Activated Protein C Resistance: Automated Detection of the Factor V Leiden Mutation by Mismatch Hybridization

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Background: A single point mutation in the factor V gene has been demonstrated to be the cause of factor Va resistance to proteolytic cleavage by activated protein C. Knowledge of the patient’s genetic disposition is of great importance in situations such as pregnancy, surgery, use of oral contraceptives, and immobilization.

Methods: We have developed a rapid, automated test for the detection of the factor V mutation that makes use of differences in thermal stability between perfect-match and non-perfect-match hybrids. A DNA fragment spanning the mutation is amplified with a biotin-labeled primer. Ruthenium-labeled oligonucleotides, perfectly matching either the biotinylated wild-type strand or the biotinylated mutation strand, are added. Heating to 95 °C and subsequent cooling lead to the formation of double-stranded DNA. Under the conditions chosen, ruthenium-labeled oligonucleotides form stable, double-stranded DNA with the biotinylated strand only if both strands perfectly match each other. The ruthenium signal is measured on a modified Elecsys 1010 system (Roche Diagnostics).

Results: The ratio between the signals obtained with perfectly matching and non-perfectly matching oligonucleotides reflects the genetic status. Analyzed samples can be divided into three nonoverlapping groups based on these ratios. We confirmed the reliability of the method by analyzing several samples of known genetic status; the results were identical in every single instance.

Conclusions: The test discriminates unambiguously between the heterozygous and the homozygous states. Because of its low costs and easy handling, the assay is suitable for use in routine laboratories of clinical chemistry.

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Proteolytic inactivation of factor Va is an important mechanism in limiting clot formation in normal hemostasis. Proteolytic cleavage of factor Va (and VIIIa) is caused by activated protein C (APC), a serine protease with potent anticoagulant properties, and protein S as a nonenzymatic cofactor. Inherited resistance of factor Va to APC is a major risk factor of familial thrombosis. A point mutation in the factor V gene, at position 1691 of the factor V mRNA, that leads to an amino acid change (Arg→Gln) at position 506 of factor V is the cause of factor V resistance to proteolytic cleavage by activated protein C (1). Although several studies have found that the prevalence of the heterozygous state of the mutation is in the range of 8% in the general Caucasian population ([1, 2]; reviewed in Ref. 3), the prevalence among healthy individuals from Japan, Asia, Australasia, and among Native Americans seems to be much lower (2, 4). With a prevalence of ~8% in the general Caucasian population and a 5- to 10-fold increased risk (5) for the development of venous thrombosis in heterozygous carriers, it is the most common hereditary risk factor for thrombosis.

Venous thrombosis is a multifactorial disease; therefore, knowledge of a patient’s genetic disposition is of great importance, especially in situations where additional risk factors for the development of a hypercoagulable state are present, such as advanced age, pregnancy, surgery, use of oral contraceptives, and immobilization. Today clinicians need information about their patients’ genetic disposition on a routine basis. To make the testing of a patient’s genetic disposition available to them, assays are needed that allow the testing to be performed in the setting of a routine clinical chemistry laboratory under the conditions of a restrained budget.

Several techniques have been described over the last few years, all of which are able to detect the mutation at position 1691 of the factor V gene. Those include multiplex PCR (6), allele-specific PCR (7), heteroduplex analysis (8), and single-strand conformational polymorphism (9). Although all of these methods have demonstrated their ability to detect this mutation, they require substan-
tional hands-on time and are not designed to analyze, in a cost-effective manner, large numbers of samples in the setting of a routine laboratory. Recently, a PCR method has been described that uses differences in thermal stability of double-stranded DNA (10) and is truly suited for routine laboratories. The appearance of a specific PCR product in this assay is monitored with fluorescein-labeled hybridization primers and requires an analyzer (LightCycler™) specifically designed for this type of PCR assays.

Here we describe a rapid, automated assay for the detection of the Leiden mutation. This PCR-based assay uses differences in thermal stability between perfect-match and non-perfect-match hybrids and uses the automated detection process of an immunoassay analyzer that is present in many clinical chemistry laboratories, thus avoiding additional investment for a specialized PCR analyzer.

