Evaluation of a Chromogenic Factor VIII-based Assay for the Detection of Factor V Leiden

Evaluation eines chromogenen Faktor VIII-basierten Assays für die Erkennung des Faktor V Leiden

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Summary: The inherited resistance to activated protein C (APC resistance) is a frequent risk factor for thrombosis and is the most common hereditary blood clotting disorder so far identified. While the commercially available activated thromboplastin time (aPTT) tests with diluted FV deficient plasma for the measurement of APC resistance have a high specificity and sensitivity, there are only a few studies with a FVIII-based chromogenic assay (*Immunochrom*® APC Response, amidolytic assay). We screened 520 Caucasian healthy individuals living in the south-west of Germany for APC resistance with a FVIII-based chromogenic assay using a fully automated measuring procedure on the Behring Coagulation Timer (BCT). By looking for the FV:Q^{506} mutation with a PCR-based DNA test we found a prevalence of the mutation in heterozygote individuals of 6.92% and for homozygote individuals of 0.19% in the study group. At a cutoff ratio of 2.2 the chromogenic assay on the BCT shows a specificity of 89.9% (49/483 false-positive APC ratios) and a sensitivity of 64.9% (13/37 false-negative APC ratios) as compared to the PCR test. In comparison to aPTT-based screening tests the factor VIII-based amidolytic assay on the BCT appears to be less efficient. Possible reasons are discussed. Perhaps other automated systems will show a better specificity and sensitivity of the Immunochrom® APC Response test like the previously evaluated manual procedure.

Keywords: Factor V/genetics; Gene Frequency; Mutation; DNA/analysis; Factor VIII; Chromogenic Compounds/diagnostic use; Thromboembolism.

Zusammenfassung: Die Resistenz gegen aktiviertes Protein C (APC-Resistenz) ist ein häufiger Risikofaktor für Thrombosen und ist die bis heute wichtigste hereditäre thrombophile Diathese. Während die auf dem Markt befindlichen aPTT-Tests zur Messung der APC-Resistenz eine hohe Spezifität und Sensitivität bei Einsatz von Faktor V-Mangelplasma zeigen, liegen nur wenige Untersuchungen zu einem FVIII-basierten chromogenen Assay (Immunochrom® APC Response) vor. Wir haben 520 gesunde Probanden (Kaukasier) aus Süd-West-Deutschland auf die APC-Resistenz mit dem FVIII chromogenen Assay am vollautomatischen Behring Coagulation Timer (BCT) untersucht. Das Vorliegen der FV:Q^{506}-Mutation wurde durch DNA-Analytik mit einem PCR-Verfahren gesichert. Wir fanden in der Studiengruppe eine Prävalenz der Mutation für heterozygote Personen von 6,92%, für homozygote Personen von 0,19%. Bei einer APC-Ratio von 2,2 als cuttoff-Wert zeigte der chromogene Assay am BCT eine Spezifität von 89,9% (49/483 falsch-positive APC-Ratios) und eine Sensitivität von 64,9% (13/37 falsch-negative APC-Ratios) im Vergleich zur PCR als Referenzmethode. Gegenüber den aPTT-Screeningtests ist der Faktor VIII-basierte Test zur Erkennung der Faktor V Leiden Mutation weniger effizient. Mögliche Ursachen werden diskutiert. Eventuell zeigen andere automatisierte Systeme eine bessere Spezifität und Sensitivität dieses Immunochrom® APC Response-Testes wie die früher evaluierte manuelle Variante dieses Tests.

Schlüsselwörter: Faktor V/Genetik; Genfrequenz; Mutation; DNA/Analytik; Faktor VIII; Chromogene Verbindungen/diagnostische Anwendung; Thromboembolie.

Venous thromboembolism is a serious health problem. It is often familial, suggesting that genetic risk factors are involved. Inherited resistance to the anticoagulant function of activated protein C (APC) has been identified during recent years as a major basis for familial thrombosis [1-4]. APC-resistance is prevalent in 21-64% of patients with venous thrombosis [5-7] and in 3-15% of the general population [8]. In at least 90% of the cases, APC-resistance is caused by a single point mutation in the factor V gene, G to A at position 1691 [9]. The mutation leads to replacement of arginine 506 with glutamine (factor V^{R506Q}, FV:Q^{506}). Mutated factor Va, activated by factor Xa, was found to be

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resistant to APC [10]. This defect was found in 94% of thrombosis-prone families with inherited APC resistance [11].

