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Two-Pair Primers PCR-CTPP Optimization to Identify rs1057910 CYP2C9 Gene Variants in Surakarta

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Abstract. Cytochrome P450 2C9 (*CYP2C9*) gene variants (rs1057910) cause inter-individual drug dose variability. Polymerase chain reaction confronting two-pair primers (PCR-CTPP) is cost effective and time-saving method. Before genotyping a large number of samples, the PCR-CTPP need to be optimized. This research aimed to optimize PCR CTPP in order to identify rs1057910 *CYP2C9* gene variant in Surakarta. Forty deoxyribose nucleic acid (DNA) samples were obtained from Universitas Sebelas Maret Hospital. The optimization steps were determining of two-pair primer ratio and the optimum PCR annealing temperature (Ta). The PCR-CTPP results were analyzed directly by agarose gel electrophoresis. The optimum Ta was at 61.5°C using outer (Forward1-Reverse2) and inner (Forward2-Reverse1) primer ratio of 1:2. From 40 DNA samples, *CYP2C9**1/*1 (92.5%) and *CYP2C9**1/*3 (7.5%) were observed based on PCR-CTPP and sequencing results. The PCR-CTPP optimization method successfully identified the rs1057910 *CYP2C9* gene variants. The results were consistent with validation using DNA sequencing.

1. Introduction

Cytochrome P450 2C9 (*CYP2C9*) has a crucial role in more than 20% drugs metabolisms. Single Nucleotide Polymorphism (SNP) variant of *CYP2C9* rs1057910 change the rate of drug metabolism, which lead to variable drug therapeutic response and adverse event [1-3]. Furthermore, SNP-variant of *CYP2C9* rs1057910 may affected the drug dose recommendation [4]. Therefore, detection of *CYP2C9* genotype can improve patient safety for warfarin dosing [5]. Gene testing could optimize medication therapy and improve therapeutic outcome [6].

Polymerase chain reaction confronting two-pair primers (PCR-CTPP) is an allele-specific polymerase chain reaction (PCR) method. It has been developed for 15 years to identify SNP-variant and genotype variation. The PCR-CTPP is similar to polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). However PCR-CTPP has shorter time and lower cost compared with PCR-RFLP because enzyme treatment step could be skipped [7-8]. In order to provide accurate protocol and high data reproducibility, it is recommended to optimize PCR-CTPP before genotyping a large number of samples [7]. This research aimed to optimize PCR-CTPP in order to identify rs1057910 *CYP2C9* gene variants in Surakarta



2. Experimental

2.1. Materials

Materials used in this study consist of two-pair primers (Table 1), promega go taq green master mix®, diamond nucleic acid dye®, agarose medium (sigma Aldrich), and promega DNA ladder 100bp®.

Table 1. Primers for CYP2C9 rs 1057910

	Primers	Sequences (Melting temperature(°C))	
Outer Primer	Forward 1 (F1)	5' CCAGGAAGAGATTGAACGTGTGATTG '3	(61.6 °C)
Inner Primer	Reverse 1 (R1)	5' TGGTGGGGAGAAGGTCAAT '3	(61.3 °C)
Inner Primer	Forward 2 (F2)	5' GCACGAGGTCCAGAGATACC'3	(61.7 °C)
Outer Primer	Reverse 2 (R2)	5' GAGTTATGCACTTCTCTCACCC'3G	(61.1 °C)

2.2. DNA samples and Ethical Consideration

Forty human Deoxyribose Nucleic Acid (DNA) samples were obtained from Universitas Sebelas Maret Hospital, which were stored in -20°C. All human DNA were obtained according to a protocol provided by local ethical committee and Universitas Sebelas Maret Hospital.

2.3. Primer, SNP target information, and optimization steps of two-pair primers PCR-CTPP

A SNP of CYP2C9 gene optimized in this study were rs1057910. Two-pair primers (Forward 1(F1)-Reverse 1 (R1) and Forward 2 (F2) – Reverse 2 (R2)) for PCR-CCTP (Table 1) were developed by Tamura (2014) [8].

