Prothrombin Mutation: Employing the Electrochemiluminescence Technology of the Elecsys® System for the Detection of the Point Mutation at Position 20210 in the 3' Untranslated Region of the Prothrombin Gene

Prothrombin Mutation: Verwendung der Elektrochemilumineszenztechnologie des Elecsys® Systems zur Detektion der Punktmutation an der Position 20210 in der 3' untranslatierten Region des Prothrombin Gens

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Summary: With an annual incidence of 1 per 1000 in the general population, venous thromboembolism is a common disease. In 1996, a mutation at position 20210 of the prothrombin gene, located in the 3' untranslated region, was identified as a risk factor for developing venous thrombosis. With a prevalence of 1-4% in the general Caucasian population, this constitutes a frequent risk factor which increases the risk for venous thromboembolism 3-fold for heterozygous carriers. Here we describe a rapid, automated assay for the reliable detection of the prothrombin mutation. The test employs off-the-shelf reagents and uses the automated detection process of an immunoassay analyzer (Elecsys®) that is present in many clinical laboratories, thus avoiding an additional investment for an exclusively dedicated analyzer. Because of its low costs and easy handling, the assay is especially suited for performance in routine clinical laboratories.

Keywords: Venous thrombosis; Prothrombin mutation; PCR-based test; Automation.

Zusammenfassung: Mit einer Inzidenz von 1 zu 1000 in der Allgemeinbevölkerung zählt die venöse Thrombose zu den häufigeren Erkrankungen. 1996 wurde eine Mutation an der Position 20210 im Bereich der 3' untranslatierten Region des Prothrombin Gens als Risikofaktor für die Entstehung venöser Thrombosen identifiziert. Mit einer Prävalenz von 1-4% in der kaukasischen Allgemeinbevölkerung stellt diese Mutation einen relativ häufig vorkommenden Risikofaktor da, der das Risiko einer venösen Thrombose bei heterozygoten Merkmalsträgern um das 3-fache erhöht. Wir beschreiben hier ein schnelles, automatisiertes Assay für die zuverlässige Detektion der Prothrombin Mutation. Der Test verwendet fertige Reagenzien und benutzt das Detektionsverfahren eines immunologischen Analysesystems (Elecsys®), das in vielen Laboratorien vorhanden ist und daher zusätzliche Investitionen für ein spezielles Analysensystem vermeidet. Auf Grund seiner geringen Kosten und der leichten Handhabung ist der Test besonders für die Verwendung in klinischen Routinelaboratorien geeignet.

Schlüsselwörter: Venöse Thrombose; Prothrombin Mutation; PCR-Test; Automation.

Being a multifactorial disease, both genetic factors and acquired conditions can increase the risk of developing venous thrombosis. While conditions such as pregnancy or immobilization have been known for decades to increase the likelihood of developing venous thrombosis, genetic factors have only been identified in recent years. To date, two genetic variations have been identified as important risk factors. In 1994, a mutation in the factor V gene (Leiden mutation) was shown to cause factor Va resistance to proteolytic cleavage by activated protein C (APC resistance) [1]. With a prevalence of about 8% in the general Caucasian population and a 5- to 10-fold increased risk [1, 2, reviewed in 3] for developing venous thrombosis in heterozygotes, it is the most common hereditary risk factor for thrombosis. In 1996, a mutation at position 20210, located in the 3' untranslated region of the prothrombin gene, was shown to increase the risk almost 3-fold [4]. The underlying mechanism by which the mutation in the prothrombin gene exerts its effects is still unknown, but heterozygous carriers of this mutation display, on average, a 27% increased prothrombin activity compared to wild type individuals, with prothrombin activity of heterozygotes and normal individuals greatly overlapping [4]. Whether increased mRNA production, splicing efficiency, or stability is responsible for these elevated prothrombin levels has not yet been proven.

