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Full Length Research Paper

Comparison of the optimized conditions for genotyping of ACE ID polymorphism using conventional and direct blood PCR

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ACE ID polymorphism is inevitable for genetic epidemiology of several cardiovascular and non cardiovascular diseases due to its direct influence on ACE activity level. In the present work, conditions were optimized for its analysis using conventional and direct blood PCR (DB PCR). Blood samples from nine normotensive male donors preserved in EDTA and lithium-heparin coated vacuatainers separately were used directly as template for DB PCR. Genomic DNA was isolated from each vacuatainer for the conventional PCR and DB PCR also. Conditions were optimized by adjusting the suitable annealing temperature, amount of $MgCl_2$ (in case of conventional PCR) and amount of blood used as DNA template for DB PCR. In case of DNA from EDTA treated blood, maximum amplification of target sequence occurred at 53°C with 2 mM concentration of $MgCl_2$ in all samples. However, when DNA from lithium heparin treated blood was used as template, 6 out of 9 samples gave amplification results with 4 mM concentration of $MgCl_2$ at the same temperature. When 1 µl genomic DNA from EDTA and lithium heparin treated blood was used as DNA template in DB PCR, all samples gave maximum yield at 53°C. DB PCR successfully amplified the target region when 1 µl blood treated with EDTA and 0.5 µl lithium heparin treated blood was used per 50 microliter reaction mixture at 51°C as annealing temperature. It can be concluded from the study that EDTA treated blood is more suitable for conventional and DB PCR.

Key words: ACE ID polymorphism, step down PCR, direct blood PCR.

INTRODUCTION

Direct involvement of Angiotensin Converting Enzyme (ACE) in Renin Angiotensin Aldosterone Syestem (RAAS), Kinin Kallikrein System, *in vivo* degradation of amyloid beta peptide, signal transduction and GPIase activity confer its important pleiotropic effects. While studying the gene responsible for ACE, Rigat and coworkers (1990) observed a polymorphism involving insertion of 287 bp sequence in intron 16 of the ACE gene (NCBI ref. SNP ID: rs1799752). If this sequence was present, allele was marked as insertion allele (I). In case of its absence, allele was tagged as deletion allele (D). Mean activity level of ACE is strongly associated with this polymorphism. It becomes double in homozygous deletion carriers (DD) and comparatively half in II carriers. ID carriers exhibit medium level of ACE activity. This piece of information caused generation of huge data about genetic basis of pathophysiology of several cardiovascular and non cardiovascular diseases like diabetes, diabetic nephropathy, diabetic retinopathy, atherosclerosis, coronary heart diseases and stroke, hypertension, Alzheimer's disease, cancer and Parkinson's disease (Sayed-Tabatabaei et al., 2006; Nawaz and Hasnain, 2009).

Study by Rigat et al. (1990) on this polymorphism was based on the PCR involving primer pair flanking the insertion region. Sooner, this method became questionable due to the frequent reports of mistyping. During mistyping, preferred amplification of the D allele used to mask ID heterozygotes as DD carrier. Many strategies

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Table 1. Primers and simple PCR strategy.

Primer	Sequence	Simple PCR Strategy
ACE Sense	5′ TCCCATCCTTTCTCCCATTTCT 3′	Initial Denaturation: 94°C (5 min)
ACE Sense Nested	5′GGTTTCACCGTTTTAGCCG 3′	35 Cycles of : 94°C (30 s) → V (30 s)→ 72°C (30 s)
ACE Antisense	5′ CATGCCCATAACAGGTCTTCAT3′	Final Extension: 72°C (5 min)

"V" indicates that it is changeable with in range starting from 60°C to 50°C.

Table 2. Step down PCR strategy for DBPCR.

Initial denaturation 94°C (5 min)
5 Cycles of: 94°C (30 s) → 60°C (30 s) → 72°C (30 s)
5 Cycles of: 94°C (30 s) → 59°C (30 s) → 72°C (30 s)
5 Cycles of: 94°C (30 s) → 58°C (30 s) → 72°C (30 s)
5Cycles of: 94°C (30 s) → 57°C (30 s) → 72°C (30 s)
5Cycles of: 94°C (30 s) → 55°C (30 s) → 72°C (30 s)
20 Cycles of: 94°C (30 s) → 51°C (30 s) → 72°C (30 s)
Final extension: 72°C (5 min)

were excogitated for the elimination of chances for mistyping through the use of 5% dimethyl sulfoxide (DMSO) and sense primer from the 5′ end of the insertion sequence along with the standard antisense primer (Shanguman et al., 1993). Other genotyping methods for this polymorphism were based on multiplex PCR and real time PCR (Evans et al., 1994; Lin et al., 2001). Both strategies were perfect but higher cost and sensitive post handling procedures did not make them ideal. Later on, Koyama et al. (2008) described a quick and easy technique involving DHPLC (Denaturing High Performance Liquid Chromatography) at non denaturing conditions to analyze the PCR product for screening ID variants (Koyama et al., 2008). This method removed the chances of mistyping and gave 100% accuracy in the confirmation of ID heterozygotes but expenses of chromatography did not prove it to be cost effective.

