction fragment sites are too small, so this method must be done by polyacrylamide gel electrophoresis. The ambiguous band formations hinder the accuracy of gel result.

The recently introduced method was simple sequence specific primer PCR method to identify the ApoE genotypes. For all PCR reactions (E2, E3, and E4), the presence of a 173-bp band indicated the presence of the specific ApoE haplotype. The control primer pair bind on two positions on chromosome 6. Therefore, with the control primer pair, two products were expected of 785 and 1598 bp. Although the 785-bp control amplification product was always present when PCR amplification occurred, the 1598-bp product was often missing in those samples with poor-quality DNA or excessive ApoE haplotype specific amplification. Nevertheless, a sample was considered negative for a particular Apo E haplotype when the haplotype specific amplicon was absent and the 785-bp control amplicons was present. Absence of haplotype-specific and control amplicons in the same reaction was indicative of PCR amplification failure. There are three reaction tubes for one sample, we must load carefully the PCR products to the wells of 2% Agarose gel as E2, E3, E4 serially for each sample. The absence or presence of haplotypes amplicons could determine the ApoE genotypes. Examples of Apo E haplotype positive PCR reactions are shown in Fig. 2 (a), (b).
This method need only conventional thermocycler and agarose gel electrophoresis apparatus. It could be done by simple steps (PCR amplification, gel separation) and does not need recognition site. So, no restriction enzyme digestion was needed. It also reduced the time consuming.

In this PCR-SSP protocol, more reagents are required because of there were three reaction tubes in one sample. It must be careful to concentrate when reagent preparation because primer concentrations were very significant in PCR-SSP reactions. Thermal profile must be adjusted to denature the DNA template and in order to bind the primers to 3'end completely. Control primer was needed to ensure that negative amplification results are truly negative and not caused by bad reagents or pipetting errors. Careful loading is required while injecting the PCR products to the wells of 2% Agarose gel as E2, E3, E4 serially for each sample, because we determined the genotype that the absence or presence of haplotypes amplicons.

Primer concentrations were very significant in PCR-SSP reactions to avoid false negative results [9]. The optimized common primer concentration was 0.5 µM and the ratio of internal control primer to specific primer was 2/5. If the amplification reaction occurs smoothly, the DNA template must be a single band [10]. Human genes were extremely complicated. Thus, the denaturation time was optimized that the hybridization between the primer and the template occurs under the most complementary conditions and it may be cycled at different denaturing and annealing temperatures.

In this study, the Apo E polymorphism in 48 subjects with both PCR-RFLP and PCR-SSP method and there was a 100% concordance between the two methods. Moreover, all samples were tested in duplicate with PCR-SSP method and the results for each sample were always concordant.

HWE demonstrating the accuracy a reliability of this methods. The allele frequencies of ε2, ε3 and ε4 allele were 8.3%, 79.2%, 12.5% respectively in this study population that showed in Table (2).

The PCR-SSP method for Apo E genotyping seems to be rapid, simple and accurate, suggesting a possible successful use of this method for an increasing range of diagnostic purposes.

In Conclusions, the new introduced method was based on sequence-specific PCR methodology can readily be applied to high-throughput ApoE genotyping. This ApoE genotyping protocol can be used in addressing the impact of ApoE polymorphism on disease risk, and notably in clinical assessments that predict the risk for a variety of vascular and neurodegenerative diseases.
Acknowledgements

We are gratefully indebted to the Professor Lt. Col. Daw Mo Mo Than, Professor and Head of Department of Biochemistry, Defence Services Medical Academy, for her kind permission and cooperation to carry out this study and for her valuable guidance and constructive comments for this research. We wish to extend my sincere gratitude to Maj. Zaw Min Htut, Lecturer of Department of Biochemistry, Defence Services Medical Academy, for his excellent technical assistance throughout the study. We are deeply honored and indebted to Capt. Aung Paing Paing Soe , other seniors and junior colleagues in the Department of Biochemistry, Defence Services Medical Academy for providing helpful suggestions.

References


Comparison between PCR-RFLP and PCR-SSP for ApoE gene Genotyping

Capt. Aung Aung
Department of Biochemistry
Defense Services Medical Academy
13 December 2019

PCR RFLP Method
- DNA purification
- PCR amplification
- Digestion with Hhal
- Analysis by PAGE

PCR SSP Method
- DNA purification
- PCR amplification
- Analysis by gel electrophoresis

100% concordance

PCR SSP method – rapid, simple and accurate
high-throughput for ApoE Gene Genotyping
Outline of Presentation

• Introduction
• Objectives
• Methods
• Results
• Discussion

Apolipoprotein E (ApoE)

• responsible for stabilizing and solubilizing circulating lipoproteins
• responsible for 5-15% of the total cholesterol level
• three ApoE isoforms (ε2, ε3 and ε4 )
• modulated cholesterol homeostasis in an isoform-dependent manner.

Reilly and Rader 2006
• *ApoE ε2* allele
  - associated with higher plasma levels of *ApoE*, decreased plasma levels of LDL cholesterol (LDL-C) and lower risk of CAD

• *ApoE ε4* allele
  - associated with lower plasma level of *ApoE*, increased plasma levels of total cholesterol (TC), LDL-C, VLDL cholesterol (VLDL-C), and greater risk of CAD when compared to *ApoE3* homozygotes.

