Multiplex Assay for Genetic Testing of Thrombophilia: A Method for Routine Clinical Care

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There is an increasing demand for genetic testing of patients with hypercoagulability. We have developed a multiplex-polymerase chain reaction (PCR) technique that incorporates the state-of-the art of genetic testing for thrombosis mutations (Factor V Leiden [FVL] and G20210A F2 and C46T F12). The sequences are detected by enzyme-linked immunosorbent assay (ELISA)-resolved colorimeter after hybridization with an amplification product labeled with digoxigenin. To evaluate the reliability of this method, we analyzed 122 deoxyribonucleic acid (DNA) samples of known genotypes for these three mutations. Six subjects initially assigned as heterozygous for FVL and two subjects assigned as normal for the G20210A F2 mutation showed discrepancies between the current techniques and our newly-developed ELISA-

based technique. When these samples were sequenced the concordance using our method was 100%. Thus, initially they were assigned incorrectly based on the available methodologies. It is noteworthy that our method is adaptable to an Automated ELISA Analyzer that allows for routine processing of both small and large numbers of DNA samples. We present a robust, rapid, reproducible, cost-effective, and simple multiplex PCR ELISA method to simultaneously detect carriers of thrombotic genetic risk factors. Testing for thrombophilia should contribute to better diagnosis, prevention, and treatment strategies, providing valuable information to assess the risk of recurrence in the proband, and in family members who are asymptomatic. *J. Clin. Lab. Anal.* 21:349–355, 2007. © 2007 Wiley-Liss, Inc.

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INTRODUCTION

Venous thrombosis is a common cause of morbidity and mortality in industrialized nations. Although there is little information on the exact prevalence, some retrospective and prospective data (1–3) suggest a minimum lifetime prevalence of 5–10% for venous thromboembolism (VTE). Thus, it is of great public health importance.

It is well established that thrombosis is a complex disease with a very high genetic component (i.e., heritability of 61%) that determines the risk of disease (4). Before 1993, the evaluation of inherited thrombophilia was limited to plasma-based functional assays for protein C (PC), protein S (PS), or antithrombin (AT) deficiency,

which together are found in less than 10% of patients with incident VTE (5). In 1994, the laboratory procedures used to test for thrombophilia changed when Bertina et al. (6) described the specific genetic defect responsible for

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activated C protein (APC) resistance—a single well-conserved G to A missense mutation at nucleotide 1691 of the *F5*—called Factor V Leiden (FVL). The FVL (OMIM 227400.001) is the most common inherited cause of thrombophilia, with an increased risk of five-fold (7), whereas homozygous FVL presented a 80-fold increased risk of developing a thrombosis (8).

Then, in 1996, a genetic variation in the 3'-untranslated region of the prothrombin structural gene (*F2*) involving a G to A transition at nucleotide position 20210 was described (9) (OMIM 176930.0009). Compared with normal homozygotes (*G/G*), heterozygous carriers (*G/A*) of the mutation have an almost three-fold increased risk of VTE (5,9,10).

Moreover, these two mutations are often associated with other genetic defects that predispose to thrombosis, such as PC and PS deficiencies (11,12). These combined defects confer a higher risk of thrombosis than either defect alone. Consequently, it has been postulated that more than one genetic risk factor may cosegregate with consequent cumulative or synergistic effect on thrombotic risk (13).

More recently, as part of a family-based linkage study, it has been documented that a polymorphism in exon 1 of the *F12* gene, a C to T transition at nucleotide 46 (OMIM 234000.0004), jointly influences FXII levels and the risk of thrombosis (14). In subsequent association studies it was confirmed that this deoxyribonucleic acid (DNA) variant represents a genetic risk factor for venous (15) and arterial thrombosis (16–18). In addition, the C46T transition also increases the risk of thrombosis in combination with FVL and G20210A in a thrombophilic family (19).

While it is important to identify novel genetic risk factors, it is equally important to have the proper methodology to test for them, since increasing numbers of patients are being referred for testing, and many more diagnoses of inherited thrombophilia are being made. Here, we present a methodology for genetic testing of thrombophilia that represents the state-of-the art for genetic diagnosis of thrombosis.

