Improved Distinction of Factor V Wild-Type and Factor V Leiden Using a Novel Prothrombin-Based Activated Protein C Resistance Assay

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Abstract

A new prothrombin-based activated protein C resistance (APC-R) test is described. In this method, the patient sample is prediluted in a plasma depleted of factor V (FV). A reagent containing APC and a specific activator of FV is added. After an incubation period, clotting is initiated by the addition of the FV-dependent prothrombin activator Noscarin. We analyzed 703 samples from patients undergoing thrombophilia screening. By using a predefined cutoff ratio of 2.5, 100% sensitivity and specificity for the detection of a factor V Leiden (FVL) mutation was found. With a cutoff ratio of 1.2, a complete but narrow distinction of FVL heterozygous (n = 192) and FVL homozygous samples (n = 27) was determined. No interference by the international normalized ratio, activated partial thromboplastin time (aPTT), protein S activity, fibrinogen and factor VIII (FVIII) levels, or lupus anticoagulant ratio was detected. The new prothrombin-based APC-R assay provides improved distinction of FV wild-type and FVL carriers compared with the aPTT-based method. By the use of an FV-dependent prothrombin activator, the assay is not influenced by FVIII concentration or lupus anticoagulants.

Activated protein C (APC) degrades factor Va (FVa) and factor VIIIa (FVIIIa) by proteolytic cleavage at specific arginine residues.1 In factor V Leiden (FVL), a single point mutation in position 506 of the factor V (FV) gene replaces an arginine to a glutamine residue. This results in a 10-fold decrease in the rate of FVa inactivation and in an APC resistance (APC-R) phenotype.2 The increase in FVAs half-life results in increased clotting. More than 95% of APC-R cases are due to the FVL mutation.3-5 This mutation leads to the most common inherited form of a primarily venous thrombophilia. Heterozygosity for FVL occurs in 3% to 8% of the general US and European populations. The frequency of homozygosity for the FVL mutation is approximately 1 in 5,000. The risk for venous thrombosis is approximately 3- to 10-fold in individuals who are heterozygous for the FVL mutation. Homozygous individuals have been reported to have an approximately 80-fold risk over baseline for thrombosis.6

The original test for APC-R used in clinical laboratories is an activated partial thromboplastin time (aPTT) performed in the presence and absence of exogenously supplied APC.3,7 In healthy patients, the APC degrades the patient’s FVas and FVIIIas and, on that basis, prolongs the aPTT. In patients with an FVL mutation, the degradation of FVas does not occur to the same extent, and, therefore, the aPTT does not become as prolonged. The ratio of the aPTT with APC vs the aPTT without APC is calculated. Healthy individuals typically have a ratio of 2.0 or greater, and individuals with FVL typically have a ratio less than 2.0. However, there is considerable overlap between healthy subjects and heterozygotes.8

In a modified APC-R assay, the patient plasma sample first is diluted 1:5 with FV-deficient plasma before the analysis and then assayed.3 The presence of the deficient plasma provides all factors except FV to offset any aPTT-related factor
deficiencies and to minimize the effect of an elevation of FVIII, which would shorten the aPTT. The modified assay also contains polybrene, which neutralizes unfractionated heparin and low-molecular-weight heparin in the specimen. Sensitivity and specificity values reported for the modified aPTT-based APC-R assay are almost 100%. In smaller evaluations, sensitivities and specificities of 100% have been reported. The discrimination gap between wild-type FV and FVL is narrow. One potential major interference of the aPTT-based APC-R assays is the presence of lupus anticoagulants in the sample. Because the tests are phospholipid-based, the lupus anticoagulant is capable of producing interference in the assay. Thus, patients with a lupus anticoagulant should be evaluated directly with a genetic assay to determine whether the FVL mutation is present.

Other functional APC-R assays have been developed; however, the aPTT-based method is still the most commonly used functional APC-R detection method.