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Although the prothrombin mutation, with a prevalence of 1-4% in the general Caucasian population [5] is less frequent than the Leiden mutation, it remains an important genetic risk for venous thrombosis. In addition, because of the high prevalence of both mutations it is likely that these mutations coexist in some patients. Both mutations should therefore be included in the diagnostic management of venous thrombotic diseases.

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. Therefore, clinicians need information about their patient's genetic disposition in order to estimate the risk of thromboembolic complications on a routine basis. In order to make this information available, assays are needed which allow rapid testing in the setting of a routine clinical laboratory under the conditions of a tight budget.

Several techniques have been described over the last few years, all of which are able to detect point mutations. These include allele-specific PCR [6, 7], heteroduplex analysis [8] and single-strand conformational polymorphism [9]. While all of these methods have proven their ability to detect point mutations, they require a lot of hands-on time and are in general not designed to meet the needs and restraints of an average clinical laboratory.

Here we describe a much faster, PCR-based, automated method for the detection of the prothrombin mutation which uses electrochemiluminescence as detection technology, employs off-the-shelf reagents and uses the automated detection process of an immunoassay analyzer that is present in many clinical chemistry laboratories. This enables these laboratories to adapt their service to the changing needs of clinicians in normal hospitals without requiring large upfront investments.

Materials and Methods

DNA isolation

Genomic DNA was isolated from 10 µl of anticoagulated whole blood (EDTA, citrate or Li-heparin), which we obtained for routine analysis from our hospital, using the Dynabeads DNA DIRECT™-kit from DYNAL, Oslo, Norway. DNA was dissolved in 30 µl of TE-buffer and stored at -20^0 C until use.

PCR

A 116 bp fragment within the 3' untranslated region of the prothrombin gene, including the mutation at position 20210, was amplified using primer A: 5'-Biotin-CAC TCA TAT TCT GGG CTC CTG-3' and primer B: Ru-5'-ATA GCA CTG GGA GCA TTG AAG C-3' [4] (synthesized by MWG-Biotech-AG, Ebersberg, Germany). Due to the underlined "A" (adenine) in primer B (which was introduced instead of a "G" (guanine) a Hind III restriction site is generated in conjunction with the mutation at position 20210.

Five µl of DNA were added to 45 µl of PCR mastermix containing 10 pM of each primer and 250 µM of each dNTP (Promega, Madison, USA) and 500 µM $MgCl_2$, (Sigma, St. Louis, USA), 1 unit Taq-polymerase (Sigma, St. Louis, USA), 1.7% (v/v) glycerol (Merck, Darmstadt, Germany) and 30 µl of sterile water. The samples were then subjected to the following cycling conditions: cycle 1- 30: 94 °C for 1 min, 62 °C for 1 min, and 72 °C for 1 min.

Digestion

Seventeen µl from each PCR sample were added to 3 µl of a Hind III-digestion master-mix, containing 10 units of Hind III (FERMENTAS, Vilnius, Lithuania) and 2 µl of buffer "R", supplied with the enzyme. The samples were digested for 30 minutes at 37° C. Samples for which the genotype had been determined independently (i.e. sequencing) were used as controls.

DNA sequencing

A 116 bp fragment within the prothrombin gene, including the mutation at position 20210, was amplified using primer A:5'- CAC TCA TAT TCT GGG CTC CTG 3' and primer B: 5'- ATA GCA CTG GGA TTG AAG C 3' (MWG-Biotech-AG, Ebersberg, Germany). The amplified DNA was sequenced with the BigDye™ DNA sequencing kit of PE-Applied Biosystems (Warrington, UK) on a 377 DNA Sequencer (PE-Applied Biosystems, Weiterstadt, Germany).

Detection

We used a modified version of the commercially available Elecsys system 1010. Due to the specialized software, this "Molecsys" system (not commercially available, Roche Diagnostics, Mannheim, Germany) is especially suited for assay development of molecular biology tests. The system employs exactly the same technology as the 1010. It is a fully automated random access analyzer; its underlying principles have been described in great detail elsewhere [11]. In principle, the system employs electrochemiluminescence (ECL) for detection which utilizes $Ru^{2+}(bipy)_3$-chelates as a label in combination with universal streptavidin-coated paramagnetic micro particles. The assay was developed in conjunction with the reagents (buffers, microbeads) supplied with the normal test kits by Roche Diagnostics.

