Original Article

Polymerase chain reaction amplification of a GC rich region by adding 1,2 propanediol

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Abstract

Background: Apolipoprotein E (ApoE) is one of the most important carriers of lipids in mammalians. The gene for this lipoprotein (ApoE) is located on chromosome 19 which is related with the pathogenesis of some nervous system disease. ApoE gene is identified as a high guanine–cytosine (GC) content fragment. Detection and amplification of these templates are extensively laborious and baffling. The aim of this study was to find a practical and feasible method for the amplification of the number of GC rich genes such as ApoE.

Materials and Methods: We experimented with simple polymerase chain reaction (PCR), nested PCR and PCR with 1-2 propanediol, dimethylsulfoxide (DMSO), and ethyleneglicol as additive substances to enhance the amplification ApoE gene and used the 40 samples of the human whole blood were collected in test tubes with a pre-treatment of ethylene diaminetetraacetic acid.

Results: According to our observations, presence of 1-2 propanediol, DMSO, and ethyleneglicol as additive substances resulted to enhanced amplification of ApoE gene. Addition of 1-2 propanediol showed the best results, caused optimization and revealed more specific and sharp bands.

Conclusion: According to our findings 1-2 propanediol are the best organic reagent for improving the amplification of ApoE gene. Optimization procedure for each GC rich sequence is recommended to be performed separately in order to identify which of the additive agent is more efficient and applicable for a particular target.

Key Words: Amplification, apolipoprotein E, guanine–cytosine rich, gene, polymerase chain reaction

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INTRODUCTION

Correlation between genetic factors and pathophysiologic aspects of numerous diseases has been previously described,[1] although achieving more accurate gene-disease association (gene role in disease onset and progress) is still under evaluation. Thereby, finding this correlation and detecting such genetic factors would provide a great opportunity for early diagnosis of disease as well as patient screening and improvement in therapeutic strategies and outcome.[2]

Apolipoprotein E (ApoE) gene is located on chromosome 19. Relationship between different alleles of ApoE gene and the incidence of some types of specific diseases is

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still under investigation.[3-5] These alleles have been generated due to single nucleotide polymorphism (SNP) mutation in 112 and 158 gene regions, special sites that are essential for ApoE-receptor binding. As a result, these alleles demonstrate different efficacy and function in brain cells (cholesterol transportation and contribution to neuron regeneration).[6-8]

ApoE gene has been identified as a high guanine–cytosine (GC) content fragment. It is confirmed that 28% of human genome sequences are categorized into GC rich genes.[9]

Detection and amplification of these templates are extensively laborious and baffling. So that undergoing these exhausting and also unyielding circumstances were inevitable for polymerase chain reaction (PCR) process. Secondary structure formation and mispriming during the course of amplification constitutes the major reason for these difficulties.[9,10]

Regarding the crucial role of gene involvement in various diseases and also high percentage of GC sequences (28% in a human genome), leading to non-specific and low yielding production through standard PCR protocols,[9,10] we have examined the amplification process of ApoE gene as a GC rich region, under three distinct PCR procedures: Simple PCR, nested PCR, PCR containing additive substances. The aim of this study was to find a practical and feasible method for the amplification of the number of GC rich genes such as ApoE.

MATERIALS AND METHODS

To perform ApoE genotyping process, 40 samples of the human whole blood were collected in test tubes with a pre-treatment of ethylene diaminetetraacetic acid. The corpuscular fraction was frozen and stored at –20°C, until DNA extraction isolation of DNA from blood samples was performed through a standard DNA extraction kit (Roche, Berlin Germany) procedure. Extracted DNA fragments were used as the template for PCR system. These steps were preceded by determination of ApoE gene by simple PCR, nested PCR, and PCR with additive substances.[11]

Simple polymerase chain reaction

The PCR mixture consisted of 200 ng of genomic DNA, 10 × reaction buffer (Bio Ran, Poland), 10 pmol of each primer.

Forward (5×-ACAGAATTCGCCCCGGCCTGGTACAC-3×)
Reverse (5×-TAAGCTTGGCACGGCTGTCCAAGGA-3×)

0.8 Mm dNTPs (Bio Ran) and 0.5 unit Taq DNA polymerase (Bio Ran) to final volume of 25 µl. PCR was performed (BioRad thermal cycler; MyCycler, USA) for 35 cycles of denaturation at 94°C for 1 min, annealing temperature was set to 64.2°C for 2 min and extension temperature was 72°C for 3 min. A final extension step of 72°C for 5 min was included. The PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining.