Normally, genomic DNA is used as template DNA for PCR based studies. It is very difficult to use blood directly for amplification as some substances in blood may inhibit the activity of DNA polymerase enzyme. Similar problem is observed when DNA isolated from blood treated with different anticoagulants like EDTA or heparin is used (Nishimura et al., 2002). In the past, heparin inhibitory effects were removed by expensive treatment of heparinnase enzyme or risky and time consuming treatment of chelex 100 resins (Taylor, 1997; Poli et al., 1993). Use of dielectorophoresis as a selective filter in a microsystem was also designed to remove the heparin from the samples, but it is quite complex (Perch-Nielsen et al., 2003). Considering all facts, we have followed modified Ueda et al. (1996) method due to its advantages like removal of chances for mistyping, cost effectiveness and easy performance (Ueda et al., 1996). This strategy was equally applied in conventional PCR and DB PCR. Conditions were made optimum for perfect genotyping by varying the temperature for annealing, amount of $MgCl_2$ (in conventional PCR) and amount of blood used as template for DB PCR. DB PCR technique was used to save time and for increasing the chances for successful study for ACE ID polymorphism using EDTA and lithium-heparin treated blood.

MATERIALS AND METHODS

Sample collection

All procedures were in compliance with the declaration of Helsinki and the study protocol was approved by the Board of Advance studies and Research University of the Punjab Lahore. Blood samples from 9 normotensive males with age 40 ± 5 were collected in EDTA coated and lithium coated vacuatainers. Written informed consent was obtained from each subject.

Molecular genetic analysis

Genomic DNA was isolated from all blood samples by using kit method (Fermentas, USA). The ACE ID polymorphism, located in intron 16 was assayed by a triple-primer method with a nested PCR primer situated within the insertion sequence of I allele (Table 1). The inclusion of a third internal PCR primer removed the chances for mistyping. Reaction mixture (50 µl/reaction) containing 1 X KCl, 200 µM dNTPs, forward primer, reverse primer, internal nested primer (0.24 µM each), Taq polymerase (0.15 U/10 µL), 1.5-4.25 mM $MgCl_2$ was added to the 1 µL genomic DNA in case of conventional PCR. Reaction mixture for DB PCR contained special buffers used for the neutralization of inhibitory agents in blood (Novagen). Reaction mixture was assembled according to the manufacturer's instructions (Novagen). Different amounts of blood samples and genomic DNA were used as template for DB PCR buffer. Initially, simple PCR program was developed for 35 cycles using Peq Lab Primus Advanced 96 thermal cycler. Annealing temperature was scrutinized by varying the temperature from 60 to 50°C. On the basis of this experiment, step down strategy was developed for enhancing the yield of PCR product (Table 2). PCR product was analyzed by using 2% agarose gel and low range marker (Fermentas) under UV. The banding pattern of the 3 possible genotypes was as follows: DD, 210 bp fragment; II, 498 and 264 bp fragments; ID, 498, 264, 210 bp fragments (Figure 1).

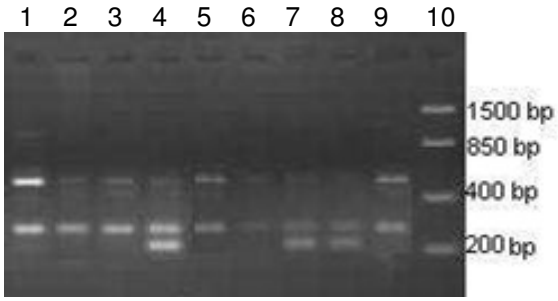

Figure 1. Succesful amplification for ACE ID polymorphism. Lanes 1, 2, 3, 5, 6 and 9 shows II allele carriers. Lanes 4, 7 and 8 shows ID allele carriers. Lane 10 shows DNA ladder.

RESULTS

Genotyping using conventional PCR

Genomic DNA was isolated from all the samples treated with EDTA and lithium heparin. In case of DNA isolated from EDTA coated vacuatainers, PCR product was observed with 2 mM $MgCl_2$ at 53°C in all the samples. For DNA from heparinated blood, only six out of nine samples could be successfully amplified with 4 mM $MgCl_2$ at the same annealing temperature (Table 3). Smear was observed due to increase in $MgCl_2$ concentration or decrease in temperature as annealing temperature.