APC resistance is currently detected by measuring the effect of APC on the prolongation of activated partial thromboplastin time (aPTT). The aPTT methods are highly variable in their capacity to discriminate between carriers and non-carriers, but when the samples are diluted with FV-deficient plasma, the sensitivity and specificity of these tests increase to 100%.

A chromogenic APC response assay, the amidolytic assay, is based on the specific interaction between APC and factor VIII [12-15]. Studies of the inactivation of factor VIIIa by APC have shown that factor V acts synergistically with the known cofactor Protein S [16]. Protein S-deficient plasma containing normal amounts of factor V shows a stronger APC response than plasma containing normal protein S and abnormal or deficient factor V. The R506Q mutation in factor V affects the ability of factor V to function as a cofactor for APC. Mutant factor V (factor V^{R506Q}) alone does not accelerate factor VIII inactivation by APC. In the presence of protein S, factor V^{R506Q} is able to accelerate APC-mediated factor VIII inactivation. Upon activation factor V loses its ability to enhance factor VIII inactivation by APC. While factor V stimulates the proteolytic degradation of factor VIII, factor Va prevents it [12].

In this study with 520 healthy individuals we compared the APC resistance using a FVIII-based chromogenic assay (*Immunochrom*® APC Response, Immuno Vienna, Austria) on the fully automatic system BCT with the DNA analysis of the factor V 1691 G→A mutation as the reference method. Our intention was to gather more information about the sensitivity and the specificity of the fully automated amidolytic assay for the detection of the factor V Leiden mutation in healthy individuals. We also determined Protein S and Protein C activities in all samples. Furthermore FVIII activity, aPTT and AT III activity were measured if homozygous or heterozygous factor V Leiden mutation was detected or discrepancies between the results of the PCR assay and the amidolytic assay were found.

Materials and methods

Structured Questionnaire

The blood of 520 healthy individuals (Caucasians) was investigated. The patients and their parents were born in Germany. The questionnaire asked about venous and arterial thromboembolism in the patients and their families and also about any operations, medical complaints, oral contraceptives, hormone substitution therapy, and other medication and drugs.

Anyone who had a previous thromboembolism, any medical complaints or was taking medication was excluded (47 persons).

Plasma Samples

Samples were obtained from 520 healthy individuals (F/M 409/111, aged 20-62 years, median 36 years) by drawing blood from a forearm vein. It was collected for DNA analysis in tripotassium EDTA tubes (Vacutainer, Becton Dickinson) and for functional assays in 0.129 mol/l trisodium citrate tubes (Vacutainer, Becton Dickinson) using a mixing proportion of blood to anticoagulant of 9 to 1. Plasma was obtained soon after drawing by centrifuging for 20 minutes at 2000 x *g* and was immediately frozen at –72 °C until use. Plasma samples were analyzed within 4 weeks from drawing.

FV:Q^{506} Mutation

DNA analysis was performed as described elsewhere (9). Briefly, a 220 bp fragment of exon 10/intron 10 of the factor V gene was amplified by PCR using 5`-CTTGAAGGAAATGCCCCATTA-3` as the 5` primer and 5`-CTTGAAGGAAATGCCCCATTA-3` as the 3` primer. Amplification involved 36 cycles of 91 °C (40 sec), 55 °C (40 sec) and 71 °C (2 min) in the presence of 2 U Taq polymerase. Subsequently the 220 bp fragment was digested during 16 h by 0.4 U MnlI at 37 °C. MnlI digests the 220 bp fragment of the normal factor V allele in three fragments of 37, 67 and 116 bp each. The factor V Leiden allele is cleaved in only two fragments of 67 and 153 bp. Finally the digestion products were separated by electrophoresis on ethidium bromide stained 2% agarose gels for 30 min at 150 V.

APC Resistance Plasma Assay

The assay was performed using a commercial kit (*Immunochrom*® APC Response, Immuno Vienna, Austria) on a Behring Coagulation Timer (BCT) in accordance with the instructions given by Immuno.

