Homogeneous Single-Tube Triplex Genotyping for Warfarin Dosing in Less Than 30 Minutes

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Background

Warfarin is an anticoagulant used for prevention of thrombisis. Warfarin is metabolized by cytochrome P450 2C9 (CYP2C9). Mutations in the metabolic pathway for warfarin need to be taken into account when calculating the proper therapeutic dosing for a given patient. A patient with a reduced capacity to metabolize warfarin is susceptible to overdosing which can lead to morbidity and mortality. Another consideration in warfarin dosing calculations is the patient's resistance to the drug. Warfarin inhibits synthesis of vitamin K dependant clotting factors. VKORC1 encodes for the main protein in the vitamin K epoxide reductase complex. Mutations in VKORC1 can lead to a patient's resistance to warfarin which reduces the drug's effectiveness at standard doses. There are three mutations that are most commonly tested: one in vitamin K epoxide reductase (VKORC1-1639C>A) and two in cytochrome P450 (CYP2C9*2, CYP2C9*3). A fast and reliable genotyping method for warfarin metabolic mutations would be useful in clinical settings. A single, closed tube triplex PCR reaction will allow medical staff to more accurately and rapidly calculate warfarin dosing.

Methods

PCR primers and unlabeled probes were designed for the three sites of interest. Each set of primers bracketed the SNP sites and the unlabeled probes were designed to overlap the SNP sites. The CYP2C9*2 and CYP2C9*3 primers were designed using Clustal alignments with the homologue genes CYP2C8, CYP2C18 and CYP2C19. The primers were placed in the variable regions, bracketing the mutations. PCR reactions were set up using LCGreen Plus® (Idaho Technology), a fluorescent double stranded DNA binding dye. Rapid cycle PCR and post PCR high resolution melting of the resulting amplicons were performed using an LC32 (Idaho Technology) or a combination of the LightCycler 2.0 (Roche) and the HR-1 (Idaho Technology) high resolution melter. The high resolution melting data were analyzed using custom software.

Primers and Probes:

MUTATIONS PRIMERS
PROBES mismatches

CYP2C9*2_C>T
Probe GAGGAGCATTGAGGACCGTGTTCAAGAGGAAGCC
138bp amplicon:
ATGACGCTGCGGAATTTtgggatggggaaGAGGAGCATTGAGGACCGTGTTCAAGAGGAAGCCcgctgccttgtggaggag
ttgagaaaaaccaagggtgggtgaccctactcCATATCACTGACCTTACTGGACTAC

CYP2C9*3_A>C
Probe GTCCcGAGATACCTTGACCTTtTCCCCA
--Probe matches mutation--
146bp amplicon:
ACCGgAGCCCCTGCATGCAAgacaggagccacatgccctacacagatgctgtggtgcacgagGTCCaGAGATACATTGACCTTcT
CCCCAccagcctgccccatgcagtgacctgtGACATTAAATTCAGAAACTATCTCATTCCC

VKORC1–1639_G>A Promoter SNP
63bp amplicon: CAAGAGAAGACCTGAAAAACAACCATTggccGggtgcggtGGCTCACGCCTATAATCCTAGCA

Thermocycling Conditions: 55 cycles

Denature 94°C for 0 s Anneal 62°C for 1 s Extend: 72°C for 5 s

Oligos [Final]	Forward Primer	Reverse Primer	Probe
CYP2C9*2	0.1 uM	0.5 uM	0.5 uM
CYP2C9*3	0.1 uM	0.5 uM	0.5 uM
VKORC1	0.5 uM	0.5 uM	

