Activated protein C resistance testing for factor V Leiden

Stephan Kadauke, Bernard Khor, and Elizabeth M. Van Cott*

Activated protein C resistance assays can detect factor V Leiden with high accuracy, depending on the method used. Factor Xa inhibitors such as rivaroxaban and direct thrombin inhibitors including dabigatran, argatroban, and bivalirudin can cause falsely normal results. Lupus anticoagulants can cause incorrect results in most current assays. Assays that include dilution into factor V-deficient plasma are needed to avoid interference from factor deficiencies or elevations, which can arise from a wide variety of conditions such as warfarin, liver dysfunction, or pregnancy. The pros and cons of the currently available assays are discussed.
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Introduction

Activated protein C (APC), in conjunction with its cofactor protein S, functions as a natural anticoagulant by inactivating activated factors V and VIII (Va and VIIIa, respectively). APC cleaves Va at three arginine sites (R306, R506, and less importantly, R679). The factor V Leiden (FV_{Leiden}) mutation is a DNA substitution (G1691A) that changes the amino acid encoded at one of these three sites (R506Q). APC cannot cleave FV_{Leiden} efficiently, thus FV_{Leiden} is "resistant" to APC, resulting in a hypercoagulable state. Furthermore, native factor V cleaved by APC at R506 also functions as a cofactor (with protein S) for APC cleavage of factor VIIIa. Thus, FV_{Leiden} causes hypercoagulability both by deficient inactivation of the procoagulant factor Va and the inability to generate the cofactor for APC-dependent inactivation of factor VIIIa [1].

Activated protein C resistance (APC-R) is the most common hereditary condition that increases the risk for venous thrombosis, accounting for 20% of unselected patients with first-episode thrombosis and 50% of familial thrombosis [2,3]. APC-R is almost always caused by FV_{Leiden} [4,5]. FV_{Leiden} is thought to have arisen from a founder mutation that occurred in an individual more than 21,000 years ago [6]. Presumably, all patients with FV_{Leiden} are descended from that one individual. The high prevalence of FV_{Leiden} suggests a survival advantage, possibly resulting from decreased bleeding [7]. In the general Caucasian population, prevalence of FV_{Leiden} heterozygosity is 5% and homozygosity is 1 in 5,000; it is uncommon in other ethnic groups [8]. The risk of venous thrombosis is increased 3-to-7-fold in individuals heterozygous for FV_{Leiden}, and 80-fold in homozygotes [9,10]. This thrombotic risk is further increased in the presence of additional risk factors, including oral contraceptive use, pregnancy, hyperhomocysteinemia, or advanced age (reviewed in [11]). Some evidence suggests that the risk of pulmonary embolism is not as great as the risk of deep venous thrombosis in individuals with FV_{Leiden} [12]. Studies have not consistently shown an increased risk for arterial thrombosis such as myocardial infarction or stroke in this population [1,13,14].

Factor VIII gene mutations causing APC-R are theoretically possible but have not yet been described. Although one group reported results that suggested that factor VIII cleavage may be impaired in a subset of patients with unexplained thrombosis, no mutation in the factor VIII gene was described which could account for this phenomenon [15].

Laboratory Testing for APC Resistance

The main clinical purpose of APC-R assays is to detect the presence of FV_{Leiden}. Testing for APC-R to detect FV_{Leiden} has undergone a remarkable evolution that has led to an increased sensitivity and specificity by eliminating interference by variable factor levels and with some assays, lupus anticoagulants (Table I).

The original APC-R assay yields a ratio between a baseline activated partial thromboplastin time (aPTT) and the aPTT after purified exogenous APC has been added [3]. APC-mediated cleavage of factor Va and VIIIa prolongs the aPTT, and this prolongation is decreased in the presence of FV_{Leiden}. Ratios >2.0 are typical of normal individuals, while FV_{Leiden} typically causes ratios <2.0 (although each laboratory must verify its own cutoff). In this test, colloidal silica is added to the patient's plasma to activate factor XII in the contact system, followed by XI, IX, VIII, X, V, and II, culminating in clot formation (Fig. 1A). In addition, an excess of calcium and phospholipid is added because these are required for several steps, including the Xa- and Va-dependent activation of prothrombin (factor II). Using a cutoff ratio of 2.0, the sensitivity is only 50–86% and specificity is 75–98% in adults [24–27]. This is in large part due to the great number of coagulation factors that participate in the clotting reaction, including fibrinogen, factors II, V, VIII, IX, X, XI, XII, and protein S [16]. An abnormal level of any of these proteins can interfere with the test result by affecting the aPTT, and the test is not accurate when the baseline aPTT is abnormal [16]. Low ratios that falsely suggest APC resistance (in the absence of FV_{Leiden}) are caused by a variety of conditions including acute phase reactions and pregnancy (due to elevated factor VIII and low protein S), and hormone replacement therapy or oral contraceptives (due to low protein S). Protein S interferes when $<20\%$ [16]. Falsely raised ratios can occur with factor deficiencies (such as with liver dysfunction, warfarin, vitamin K deficiency, disseminated intravascular coagulation) or anticoagulant therapies. Lupus anticoagulants can artificially interfere by sequestering phospholipids required for clotting [17]. Heparin,

Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts

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***Correspondence to:** Elizabeth M. Van Cott; GRJ235, 55 Fruit Street, Boston, MA 02114.
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TABLE I. Features of Commercially Available APC Resistance Assays

Assay name(s)	Clotting mixture	APC source	Interferences: Factor levels	LA	Xa inh.	DTI
Chromogenix Coatest APC Resistance (the "original" assay)	Colloidal silica Phospholipids Calcium	Purified APC	+ [16]	+ [17]	+	(+)
Chromogenix Coatest APC Resistance V (the "modified" or "second-generation" assay)	Colloidal silica Phospholipids Calcium Factor V-deficient human plasma Polybrene (heparin inhibitor)	Purified APC	− [18]	+	+ [19]	+ [20]
Siemens ProC Ac R (formerly also available as LifeTherapeutics GradiLeiden)	RVV-X Phospholipids Calcium Heparin inhibitor	*A. c. contortrix* protein C activator (activates protein C in patient plasma)	+ [21]	(−) [21]	(+)	(+)
Aniara Hemoclot Quanti V-L	Purified factor Xa Phospholipids Calcium Purified fibrinogen, factor II, and protein S Heparin inhibitor	Purified APC	−	+	+	(+)
Pentapharm Pefakit APC-R Factor V Leiden (also available as Sekisui Acticlot Protein C Resistance)	RVV-V Factor V-deficient human plasma Noscarin, a prothrombin activator (Va dependent, calcium, and phospholipid independent) Polybrene (heparin inhibitor)	Purified APC	− [22]	− [22]	(−) [19]	+ [23]

Factor levels, factor deficiency or elevation; LA, lupus anticoagulant; Xa inh., factor Xa inhibitors (e.g., rivaroxaban); DTI, direct thrombin inhibitors (e.g., arbatroban, dabigatran, bivalirudin); Interferences: the plus sign "+" denotes that the presence of the indicated factor has been shown to interfere with the interpretation of the assay, minus sign "−" denotes that the indicated factor has been shown not to interfere with the assay. The plus or minus signs in parentheses "(+)", "(−)" denote that the indicated factor is expected (or not expected) to interfere with the assay but this has not yet been fully investigated. Information about interferences was derived from manufacturer's package inserts and/or published literature. Please also note the following: (1) Factor deficiencies may be due to vitamin K deficiency, warfarin, liver dysfunction, genetic defects, hyperestrogenic states (pregnancy, OCPs decrease protein S), proteinuria, amyloidosis, consumption (e.g., DIC), and acute phase reactions (decrease protein S). Increased factor levels may be due to acute phase reactions (fibrinogen and factor VIII). (2) Low factor V level, which could be due to liver disease, DIC, or genetic deficiency, interferes with the interpretation of all APC-R assays.