The plasma samples were diluted 1:21 with dilution buffer (Imidazol/NaCl, Albumin 0.2%, pH 7.4). This was preincubated with 100 µl of Immunochrom reagent A (Phospholipid, Albumin) and 50 µl buffer for 2 min at 37 °C. After the addition of 100 µl of Immunochrom reagent B (F IXa, F X, Ca^{2+}, Albumin and a trace amount of thrombin) the mixtures were further incubated for 5 min, then 200 µl of factor Xa chromogenic substrate (10 µmol/ml F Xa-1, 0,01 µmol/ml α-NAPAP) diluted (1+2) in a reaction buffer (TRIS/Na_2 EDTA/NaCl, pH 8.3) was added. The mixture was further incubated for 5 min at 37 °C, and acetic acid (100 µl of a 50% solution) was then added to terminate the chromogenic substrate conversion. After the reaction was stopped, the absorbance was measured at 405 nm. The amount of factor Xa generated in the incubation mixture depends on the concentration of factor VIII. When APC is present, the factor VIII in the sample is partially inactivated and

Nonstandard abbreviations: APC, activated protein C; aPTT, activated partial thromboplastin time; FV, factor V; FVIII, factor VIII; NAPAP, Nα-(2-naphthylsulfonyl-glycyl)-4-amidinophenylalanine piperidide

therefore less factor Xa is generated, i.e. lower absorption values are observed.

Frozen plasma samples were assayed within 3 hours after thawing. The coefficient of variation (CV) of the APC ratio for a pool of 140 vials (the first 140 healthy individuals of the study without factor V Leiden) was determined to be 6.0%. The coefficient of variation observed with 20 portions over 20 days was 6.4%.

Functional and Immunological Assays

Protein S and Protein C activities were measured by functional assays (Behring® Protein S Reagent, Behring® Protein C Reagent), AT III and Factor VIII by a chromogenic assay (Behring® Antithrombin III (A), Behring® factor VIII) on the Behring Coagulation Timer (BCT). Protein S Clotting Test from Boehringer Mannheim® was used as a second functional assay, total and free protein S antigen were determined with a commercially available kit (Electroimmunodiffusion Protein S from Immuno®).

Statistical Analysis

The chi-squared test was used to assess the APC ratio in comparison to the factor V 1691 G→A mutation. It was also used for comparison of the females with and without oral contraceptives and for comparison of Protein S, Protein C, AT III and FVIII activity. Potential relationships between the variables were evaluated by linear regression.

Results

520 healthy individuals were studied. The median age was 36 years (range 20-62), 409 were female (78.7%) and 111 were male (21.3%). Of the 409 women studied, 171 (41.6%) were taking oral contraceptives and 238 (58.4%) were not taking oral contraceptives.

Screening of the 1040 alleles by DNA analysis resulted in heterozygosity for the factor V Arg^{506}→Gln mutation in 36/520 individuals and homozygosity in 1/520 individuals, yielding a total prevalence of 3.65% for one allele. The prevalence for heterozygosity was 6.92%, for homozygosity 0.19%.

APC ratios were determined between 1.53 and 4.83 (figure 1) with median 2.69 and mean 2.80 (n=520), Protein S activities between 31.0% and 240% with median 103%, Protein C activities between 39% and 190% with median 106%. The median APC ratio from factor V Leiden-negative individuals (n=483) was 2.74 (range 1.55-4.83); the median APC ratio from factor V Leiden-positive individuals (n=37) was 2.02 (range 1.53-2.65). This is significant ($p < 0.001$).

49/483 (10.1%) individuals without factor V Leiden mutation had an APC ratio < 2.2. Of these, 37/49 (75.5%) had a normal factor VIII concentration between 50% and 150%, and 12/49 (24.5%) had an increased factor VIII concentration of > 150%.

Figure 2 demonstrates APC ratio test sensitivity and specificity as a function of the test cut-off critical values for the factor V Leiden-negative individuals studied (n=483). Specificity and sensitivity as a function of the cut-off value used in the APC resistance assay are plotted. The reference range (given as 95 percentile of the first 140 healthy individuals without factor V Leiden) was 2.21-3.51. At a cut-off of 2.2, the test has a specificity of 89.9% but a sensitivity of only 64.9%. Increasing the cut-off to 2.8 increases the sensitivity to 100%, but at a cost of decreased specificity (46.8%).

