Automated Detection of the Factor V Leiden Mutation Using the LCx Microparticle Enzyme Immunoassay

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The factor V Leiden mutation, a G→A transition at position 1691 in exon 10 of the gene that codes for factor V, produces an Arg506Gln substitution and is the most common genetic risk factor for venous thrombosis. We have developed a rapid, sensitive, and specific method to detect the factor V Leiden mutation in genomic DNA from whole blood by PCR amplification and microparticle enzyme immunoassay detection using the Abbott LCx instrument. We compared this automated method with the standard procedure using restriction endonuclease digestion of PCR products followed by gel electrophoresis in blinded experiments. In 130 patients (from Veterans Affairs medical centers) with deep venous thromboses, including 24 heterozygotes with the factor V Leiden mutation, there was complete agreement between the two methods. The assay was also able to distinguish heterozygotes from homozygotes. This method, which carries a low potential for cross-contamination of samples, should be a useful routine test for the factor V Leiden mutation in clinical laboratories with sufficient demand for molecular diagnostic assays using the LCx instrument.

The factor V Leiden mutation, a G→A transition at position 1691 in exon 10 of the gene that codes for factor V, produces an Arg506Gln substitution (1). The Arg506Gln substitution eliminates one of the three sites in the factor Va molecule that are cleaved by activated protein C (2) and is responsible for almost all hereditary cases of activated protein C resistance. Studies have shown that heterozygosity for the factor V Leiden mutation confers a 3.5- to 8-fold increased risk for an initial episode of deep venous thrombosis or pulmonary embolism as compared with genetically unaffected patients (3, 4). Homozygosity confers an 80-fold increased risk for venous thrombosis (5). The prevalence of this mutation in general Caucasian populations varies between 1% and 8.5%. The mutation is apparently not present in African, Chinese, Japanese, or Native American populations (6). Among patients with an initial episode of deep venous thrombosis or pulmonary embolism, the prevalence of factor V Leiden is between 12% and 50%, whereas <10% of patients have hereditary deficiencies of antithrombin III, protein C, or protein S. The factor V Leiden mutation is, therefore, the most common genetic risk factor found in thrombosis patients.

Although many methods are available to detect the factor V Leiden mutation, an automated assay procedure suitable for use in the routine clinical laboratory would be desirable. We developed a rapid, sensitive, and specific method for the factor V Leiden mutation, using PCR amplification and microparticle enzyme immunoassay (MEIA)5 detection, using the commercially available LCx instrument (Abbott).

Materials and Methods

PATIENTS
One hundred and thirty male veterans from 21 Veterans Affairs medical centers with at least one episode of deep venous thrombosis or pulmonary embolism occurring

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5 Nonstandard abbreviations: MEIA, microparticle enzyme immunoassay; bp, base pair; and cps, counts per second.
between 1990 and 1995 were included in this study. Their mean age was 63 years. Thromboembolism was objectively confirmed by at least one of the following tests: positive impedance plethysmography, venous duplex ultrasound, venogram, and positive pulmonary angiogram, or a high-probability lung scan with appropriate clinical history. Patients were ineligible if their factor V Leiden genotype was known; if the thrombosis was precipitated by surgery, immobilization, or malignancy; or if they were known to have a lupus anticoagulant/antiphospholipid antibody syndrome or a deficiency of antithrombin III, protein C, or protein S.

INFORMED CONSENT

Informed consent was obtained from all patients. The study was approved by the Human Studies Committee of Brockton-West Roxbury Department of Veterans Affairs Medical Center and Beth Israel Deaconess Medical Center (Boston, MA).

DNA ISOLATION

Blood was collected by atraumatic venipuncture into tubes containing 0.129 mol/L buffered trisodium citrate. The ratio of blood to anticoagulant was 9:1 (by volume). Cellular elements were stored at −80 °C after centrifugation at 4 °C for 15 min at 2000g. Genomic DNA from the 130 patients with venous thromboses; two individuals known to be homozygous or heterozygous for the factor V Leiden mutation, respectively; and a control subject known to be homozygous or heterozygous for the factor V Leiden mutation, were extracted (103 samples) or the QIAamp Blood kit from Qiagen (27 samples) according to the manufacturers’ instructions. DNA concentrations were measured spectrophotometrically at 260 nm.