Strategies in optimizing tetra primer Amplification Refractory Mutation System (ARMS) Polymerase Chain Reaction (PCR) by Suhda (2016) and Medrano (2014) were used in this study. The optimizing steps were determining ratio of outer and inner primer as well as the annealing temperature (Ta), while minimizing unspecific band [10-11]. The total volume of PCR reaction was 10 µl, and it was contained of 5 µl Promega Go Green Taq® ready mix, optimized ratio of outer primer and inner primer (each primer has concentration 10 nm/µl), and 1-2 µl genomic DNA (1-3 µg/ml). DNA amplification was applied by using thermal cycler PCRmax®. Two different ratios of outer and inner primer concentration was applied by using variation ratios of outer and inner primer concentration in 1:1, 1:2, 1:4, 1:8, 1:10 with gradient of Ta (59-63°C). PCR condition were as follows: 95°C(5 minutes) for initial denaturation; followed by 35 cycle of denaturation at 95°C (60 seconds), annealing (60 seconds) with different temperatures, polymerization at 72°C (60 seconds); and final extension at 72°C (10 minutes). For result visualization, a mixture of 2.5 µl PCR product was run in 2% gel agarose stained with diamond nucleic acid dye, against DNA marker 100-1500 bp.

2.4. Validation assay from two-pair primers PCR-CTPP result

Validation assay was conducted with DNA sequencing. For DNA sequencing, conventional PCR was generated to amplify region contains rs1057910, using a pair of primer (F1 – R2) (Table 1). Fifty microliter (50 ul) of PCR product were sent to Genetica Science for the process. The homology of DNA sequences were compared with the sequence in Gen Bank: KF248054.1.

3. Results and Discussion

PCR-CTPP optimization for rs1057910 detection was shown in Figure 1 and 2. The optimum final ratio of outer (F1 – R2) to inner (F2- R1) primer concentrations was 1:2 and the optimum Ta was at 61.5°C. It was determined by the thickest and darkest DNA fragments at desired PCR band size, while minimizing unspecific DNA fragments. The optimum Ta was observed around the melting temperature (Tm) of each primer (Table 1 and Figure1).

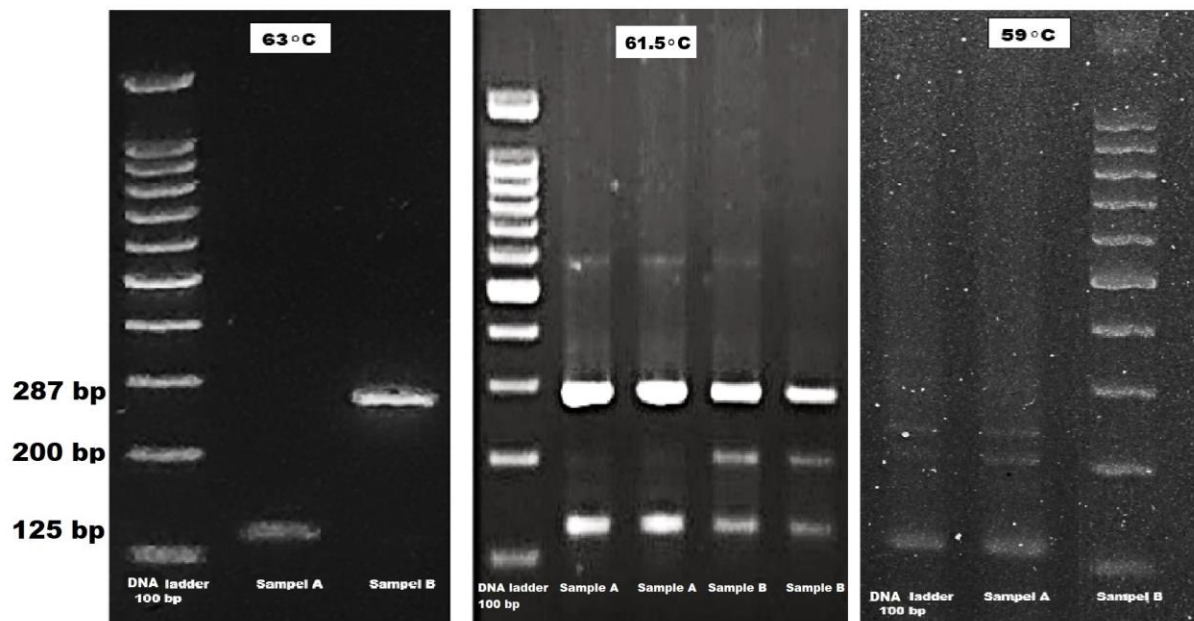


Figure 1. PCR-CTPP optimization for rs1057910 with variation annealing temperature .