MATERIAL AND METHODS

Subject Samples

Our study included 122 subjects who were selected based on their genotype for the FVL, the G20210A *F2* gene and the C46T *F12* gene; without taking into account their clinical status since our goal was to validate this new methodology.

Blood Collection and Isolation of DNA

Whole blood was collected from the antecubital vein and immediately anticoagulated with 1/10 volume of

0.129 M sodium citrate. DNA was isolated from the peripheral blood leukocytes by standard procedures (20) using a commercial kit QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) following the recommendation of the suppliers from 200 µL of frozen whole blood (yields 3–12 µg of DNA). The DNA was resuspended at a concentration of 40 µg/mL (buffer of the DNA extraction kit), stored at 2–8°C for the polymerase chain reaction (PCR) procedure and immediately stored frozen at –20°C.

FVL Detection

The *FVL* genotype was detected by FV-PROT Strip Assay (Vienna Labs) and by direct sequence analysis of the PCR fragment corresponding to this mutation using the forward primer 5'-TCAGGCAGGAACAACAC-CAT-3' and the reverse primer 5'-TGTTATCA-CACTGGTGCTAA-3'. Sequencing methodology is explained below.

G20210A *F2* Gene Detection

The amplification of the PCR fragment (297 bp) was performed using the forward primer 5'-CCGC TGGTATCAAATGGG-3' and reverse primer 5'-CC AGTAGTATTACTGGCTCTTCCTG-3' in a total volume of 25 µL. The conditions were: 1.5 mM MgCl₂, 0.02 mM dNTPs, 3 pg each primer, one unit of Taq polymerase (Applied Biosystems), and 3 µL of genomic DNA. The amplification protocol included a denaturation step at 96°C for 10 min; 40 cycles (30 sec at 94°C, 1 min at 57°C, and 1 min at 72°C); and a final extension at 72°C for 10 min. Each sample was tested by a PCR endonuclease method using *Hind III* restriction enzyme.

C46T *F12* Gene Detection

The analysis of C46T genotype in *F12* was performed by real-time PCR and a fluorescence resonance energy transfer. The protocol was carried out on a Light Cycler instrument (Roche) in the conditions described previously (21). PCR was performed by rapid cycling with Light Cycler Fast Start Reaction Mix Hybridization Probes (Roche). During amplification, the fluorescence was measured and plotted against temperature to generate melting curves for each sample.

DNA Sequencing

Doubtful results were retested by sequencing using a new PCR fragment from the same sample that was used for genotyping. Briefly, the PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) to prepare them for sequencing. The sequence reaction was performed in a 10-µL reaction volume containing 2 µL

of purified DNA, 3 μ L of DNA Sequencing Kit (BigDye Terminator Cycle Sequencing Ready Reaction; Applied Biosystems), 5% dimethylsulfoxide (DMSO) vol/vol, and 0.32 μ M of the sequencing primer. The sequence program started with 3 min at 94°C, followed by 25 cycles consisting of 10 sec at 96°C, 5 sec at annealing temperature of 50°C, and finally 4 min at 60°C. Sequence analyses were performed using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) following the instructions of the supplier.

PCR-Multiplex Amplification and Enzyme-Linked Immunosorbent Assay (ELISA) Detection

The targeted gene fragments (*F5*, *F2*, and *F12* genes) were simultaneously amplified by a multiplex-PCR reaction using the primer pairs described previously for FVL and G20210A and for the C46T the primer pair described by Tirado et al. (21). PCR conditions were the same as described previously in a total volume of 50 μ L. The amplification protocol included a denaturation step at 96°C for 10 min; 40 cycles (30 sec at 94°C, 1 min at 57°C, and 2 min at 72°C), and a final extension at 72°C for 10 min. In each amplification negative controls were included for each mutation.

The amplification reaction included dUTP-digoxigenin (0.625 nmol) instead of dTTP random and the amplification product was labeled with digoxigenin molecules for detection by an ELISA procedure.