To achieve the most appropriate $MgCl_2$ concentration and annealing temperature, altering concentration and temperature gradient process were utilized.[11]

Nested polymerase chain reaction

In this method, we employed two pairs of primers for optimizing the procedure. Incorporating first primer produced 512 bp length fragments, then this product and primer number 2 utilized in second step. Finally, this procedure resulted in 219 bp DNA fragment.

First step primer
Forward : 5×-CTGGGAACTGGCACTGGGTG-3×
Reverse : 5×-ACGCGGGCCTGTTCCACC-3×

Second step primer
Forward : 5×-AGGAGACGCGGGCACGGC-3×
Reverse : 5×-CCTCGCGGCCCCGGCCTCCTCACCT-3×

Temperature cycle and amount of used substances were completely similar to simple PCR protocol.

Polymerase chain reaction with additive agents

This step was run using 1.5 µl of additive agents (1, 2 propanediol, glycerol, and dimethyl sulfoxide [DMSO]). In accordance with comparisons have been made, rather than inclusion of glycerol, DMSO and even concomitant usage of all substances in the same tube, products of this step (using 1.5 µl 1, 2 propanediol) were completely specific.

This step was accomplished using simple PCR procedure and 1.5 µl of additive substances (1, 2 propanediol, glycerol oil, and DMSO).[12]

Amplified DNA was then analyzed by restriction pattern of ApoE alleles. For this purpose 8 ml of the PCR product was digested with 10 units *Hhal* enzyme (Fermentase; Gdansk, Poland) for 4 h. The digested fragments were separated by electrophoresis on a 15% polyacrylamide gel at 100 volt for 3 h and polymorphic patterns were then visualized by ethidium bromide.[11]

RESULTS

According to our observations, conventional standard

methods did not demonstrate remarkable and reproducible results. In addition, during first and second steps of nested PCR course, we observed only non-specific and smear bands. As illustrated in Figures 1, 2 and 3 any of the optimizing methods mentioned above, were not of high efficacy and applicability. Acquired results were similar in both methods.

Figure 4 demonstrates changes in PCR products in the presence of additive substances. First line illustrates products of PCR content and 1.5 µl DMSO. Line number 2 illustrates products of PCR content and 1.5 microliter of 1,2 propanediol and line number 3 illustrates products of PCR content and 1.5 microliter of ethylene glycol. Line number 4 illustrates products of a PCR content and 1.5 microliter of ethylene glycol and 1.5 microliter of 1,2 propanediol.

Dramatic changes in specificity and efficiency of included substances is illustrated in Figure 4.

Line numbers 5-7 illustrate PCR reaction by utilizing without 1,2 propanediol and ethylene glycol and DMSO. Desired fragment length was 219 bp and yielded fragments were in a predetermined range.

DISCUSSION

Polymerase chain reaction is now considered as an indispensable method in genetic research. Despite numerous developments and progresses have been made, the PCR process of GC rich DNA templates generally lead to inadequate consequences, due to a high percentage of guanine-cytosine bases[13] and after that, forming more triple hydrogen bond compared with binary hydrogen bond. These types of templates are more prone to form secondary intra-molecular

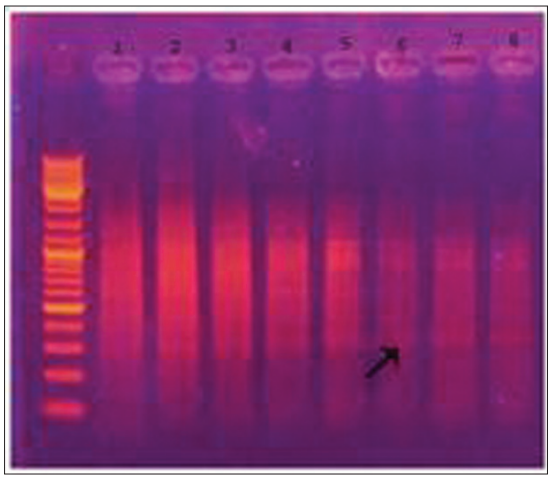

Figure 1: Simple polymerase chain reaction (PCR) (200 ng of genomic DNA, 10 × reaction buffer, 10 pmol of each primer and as illustrated, there is only inadequate PCR products: Non-specific and smear bands)