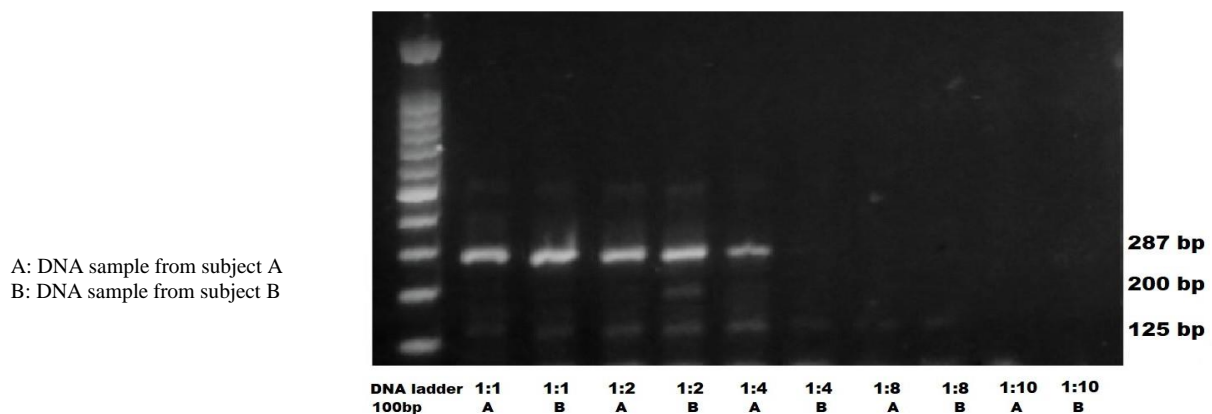


Figure 2. PCR-CTPP optimization for rs1057910 with variation outer (F1-R2) and inner (F2-R1) inner PCR ratios (1:1;1:2;1:4, 1:4;1:8;1:10) in annealing temperature at 61.5°C.

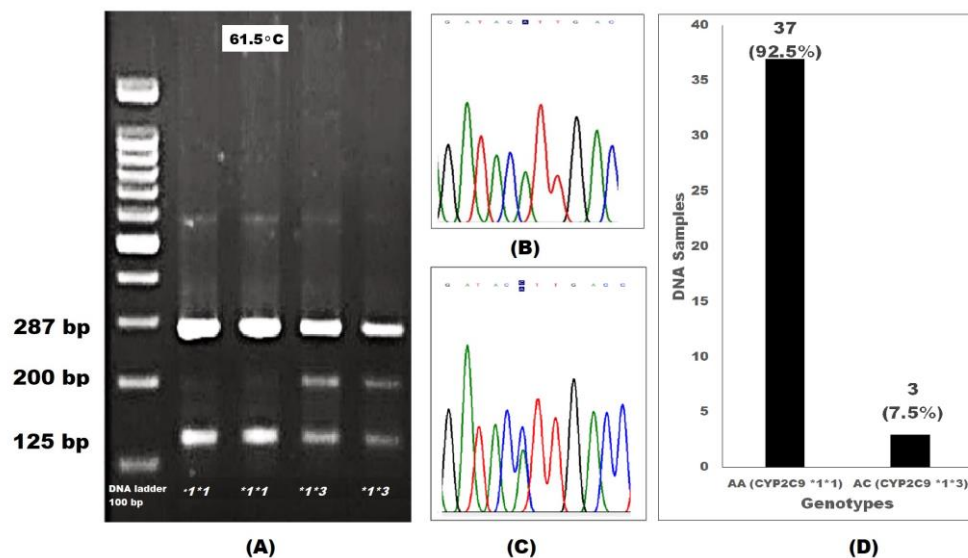


Figure 3. Genotyping results: (A) PCR-CTPP; (B) Sequencing chromatogram genotype AA (*CYP2C9*1*1*); (C) Sequencing chromatogram genotype AC (*CYP2C9*1*3*); (D) Genotyping result in 40 DNA samples

An allele-specific PCR is fast and inexpensive methods for genotyping [9-11]. However, the optimization steps could be time-consuming and require hard work [8,11]. First step in optimization of PCR-CTPP is producing clear and specific PCR products. Producing a balanced amplification of allele-specific PCR-CTPP band is a challenging process. It is not rare that PCR-CTPP does not produce clear and specific band. The balance in T_m of each primer is crucial for the strength of DNA bands. When combinations of four primers are failed to produce clear PCR-CTPP products, so a modified method, named one primer amplification of PCR-CTPP products (OPA-CTPP), can be used to optimize the PCR. PCR-CTPP products tend to be balanced if T_m of four primers are similar [8,9]. In this study, T_m of four primers were similar (Table 1). We identified that the optimum T_a (61 °C) was close to the T_m of each primer (Figure 1 and 2).