Biotin-labeled probes were designed, one for the wild-type allele (*wt*) and the other for the mutant allele (*mut*). These probes were added to a streptavidin-coated 96-well ELISA plate and 25 μ L of PCR product. Following a short incubation period, unbound products were washed away and a conjugated antibody for peroxidase was added. The reaction was completed when green color developed in the presence of POD substrate (ABTS).

ELISA Analysis

The results were determined colorimetrically using an ELISA plate reader (Multiskan Labsystems).

Optical densities (ODs) at 405 nm were analyzed according to standard cutoff by *mut/wt* ratio values:

- . $R = \text{OD}(\text{nor}) - \text{OD}(\text{negative nor}) / \text{OD}(\text{mut}) - \text{OD}(\text{negative mut})$
- . OD(nor) of each sample is the value of its corresponding well with the wild-type probe.
- . OD(mut) of each sample is the value of its corresponding well with the mutant probe.
- . OD(negative nor) of each sample is the value of the negative control well with the wild-type probe.

- . OD(negative mut) of each sample is the value of the negative control well with the mutant probe.

Table 1 shows the value of the OD for the different genotypes.

Automatic ELISA Detection

We used an Automated Triturus[®] ELISA Analyser (Diagnostic Grifols) for genotyping. It is an open and completely automated ELISA immunoassay analyzer that can perform a variety of different tests on a series of samples and processes several batches simultaneously.

After the PCR amplification, this system allowed us to do reagent dispensing, washing, incubation, and reading of microplates, as well as the calculation and final interpretation of the ELISA results without manual intervention.

Software Development

In addition to the Triturus analyzer's software package that included an Automatic Self-Test, On Board Quality Control Protocols, Customized Quality Control Reports, Easy to Read Results Reports, Multiple and Serial Sample Dilution Options, we have incorporated a software (Software F12) designed to obtain easily and immediately the genotypes of each factor for every patient sample (valid for manual and automatic analysis).

Briefly, the software is able to assign the value of each genotype from data generate by the automatic reader (Triturus) or manual ELISA reader. The application has the option to export data from text files (useful if the data have been generated from a manual array), assigning the result to the patient identification (ID) number. In addition, the software allows the possibility to search results by date, genotype, or patient ID number. This software is fitted with security passwords to save data and it satisfies the entire legal requirements about confidentiality.

TABLE 1. O.D. Readings analyzed according to standard cutoff and *mut/wt* ratio values

Mutation	OD 405 nm		
	Normal (<i>wt</i>)	Ratio <i>mut/wt</i>	Mutated (<i>mut</i>)
FVL	≥ 4	1.2–2.2	≤ 0.8
G20210A <i>F2</i>	≥ 5	0.5–1.5	≤ 0.2
C46T <i>F12</i>	≥ 3.5	$1.0 \geq X \geq 2.0$	≤ 0.35

RESULTS

We analyzed 122 samples of DNA. Each sample was tested for three genetic mutations for venous thrombosis (FVL, 20210A/G *F2*, and C46T *F12*) with a newly developed multiplex-PCR ELISA detection system. Table 2 shows the distribution of the genotypes for these three mutations in each sample. Negative and positive controls for each genotype were included in each analysis. Under our conditions, the color signal appeared for each sample, whereas no color signal was detected in the negative control (Fig. 1).

The OD for each genotype (Table 1) allowed us to discriminate precisely between homozygotes for the normal allele, heterozygotes, and homozygotes for the mutant allele for each polymorphism, even when different samples showed different amplification efficiencies.

To evaluate the reliability of the ELISA technique, the results were compared with those obtained by standard techniques such as direct sequencing (for FVL), restriction analysis (for G20210A *F2*), strip assay (for FVL/G20210A *F2*), or real-time PCR (for *F12*).