Table 1 shows false-negative and false-positive APC ratios at a cut-off value of 2.2.

We found one homozygote factor V Leiden mutation in a female (37 years, using an oral contraceptive). She has not suffered venous thrombosis so far (APC ratio etc. see table 2). Protein S activity was determined by two different tests: The results indicate a Protein S type II defect with decreased activity and normal Protein S antigen (free and total). Both her parents are heterozygotes. One sister is a heterozygote, another sister does not have factor V Leiden mutation.

Discussion

Factor V Leiden mutation is recognized as the most frequent inherited cause of venous thrombophilia, underlying a poor anticoagulant response to activated

Table 1 Specificity and sensitivity of the amidolytic assay of the APC ratio at a cut-off of 2.2.

APC ratio	Factor V Leiden (n = 520) negative (n = 483)	positive (n = 37)
> 2.2	434 (89.9%)	13 (35.1%)
< 2.2	49 (10.1%)	24 (64.9%)

Table 2 APC ratio, Protein S, Protein C activity and mass concentrations (Antigen=AG) of one female (37 years, patient number 258) who was homozygote in factor V Leiden mutation. We found an APC ratio of 1.69 and a Protein S defect type II with an activity of 20% (pathological values are in bold type). She was taking oral contraceptives up to the end of the study.

	patient no. 258	reference values
APC ratio	**1.69**	>2.2
Protein S activity	**20%**	70-140%
Protein S AG (total)	97%	72-125%
Protein S AG (free)	35%	21- 48%
Protein C activity	100%	70-140%
Protein C AG	73%	70-140%
AT III activity	121%	80-120%
F VIII activity	78	50-150