The “gold standard” DNA assay for the FVL mutation involves the use of the polymerase chain reaction (PCR) method. The testing by PCR allows specific identification of patients with FV wild-type, FVL heterozygosity, and FVL homozygosity. The genetic tests are much more expensive and labor-intensive than the clot-based screening assay. Owing to the need for special instrumentation, genetic testing usually also has significantly longer turnaround times than functional APC-R testing.

In this article, we describe a new functional prothrombin-based APC-R test with improved sensitivity and specificity. We also describe optimization and validation data.

Materials and Methods

Principle of the Prothrombin-Based APC-R Test

Pefakit APC-R Factor V Leiden (Pentapharm, Basel, Switzerland) is a plasma-based functional clotting assay. It relies on an FVa elimination step and an FVa detection step. The analysis is performed at a temperature of 37°C.

First the plasma sample (30 µL) is mixed with a standardized plasma sample that has been depleted of FV (FV dilution plasma, 20 µL) and a reagent that contains APC and a snake venom specifically activating the FV in the plasma sample (Russell viper venom–factor V [RVV-V] isolated from Daboia russelli venom). During an incubation period of 180 seconds, the activated FV is inactivated by the added APC. The velocity of the inactivation of the FVa molecules depends on their binding kinetics to APC and, thus, is decelerated significantly in the case of an FVL mutation.

Subsequently, a reagent that contains an FV-dependent prothrombin activator (Noscarin from the venom of Notechis scutatus scutatus) and EDTA (50 µL) is added. The prothrombin activator converts prothrombin to thrombin and, thus, induces clotting of the sample. The clotting time is recorded. If the FVa molecules in the sample have been eliminated during the incubation step, the velocity of prothrombin activation is slow, and, therefore, the clotting time is long. If the FVa elimination has been incomplete (eg, due to an FVL mutation), the velocity of prothrombin activation is high and the clotting time is short.

Interference from unfractionated or low-molecular-weight heparin in the sample is precluded by the addition of a heparin inhibitor (the polycation polybrene).

A second determination is performed under identical assay conditions, with the exception that no APC is added to the first reagent. Thus, the baseline clotting time is determined without the inactivation of the FVa molecules by APC. This determination is called the APC(–) measurement, as opposed to APC(+) for the test with the FVa elimination by APC. A ratio between the APC(+) and the APC(–) analysis is calculated.

Figure 1

Principle of the prothrombin-based activated protein C (APC) resistance assay. During the elimination phase (A), the plasma sample reacts with an enzyme (Russell viper venom–factor V [RVV-V]) specifically activating factor V (FV) and APC. Subsequently, residual (noninactivated) FVa molecules are detected using the FV-dependent prothrombin activator Noscarin (B, detection phase). FVai, inactivated factor V; FVL, factor V Leiden.
Standard Methods

The aPTT based APC-R test with 1:5 sample predilution in FV-deficient plasma was performed as instructed by the manufacturer (Coatest APC-R-V, Chromogenix, Milano, Italy). Determinations of FVIII activity (aPTT-based, Instrumentation Laboratory, Kirchheim, Germany), protein S (PS) activity (Staclot Protein S, Stago, Asnières, France), fibrinogen concentration (Clauss method; Fibrinogen kit, Trinity, Wicklow, Ireland), prothrombin time (PT; Thromborel S, Dade-Behring, Marburg, Germany), and aPTT (Pathromtin SL, Dade-Behring) were analyzed using standard techniques. The lupus anticoagulant index was calculated using the aPTT-dependent method (Lupus Anticoagulant, Technoclone, Vienna, Austria).

Examinations

The influence of the concentration of the major components of the prothrombin-based APC-R assay was examined using plasma samples with wild-type FV, heterozygous FVL, and homozygous FVL. The concentration ranges examined were as follows: APC, 0 to 10 µg/mL; Noscarin, 1 to 10 U/mL; RVV-V, 0 to 20 U/mL; and EDTA, 0 to 100 mmol/L. One component was varied at a time. Basic concentrations were as follows: APC, 8 µg/mL; RVV-V, 20 U/mL; Noscarin, 5.5 U/mL; and EDTA, 70 mmol/L.