TABLE 2. Number of individuals and frequencies (%) for the FVL, the G20210A *F2*, and the C46T *F12* in a total 122 sample

	FVL (%)	G20210A <i>F2</i> (%)	C46T <i>F12</i> (%)
Homozygote normal	106 (87)	101 (83)	71 (58)
Heterozygote carrier	158 (13)	21 (17)	44 (36)
Homozygote mutant	0	0	7 (6)

It is important to note that six subjects initially assigned as heterozygous for FVL and two subjects assigned as normal for the G20210A *F2* mutation showed discrepancies between the current techniques and our newly developed ELISA-based technique. Sequencing of these samples revealed that the concordance with our method was 100%. Thus, they were misclassified initially based on the standard methodology.

Table 2 shows the genotypic frequencies of each DNA variant. For FVL and PT20210 mutations, 106 individuals were homozygous for the *G* allele, 16 were heterozygous, and 102 were homozygous for the *G* allele, 20 were heterozygous, respectively. No homozygotes for the *A* alleles were identified in either FVL nor G21210A *F2*. A total of 71 individuals were homozygous for the *C* allele of the 46C/T *F1*, 44 were heterozygous, and seven were homozygous for the *T* allele.

With the increased demand for testing thrombophilia at the molecular level there is a need for an accurate, simple, rapid, and cost-saving procedure for genotyping DNA samples. To this end, we were able to automate the ELISA analytical method, which allowed us to perform all steps of microplate ELISA assays including sample dilution, reagents addition, washings, incubation, reading, and data analyses. The method is extremely flexible and maximizes output for laboratories with a large workload and different testing groups. This new procedure can genotype 48 samples in a single working day. In contrast, the standard restriction

Fig. 1. Visual genotype in ELISA plate. The normal (*wt*) and mutant (*mut*) probes are dispensed as shown at each arrow and the samples are dispensed in each column (1–12). In the plate, samples with different FVL, G20210A *F2*, and C46T *F12* genotypes and one negative control have been included. Coloration in the *mut* well alone indicated the presence of a homozygous for the tested mutation, while coloration in the *wt* well indicated the absence of the mutation, or normal genotype. Coloration in both wells indicated that the tested individual is heterozygous for the mutation.

analysis protocol requires 2 hr for the PCR amplification, then an overnight digestion, and 2 hr for electrophoresis before the results are available. Since the restriction analysis protocol requires several manual steps, it is subject to a high error rate. Nevertheless, another methodology that has become popular for thrombophilia testing is real-time PCR (21). The limitation of this technique is that one can genotype only 32 samples at the same time (including negative and positive controls). In addition, real-time PCR is very sensitive to the quality of the DNA and the salt concentrations, with different samples showing different amplification efficiencies, which in turn can alter the results.

It is important to note that since there was no overlap between the OD of each genotype by our ELISA technique (Table 1) we developed software (F12 Software) that automatically assigned the genotype of each sample. Briefly, the software was able to assign the value of each genotype from data generated by the automatic reader (Triturus) or manual ELISA reader. The application has the option to export data from text files, assigning the result to the patient ID number. The software also allows the possibility to search saved results by date, genotype, or patient ID number. In addition, this software satisfies the entire legal requirement for confidentiality.

To validate this software, we analyzed data from an excel file generated from a manual ELISA reader and from the Triturus (automatic ELISA system). The concordance between these methods (manual and automatic assignment of the genotype) was 100%. In addition, the software allowed us to identify those samples that were not amplified by PCR.

DISCUSSION

The FVL and G20210A mutations are the only genetic risk factors that are strongly, consistently, and independently related to thrombotic disease. However, the increased interest in the genetics of VTE has stimulated studies to identify new genetic risk factors and to develop new detection methods and platforms for DNA analysis in the clinical and research laboratory (22).

A novel genetic risk factor for venous and arterial thromboses (a C46T in exon 1 of the *F12* gene) has been described in the Spanish population using family-based linkage analysis (14) and population-based association studies (15–18). In addition, this DNA variant increases the risk of thrombosis in combination with FVL and G20210A in thrombophilic families (19). Nevertheless, as these variants are common in Caucasian populations, coinheritance of more than one predisposing mutation is

relatively frequent. Thus, there is a need for comprehensive genetic testing to accurately detect and evaluate risk.