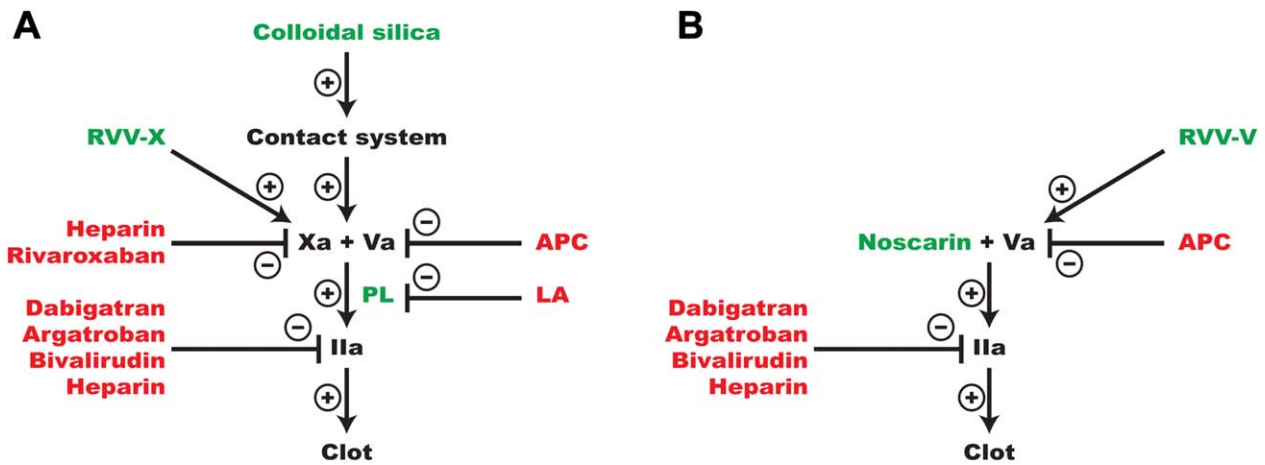

Figure 1. Simplified overview of major components of APC resistance assays discussed in this article. Components which either activate (+) or inhibit (−) the indicated clotting factor [or phospholipid (PL), in the case of lupus anticoagulant (LA)] are shown. (A) Interactions of components of the first four assays listed in Table I are shown. (B) Interactions of components of the fifth assay listed in Table I are shown. RVV-X, RVV factor X activator; RVV-V, RVV factor V activator. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

rivaroxaban, and direct thrombin inhibitors (dabigatran, argatroban, bivalirudin) interfere with the original APC-R assay (Fig. 1A). Platelet contamination of plasma specimens can also lead to altered results with subsequent freezing and thawing [28]. These interferences can result in misclassification. Therefore, a significant effort has been made to modify the APC-R assay to limit interferences.

The "modified," second-generation APC-R assay (e.g., Coatest APC resistance V, Chromogenix AB, Sweden) effectively eliminates the influence of abnormal factor levels [18] by adding a step in which the patient plasma is diluted in factor V-deficient but otherwise normal human plasma. In addition, a heparin neutralizer is added. These modifications increase sensitivity and specificity of FV_{Leiden} detection to almost 100% [29,30]. The plasma dilution step essentially eliminates interference from warfarin, liver dysfunction, low protein S, low factor levels, acute phase reactions, oral contraceptives, and pregnancy. Although the heparin neutralizer is effective for up to 1 U/mL heparin, greater levels can lead to a falsely elevated APC-R ratio (unpublished observations). The effects of specimen freezing and

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platelet contamination are also decreased ([31] and manufacturer's data). Platelet contamination does not interfere unless >20,000 platelets/μL are in the plasma. Direct thrombin inhibitors and rivaroxaban can give falsely elevated APC-R results [18–20,23,32]. Lupus anticoagulants occasionally decrease the APC-R ratio in the absence of FV_{Leiden}, however, some evidence suggests that this might reflect clinically significant acquired APC resistance [33].

Proposals to reduce lupus anticoagulant interference include using higher plasma dilutions such as 1:10 or 1:20 (per manufacturer) or 1:40 and adding excess phospholipid to neutralize the lupus anticoagulant (reviewed in [11]).

Using a normalized ratio appears to reduce intra-assay and interassay variability. This ratio is obtained by dividing the result of the patient's APC-R assay by that of normal pooled plasma. However, some studies report that the use of the normalized ratio has not improved the ability of the assay to distinguish APC resistance from normal [11].

More recently, APC-R assays have been developed that use Russell viper venom (RVV) from the snake *Daboia russelli*. The RVV time is analogous to the aPTT, except that the clotting cascade is initiated by the RVV factor X activator (RVV-X). This sidesteps the contact pathway and thereby eliminates interference by deficiency or elevations of factors VIII, IX, XI, and XII (Fig. 1A). The ProC Ac R assay (Siemens, Washington, DC) involves a dilute RVV time performed with and without APC (treating the specimen with a snake venom from *Agkistrodon contortrix* activates endogenous protein C). There is no dilution into factor V-deficient plasma, therefore low factors II or V <10%, factor X <20%, or protein C <50% [21] interfere (according to the manufacturer). Low fibrinogen, direct thrombin inhibitors, and direct factor Xa inhibitors such as rivaroxaban might also interfere, but we are not aware of data investigating these possibilities. The manufacturer claims that warfarin does not interfere, but if factor II, X, or protein C fall below the indicated thresholds, then we expect warfarin would interfere. The reagent contains a heparin neutralizer, as well as phospholipid intended to remove lupus anticoagulant interference (per manufacturer) [21].

