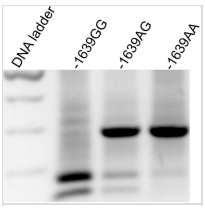

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Characterization of the VKORC1 and CYP2C9 genotypes

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ABSTRACT

Vitamin K antagonists are anticoagulants which represent widely prescribed drugs for prevention and treatment of thromboembolic disorders.

The molecular target of these drugs is vitamin K epoxide reductase enzyme and their metabolism is performed by the cytochrome P-450 2C9 enzyme, both of which are encoded by polymorphic genes - vitamin K epoxide reductase complex subunit 1 (*VKORC1*) and cytochrome P-450 2C9 (*CYP2C9*), respectively.

Identification of *VKORC1* and *CYP2C9* genotypes was recommended by the U.S. Food and Drug Administration in 2007, in order to guide the initial dosing of warfarin, the first oral vitamin K antagonist drug, to achieve optimum anticoagulation and prevent hemorrhagic events.

This protocol is designed for undergraduate students of pharmacology or medicine, or anyone receiving training in pharmacogenomics/pharmacogenetics and understanding a relationship between a gene and drug response for the first time.

The protocol represents a step-by-step guide to using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method in determining *VKORC1 -1639G>A*, *CYP2C9*1/*1*, *CYP2C9*1/*2*, *CYP2C9*1/*3*, and *CYP2C9*2/*3* genotypes.

Based on the combination of the *VKORC1 -1639G>A* and *CYP2C9* genotypes, that is, the pattern of DNA fragments, which are visualized using agarose gel electrophoresis and DNA staining, a conclusion on an individual's sensitivity to vitamin K antagonists and appropriate drug dose is reached.

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KEYWORDS

Vitamin K Epoxide Reductase, Vitamin KO Reductase, VKORC1, Cytochrome P-450, CYP2C9

IMAGE ATTRIBUTION

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GUIDELINES

This protocol uses human whole blood as a starting material for extracting human, genomic DNA. Human whole blood could be a source of hepatitis B (HepB) virus infection (if taken from persons with HepB virus infection) to the individuals who work with human blood and, as such, represents health and safety or biological hazard.

MATERIALS TEXT

Agarose - Sigma A9539-100G;
AvaII restriction endonuclease enzyme - New England BioLabs R0153S;
Bromophenol blue - Sigma B0126-25G;
Boric acid - Sigma B6768-500G;
DNA dye - Ethidium bromide - Sigma E1510-10ML;
DNA ladder (1) - BenchTop 100bp DNA Ladder - Promega G8291;
DNA ladder (2) - MiniSizer 50bp DNA Ladder - Norgen 11200;
dNTPs - dATP, dCTP, dGTP and dTTP - at a stock concentration of 100mM each - Promega U1330;
EDTA - Sigma EDS-500G;
Ethanol - Sigma 32205-1L;
Glycerol - Sigma G5516-500ml;
HCl - Sigma 30721-2.5L;
Isopropanol - 2-propanol - Sigma 59304-500ml;
KpnI restriction endonuclease enzyme - Promega R6341;
NaCl - Honeywell-Fluka 31434-1KG;
NsiI restriction endonuclease enzyme - New England BioLabs R0127S;
MspI restriction endonuclease enzyme - New England BioLabs R0106S;
Proteinase K - Sigma P2308;
SDS - Sigma L3771-100G;
GoTaq G2 DNA Polymerase - Promega M7845;
Trizma base - Sigma RDD008-1KG.

SAFETY WARNINGS

Ethidium bromide, which is used during the preparation of agarose gels, is a DNA intercalating dye and it can, therefore, act as a mutagen. It should be handled with care and only when wearing gloves and safety glasses.

BEFORE STARTING

In order to protect the individuals from the potential HepB virus infection while working with human blood, the U.S.A. Centers for Disease Control and Prevention (CDC) issued a recommendation for HepB vaccination to be carried out in individuals before they start working with human blood. Vaccine-induced antibody titer to the HepB virus of ≥10 mIU/mL should be present in individuals before commencing work with human blood.

Genomic DNA extraction

1 Patients' whole blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and stored at -20°C until use. Genomic DNA extraction from the human whole blood, that is, leukocytes, was carried out according to the protocol described by Subbarayan PR and colleagues (doi: 10.2144/02336bm10), with modifications.

Upon thawing of the blood, for every patient's blood sample, five (5) sterile tubes were labeled and 300 µl of the whole blood was added. This was the amount of the starting material that yielded ample amount of human, genomic DNA at the end of the DNA extraction protocol.

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2 Concentrations of human, genomic DNA was determined by using µDROP plate (N12391) and a Multiscan GO spectrophotometer (Thermo Fisher Scientific).