The sensitivity and specificity of the prothrombin-based APC-R method were examined by analyzing citrated plasma samples from 686 patients undergoing laboratory thrombophilia examinations. In addition, citrated plasma samples from 17 patients with a known homozygous FVL mutation were assayed. FVL status was determined by using the PCR technique.

The results of the APC-R test were correlated with the PT (n = 386), aPTT (n = 386), FVIII activity (n = 326), PS activity (n = 394), fibrinogen concentration (n = 380), or the aPTT-based lupus anticoagulant test (n = 86). In a retrospective analysis, the results of the aPTT-based APC ratio (with plasma predilution in FV-deficient plasma) for 586 patients with a known FV genotype were analyzed.

Analyses were performed on the Behring coagulation system (BCS, Dade-Behring; for APC-R assays, FVIII, and PS), the Amax (Trinity; for PT, aPTT, and fibrinogen concentration), and the ACL 9000 (Instrumentation Laboratory; for the lupus anticoagulant test). The new test was performed using an instrument with optical detection (BCS). However, the assay was tested successfully on various instruments with optical or mechanical detection. The same cutoff value (ratio, 2.5) could be applied on all devices for differentiation of FVL and FV wild-type samples.

Results

Figure 2 shows the effect of various concentrations of the main components of the assay. Rising concentrations of APC led to steeply increasing clotting times in the FV wild-type sample, indicating the inactivation of the FVa molecules (Figure 2A). In contrast with this, the clotting times of the heterozygous FVL sample were only slightly prolonged, whereas clotting was not delayed in the homozygous FVL sample, indicating the reduced or absent inactivation of the FVa by the added APC. With rising concentrations of APC, the differentiation between the different FV genotypes was improved.

In contrast, rising concentrations of Noscarin (Figure 2B) led to decreasing clotting times in all 3 samples and to impaired differentiation between them. Therefore, the Noscarin concentration should not be too high.

Figure 2C depicts the importance of RVV-V in the assay. Only when RVV-V is added can the different samples be separated and characterized effectively. Concentrations of more than 10 U/mL of RVV-V do not prolong the clotting times significantly more, indicating a nearly complete FV activation with 10 U/mL of RVV-V.

Figure 2D shows increasingly prolonged clotting times of the FV wild-type sample when rising concentrations of EDTA are used. Also, the clotting times of the FVL samples are prolonged with rising EDTA levels but to a lesser extent, leading to better distinction when higher amounts of EDTA are applied.

Figure 3 shows the percentile distributions of the APC ratios for the patient samples separated by FVL genotype. In
Figure 3A, the APC ratio of the prothrombin-based test is shown. With a predefined cutoff of 2.5, 100% sensitivity and specificity for the detection of an FVL mutation were found. No APC ratios between 2.2 and 3.0 were determined, leading to a broad distinction between the FV wild-type (n = 484) and FVL samples (n = 219). With a cutoff ratio of 1.2, a complete but narrow distinction of FVL heterozygous (n = 192) and FVL homozygous (n = 27) samples was found.

In Figure 3B, the percentile distribution of a ratio between the APC(+) determination of the individual samples to the APC(−) measurement of an FVL-negative control plasma sample in the same population as in Figure 3A is shown. A broad overlap in the ratios of FV wild-type, heterozygous FVL, and homozygous FVL was determined, indicating the need for the determination of the clotting time with and without addition of APC for the analysis.

In Figure 3C, the percentile distribution of the aPTT-based APC ratios is shown. Of the patients, 213 had a wild-type FV, 314 a heterozygous FVL mutation, and 41 a homozygous FVL genotype. With a cutoff value of 2.0, 356 patients were identified as APC-resistant and 230 were detected as normal. Of the 356 positive results, 353 were truly APC-resistant (specificity, 99.2%). In addition, 353 of 355 FVL carriers were detected (sensitivity, 99.4%).