Results

The basic principle of the assay is outlined in Figure 1. Briefly, a DNA fragment spanning the mutation is amplified with a biotin-labeled 3' primer and a ruthenium-labeled 5' primer. After amplification, the sample is split into two aliquots. One aliquot is digested with Hind III which cuts the fragment only if the mutation is present thus removing the ruthenium-labeled 5' part

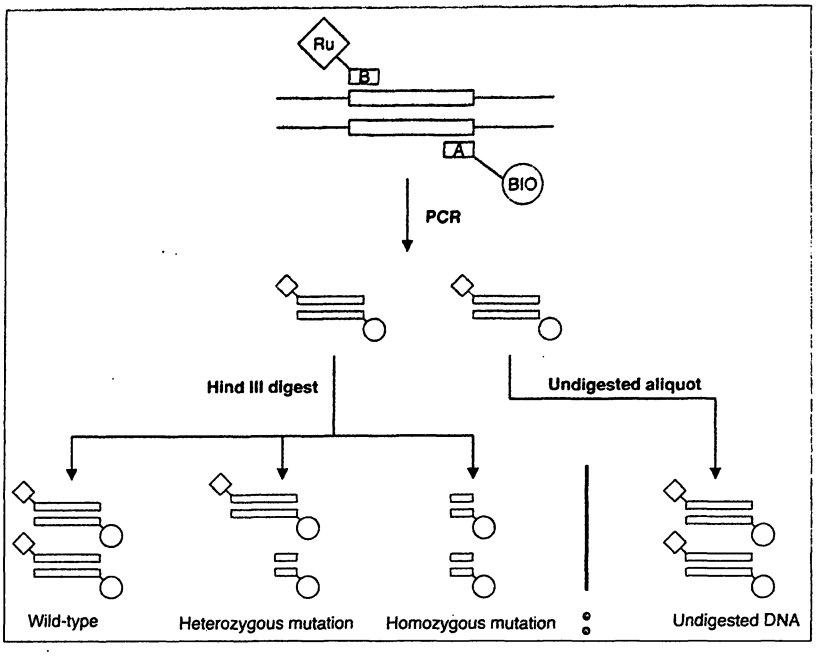

Figure 1 The prothrombin assay. A 116 bp DNA fragment spanning the mutation is amplified from genomic DNA with a biotin-labeled 3' primer and a ruthenium-labeled 5' primer. After amplification, the sample is split into two aliquots. One aliquot is digested with Hind III which cuts the fragment only if the mutation is present, thus removing the ruthenium-labeled 5' part of the fragment from the biotinylated 3' part. The other aliquot remains unaltered. Biotinylated DNA binds 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 measuring the electrochemiluminescence signal which is generated by the ruthenium complex in the detection chamber. The samples are compared by calculating the ratio of the two signals.

of the fragment from the biotinylated 3' part. The other aliquot remains unaltered. Biotinylated DNA binds 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 measuring the electrochemiluminescence signal, which is generated by the ruthenium complex in the detection chamber. The genotype is determined by calculating the ratio between the signals from the digested and the undigested sample.

DNA preparation

We decided to use the Dynabeads DNA DIRECT™ kit which allows the preparation of 10 samples within 15 minutes from blood samples anticoagulated with either heparin, citrate or EDTA. Using the Dynabeads DNA DIRECT™ kit, we were able to obtain DNA from samples which had been stored up to four days at room temperature.