Materials and Methods

Determination of Melting Temperatures

Candidate oligonucleotides were determined using the software DNASTAR™ (Ver. 4.1; DNASTAR) for the prediction of melting temperatures. Based on the results obtained with DNASTAR, two candidate oligonucleotides were synthesized for further evaluation: oligonucleotide 714 (5’-ruthenium-GGA CAG GAG AGG AAT A-3’), which perfectly matches the mutation, and oligonucleotide 715 (5’-ruthenium-GGA CAG GAA AGG AAT A-3’), which perfectly matches the wild-type (MWG-Biotech). The ultraviolet absorbance of the oligonucleotides, in combination with the 95-bp complementary strand, was measured in water at a concentration of 10 μmol/L, using different temperatures and a wavelength of 260 nm with a Unicam UV2 (Unicam Ltd.), equipped with a Peltier element. A ramp rate of 1.5 °C/min was used in all experiments.

DNA Isolation

Genomic DNA was isolated from 10 μL of anticoagulated whole blood (EDTA, citrate, or lithium-heparin), using the Dynabeads DNA DIRECT™ kit from DYNAL. DNA was dissolved in 30 μL of Tris-EDTA buffer and stored at −20 °C until use.

PCR

A 95-bp fragment within the factor V gene, including the mutation at position 1691, was amplified using primer A (5’-biotin-CTT CAA GGA CAA AAT ACC TGT ATT-3’) and primer B (5’-ATG AGA GAC ATC GCA TCT GGG C-3’), both from MWG-Biotech. The amplified DNA was sequenced with the Big-Dye™ DNA sequencing kit from Perkin Elmer-Applied Biosystems on a 377 DNA Sequencer (Perkin Elmer-Applied Biosystems).

DNA Sequencing

A 95-bp fragment within the factor V gene, including the mutation at position 1691, was amplified using primer A (5’-CTT CAA GGA CAA AAT ACC TGT ATT-3’) and primer B (5’-ATG AGA GAC ATC GCA TCT GGG C-3’), both from MWG-Biotech. The amplified DNA was sequenced with the Big-Dye™ DNA sequencing kit from Perkin Elmer-Applied Biosystems on a 377 DNA Sequencer (Perkin Elmer-Applied Biosystems).

Detection

We used a modified Elecsys system 1010 (Molecysys), especially suited for molecular biology tests (not commercially available; Roche Diagnostics) in conjunction with the reagents (buffers and microbeads) supplied with the routine test kits by Roche Diagnostics. The system is a fully automated random access analyzer; its underlying principles have been described in detail elsewhere (11). In principle, the system uses electrochemiluminescence (ECL) for detection, which utilizes ruthenium-tris-2,2’-biphenyl chelates as a label in combination with universal streptavidin-coated paramagnetic microparticles.

Results

The basic principle of the assay is outlined in Fig. 1. Briefly, a DNA fragment spanning the mutation is amplified with a biotin-labeled primer. After amplification, the sample is split into two aliquots. Ruthenium-labeled oligonucleotides, perfectly matching either the biotinylated wild-type strand or the biotinylated mutation strand, are added to the aliquots. The aliquots are then heated to 95 °C to allow separation of the double-stranded DNA. Subsequent cooling to room temperature leads again to the formation of double-stranded DNA. Under the conditions chosen, ruthenium-labeled oligonucleotides form stable, double-stranded DNA with the biotinylated strand only if both strands match each other perfectly. Biotinylated DNA is then bound via streptavidin to paramagnetic beads, which keep the biotinylated DNA in the detection chamber of the analyzer while all other components are washed away. The amount of ruthenium bound to the biotinylated DNA is determined for both samples by measurement of the ECL signal generated by the ruthenium complex in the detection chamber. The samples are compared by calculating the ratio of the two signals.

DNA Preparation

We used the Dynabeads DNA DIRECT kit, which allows the preparation of 10 samples within 15 min from blood samples anticoagulated with heparin, citrate, or EDTA. In almost all cases, blood samples reach the laboratory without being cooled during transport; thus DNA may...
degrade quite rapidly during transportation. Using the Dynabeads DNA DIRECT kit, we were able to obtain DNA from samples that had been stored up to 4 days at room temperature.