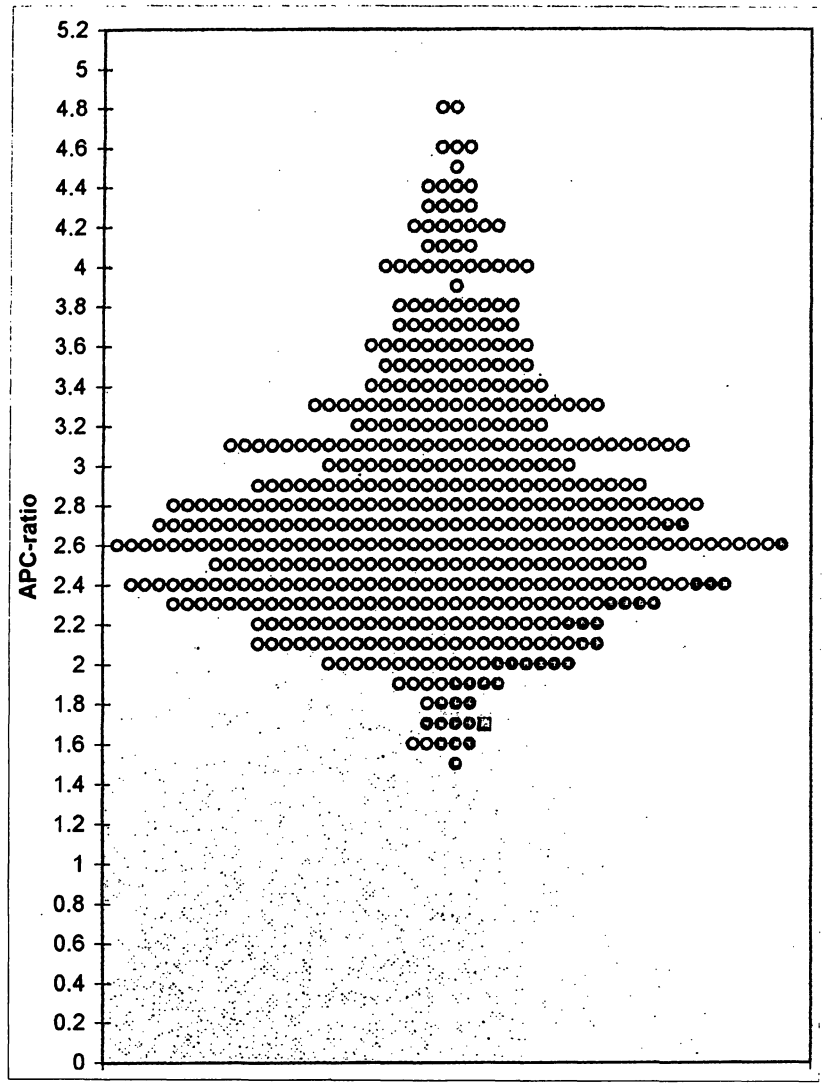

Figure 1 APC ratios of the 483 individuals without factor V Leiden mutation (O), of the 36 individuals with heterozygote (◘) and the one individual with homozygote factor V Leiden mutation (■)

protein C (APC resistance). We compared the results of factor V 1691G→A mutation DNA analysis as the reference method to a factor VIII chromogenic assay (Immunochrom® APC Response Test) in a healthy population of 520 individuals (Fig. 1). We found a total prevalence of 3.65% for one allele in South-West Germany, which concurs with the study of *Schröder* et al. [17] who recently found an allele frequency in North-Eastern Germany of 3.56% (n = 814 anonymous individuals) and *Schwender* [18] who described an axis from the north to the south through Germany for the prevalence, decreasing to the east and west. The comparison of the FVIII chromogenic assay to the reference method of DNA analysis shows the following results:

At a cut-off of 2.2 we found 13/37 false-negative APC ratios and 49/483 false-positive APC ratios. The sensitivity of the APC ratio was 64.9% with a specificity of 89.9% (Fig. 2). Increasing the cut-off to higher values increases the sensitivity, but at a cost of decreased specificity. At a cut-off of 2.8 the sensitivity would be 100% (specificity only 46.8%). All individuals with a factor V Leiden mutation would be detected, but also 257/520 false-positive APC ratios (53.2%), which indicates the low specificity. As the factor V Leiden DNA test is by definition the standard, the sensitivity of the test is 100%, assuming that DNA isolation, amplification and electrophoresis of all examined individuals are performed correctly.

A reason for the false-positive APC ratios could be an increase in factor VIII activity > 150%. 49/483 (10.1%) individuals without factor V Leiden mutation have an APC ratio < 2.2. Of these 37/49 (75.5%) have a normal factor VIII concentration between 50% and