All human, genomic DNA samples were set-up into ready-to-use DNA samples for subsequent polymerase chain reactions (PCRs) by creating dilutions or stocks of 50 ng/µl. 200 ng, that is, 4 µl of every 50 ng/µl DNA stock, was used in every PCR reaction.

Identification of the *VKORC1 -1639G>A* genoytpes

3 Identification of the *VKORC1 -1639G>A* genotypes was performed according to the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method-based protocol published by Sconce EA and colleagues (doi: 10.1182/blood-2005-03-1108).

4 PCR reactions were assembled as follows: reactions of 25 µl contained 1) sterile, milliQ-H_2O; 2) 1xPCR buffer (for GoTaq G2 DNA polymerase, Promega), from a stock of 5xPCR buffer; 3) 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (U1330, Promega), from a stock of 2.5 mM; 4) 1 µM forward primer, from a stock of 25 µM; 5) 1 µM reverse primer, from a stock of 25 µM; 6) 200 ng of genomic DNA and 7) 2.5 U GoTaq DNA polymerase (M7845, Promega).

Sequences of the PCR primers were as follows: 1) Forward primer: 5'-GCCAGCAGGAGAGGGAAATA-3'; 2) Reverse primer: 5'-AGTTTGGACTACAGGTGCCT-3'.

PCR cycles used for the amplification of the *VKORC1* gene promoter, which were based on the touchdown PCR protocol published by Korbie and Mattick in 2008, were as follows:
1) 95°C for 3 min (1 cycle);
2) 95°C for 2 s, 60°C for 2 s, 72°C for 30 s (2 cycles);
3) 95°C for 2 s, 59°C for 2 s, 72°C for 30 s (2 cycles);
4) 95°C for 2 s, 58°C for 2 s, 72°C for 30 s (2 cycles);
5) 95°C for 2s, 57°C for 2s, 72°C for 30 s (2 cycles);
6) 95°C for 2 s, 56°C for 2 s, 72°C for 30 s (2 cycles);
7) 95°C for 2 s, 55°C for 2 s, 72°C for 30 s (2 cycles);
8) 72°C for 7 min (1 cycle);
9) 4°C for 2 h (1 cycle).

The expected size of the PCR product is 290 bp.

5 In order to characterise the *VKORC1 -1639G>A* genotypes by using the PCR-RFLP method, the following reactions were set up with MspI restriction endonuclease enzyme:
1) 25 µl of the PCR product;
2) 3 µl of the 10xCutSmart Buffer (B7204S, New England Biolabs);
3) 1 µl of sterile, milliQ-H_2O;
4) 1 µl of MspI enzyme (R0106S, New England Biolabs). Total reaction volume: 30 µl.

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Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTris, boric acid, EDTA (TBE) buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

The following pattern of DNA fragments can be expected after the MspI restriction endonuclease enzyme reaction:

1) GG or *VKORC1 -1639GG* genotype is characterized by two (2) DNA fragments of 168 and 122 base pairs (bp);

2) AG or *VKORC1 -1639AG* genotype is characterized by three (3) DNA fragments of 290, 168 and 122 bp;

3) AA or V*KORC1 -1639AA* genotype is characterized by one (1) DNA fragment of 290 bp.

Identification of the *CYP2C9* genoytpes

6 Identification of the *CYP2C9* genotypes (**1/*1*, **1/*2*, **1/*3*, and **2/*3*) was performed according to the PCR-RFLP method-based protocol published by Sullivan-Klose TH and colleagues (doi: 10.1097/00008571-199608000-00007) and Yasar U and colleagues (doi: 10.1006/bbrc.1998.9992).

7 PCR reactions were assembled as follows: reactions of 25 µl contained 1) sterile, milliQ-H_2O; 2) 1xPCR buffer (for GoTaq G2 DNA polymerase, Promega), from a stock of 5xPCR buffer; 3) 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (U1330, Promega), from a stock of 2.5 mM; 4) 1 µM forward primer, from a stock of 25 µM; 5) 1 µM reverse primer, from a stock of 25 µM; 6) 200 ng of genomic DNA and 7) 2.5 U GoTaq DNA polymerase (M7845, Promega).

7.1 Sequences of the PCR primers for AvaII restriction endonuclease enzyme

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reactions were as follows: 1) AvaII-Forward primer: 5'-TACAAATACAATGAAAATATCATG-3'; 2) AvaII-Reverse primer: 5'-CTAACAACCAGACTCATAATG-3'.

PCR cycles used with the above primers were as follows:
1) 94°C for 5 min (1 cycle);
2) 94°C for 60 s; 55°C for 90 s; 72°C for 30 s (35 cycles);
3) 72°C for 7 min;
4) 4°C for 2 h (1 cycle).

The expected size of the PCR product is 691 bp.