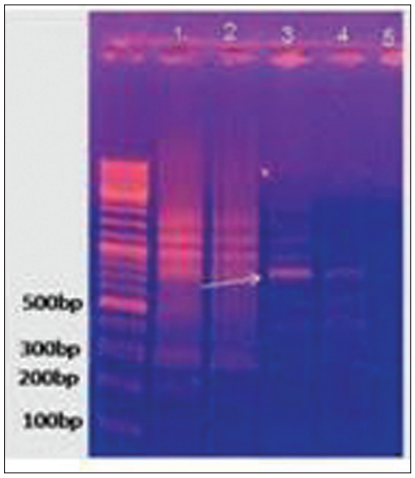

Figure 2: Nested polymerase chain reaction: The first step (as illustrated the protocol was not successful for ApoE: Non-specific bands)

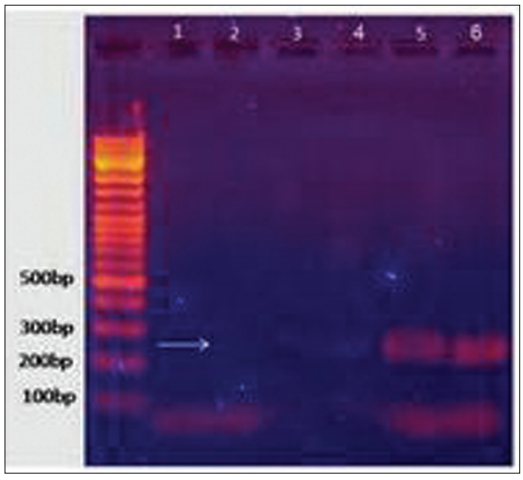

Figure 3: Nested polymerase chain reaction. The second step (as illustrated the process resulted in completely non-efficient bands)

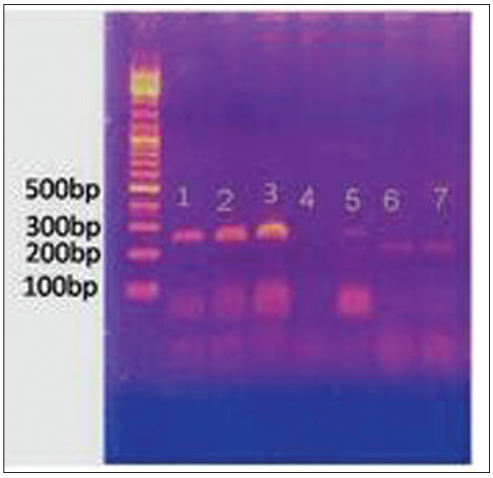

Figure 4: Polymerase chain reaction products using 1,2 propanediol as additive substance (line 2) (this figure illustrates dramatic changes in specificity and efficiency of included substances)

structures and higher melting temperatures during PCR system. It is widely accepted that setting annealing temperature of the procedure is of vital importance in PCR optimization. It is believed that the main cause of ineffective amplification is the formation of secondary structures resulting in inappropriate primer binding sites.[14-16]

1,2 Propanediol is a co-solvent that destabilizes DNA by disrupting base pairs and leads to subsequent decrease in melting temperature of DNA. 1,2 Propanediol is known to improve the amplification of some GC rich DNA sequences, including some resistant to amplification by standard PCR technique.[14,16-18] While 1,2 propanediol improved the yield and specificity of amplification for these targets it may not necessarily be advantageous for others. In these cases other PCR additives such as betaine, formamide, glycerol, or trimethylammonium hydrochloride can be considered beneficial.

This fact that it is practically impossible to identify which if any PCR additive is best for a particular target makes it essential to test several different agents separately or in combination. It should be noted that although these additives can increase the yield and specificity of PCR reactions, their effects on the melting temperature of DNA alter the optimal annealing temperature for a specific primer/template system. As a result, optimization of the thermal cycling parameters may be required, which often requires the design and performance of several experiments.

Consistent with comparisons across this study, To overcome problems related to other methods such as a slowdown[10] or touchdown PCR and Triple master,[9] incorporation of additive substances is suggested. Most of the previously established optimization procedures (besides being time and labor consuming and involving high cost) have their own practical limitations[10] that the method introduced in this study does not. Amplification feasibility of GC rich gene was also remarkable. Furthermore, these additives are highly compatible with other biological agents, which make them ideal for any gene synthesis assay.

In conclusion, this technique might provide feasible circumstances for more efficient amplification of GC rich templates. Undoubtedly, to ascertain type and amount of additive agents, further study for each particular fragment is needed.

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