Reilly and Rader 2006
General Methods used to detect ApoE Gene Genotyping

- Hybridization of allele-specific probes
- PCR plus sequencing or mass spectrometry
- Real Time PCR-based techniques
- HRM (high resolution melt)
- FRET (Fluorescent Resonance Energy Transfer)
- PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)
- Sequence Specific Polymerase Chain Reaction (PCR-SSP)
- TaqMan probe assay

Rationale of the study

Current Study

Just conventional PCR
How can you do High throughput?
PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

- a conventional method
- the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes

Hixson and Vernier, 1990

Sequence specific PCR (PCR-SSP)

- based on the principle that Taq DNA polymerase was more specific for the oligonucleotide primers that completely match the target gene
- Thermal profile must be adjusted to denature the DNA template and in order to bind the primers to 3'end completely

Pantelidis et al., 2003
Principle of PCR SSP

Objectives

1. To determine the genotypes frequencies of *ApoE* gene polymorphism in study population by PCR-RFLP methods
2. To determine the genotypes frequencies of *ApoE* gene polymorphism in study population by sequence specific PCR methods
3. To compare and evaluate the difference between PCR-RFLP and PCR-SSP method for *ApoE* gene genotyping
Objectives

1. To determine the genotypes frequencies of ApoE gene polymorphism in study population by PCR-RFLP methods
2. To determine the genotypes frequencies of ApoE gene polymorphism in study population by sequence specific PCR methods
3. To compare and evaluate the difference between PCR-RFLP and PCR-SSP method for ApoE gene genotyping

METHODS

DNA Extraction

Sample Preparation

Phenol Chloroform Method

- 12.5 μL of nuclease free water
- 5μL of 10 X PCR buffer
- 2.5μL of DMSO
- 1.25μ L of MgCl₂
- 0.5μL of each deoxynucleotide triphosphate
- 0.5μLof each primer
- 0.25μL of Taq polymerase
- 2μl of genomic DNA
METHODS

**PCR Amplification**
- 5′GCACGGCTGTCAAGGAGCTGC AGGC 3′
- R 5′GGC GCT CGC GGA TGG CGC TGAG 3′
- 5 cycles of 1 min of 95°C and 3 mins of 72°C
- 30 cycles of 1 min for 95°C, 1 mins for 65°C, 1 mins for 72°C
- 5 mins for 72°C

**Digestion**
- 10U of restriction enzyme (HhaI) in final volume of 20 µL containing 8 µL of PCR product and buffer C 2.0µL and BSA 0.2µL.
- incubated at 37°C for overnight digestion

**Analysis**
- 25 bp marker
- 8% Polyacrylamide gel electrophoresis
## METHODS

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence 5' - 3'</th>
<th>Primer identifies</th>
<th>Forwards or reverse orientation</th>
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<tr>
<td>primer 1</td>
<td>CGG ACA TGG AGG ACG TGT</td>
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<td>GCA TCT TGC TCT GTG CAG AT</td>
<td>(control) HLA-DRB 1 region</td>
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### Objectives

1. **To determine the genotypes frequencies of *ApoE* gene polymorphism in study population by PCR-RFLP methods**

2. **To determine the genotypes frequencies of *ApoE* gene polymorphism in study population by sequence specific PCR methods**

3. **To compare and evaluate the difference between PCR-RFLP and PCR-SSP method for *ApoE* gene genotyping**
- The black colour sequence is ApoE exon 4.
- The blue colour sequence is its complementary sequence.
- The red colour sequence is primer.

RESULTS

2% agarose Gel Electrophoresis
100 bp ladder
Objectives

1. To determine the genotypes frequencies of ApoE gene polymorphism in study population by PCR-RFLP methods

2. To determine the genotypes frequencies of ApoE gene polymorphism in study population by sequence specific PCR methods

3. To compare and evaluate the difference between PCR-RFLP and PCR-SSP method for ApoE gene genotyping

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of cases (%)</th>
<th>No of case (%)</th>
<th>Accuracy</th>
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<tr>
<td>E3/E4</td>
<td>11(22.9)</td>
<td>11(22.9)</td>
<td></td>
</tr>
<tr>
<td>E4/E4</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>48(100)</td>
<td>48(100)</td>
<td></td>
</tr>
</tbody>
</table>
PCR RFLP

- various steps in PCR RFLP
- requires only a single PCR reaction per sample
- restriction enzyme digestion step
- overnight digestions.
- resolved by PAGE
- limiting the number of samples

PCR SSP

- need only conventional thermocycler and agarose gel electrophoresis apparatus
- simple steps (PCR amplification, gel separation)
- not need recognition site
- reduced the time consuming
• worldwide frequency of ε2, ε3 and ε4 allele was 8.4 %, 77.9 % and 13.7 %, respectively (Farrer et al 1997)

• Chinese Han population was 7.90 %, 83.94 %, 8.16 %, respectively (Zhong, L et al; 2016)

• In this study population, the allele frequencies of ε2, ε3 and ε4 allele were 8.3%, 79.2%, 12.5% respectively

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Thank you