DNA digestion with Hind III

The assay is based on the appearance of a Hind III restriction site. The mutation carries an adenine instead of a guanine at position 20210 and generates, in conjunction with the degenerated primer B, the recognition sequence for Hind III (AAGCTT). The 116 bp fragment amplified from mutated DNA can be cut by Hind III, generating a 23 bp and a 93 bp fragment, thus removing the Ru-label from the biotinylated part of the fragment. If wild-type DNA is amplified, Hind III will not cut the DNA and the Ru-label is not removed.

Validity of the new method

In order to demonstrate the validity of our results, we decided to have the DNA from 5 randomly picked wild-type samples, five heterozygous, and two homozygous samples analyzed, not only by this method but in addition by DNA sequencing. The results are listed in Table 1, demonstrating total agreement of our method with the independently obtained results.

Intra-assay and inter-assay imprecision

DNA from a single sample was split into 10 aliquots, which were subsequently amplified by PCR and analyzed by the standard procedure: each sample was split into two secondary aliquots. One of these secondary

Table 1 Comparison of DNA sequencing and obtained ratios

Sample No.	Sequencing	Ratio
1	Wild-type	1.04
2	Wild-type	0.94
3	Wild-type	1.06
4	Wild-type	1.00
5	Wild-type	1.04
6	Heterozygous	0.69
7	Heterozygous	0.66
8	Heterozygous	0.61
9	Heterozygous	0.78
10	Heterozygous	0.67
11	Homozygous	0.13
12	Homozygous	0.15

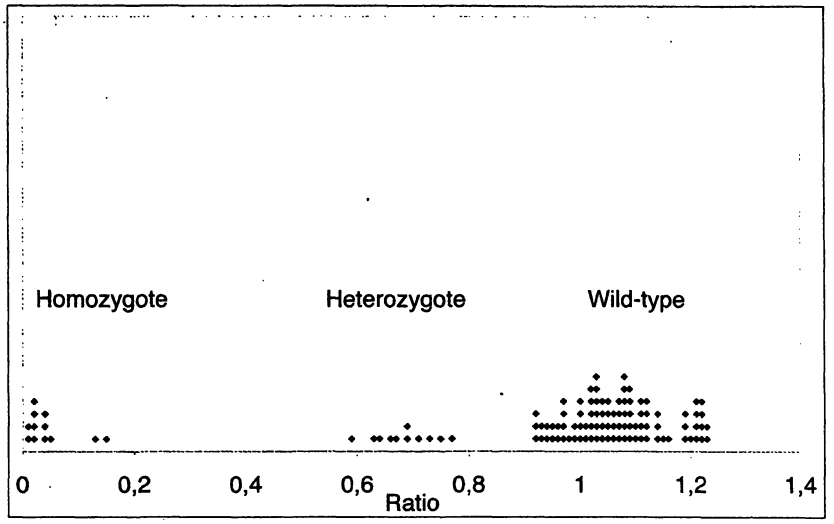

Figure 2 Ratio distribution. The results from 53 blood samples which were analyzed by different technicians over a period of 6 months are summarized. The samples can be divided into three non-overlapping groups based on the obtained ratios.

aliquots was digested with Hind III. All 20 secondary aliquots were measured on the analyzer in the same run, and the ratios were calculated. From these data intra-assay imprecision was calculated. Further, DNA from a single sample was split into 10 aliquots, which were subsequently amplified by PCR and stored at -20 °C. For 10 days within a 14-day period, a single aliquot was thawed, and two secondary aliquots were generated and analyzed ± Hind III digestion. These data were used to calculate inter-assay imprecision. The results are summarized in Table 2.

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

In order to evaluate the reproducibility of the analytical procedure, the ratios from 53 blood samples, analyzed by different technicians over a period of 6 months, were plotted. As outlined in Figure 2, the results fall into one of three distinctive groups which do not overlap, demonstrating the reliability of the technique.