7.2 In order to characterise the *CYP2C9 *1/*1* or **1/*2* genotypes by using the PCR-RFLP method, the following reactions were set up with AvaII restriction endonuclease enzyme:
1) 10 µl of the PCR product;
2) 2 µl of the 10xCutSmart Buffer (B7204S, New England Biolabs);
3) 7 µl of sterile, milliQ-H_2O;
4) 1 µl of AvaII enzyme (R0153S, New England BioLabs). Total reaction volume: 20 µl.

Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTBE buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

The following pattern of DNA fragments can be expected after the AvaII restriction endonuclease enzyme reaction:

1) **1/*1* or *CYP2C9 *1/*1* genotype is characterized by two (2) DNA fragments of 527 and 164 base pairs (bp).
In addition, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4).

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Furthermore, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6).

2) **1/*2* or *CYP2C9 *1/*2* genotype is characterized by three (3) DNA fragments of 691, 527 and 164 bp.
In addition, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4).
Furthermore, the **1/*1* genotype is characterized by one (1) DNA fragment, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6).

7.3 Sequences of the PCR primers for NsiI restriction endonuclease enzyme reactions were as follows: 1) NsiI-Forward primer: 5'-AATAATAATATGCACGAGGTCCAGAGATGC-3'; 2) NsiI-Reverse primer: 5'-GATACTATGAATTTGGGACTTC-3'.

PCR cycles used with the above primers were as follows:
1) 94°C for 5 min (1 cycle);
2) 94°C for 60 s; 60°C for 90 s; 72°C for 30 s (35 cycles);
3) 72°C for 7 min;
4) 4°C for 2 h (1 cycle).

The expected PCR product is running between 100 and 200 bp markers when compared to a DNA ladder.

7.4 In order to characterise the *CYP2C9 *1/*3* or **2/*3* genotypes by using the PCR-RFLP method, the following reactions were set up with NsiI restriction endonuclease enzyme:
1) 10 µl of the PCR product;
2) 2 µl of the 10xNEBuffer 3.1 (B7203S, New England Biolabs);
3) 7 µl of sterile, milliQ-H_2O;
4) 1 µl of NsiI enzyme (R0127S, New England BioLabs). Total reaction volume: 20 µl.

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Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTBE buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

The following pattern of DNA fragments can be expected after the NsiI restriction endonuclease enzyme reaction:

1) **1/*3* or *CYP2C9 *1/*3* genotype is characterized by two (2) DNA fragments of 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme.
In addition, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction.
Furthermore, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6).

2) **2/*3* or *CYP2C9 *2/*3* genotype is characterized by three (3) DNA fragments of 691, 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme.
In addition, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction.
Furthermore, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction (see sub-steps 7.5 and 7.6).

7.5 Sequences of the PCR primers for KpnI restriction endonuclease enzyme reactions were as follows: 1) KpnI-Forward primer: 5'-AATAATAATATGCACGAGGTCCAGAGGTAC-3'; 2) KpnI-Reverse primer: 5'-GATACTATGAATTTGGGACTTC-3'.

PCR cycles used with the above primers were as follows:
1) 94°C for 5 min (1 cycle);

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2) 94°C for 60 s; 60°C for 90 s; 72°C for 30 s (35 cycles);
3) 72°C for 7 min;
4) 4°C for 2 h (1 cycle).

The expected PCR product is running between 100 and 200 bp markers when compared to a DNA ladder.

7.6 In order to characterise the *CYP2C9 *1/*3* or **2/*3* genotypes by using the PCR-RFLP method, the following reactions were set up with KpnI restriction endonuclease enzyme:
1) 10 µl of the PCR product;
2) 2 µl of the 10xBuffer J (R009A, Promega);
3) 0.5 µl Bovine Serum Albumin Acetylated, 10mg/ml (R3960, Promega);
4) 6.5 µl of sterile, milliQ-H_2O;
5) 1 µl of KpnI enzyme (R6341, Promega). Total reaction volume: 20 µl.

Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTBE buffer and agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

The following pattern of DNA fragments can be expected after the KpnI restriction endonuclease enzyme reaction:

1) **1/*3* or *CYP2C9 *1/*3* genotype is characterized by two (2) DNA fragments of 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme.
In addition, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4).
Furthermore, the **1/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction.

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2) **2/*3* or *CYP2C9 *2/*3* genotype is characterized by three (3) DNA fragments of 691, 527 and 164 bp that are achieved by AvaII restriction endonuclease enzyme.
In addition, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the NsiI restriction endonuclease enzyme reaction (see sub-steps 7.3 and 7.4).
Furthermore, the **2/*3* genotype is characterized by two (2) DNA fragments, running between 100 and 200 bp markers when compared to a DNA ladder, which is the result of the KpnI restriction endonuclease enzyme reaction.

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