DETECTION OF THE FACTOR V LEIDEN MUTATION

Detection of the factor V Leiden mutation by PCR and LCx MEIA. Two adamantane-labeled oligonucleotide primers adapted from Ridker et al. (4) (Table 1) were used to amplify a 223-bp fragment, which included the last 136 bp of exon 10 and the first 87 bp of intron 10. PCR amplifications were performed using a DNA Thermal Cycler (Perkin-Elmer Cetus) (9). The reaction mixtures contained 50 ng of genomic DNA, 5 U of Taq DNA polymerase (Perkin-Elmer Cetus), 0.25 μmol/L of each adamantane-labeled oligonucleotide primer (Abbott), 150 μmol/L of each dNTP, 1 mmol/L MgCl₂, 10 mmol/L Tris-HCl, pH 8.3 (at 25 °C), 50 mmol/L KCl, and 0.01 g/L of autoclaved gelatin. In addition, each reaction mixture contained 5 mmol/L of either the wild-type (5′-GACAGGGAG-GAAA-Biotin-TTTTTT-Biotin-3′) or the mutant (5′-GACAGGAGAAAATACCTGTAAGCT-3′) biotinylated probe, which had annealing temperatures 15 °C lower than the adamantane-labeled primers. A polyT linker was introduced between the two molecules of biotin to facilitate streptavidin capture in the MEIA. Although both PCR primers were labeled with adamantane, it would have been sufficient to label only the primer used to amplify the DNA strand complementary to the biotin-labeled probe. Each sample was subjected to a first cycle of 4 min denaturation at 97 °C, followed by 35 cycles of denaturation (1 min at 94 °C) and annealing (1 min at 64 °C). Single-stranded PCR product was obtained by a 5-min denaturation step at 97 °C in the last cycle. The biotinylated probes in the samples were preferentially annealed to single-stranded PCR product by rapid cooling to 15 °C and incubation for 5 min. When convenient, samples could be left overnight at 12 °C. PCR reactions were run in duplicate for each probe type in a volume of 200 μL.

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Table 1. Sequences of the oligonucleotide primers used for PCR amplification of a fragment of the factor V gene.

<table>
<thead>
<tr>
<th>Primers for HindIII digestion (8)</th>
<th>Nucleotides</th>
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<tbody>
<tr>
<td>+ 5′-CCACATACAGTGACG-3′</td>
<td>1600–1619</td>
</tr>
<tr>
<td>− 5′-GGTCTTCAAGGACAAATACCTGTAAGCT-3′</td>
<td>1692 to −22 in intron 10</td>
</tr>
<tr>
<td>Primers for MEIA detection [primers adapted from Ridker et al. (4)]</td>
<td></td>
</tr>
<tr>
<td>+ 5′-Adamantane-CCCCACAGAATATG-3′</td>
<td>1566–1587</td>
</tr>
<tr>
<td>− 5′-Adamantane-TCGCTTATTAGGAG-3′</td>
<td>−66 to −87 in intron 10</td>
</tr>
</tbody>
</table>

* +, upstream primer; −, downstream primer. The positions of the primers are indicated on the right, according to Jenny et al. (28).
After PCR, the unopened tubes were transferred to the automated LCx instrument for detection of the amplified product or amplicon by MEIA (10, 11). Biotin-labeled probes that hybridized to the wild-type or mutant single-stranded PCR product were captured on microparticles coated with streptavidin. After extensive washing, the captured probe-amplicon complex was detected by an anti-adamantane-alkaline phosphatase conjugate that, in the presence of the substrate methylumbelliferyl phosphate, generates a fluorescent product at a rate proportional to the amount of captured PCR product. The rate of increase of fluorescence is quantified by the LCx and expressed as counts per second (cps)/s. The LCx can accommodate up to 24 patient specimens in a single analytical run. If the time for sample preparation is included, results can be obtained in $\sim 5$ h, including 90 min for DNA extraction, 120 min for PCR amplification, and 60 min for MEIA detection. The different steps of the MEIA method are summarized in Fig. 1.