29 of the samples displayed ratios between 0.92 and 1.23, with a mean ratio of 1.07 (± 0.09, cv 8.7%) representing wild-type samples. 12 samples displayed ratios between 0.59 and 0.77 with a mean ratio of 0.68 (± 0.05, cv 7.2%) and were thus identified as being heterozygous for the mutation at position 20210 of the prothrombin gene. The mean ratio of the heterozygous samples is significantly higher than theoretically expected (0.68 vs. 0.5). This is due to the formation of heteroduplex DNA during PCR amplification: a single strand of wild-type DNA and a single strand of mutated DNA form double-stranded DNA (heteroduplex DNA). This DNA cannot be cut by Hind III, thus leading to a ratio > 0.5.

Since, only two homozygous samples were available, we obtained the required results by repeatedly analyzing the samples on different days. These samples displayed ratios from 0.01 to 0.15 with a mean ratio of 0.05 (± 0.04, cv 96%). Together, the results allow a clear separation of heterozygotes from the wild-type and homozygotes.

Table 2 Intra- and inter-assay imprecision

	Mean Ratio	SD	CV (%)
Intra-Assay Imprecision			
Wild-type	1.08	0.10	9.3
Heterozygous	0.63	0.05	7.6
Homozygous	0.03	0.01	52.2
Inter-Assay Imprecision			
Wild-type	1.07	0.14	12.9
Heterozygous	0.65	0.07	11.5
Homozygous	0.04	0.02	45.7

SD = standard deviation
CV = coefficient of variation

Costs

Since our intention was to design an assay for the average clinical laboratory, we focused not only on handling and reliability but we also looked at cost-effectiveness as well. Apart from a thermal cycler, no special equipment is needed. The costs of the consumables (enzymes, reagents and plastic ware) for one blood sample (digested and undigested aliquot) are less than 3 $ (US).

Discussion

A plethora of assays now exists, employing various techniques for the detection of point mutations, the

majority of which has been developed in the research laboratory for the research laboratory. Just recently, with an emerging market for genetic testing, dedicated automated systems have become available which are designed to analyze a large number of samples in a short period of time. Though **both types** of assays have proven their ability to detect point mutations, they are in most cases not suitable for the average clinical laboratory.

Our intention was to focus strictly on the development of a reliable and easy-to-use assay, which is truly suited for use under the conditions and restraints present in the setting of an average clinical laboratory. To reach this goal, chemicals and equipment usually available in such laboratories had to be integrated into the analytical procedure, and manual work had to be avoided whenever possible. Consequently, we decided to use a DNA preparation method, which is very quick and allows the handling of several samples simultaneously. For the same reason, quantification of DNA or cells was omitted. Instead, we decided to split every sample and carry the undigested sample through the whole assay, allowing on one hand control of all assay steps and on the other hand avoiding any quantification steps after DNA preparation and after PCR amplification. In addition, the undigested sample is the 100% base for the calculation of the ratio of each sample, thus avoiding extra handling steps. This design allows the medical technologist to work efficiently without compromising on quality or security.

For the detection of the PCR product itself, we used a modified Elecsys 1010 system from Roche Diagnostics, because the instrument is a widely distributed analyzer system, thereby taking advantage of the possibility of using existing shelf reagents. Moreover, this analytical system allows the determination of the genotype with high reliability and low costs. The system employs electrochemiluminescence (ECL) for detection, which is very sensitive and, since detection is linear over almost five orders of magnitude, allows the simultaneous processing of samples with varying amounts of amplified DNA. In addition, the assay is fast enough to allow same-day reporting which leads to a rapid therapeutic response.

Nowadays, almost all clinical laboratories feel increasingly the impact of a tight budget. The fact that this assay, apart from the PCR step, does not require any specialized equipment, keeps the costs to less than 3 $ (US).

The recently described fluorescence-based PCR method (LightCycler™) [12] is certainly faster than the method described here, but it requires an analyzer exclusively dedicated to PCR-based testing which represents an additional investment of 40,000 to 50,000 $ (US). In many clinical laboratories this investment cannot be justified due to low sample numbers. Assays, comparable to the test described here, may help genetic analyses in the future to become an affordable part of the daily workload of an average clinical laboratory.

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