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Genotyping for CYP2C9 and VKORC1 alleles by a novel point of care assay with HyBeacon® probes

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ABSTRACT

Background: Coumarin anticoagulants such as warfarin are used to treat and prevent thromboembolic events in patients. The required dosage is difficult to predict and the risk of over or under anticoagulation are dependent on several environmental and clinical factors, such as concurrent medication, diet, age and genotype for polymorphisms in two genes *CYP2C9* and *VKORC1*.
Methods: A novel fluorescent PCR genotyping assay using HyBeacon® probes, was developed to enable clinical staff to genotype the *CYP2C9*2* and *CYP2C9*3* alleles and the *VKORC1* G-1639A polymorphism directly from unextracted blood samples. A prototype PCR instrument, Genie 1, suitable for point of care use was developed to carry out the assays. The panel of tests was validated by analysing blood samples from 156 individuals and comparing genotypes with data obtained using DNA samples from the same individuals. The accuracy of genotypes obtained with the Genie 1 was compared against results from well validated real time PCR and PCR-restriction fragment length polymorphism analysis.
Results: Identical results were obtained for the newly developed HyBeacon® method and the validation method in all cases except for one where no result was obtained for the *VKORC1* polymorphism on the Genie instrument. The samples used for validation represented all six possible **2* and **3* allele-related *CYP2C9* genotypes and all three *VKORC1* G-1639A genotypes.
Conclusions: We observed excellent accuracy for the newly developed method which can determine genotype in less than 2 h.

1. Introduction

Oral anticoagulants such as warfarin, acenocoumarol and phenprocoumon are prescribed widely for the treatment of thromboembolic disorders. Prescribing these coumarin based derivatives is difficult due to their narrow therapeutic range with patients at risk of haemorrhage if given too high a dose. Factors such as height, weight and concurrent medications all affect dose requirement. It is also now very well established that genetic factors play a role in the variability of a patient's response. Several polymorphisms in the genes encoding the cytochrome P450 2C9 (*CYP2C9*) and vitamin K epoxide reductase (*VKORC1*) contribute to this variability. It is estimated that these single nucleotide polymorphisms (SNPs) account for about 40% of the variability in response to coumarin dosing regimes, with patient age and height or weight contributing an additional 15% [1–4]. In 2007, the US Food and Drug Administration (FDA) Center for Drug Evaluation and Research updated the label of warfarin to include information on genetic testing [5]. This has prompted clinical trials to demonstrate the utility of pharmacogenetic testing in coumarin anticoagulant prescription. Until now, published results from clinical trials involving genotyping have related to warfarin only, and have mainly involved small numbers of patients; they are therefore underpowered to identify significant differences in outcomes between cases and controls. These studies have generally not involved determination of a patient's genotype prior to start of treatment [6–11] with the exception of one recent study involving 230 patients [12]. It has been estimated that to detect a 10% improvement in time within range for the target international normalised ratio (INR) as a result of including genotyping during initial warfarin dosing, it would be necessary to study 442 cases and 442 controls [13] The only study completed up to the present with numbers of cases in excess of these estimates [9] found that more frequent monitoring of patients based

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on genotype led to a significant decrease in hospitalisation rates but in that study, genotyping results to enable decisions about clinical management were available only 11 to 60 days (median 32 days) after the start of treatment. Several clinical trials are currently in progress, including the European EU-PACT which is aiming to recruit almost 3000 patients. In addition to warfarin, EU-PACT is assessing genetic testing in patient groups being prescribed acenocoumarol and phenprocoumon, and is seeking to genotype patients prior to the initiation of treatment [13].

A range of genotyping assays for SNPs in *CYP2C9* and *VKORC1* genes relevant to coumarin anticoagulants have been used previously, including PCR-restriction fragment length polymorphism (RFLP) analysis [1], pyrosequencing [14,15], Invader [12] and several fluorescence based real time PCR methods [16–18]. Recently, several commercial platforms with regulatory approval have been developed including Osmetech eSensor, Nanosphere, Infiniti and ParagonDx [19]. In general, studies comparing the validity of the various methods have shown very high levels of accuracy and good agreement in results across platforms [15,18,19]. However, all the available technologies require initial DNA extraction from blood and take a minimum of 3 to 4 h for the PCR step to be completed. All are designed for use in a laboratory rather than as a point of care test (POCT) which further increases the time taken from collecting a blood sample to obtaining a result.

As reviewed recently [20,21], a number of studies suggest that knowledge of patient genotype prior to initiation of dosing with coumarin anticoagulant is likely to decrease the risk of serious bleeding, though this has not yet been demonstrated by a formal clinical trial. Some initiation of coumarin anticoagulant dosing is elective (e.g. for chronic atrial fibrillation or prior to planned surgery) but for patients diagnosed with, for example, deep vein thrombosis or pulmonary embolism, dosing cannot be delayed. A rapid POCT is needed if initial dosing of such patients is to be based on their genotype. To enable a rapid determination of the relevant genotypes prior to initial dosing with coumarin anticoagulants at the point of care, we have developed a novel genotyping assay using HyBeacon® probe technology to detect and identify specific *CYP2C9* and *VKORC1* alleles. This assay uses HyBeacon® probe technology that provides a homogeneous method for fluorescence-based sequence detection, allele discrimination and DNA quantification [22–25]. We now describe the test and its validation.