**Results**

**Optimization of Reaction Conditions**

We first performed experiments to define PCR conditions that would readily distinguish heterozygosity and homozygosity for the sequence encoding factor V Leiden from the wild-type sequence. In patients with and without the factor V Leiden mutation, optimal conditions were defined by high and low rates of fluorescence with the mutant probe, respectively, and conversely low and high fluorescence rates with the wild-type probe, respectively. The experimental conditions evaluated included number of PCR thermal cycles (30–40), annealing temperature (55, 60, 62, and 64 °C), annealing time, Mg$^{2+}$ concentration (0.5–4 mmol/L), and dNTP concentration (63–250 μmol/L). The best results were obtained using 35 cycles with 1-min annealing at 64 °C, 150 μmol/L of each dNTP, and 1 mmol/L Mg$^{2+}$ (data not shown), and these conditions were used to analyze all patient samples.

We also determined the lowest amount of genomic DNA that could be used in the PCR reaction. No detectable amplification was observed when 1 ng was used, but PCR product was detectable with as little as 5 ng; the fluorescent signal reached a plateau at 50 ng (Fig. 2). Similar results were obtained with the mutant probe (data not shown); therefore, all additional experiments were performed with 50 ng of genomic DNA.

**MEIA Results in Patients with Known Factor V Leiden Status**

Using the wild-type and mutant biotinylated probes and the conditions described previously, we were able to accurately differentiate patients who were heterozygous or homozygous for the factor V Leiden mutation from a control who did not have the mutation (Fig. 3). The wild-type probe specifically hybridized with the adamantane-labeled PCR product from the wild-type gene sequence and gave a high fluorescent signal for a single patient without the mutation ($840 \pm 175$ cps/s, n = 10

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**Fig. 1. Schematic representation of the LCx MEIA after PCR amplification.**

PCR products spanning 223 bp of the factor V gene containing nucleotide 1691 were obtained using adamantane-labeled primers. After completion of the PCR reaction, double-stranded products are denatured, and the single adamantane-labeled strands hybridize with biotinylated probes specific either for the wild-type or the mutant sequence. The single-stranded wild-type PCR product that had annealed to the biotinylated wild-type probe is then captured on streptavidin-coated microparticles. The captured probe-amplicon complex is detected by an anti-adamantane-alkaline phosphatase conjugate that, in the presence of an alkaline phosphatase substrate (methylumbelliferyl phosphate; MUP), generates a fluorescent product at a rate (count/sec/sec) proportional to the amount of captured amplicon. The single-stranded PCR product carrying the mutation does not hybridize to the wild-type biotinylated probe (Normal probe); it therefore is not linked to the microparticles coated with streptavidin and is washed away during the extensive washing step, generating fluorescence no greater than the background.

**Fig. 2. MEIA fluorescence signal relative to the amount of DNA in the PCR reaction.**

DNA from a subject without the mutation was used with the wild-type probe. The detection of the fluorescent product in the MEIA assay is expressed in cps/s. The error bars represent the SD of five independent reactions performed in duplicate.
determinations or two independent PCR reactions performed in five separate assays) and for a single heterozygote (550 ± 163 cps/s) with the factor V Leiden mutation. However, the wild-type probe did not hybridize to the mutant sequence, as can be seen in a homozygous patient, in whom the fluorescent signal was equivalent to background obtained without PCR product (21 ± 3 vs 18 ± 3 cps/s). Conversely, the biotinylated mutant probe produced a high signal with amplified DNA from homozygous (847 ± 260 cps/s) and heterozygous patients (635 ± 235 cps/s) without hybridizing to the PCR product from the patient without the mutation (24 ± 12 cps/s). We observed an SD of ~30% in the fluorescent signals obtained for each patient; however, the genotype of an individual patient was always readily apparent when both the wild-type and the mutant probes were used. As a system control, a sample from a known Factor V Leiden heterozygote was included in each analytical run. The MEIA signals for identical samples within a single assay were virtually identical (data not shown).