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CYP2C9    GGAAAGAAATGGAAGGAGATCCGGCGTTTCTCCCTCATGACGCTGCGGAATTTTGGGATG
CYP2C19   GGAAAGAGATGGAAGGAGATCCGGCGTTTCTCCCTCATGACGCTGCGGAATTTTGGGATG
CYP2C8    GGAAAGAGATGGAAGGAGATCCGGCGTTTCTCCCTCACAACCTTGCGGAATTTTGGGATG
CYP2C18   GGAAAGAGATGGAAGGAGATCCGGCGTTTCTGCCTCATGACTCTGCGGAATTTTGGGATG
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CYP2C9    GGGAAGAGGAGCATTGAGGACCGTGTTCAAGAGGAAGCCCGCTGCCTTGTGGAGGAGTTG
CYP2C19   GGGAAGAGGAGCATTGAGGACCGTGTTCAAGAGGAAGCCCGCTGCCTTGTGGAGGAGTTG
CYP2C8    GGGAAGAGGAGCATTGAGGACCGTGTTCAAGAGGAAGCTCACTGCCTTGTGGAGGAGTTG
CYP2C18   GGGAAGAGGAGCATCGAGGACCGTGTTCAAGAGGAAGCCCGCTGCCTTGTGGAGGAGTTG
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CYP2C9    AGAAAAACCAAGGGTGGGTGACCCTACTCCATATC----ACTGACCTTACTGGACTACTA
CYP2C19   AGAAAAACCAAGGGTGGGTGAACATACTCTCTATC----ACTGACCTTTCTGGACTGCTC
CYP2C8    AGAAAAACCAAGGGTGGGTGACTCTACTCTGCGTC----ATTGACCTTAACAGTTACCTG
CYP2C18   AGAAAAACCAATGGTGGGTGACTTTTTTTTTTCCTGAAAAATGTTTTCTAAAATTTATTT
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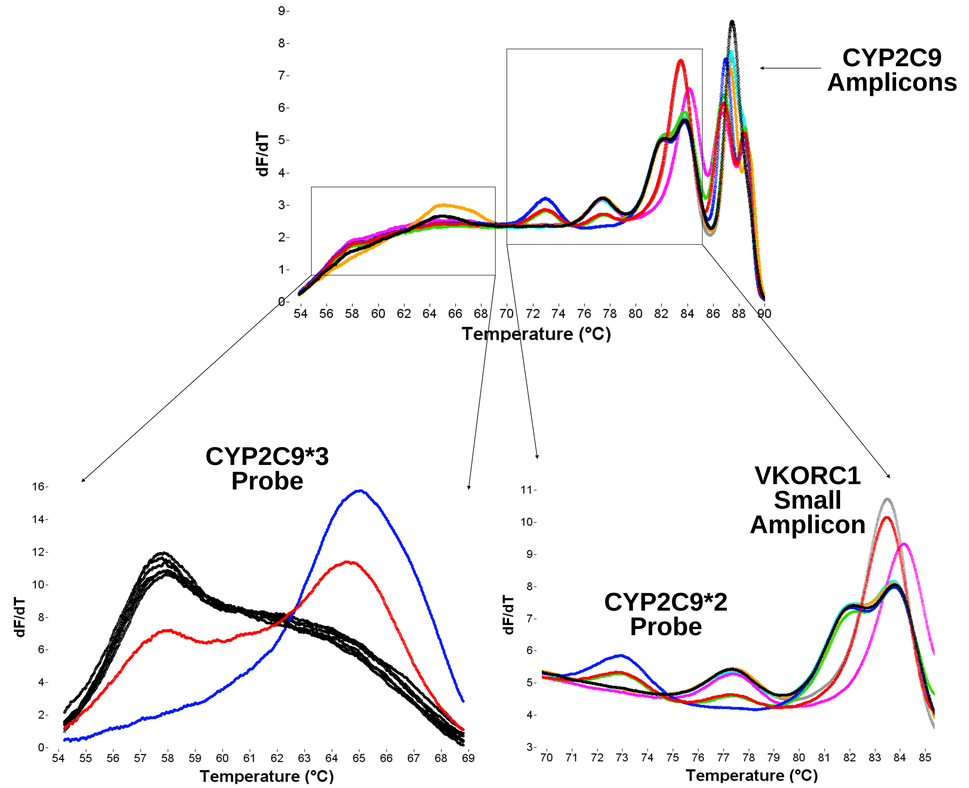
CYP2C9*2 Clustal alignment with homologue genes. Primers in green and SNP site in red.

Results

Genotyping by High Resolution



Analysis of the high resolution melting data was performed in 2 parts. The high Tm CYP2C9*2 probe and the VKORC1 small amplicon were analyzed together. The lower Tm CYP2C9*3 probe was analyzed by itself to better separate signal from background. A blinded panel of 40 DNA samples of known genotypes were assayed for each of the three SNPs in triplex reactions. All 40 blinded samples were correctly genotyped.

Conclusions

This is rapid and inexpensive method for genotyping the three most common mutations used for warfarin dosing calculations. A single, closed tube system reduces time, consumables and amplicon contamination. Amplification and melting required only 27 minutes.