A factor Xa-based clotting time is another type of APC-R assay (Hemoclot Quanti V-L, Aniara, Mason, OH). Factor deficiencies do not interfere with this assay, as specimens are diluted in a proprietary reagent containing purified factor II, fibrinogen, protein S, and APC. Factors VII, VIII, IX, XI, and XII are not involved in factor Xa-based clotting times, therefore presumably they do not interfere. Low factor V does interfere. The reagent contains a heparin neutralizer. The effect of lupus anticoagulants, direct thrombin inhibitors, or direct factor Xa inhibitors is not currently known; however, as the method is clot-based, it is plausible that these could interfere.

Another APC-R assay (Pefakit APC-R FVL, Pentapharm, Basel, CH, and Acticlot Protein C Resistance, Sekisui, Lexington, MA) goes even further with eliminating interferences. Factor deficiencies or elevations are overcome by dilution into factor V-deficient plasma. The role of factor Xa is replaced by Noscarin, a factor Va-dependent but phospholipid-independent prothrombin activator derived from the Tiger snake *Notechis scutatus*. Phospholipid and calcium are not present in the reagent. Phospholipid independence should eliminate interference by lupus anticoagulants [19,22] (Fig. 1B). Factor V is activated by RVV factor V activator (RVV-V, not to be confused with RVV-X) in the presence or absence of exogenous APC. Direct thrombin inhibitors falsely increase the ratio ([23] and manufacturer's data). In one study, rivaroxaban did not affect the ratio result in the one patient with a heterozygous FV_{Leiden} mutation, but falsely increased the ratio in all nine normal individuals (i.e., rivaroxaban made normal results appear even more normal but had no effect on the abnormal result) [19]. Further study is needed. Low factor V levels can interfere with this assay.

Mutations in factor V other than FV_{Leiden} that can cause APC-R have been described, but these appear to be very rare. For example, in the Coagulation Laboratory at Massachusetts General Hospital in Boston, Massachusetts, APC-R testing has been performed in 42,125 cases between 2001 and 2013 (using the "modified" aPTT-based method with dilution into factor V-deficient plasma). All specimens with values less than 2.0 have tested positive for FV_{Leiden} or, occasionally, a lupus anticoagulant.

APC-R Testing in Pediatrics

The original APC-R assay has a higher normal range for newborns than for older children and adults, decreasing to adult range by age 6 months [34]. As with adults, the assay is greatly improved by diluting the patient plasma 1:5 in factor V-deficient plasma. In newborns, a 1:11 dilution performed better than a 1:5 dilution [35]. In children, age 3 months to 16 years, the 1:5 dilution in factor V-deficient plasma correlated well with DNA analysis, but the nondiluted (original) method did not, even when the normalized ratio was used [36]. A 1:11 dilution in these children was not investigated.

Practical Considerations for Laboratories and Clinicians

APC-R testing should not be performed on patients currently receiving direct thrombin inhibitors or rivaroxaban, as falsely normal results may occur with most assays. If necessary, specimens can be tested for direct thrombin inhibitors with a thrombin time, and for factor Xa inhibitors with an anti-Xa assay. As there are currently no FDA-approved calibrators for rivaroxaban or apixaban, an anti-Xa assay calibrated with heparin, low-molecular weight heparin, or fondaparinux can be used to qualitatively detect the presence or absence of rivaroxaban and apixaban.

The presence of FV_{Leiden} can also be determined by DNA testing, which is not affected by any of the interferences described above. However, APC-R assays are advantageous as they are easily automated, cost effective [37,38], and may detect rare causes of APC resistance other than FV_{Leiden}. They are also necessary for detecting pseudohomozygous FV_{Leiden} [11] and for assessing phenotypic FV_{Leiden} thrombophilia in bone marrow or liver transplant patients [39]. APC-R assays that dilute patient plasma into factor V-deficient plasma (or the relevant equivalent for the assay) are much more accurate for detecting FV_{Leiden} than are assays that do not. Although concordance of APC-R assays with DNA analysis is extremely high when the APC-R assay includes a dilution step, it is reasonable for laboratories to perform DNA analysis on patients with abnormal APC-R results, as this practice helps confirm that there has been no specimen mix-up during testing [39]. With most APC-R assays, lupus anticoagulants can cause falsely normal results (Table I). Therefore, if DNA analysis is not available, any patient with an abnormal result should be tested for a lupus anticoagulant.

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