**Comparison of factor V Leiden mutation detection by MEIA and restriction enzyme digestion**

We compared the MEIA LCx assay with a standard method in blinded experiments, using restriction enzyme digestion and polyacrylamide gel electrophoresis. Of the 130 samples from a cohort of patients with deep venous thromboses, 24 were heterozygous for the factor V Leiden mutation (18.5%), and 106 did not have the mutation, as determined by restriction enzyme digestion and polyacrylamide gel electrophoresis. With the LCx MEIA, we successfully identified the same 24 patients heterozygous for the factor V Leiden mutation and the 106 patients without the mutation. The background signals (negative control), resulting from the detection of wild-type or mutant biotinylated probe without any added genomic DNA were 18.1 ± 3.4 cps/s and 18.5 ± 3.6 cps/s, respectively (mean ± SD, n = 130). In patients without the mutation, the signals were high with the wild-type probe (694 ± 256 cps/s, n = 106), but similar to background with the mutant probe (21.2 ± 3.5 cps/s; Fig. 4). Heterozygous patients were easily identified by intermediate signals with both probes (480 ± 170 cps/s with the wild-type probe and 598 ± 187 cps/s with the mutant probe, n = 24). None of the patients in this cohort was homozygous for the factor V Leiden mutation. The results of MEIA were therefore in perfect agreement with the standard method, leading to a sensitivity and a specificity of 100%.

To extract genomic DNA from leukocytes in blood, we used phenol-chloroform extraction or the Qiagen kit. The magnitude of the MEIA signal in patients without the mutation, using the wild-type probe, were somewhat higher after phenol-chloroform extraction (after adjusting the amount of added genomic DNA to 50 ng for the PCR amplification; 727 ± 254 cps/s, n = 85) than with the Qiagen kit (without measuring the amount of added DNA; 561 ± 222 cps/s, n = 21). This difference was also observed in heterozygous samples [612 ± 176 cps/s and 519 ± 162 cps/s (n = 18) when phenol-chloroform was used, compared with 557 ± 232 cps/s and 362 ± 144 cps/s (n = 6) when the Qiagen kit with the mutant and wild-type probes was used]. In Fig. 4, one sample ex-

![Fig. 3. Detection of homozygosity and heterozygosity for the factor V Leiden mutation with wild-type (open column) and mutant biotinylated probe (striped column) by the LCx MEIA method.](image)

The results obtained in an individual without the mutation (Normal) and the background signals in the absence of DNA are also shown. The error bars represent the SD of 10 determinations or two independent PCR reactions performed in five separate assays.

![Fig. 4. MEIA signals with the mutant and wild-type biotinylated probes in 130 patients with venous thromboses.](image)

Squares represent patients without the factor V Leiden mutation whose DNA has been extracted by phenol-chloroform extraction (□) or with the Qiagen kit (■). Triangles are heterozygous patients with the mutation whose DNA has been extracted by phenol-chloroform (▲) or with the Qiagen kit (▲). The (+) represents a homozygous patient who has been included for illustrative purposes but is not from the cohort of 130 patients with venous thromboses.
tracted with the Qiagen kit was an outlier with lower, but clearly positive signals, when either the wild-type or the mutant probe was used (99 and 150 cps/s, respectively), which suggested heterozygosity for the factor V Leiden mutation. Increasing the amount of DNA for this sample increased the signals to 152 and 455 cps/s with the two probes, suggesting that the yield of DNA in this specimen was low. All other DNA specimens prepared with the Qiagen kit gave signals >200 cps/s with both probes.