Second step in the optimization process is determining optimum primer concentration ratios. Outer primer (F1-R2) is used to determine DNA sequence of interest. Inner primer (F2-R1) is identified allelic variation [8, 10,11]. Inner primers band was the weakest band. Balancing ratio of outer and inner primer concentration was important. Optimizing four primers could be applied by using variation ratio of outer (F1-R2) and inner (F2-R2) primer in 1:10, 1:8, 1:4, 1:2, and 1:1 [10, 11]. In this study, the optimum band for *CYP2C9*1*1* was in T_a 61.5 °C with outer (F1-R2) and inner (F2-R1) primers ratio of 1:2 (Figure 2).

Two pair of primers in PCR-CTPP generated two allele specific bands. A pair of primers (F1-R1) produce a 125 bp band size representing A allele (*CYP2C9*1*1*) and another pair of primers (F2-R2) produce a 200 bp band size representing C allele (*CYP2C9*3*3*) [8, 12]. Those two different size DNA fragments were distinguished by 2% agarose gel electrophoresis and the PCR-CTPP result can be visualized directly using the gel.

PCR-CTPP result required a validation assay [7]. In this study, the PCR-CTPP results were confirmed with DNA sequencing (Figure 3). The PCR-CTPP results were consistent with validation using DNA sequencing. From 40 DNA samples, *CYP2C9*1*1* (92.5%) and *CYP2C9*1*3* (7.5%) were identified by PCR-CTPP and sequencing results. AA homozygous (*CYP2C9*1*1*) had two fragments (125-287 bp) in the PCR-CTPP result and one peak in the sequencing chromatogram (Figure 3A and 3B). Meanwhile, AC heterozygous (*CYP2C9*1*3*) had three different sized of DNA fragments and double peak in the sequencing result (Figure 3A and 3C).

As limitation of this study, *CYP2C9**3*3 could not be verified, because Indonesian tend to have no *CYP2C9**3*3. Genotype AA (*CYP2C9**1*1) was identified in 96.31% of 244 samples in Jakarta, Indonesia and 96,76% of 309 samples in Bandung, Indonesia. The rest (less than 4%) were genotype AC (*CYP2C9**1*3). *CYP2C9**3*3 were not identified in Jakarta and Bandung [13, 14]. PCR-CTPP will amplify two bands of DNA fragments for *CYP2C9**3*3, which were 200 and 287 bp.

CYP2C9 has an important role in S-warfarin metabolism. S-warfarin has three to five fold greater anticoagulation activity than R-warfarin. Mutant allele of *CYP2C9* gene is associated with a reduction of S-warfarin metabolism and clearance, so it can increase blood level of S-warfarin and prothrombin time–international normalized ratio (PT-INR). Patients with genotype variation of *CYP2C9* (*CYP**1*3 and *CYP**3*3) achieved the PT-INR target more rapidly than in those with *CYP**1*1 (the wild type variant). Bleeding risk was also higher in those with the genotype variation [12-14].

CYP2C9 also has important role in metabolizing sulfonylurea, such as glipizide, glyburide, and glimepiride. *CYP2C9**3 are associated with reduction of the sulfonylurea clearance. *CYP2C9**3 might have a greater incidence of hypoglycemia. Carrier of the *CYP2C9**3 may have a lower sulfonylurea dose compared with wild-type homozygote [15].

Providing a simple, fast, and cost saving method is useful for gene testing. Two-pair primers PCR-CTPP was relatively economic, time-saving, and simple method for genotyping *CYP2C9*.

4. Conclusion

The PCR-CTPP method successfully identified the rs1057910 *CYP2C9* gene variants in optimum Ta 61.5°C using outer (F1-R2) and inner (F2-R1) primer ratio of 1:2. The PCR optimization results were consistent with validation using DNA sequencing.

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