2. Materials and methods

2.1. Sample preparation

For the point of care genotyping assay, blood (5 µl) was collected using a microsafe pipette (Alpha Laboratories) either directly from a finger pricked with a sterile lance or from blood already in a collection tube. The blood was then mixed with 100 µl 0.32 M sucrose in 10 mM Tris–HCl pH7.4 containing 1% (v/v) Triton X-100 in a sterile microfuge tube. One microliter of the diluted blood was transferred as template into a 17 µl PCR reaction with a 1 µl microbiology loop.

For assay validation, DNA was prepared from blood using either a previously described method [26] or commercial kits (Qiagen and Applied Biosystems).

2.2. PCR primers and probes

Primers and probes for the POCT are listed in Table 1. Primers and probes were designed using information from the genomic sequence of chromosomes 10 (CYP2C9; NC_000010.10) and 16 (VKORC1; NC_000016.9) and dbSNP information (http://www.ncbi.nlm.nih.gov/snp/) for rs1799853, rs1057910 and rs9923231. Extensive BLAST searches were carried out to ensure that primers were specific to target sequences and did not cross-hybridise to CYP2C9 homologues. Probe design was carried out to allow complete complementarity to one of two alleles in each case. The second allele for each sequence therefore had a single mismatch to the probe at the SNP position. This caused the melting temperature to be decreased when compared with the matched allele, allowing distinct derivative peaks to be generated from each allele.

Table 1
Primers and Probes for point of care genotyping.

Assay	Primer name	Primer sequence 5′ → 3′
A. Primers		
*CYP2C9*2*	C9*2CF3	CCTCCTAGTTTCGTTTCTCTTCCTGTTAGGAATT
	C9*2CR4	GTAGAGAAGATAGTAGTCCAGTAAGGTCAGTGATATG
*CYP2C9*3*	C9*3F2	TGCATGCAAGACAGGAGCCACAT
	C9*3R2	GGAGAAACAAACTTACCTTGGXAATGAGA X = Inosine
VKORC1 For blood only	VKOF2	GGAGCCAGCAGGAGAGGGAAATA
	VKOR3	CGGCCTCCCAAAATGCTAGGATT
VKORC1 For purified DNA only	VKOF3	CAGAAGGGTAGGTGCAACAGTAAGGGATCC
	VKOR4	CTGACCTCAAGTGATCCACCCACCTCG
Assay	Probe name	Probe sequence 5′→ 3′
B. Probes		
*CYP2C9*2*	C9*2C2	2GCATFGAGGACCGFGTTCAAG3
*CYP2C9*3*	2C9*3C5	2TCCAGAGATACCTFGACCTFCTCCC3
VKORC1	VKORC1	2CATFGGCCAGGFGCGGT3

F = FLUOROSCEIN dT, 2 = 5′ Trimethoxystilbene, 3 = 3′ PHOSPHATE.
These probes were also used for the LightTyper assay.

The HyBeacon® probes [27] were synthesised by ATDBio Ltd and were each labelled using fluorescein dT monomers (Glen Research, Virginia, USA). To ensure similar levels of amplification efficiency from two distinct sample types, it was necessary to use two different sets of primers for the *VKORC1* assay (see Table 1). The same probe was used in both cases to ensure consistency in genotyping.

2.3. PCR assays

PCR was performed in 17 µl reactions containing 0.7 µl HemoKlen Taq in 1X supplied buffer (New England Biolabs), 0.2 mM dNTP (New England Biolabs), 0.1 µM forward primer, 10 µM reverse primer, 0.2 µM HyBeacon® probe (see Table 1). Amplification was performed in a Genie 1 PCR instrument (Optigene Ltd) (Fig. 1). Thermocycling was carried out with an initial step of 95 °C for 4 min followed by 50 cycles of 94 °C for 3 s and 64 °C for 1 min, using a 5 °C/s ramp rate. No separate extension step was required. Following amplification, results were obtained by performing melting curve analysis from 40 °C to 75 °C at a ramp rate of 0.1 °C/s and measuring the fluorescence changes as the HyBeacon® probes dissociate from the amplified target DNA.

Strips of 8 low profile 0.2 ml tubes (Starlab) containing pre-prepared mastermix were used for all the POCT analysis. Three assays (for *CYP2C9*2* (rs1799853), *CYP2C9*3* (rs1057910) and *VKORC1* -1639 G>A (rs9923231) were performed simultaneously in separate tubes (diagnostic PCR; tubes 1 to 3)) together with 3 separate control assays (tubes 4 to 6) which included 1.5 ng DNA from known heterozygotes for each SNP and two “no DNA” template controls (tubes 7 and 8).

Mastermix strips including control DNA were prepared in advance and stored at −20 °C in the dark. To genotype an individual for *CYP2C9* and *VKORC1* SNPs, a strip was thawed, 1 µl blood diluted as described above was added to the 3 diagnostic PCR tubes (1 to 3) and amplification/melting analysis performed. Analysis of the fluorescence derivative profile was performed on a PC connected to the Genie 1 instrument using software developed for this purpose by Optigene Ltd.