Discussion
Several methods are available for the detection of the factor V Leiden mutation. Screening can be accomplished by performing a clotting assay on patient plasma diluted in factor V-deficient plasma; the presence of the mutation is suspected if there is inadequate prolongation of the activated partial thromboplastin time after the addition of exogenous activated protein C, compared with the baseline activated partial thromboplastin time (12, 13). Samples testing positive are then confirmed genetically. The initial report of the factor V Leiden mutation used PCR amplification of the region of the factor V gene containing the base substitution followed by restriction digestion with MnlI and gel electrophoresis, or dot-blot hybridization of the PCR product with biotinylated wild-type or mutant oligonucleotide probes (1). Other PCR-based assays have used primers that introduce a NlaIII (14) or Hind III (8) restriction site when the mutation is present or a TaqI site when the wild-type sequence is present (15). PCR amplification using sequence-specific primers (allelespecific PCR) avoids the need for a lengthy restriction enzyme digestion followed by a potentially long electrophoresis step to separate products of similar and low molecular size (16, 17); the specificity of such methods may, however, be lost if the discriminating 3’ end is degraded in any way. Mutation detection methods using single-strand conformation polymorphism (18, 19) or heteroduplex technology analysis (20) have also been developed. The above methods, however, are not suitable for the routine clinical laboratory because they are relatively time-consuming, require technical skills such as electrophoresis, and do not lend themselves to the analysis of one or only a few samples at the same time.

A few attempts have been made to produce automated assays. Allele-specific PCR combined with analysis by capillary electrophoresis led to the development of a nonradioactive assay that is reproducible and semiautomated (21). An ELISA-based oligonucleotide ligation assay (22) and colorimetric minisequencing assay (23) could both be automated, but require working with microtiter plates with the attendant risk of sample cross-contamination.

The Abbott LCx system for the detection of amplicons after PCR or ligase chain reaction amplification is in routine use in clinical laboratories for the detection of pathogenic microorganisms like Neisseria gonorrhoeae (24), Chlamydia trachomatis (25), or GB virus C (26). This system uses MEIA for signal detection after PCR and has not been used previously to identify point mutations in human genomic DNA.

We have developed an assay that uses the LCx system to detect the factor V Leiden mutation. The entire assay, including DNA extraction, can be completed within 5 h. The procedure involves purification of genomic DNA and PCR amplification, followed by transfer of the closed PCR tubes to the automated LCx instrument for MEIA. The agreement of this method with restriction enzyme digestion for the factor V Leiden mutation is 100%. Determination of a patient’s genotype does not require professional interpretation of the data. Homozygous patients are defined by high signals with the mutant probe and signals equivalent to background with the wild-type probe, patients without the mutation are identified by high signals with the wild-type probe but signals similar to background with the mutant probe, and heterozygous patients are identified by intermediate counts with both probes. Because the detection method is extremely sensitive and requires as little as 5 ng of genomic DNA per reaction, it can be performed with DNA extracted from <200 μL of blood by either phenol-chloroform or a commercial kit for DNA extraction. A major advantage of this method is the low potential for cross-contamination of samples because the reactions take place in self-contained reaction vials.

We set up four tubes for each DNA sample, two with the biotinylated probe specific for the wild-type sequence and two with the biotinylated probe specific for the mutant sequence. It would, however, be possible to initially screen patient DNA with only the mutant probe. A second PCR reaction containing the wild-type probe could be run only for patients who are positive with the mutant probe to differentiate heterozygotes and homozygotes with factor V Leiden.

In summary, we have demonstrated the feasibility of using PCR in conjunction with the LCx MEIA detection method for detecting factor V Leiden, a frequently encountered point mutation in venous thrombosis patients. The Abbott LCx instrument could be used to detect another frequent prothrombotic risk factor, namely the G→A 20210 mutation in the 3’ untranslated region of the prothrombin gene (27). It is our belief that such assay procedures could be undertaken in routine clinical laboratories provided the demand for molecular diagnostic assays using MEIA detection was sufficient to make it cost-effective.

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References