Genotyping using DB PCR

When DB PCR was used with genomic DNA as template, all the samples showed positive amplification at 53°C. When 1 µl blood treated with EDTA per 50 µl reaction mixture was used as template, all the samples were successfully amplified at 51°C. The only observed problem was the presence of weak bands on the gel. Step down strategy helped in increasing the PCR product as well. For 1 µl blood treated with lithium heparin, no PCR product could be observed using same conditions. When amount of blood was decreased to 0.5 µl, successful amplification was observed in 6 out of nine samples.

DISCUSSION

ACE polymorphism is defined on the basis of presence or absence of 287 bp segment in intron 16 of gene for ACE. If this segment is absent, allele is called as deletion allele (D). In case, it is present, allele is called as insertion allele (I). In homozygous D allele carriers, ACE activity level becomes double as compared to II carriers. ID carriers show medium level of ACE activity. Increased ACE activity level may disturb RAAS (rennin angiotensin aldosterone system), kinin- kallikrein system, GPIase activity and signal transduction (Nawaz and Hasnain, 2009). Considering role of ACE polymorphism in activity of angiotensin converting enzyme, clinical geneticists have focused over its role in association with the genetic epidemiology of several diseases. The only way to scrutinize this gene variation is based on PCR. In the present work, conditions are optimized for this gene variation using conventional and DB PCR. Genomic DNA isolated from blood treated with EDTA and lithium heparin was used as DNA template in conventional PCR. Same genomic DNA was also used in DB PCR. Blood contains many inhibitory agents for Taq polymerase. So, it needs special buffers to be used directly as DNA template in reaction mixture. Blood treated with EDTA and lithium heparin was used as DNA template only in DB PCR.

Results depict that suitable annealing temperature for conventional PCR was 53°C. DNA isolated from EDTA and lithium heparin treated blood gave amplification results at different concentrations of $MgCl_2$. Amplifications occur at 2 mM and 4 Mm concentration of $MgCl_2$ for genomic DNA isolated from EDTA and lithium heparin treated blood respectively. It was also noteworthy that rate of successful amplification for samples involving DNA isolated from lithium heparin blood was low as compared with the samples involving DNA isolated from EDTA treated blood. These results suggest that trace amount of heparin might remain attached with genomic DNA causing hindrance in Taq polymerase activity. This activity can be restored by increasing $MgCl_2$ concentration. While the genomic DNA isolated from blood treated with EDTA have not enough amount of EDTA bound with it causing inhibition to Taq polymerase. It can also be concluded from this experiment that annealing temperature is not disturbed due to trace amounts of lithium heparin or EDTA bound with genomic DNA.

Results for DB PCR involving genomic DNA isolated from blood treated with EDTA and lithium heparin were successfully amplified at 53°C. Success rate for amplification was 100 percent in all samples. It suggests that special buffers used in this type of PCR have removed the inhibitory effects of trace amounts of anticoagulants bound with genomic DNA. Using the same PCR program (53°C as annealing temperature), when blood treated with these different coagulants were added directly as DNA template, weak bands appeared on agarose gel. For obtaining strong bands, annealing temperature was lowered in addition of step down strategy. This modification was effective causing production of enough amount of PCR product suitable for genotyping at 51°C as annealing temperature. These results suggest that annealing temperature varies depending on the type of PCR. In case of conventional PCR, it was 53°C while for DB PCR, it was 51°C. Buffers used in DB PCR lower the pH of reaction mixture for blocking the inhibitory agents present in the blood. This variation in microenvironment

Table 3. Effect of different concentrations of $MgCl_2$ on PCR amplification and different anticoagulants at 53°C.

DNA from blood with different anticoagulants	No. of Successful PCR amplifications with different $MgCl_2$ conc. in conventional PCR						
	1.5 mM	2.0 mM	2.5 mM	3.0 mM	3.5 mM	4.0 mM	4.5mM
EDTA (n = 9)	5	9	U	U	U	U	U
Lithium heparin (n = 9)	-VE	-VE	-VE	-VE	3	6	U

-VE indicates no amplification; digits show the number of successful amplifications, U indicates unspecific amplification.

of PCR tube might change the thermal conditions for proper annealing in DB PCR. Possibly, lowering the temperature for annealing purpose caused decrease of stringency for Taq polymerase resulting in the higher production of PCR yield. Step down strategy improved PCR yield in the same way as was observed by Chiang and fellows (1998). During experimentation, it was realized that agitation of PCR tubes having reaction mixture for DB PCR can decrease the PCR product. Similar problem was faced by Bu and coworkers (2008). They solved it by increasing the amount of DNA polymerase. In above mentioned experiments, problem was solved just by avoiding the agitation and preparation of reaction mixture on ice.

These findings indicate that optimization conditions vary for the amplification of same DNA sequences using different PCR types. Moreover, blood can be used as DNA template directly which surely improves and speeds up the genotyping process.

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