As depicted in Figure 4A, Figure 4B, Figure 4C, Figure 4D, and Figure 4E, the prothrombin-based APC ratio is not influenced by variations in international normalized ratio (INR) or aPTT values or by PS, fibrinogen, or FVIII levels. Also, the presence of lupus anticoagulants in the samples did not affect the APC ratio. The correlation coefficients were as follows: 0.06, APC ratio vs INR; 0.06, APC ratio vs aPTT; 0.09, APC ratio vs PS; –0.10, APC ratio vs fibrinogen level; –0.07, APC ratio vs FVIII level; and 0.07, APC ratio vs lupus anticoagulant index.

Discussion

We have described a new functional assay for the detection of APC-R. The test is based on an invention by Stocker et al. The method has significant biochemical differences from the commonly applied aPTT-based method.

Prothrombin-Based Detection of FVa Inactivation

First, the FVa inactivation by APC is detected by an FV-dependent prothrombin activator. This makes the assay independent of FVIII activity, which was confirmed in the examinations (Figure 4E).

Absence of Free Calcium Ions in the Assay

In addition, the assay is performed in the absence of free calcium ions. The citrated plasma sample is not recalcified, and additional EDTA is added. No phospholipids are present in the reagent because the formation of phospholipid-based complexes would not take place in the absence of free calcium ions. In theory, this should eliminate the influence of lupus anticoagulants on the assay and was confirmed in our examinations. This is very significant for the laboratory detection of APC-R because of the high prevalence of FVL and lupus anticoagulants in thrombophilic populations. Galli et al showed that test methods proposed as less lupus sensitive (tissue factor–based APC-R assay, using FV-deficient plasma and 1:40 diluted test plasma/dilute RVV test–based assay with highly concentrated phospholipids) can yield false-positive results when lupus anticoagulants are present. Also the recent finding of Tripodi et al that residual platelets in the sample can significantly alter the results of functional APC-R tests confirms the advantage of an assay method that is not phospholipid-based.
Concentration-Dependent Effect of Assay Components

The effect of varying concentrations of the main reagent components (Figure 2) on the test outcome confirms the theoretical concept of the assay. Rising doses of APC led to an increasing elimination of FV wild-type molecules and, thus, to improved distinction of FV wild-type and FVL samples. APC binds more readily to FVa than to FV, explaining the prolonged clotting times detected using rising concentrations of RVV-V. The short clotting times obtained when no RVV-V was added show that FV and FVa but not inactivated FVAs accelerate the enzymatic activity of Noscarin. As previously reported by Tans et al., the addition of rising amounts of EDTA slows prothrombin activation. When used for the prothrombin-based APC-R assay, rising amounts of EDTA improve the discrimination of FV wild-type and FVL samples.

Results for Patient Samples: Prothrombin-Based Method

The analysis of patient data showed 100% sensitivity and specificity for the detection of an FVL mutation using a predefined cutoff of 2.5. There was a very broad distinction of the APC ratios of FVL and FV wild-type samples. Also, a complete

Figure 4 No interference of international normalized ratio (INR) (A) values, activated partial thromboplastin times (PTT) (B), protein S activity (PS) (C), fibrinogen concentration (Fg) (D), factor VIII (FVIII) activity (E), and lupus anticoagulant index (LAC) (F) toward the prothrombin-based activated protein C ratio was found. Open circles, factor V wild-type samples; closed circles, factor V Leiden samples. Fg and FVIII are given in conventional units; conversions to Système International units are as follows: Fg (g/L), multiply by 0.01; FVIII (proportion of 1.0), multiply by 0.01.
but narrow distinction of heterozygous and homozygous FVL carriers was found.

Regarding these positive results, we questioned whether the formation of a ratio between the APC(+) and APC(–) clotting times is necessary for the detection of APC-R. However, as shown in Figure 3B, there is a large overlap between FVL and FV wild-type samples when only the APC(+) clotting times are considered (in the Figure, the clotting time was expressed as a ratio to the APC(–) clotting time of an FVL-negative control plasma sample).