**Design of the Detection Oligonucleotides**

Because the assay is designed for clinical laboratories, costs and reliability were of major concern. Therefore, we tried to avoid the use of enzymes because they can fail and are expensive. In addition, we tried to reduce labor to a minimum by mechanization of the procedure and by the design of the assay so that all DNA quantification steps, steps to verify proper DNA isolation or PCR amplification, were therefore obsolete. To achieve this without compromising reliability, we designed the assay in such a way that the result itself reflects any problem that might occur during the whole procedure. To achieve this we worked with two oligonucleotides: one perfectly matching the wild type, the other matching the mutant.

Because the wild type and mutant differ only in a single nucleotide at position 1691 of the factor V gene, the performance of the assay depends on the ability of the chosen oligonucleotides to form a stable double strand only with the perfectly matching biotinylated 3’ strand of the amplified DNA. As soon as a single mismatch is present in the mixture, formation of double-stranded DNA is prevented. Our aim was to obtain oligonucleotides with a melting temperature of 28 – 30 °C because the detection chamber of the instrument is kept at 28 °C.

Following the predictions of the DNASTAR software, two detection oligonucleotides were synthesized: oligonucleotide 714, which is complementary to the mutation, and oligonucleotide 715, which perfectly matches the wild type. Both are 16 bp long and differ only at the ninth nucleotide (position 1691 of the factor V gene); both carry the ruthenium label at the 5’ end. In conjunction with the perfectly matching complementary, wild-type strand, oligonucleotide 715 displays a melting temperature of 40 °C (Fig. 2A, right curve), whereas the melting temperature is reduced to 29 °C if the same oligonucleotide is hybridized with the complementary, mutated strand (Fig. 2A, left curve). Oligonucleotide 714 displays a melting temperature of 34 °C if hybridized with the perfectly matching complementary, mutated strand (Fig. 2B, right curve). Hybridization of this oligonucleotide to the complementary, wild-type strand reduces the melting temperature to 28 °C (Fig. 2B, left curve).

**Melting of Double-Stranded DNA**

After PCR amplification, double-stranded DNA must be melted to allow the detection oligonucleotides to hybridize with the amplified DNA. Strand separation can be achieved either by heating the DNA to 95 °C for 5 min or by chemical denaturation with NaOH. In both cases, 30 μL of PCR product was incubated with 15 pmol detection oligonucleotide, leading to a roughly equimolar concentration of amplified DNA and detection oligonucleotide. Samples were vortex-mixed and then either heated for 5 min at 95 °C (thermal denaturation) or incubated with 12 μL of 0.1 mol/L NaOH for 5 min with subsequent neutralization to pH 7.5 with 11 μL of 0.1 mol/L HCl (chemical denaturation). Both methods worked well in our hands. All results reported here were obtained with...
thermal denaturation because this avoids the handling of strong acids and bases and abolishes one pipetting step. In addition, thermal denaturation, in conjunction with a thermal block, allows the handling of a large number of samples.

IDENTIFICATION OF WILD-TYPE, HETEROZYGOUS, AND HOMOZYGOUS SAMPLES

Once samples reach room temperature, they can be processed directly on the analyzer (leaving the samples at room temperature for another 30 min does not have an effect on the results). Nine minutes after the sample is placed on the analyzer, the results are available. For each blood sample, two results are obtained: one for the aliquot with the detection oligonucleotide 714 and one for the aliquot with oligonucleotide 715. If the instrument cannot detect hybridization complexes in either of the aliquots, the procedure does not work properly and the sample must be repeated. If the aliquot with detection oligonucleotide 714 displays a much stronger signal than the aliquot with oligonucleotide 715, the patient is a homozygous carrier of the Leiden mutation. If the signal of oligonucleotide 715 is much stronger than the signal of oligonucleotide 714, the patient does not carry the Leiden mutation. To compensate for variations in DNA yield and/or in the amplification process, the signals from both aliquots are compared by calculating the ratio of the two signals, i.e., that ratio of the signal obtained with the wild-type detection oligonucleotide to the signal obtained with the mutant detection oligonucleotide. In the case of wild-type DNA, a ratio >1 is expected, whereas in the case of the mutation (homozygote), a ratio close to 0 is expected. The ratio for heterozygote samples is between these two values.