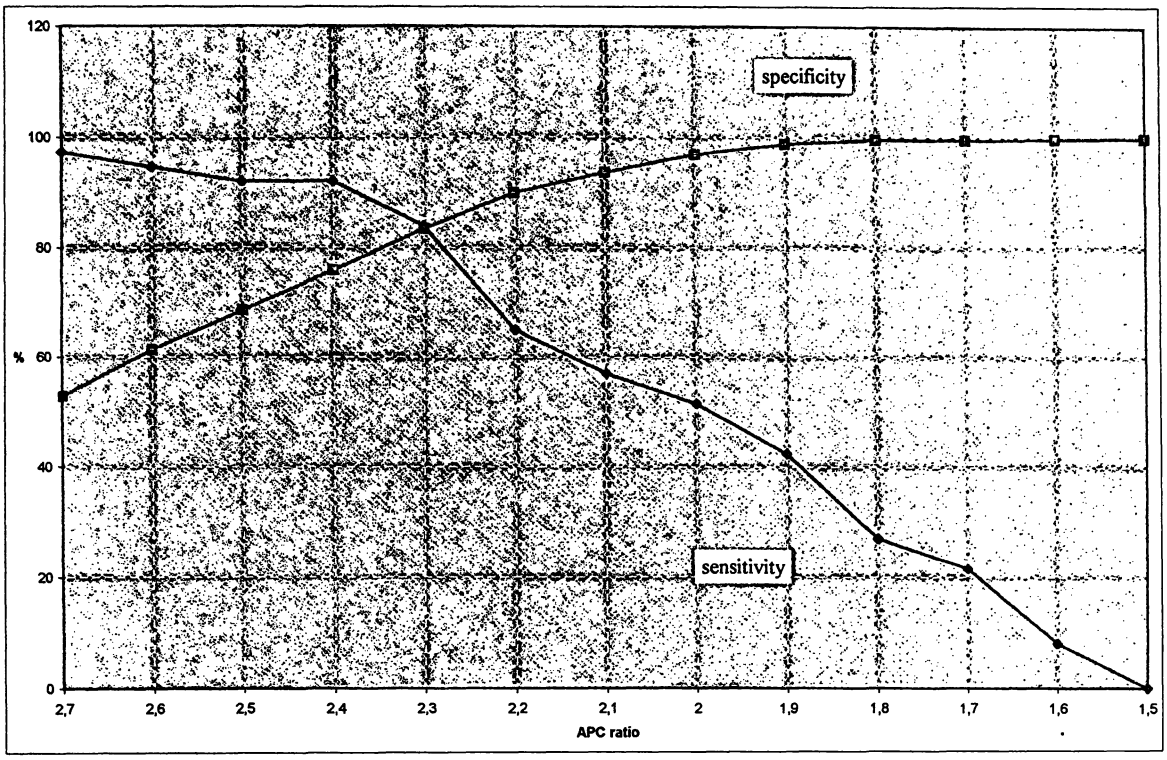

Figure 2 Test cut-off value analysis. Specificity and sensitivity as a function of the cut-off value used in the APC resistance assay are plotted.

150%. and 12/49 (24.5%) have an increased factor VIII concentration of > 150%. If we consider the 12 false-positive APC ratios due to the increased factor VIII activity, 37/483 (7.7%) false-positive APC ratios remain. Other studies show that 67% of patients who are non-carriers of the Leiden mutation and who have FVIII activities > 150% have a normal APC ratio. An increased FVIII activity does not always cause a reduced APC response [19].

Tripodi et. al. [20] evaluated thirteen plasma-based methods for their diagnostic efficacy in comparison with DNA analysis. They found that the FVIII-based method does not discriminate between carriers and non-carriers. Finally, all methods except the FVIII-based method are able to distinguish heterozygotes from homozygotes, but only 30 plasma samples from carriers were examined (heterozygotes, n = 27; homozygotes, n = 3) and 30 plasma samples from non-carriers of the FV:Q^{506} mutation. Our results corroborate these discriminations by using a much higher collective. Equally *Siegert* et al. [19] found in their study with 157 patients, 7/66 false positive APC-Response (10.6%) which corresponds to our results (49/483 individuals; 10.1%). (APC-resistance was compared in three different tests.) A possible explanation for this is that the Immunochrom® APC Response Test could detect a thrombophilic situation in individuals without factor V Leiden mutation better than other aPTT-tests [21]. Perhaps an abnormal APC ratio in these cases shows a hypercoagulability or is caused by other genetic defects.

The original APC-resistance test, which is based on an aPTT-reaction, has a sensitivity and specificity for the presence of the FV:Q^{506} allele of 85-90% or better [22]. Clinical experience with this test has been obtained mainly for APC resistance. Different instruments give different clotting times. Variations in plasma levels of protein C have no influence on the APC ratio because a standardized amount of APC is added. Patients with protein S deficiency have no or only a very minor influence on the APC response in the aPTT-based assay. For analysis of plasma from individuals with other coagulation defects (lupus anticoagulants, coagulation factor deficiencies, oral anticoagulants, heparin) a modified APC resistance test was developed. This modified test (the patients plasma is diluted in FV-deficient plasma) shows a sensitivity and a specificity of nearly 100% [23]. The procedure eliminates the difference in APC ratios between fresh and frozen plasma and also makes the APC resistance quite in-

sensitive to preanalytical variation in sample handling. A comparison of the FVIII-based test to aPTT-tests was not the aim of our study.

We conclude that the sensitivity and specificity of the Immunochrom® APC Response test using the fully automated system BCT is not as good as the reference method we used. Perhaps other automated systems show a better specificity and sensitivity of the Immunochrom® APC Response test like the previously evaluated manual method for this test [13, 14]. Nevertheless the amidolytic assay can have the advantage of detecting a thrombophilic situation in individuals without factor V Leiden mutation. This should be evaluated in future studies.

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