Results of Patient Samples: aPTT-Based Method

With the aPTT-based APC-R test, a sensitivity of 99.2% and specificity of 99.4% were found. This corresponds well to the reported sensitivities and specificities by Trossaert et al.\textsuperscript{10} In their study, a sensitivity of 98.36% and a specificity of 98.41% after studies in 436 patients (positive predictive value, 97.3%); negative predictive value, 98.8%)\textsuperscript{10} were reported. In other studies, 100% sensitivity and specificity were found\textsuperscript{11,12}; however, these examinations had a smaller sample than the study by Trossaert et al\textsuperscript{10} and the present study.

Exclusion of Interference

The analysis of the correlation of the prothrombin-based APC ratio with INR, aPTT, and PS, fibrinogen, and factor FVIII levels excluded interference of these parameters on the assay (Figure 4). Of the patients tested, 92 were receiving coumarin, as shown by the high INR values. As mentioned, no influence of lupus anticoagulants was detected.

Diagnostic Algorithm

Compared with PCR testing, functional tests have a significantly lower price and usually much shorter turnaround times. Regarding the 100% sensitivity and specificity for the detection of FVL found in the present study with more than 700 analyzed samples, it seems reasonable to abstain from PCR testing for the majority of samples.

A diagnostic algorithm is proposed and summarized in Figure 5. Samples with an APC ratio of more than 2.5 (analyzed with the prothrombin-based method) have an APC-sensitive phenotype and should have a wild-type genotype for the 506 amino acid position of FV. In our study, this was correct for all samples. Samples with an APC ratio of less than 2.5 have an APC-resistant phenotype and, owing to the very high prevalence of FVL, have a higher than 95% probability of having an FVL mutation (in white subjects). In the new test, all samples with a ratio of less than 2.5 were positive for FVL. Regarding the relatively narrow discrimination gap between the ratios of heterozygous and homozygous FVL carriers found in our study, we suggest that patient samples with a prothrombin-based APC ratio of less than 1.5 be analyzed subsequently by the PCR technique.

Economic Implications

Of the 703 samples tested, only 35 had an APC ratio of less than 1.5 in the prothrombin-based method. When total costs of $6 (all amounts are in US dollars) for the functional analysis and $48 for the PCR test (including costs of labor and additional instrumentation) are assumed, the total costs for the diagnosis of 703 patient samples by functional testing would have been $5,898 ([703 samples × $6] + [35 PCR tests × $48]), or about $8.40 per sample. If samples from all FVL-positive patients (n = 219) and 5% of patients with the FV wild-type would have been analyzed by PCR (estimated situation for the aPTT-based method, ie, all samples less than and near the cutoff analyzed by PCR as recommended by Tripodi\textsuperscript{23}), 243 PCR tests would have been performed, resulting in total costs of $15,882 ([703 samples × $6] + [243 PCR tests × $48]), or $22.60 per sample.

In our study, all patients whose samples had an APC ratio of less than 2.5 were stratified correctly as carriers of the FVL mutation. However, being a functional method, the new test theoretically also detects other kinds of FV mutations leading to an APC-resistant phenotype. The prevalence of such other FV mutations is extremely low,\textsuperscript{24,25} and until now, there is no evidence that an FVL mutation has different prognostic implications from those of any other mutation of FV leading to an APC-resistant phenotype.
Limitations and Conclusion

As a limitation of our study, the 2 functional tests were compared based on different populations and were analyzed in different periods. Owing to the high number of samples examined, the additional performance of the aPTT-based APC-R assay in all samples would have increased significantly the amount of labor and costs required for the study. A direct head-to-head comparison between the prothrombin-based and aPTT-based APC-R assays is being performed in another evaluation by a different institution, and results will be published separately.

The novel APC-R test provides improved sensitivity and specificity for functional APC-R testing compared with currently available techniques.

References