AGREEMENT WITH OTHER METHODS

To demonstrate the validity of our results, we randomly picked 15 samples (5 of each genotype) and sequenced them. Five samples that were identified as wild-type samples according to the results of sequencing displayed ratios between 0.16 and 0.28. Ratios for heterozygous samples ranged from 0.57 to 0.82, and samples homozy-
gous for the Leiden mutation led to ratios between 1.47 and 2.04. The results are listed in Table 1, demonstrating total agreement between the two methods.

**Intra- and interassay variation**

A single DNA sample was split into 10 primary aliquots, which were subjected to PCR amplification. The amplified aliquots were then further split into two secondary aliquots, which were hybridized with either oligonucleotide 714 or oligonucleotide 715. Intraassay variation was determined by measuring all 20 secondary aliquots in a single analytical run on the Molecsys. The results were used to calculate the ratios.

To determine the interassay variation, a single DNA sample was split into 10 aliquots, which were subjected to PCR amplification and then stored at \(-20^\circ \text{C}\). On 10 different days within a period of 14 days, a single sample was removed from the freezer. The sample was split into two aliquots, which were hybridized with either oligonucleotide 714 or oligonucleotide 715 and then measured immediately on the Molecsys. The results, summarized in Table 2, were used to calculate the ratios and to demonstrate that the precision of the assay is high enough to allow an unambiguous determination of genetic status.

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**Table 1. Comparison of DNA sequencing and obtained ratios.**

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<th>Sample no.</th>
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<th>Ratio</th>
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<tr>
<td>1</td>
<td>Wild type</td>
<td>0.17</td>
</tr>
<tr>
<td>2</td>
<td>Wild type</td>
<td>0.28</td>
</tr>
<tr>
<td>3</td>
<td>Wild type</td>
<td>0.22</td>
</tr>
<tr>
<td>4</td>
<td>Wild type</td>
<td>0.16</td>
</tr>
<tr>
<td>5</td>
<td>Wild type</td>
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</tr>
<tr>
<td>6</td>
<td>Heterozygous</td>
<td>0.63</td>
</tr>
<tr>
<td>7</td>
<td>Heterozygous</td>
<td>0.82</td>
</tr>
<tr>
<td>8</td>
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<tr>
<td>12</td>
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<td>13</td>
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<td>1.47</td>
</tr>
<tr>
<td>14</td>
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</tr>
<tr>
<td>15</td>
<td>Homozygous</td>
<td>1.64</td>
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**Table 2. Intra- and interassay imprecision.**

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<th>Mean ratio</th>
<th>SD</th>
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<td><strong>Intraassay imprecision</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Wild type</td>
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<td>Heterozygous</td>
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<tr>
<td>Homozygous</td>
<td>1.96</td>
<td>0.25</td>
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<tr>
<td><strong>Interassay imprecision</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild type</td>
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<td>0.1</td>
<td>41</td>
</tr>
<tr>
<td>Heterozygous</td>
<td>0.69</td>
<td>0.1</td>
<td>15</td>
</tr>
<tr>
<td>Homozygous</td>
<td>2.16</td>
<td>0.4</td>
<td>19</td>
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</table>

**Ratios for the wild-type, heterozygous, and homozygous states of the mutation**

To evaluate whether the ratios still fall into one of three distinctive groups that do not overlap even when large numbers of samples are analyzed, we chose 30 wild-type and 30 heterozygous samples on a random basis and used all of our homozygous samples. All samples had been analyzed before by an independent method (12).

The samples can be divided into three nonoverlapping groups based on the obtained ratios (Fig. 3): 30 samples displayed ratios between 0.12 and 0.31, with a mean ratio of 0.19 (± 0.05; CV, 26%). All of these samples had been identified beforehand as wild type. All 30 heterozygote samples displayed ratios between 0.52 and 0.90, with a mean ratio of 0.68 (± 0.1; CV, 15%). Sixteen samples from homozygous patients displayed ratios from 1.47 to 2.51, with a mean ratio of 1.94 (± 0.29; CV, 15%). Fig. 3 demonstrates that heterozygotes can be clearly distinguished from the wild type and homozygotes. Furthermore, the reliability of the method is confirmed by the fact that whenever samples—for various reasons—were analyzed not only with the method described here but with another method as well, the results were identical in every instance.