It is important to note that genetic defects that cause thrombophilia are clinically relevant not only to the patient, but also to asymptomatic relatives and offspring that may carry the same genetic factors (11,12). In addition, the mode and duration of treatment are determined by the type of abnormality and the use of more intense prophylaxis in thrombotic risk situations. Currently, there are no studies that unequivocally establish when and how thromboembolic prophylaxis should be undertaken. Further, there is no information about whether the hemorrhagic risk of long-term anticoagulant treatment outweighs the benefit of antithrombotic prophylaxis. At present, the most reasonable course of action is to carry out thromboprophylaxis in risk situations (23,24). We expect that oncoming multicenter case-control studies will throw light on this question. Thus, it would be advantageous to have at one's disposal an accurate, reliable, and economical technique to test for genetic risk factors for thrombosis. However, it is crucial to distinguish between the tests of proven value (which can modify the therapeutic approach) from those of unproven value.

Unfortunately, there are problems with the results from various laboratories, including misclassifications and inaccuracies. Many laboratories cannot determine the analytic validity of genetic testing and thus show wide discrepancies in their results. Additional studies are needed to determine the frequency of these discrepancies (i.e., are they concentrated in poorly-performing laboratories) and whether the technical errors are correctable (or even detectable). Another major problem of genetic testing has been the incidence of dubious genotypes resulting from the manual, labor-intensive techniques, and the difficulty in reading the output. These difficulties have hampered the use of genotyping data in assessing the genetic risk both in patients and in their relatives. As a result, some clinicians are not fully convinced that it is clinically useful.

There are a variety of technical approaches for thrombosis risk assessment including molecular diagnostic testing for FVL and G20210A *F2*. Several authors have reported on the use of multiplex PCR–restriction fragment length polymorphisms (PCR–RFLP) approaches for the combined detection of both mutations (25–30). However, these multistep, labor-intensive, manual, direct mutation assays are not always precise. Obviously, as the number of manual steps increases the possibility of error increases. Thus, because of the high cost and lack of automation, these methods are difficult to incorporate into routine clinical testing. Other semiautomated, homogeneous, one-step

direct mutation methods have been developed (21,31). But, many of them do not evaluate simultaneously more than a single prothrombotic mutation.

The potential benefits of a homogenous detection system have been recognized, and several methods for improved sample throughput for typing diallelic systems have been described (32–34). In addition, the use of multiple genetic tests markedly improves the ability to predict disease risk. For example, the concurrent use of a panel that test for three genetic tests (FVL, G20210A, and PC deficiency) increases the predictive value of detecting venous thrombosis at least eight-fold (35).

The objective of our study was to devise an accurate, simple, and cost-effective method to simultaneously detect the FVL, G20210A, and C46T mutations in human DNA samples. This technique is ideally suitable for use in molecular diagnostic laboratories. Our multiplex ELISA-PCR technology was able to meet these criteria and, since ELISA technology has been generally applied in diagnostic laboratories, our method represents a valid alternative to current standard procedures, especially when simultaneous determination of multiple genetic mutations is required.

A decided advantage of our procedure is that amplification and genotyping are performed in a closed system without manual intervention, since postamplification manipulation is avoided by automating the whole process. Thus, sample-tracking and the risk of end-product contamination are eliminated. In addition, this genotyping method, together with the advances in the automation of the genomic DNA extraction and amplification setup, significantly reduces the cost of the DNA-based tests. In addition, new prothrombotic gene mutations that may be identified in the future can be added to the gene panel easily. Finally, our method takes into account the future evaluation of thrombophilia that may ultimately include an individualized comprehensive risk assessment based on the sequence pattern of multiple relevant prothrombotic target genes.

In conclusion, the technique that we have developed is accurate, rapid, efficient, and economical. In addition, the method is adaptable to the clinical molecular diagnostic laboratory since it offers the possibility of rapid genotyping of the three most prevalent and relevant genetic risk factors that are routinely screened for patients with thrombophilia.

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