We deduced from these results, obtained with samples of known genetic status, that results can only be accepted if they are within ± 2 SD. Therefore, the ratios must fall into one of three categories: <0.3, between 0.5 and 0.9, or >1.3. If we obtain a ratio outside of these ranges, the sample must be repeated. Regardless of the ratios, the test must be repeated if the ratios of the controls (samples with known genetic status) are not within the expected range.

**Costs**

Because our intention was to design an assay that can be used as a screening test, we focused not only on handling
and reliability but looked at cost-effectiveness as well. The costs of the consumables (reagents and plastic ware) for one blood sample (two tests) are less than $3 (US).

**Discussion**

Our intention was to develop a reliable and easy-to-use assay for the detection of the mutation at position 1691 of the factor V gene, which leads to an amino acid change (Arg→Gln) at position 506 of the factor V, by using a technique suitable for the detection of other mutations as well. The assay must be suitable not only for the specialist but also for the routine clinical chemistry laboratory. To reach this goal, chemicals and equipment usually available in such laboratories had to be integrated into the assay; on the other hand, toxic substances, special procedures, and manual labor had to be avoided whenever possible. We used a DNA preparation method that yields DNA of only average purity but is very quick and allows the handling of several samples simultaneously.

The rationale for using differences in the thermal stability of double-stranded DNA as a detection principle was based on (a) reliability, because the use of additional enzymes and their potential failure is avoided; (b) costs, because we save the costs of restriction enzymes or other enzymes; and (c) time saving, because thermal denaturation and subsequent detection are very fast.

Again, ease of handling and cost-effectiveness were the reasons we decided against any DNA quantification steps and against verification of proper PCR amplification. This was possible because we split every sample and incubated one of the aliquots with the detection oligonucleotide that perfectly matches the wild-type, whereas the other aliquot was incubated with the oligonucleotide that matches the mutant. Therefore, regardless of the genotype, at least one of the aliquots displayed a strong signal so that the result itself allowed immediate validation of the whole procedure. Thus, extra handling steps and additional reagents or equipment have been avoided without compromising quality and security.

For the detection of the hybridization complex itself, we used a modified Elecsys 1010 system from Roche Diagnostics, a widely distributed immunoassay analyzer system, thus enabling us to take advantage of this system in terms of shelf reagents, small day-to-day variation, and low costs. The system uses ECL for detection, which is very sensitive, and because detection is linear over almost five logs, it allows the simultaneous processing of samples with very different amounts of amplified DNA.

The reliable identification of patients who are heterozygous for a mutation is often a problem and was of primary importance for the design of this assay because heterozygotes represent the vast majority of patients carrying this mutation. The results demonstrate that the heterozygous genotype can be distinguished equally well from the wild-type and the homozygous genotypes. In addition, with this assay, results can be reported on the same day the sample arrives in the laboratory, thus allowing a rapid therapeutic response.

Currently, almost all clinical laboratories increasingly feel the constraints of a tight budget and a very cost-conscious administration. The fact that the assay, apart from the PCR step, does not require any additional enzymes or equipment and that the type of analyzer and most of the reagents are already used in the laboratory for many different immunoassays keeps the costs to less than $3 US. This price tag for consumables allows use of this assay as a screening test, although the earlier enthusiasm for screening larger cohorts, such as women taking oral contraceptives, has disappeared (13).

Here we demonstrate that differences in thermal stability can easily be used as an assay technique in the setting of a routine laboratory by use of an immunoassay analyzer that is already present in the laboratory. Apart from reliability and cost efficiency, the technique itself is very versatile because it can be used for the detection of other point mutations as well as short deletions or insertions. In addition, the technique can be rapidly adapted to other detection principles, analyzers, and assay formats such as microtiter plates, although it may be necessary to increase the incubation time.

The recently described fluorescence-based PCR method is faster than the method described here, but it requires an extra analyzer (LightCycler) exclusively for this type of PCR assay, which represents an additional investment of $40 000 to $50 000 (US).

For the detection of other point mutations, which can either be linked to certain diseases or represent an increased risk under certain circumstances, the necessity to develop and use simple, fast, cost-effective, and therefore automated, methods for the detection of point mutations will increase. Assays similar to this APC resistance assay may help genetic analysis in the future to become a part of the daily workload of an typical clinical chemistry laboratory.

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