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# Comparison of clot-based and chromogenic assay for the determination of protein c activity

Tariq M. Roshan, Nancy Stein and Xiu Y. Jiang

**Activated protein C inactivates factor Va and VIIIa. Deficiency of this natural anticoagulant may result in recurrent venous thrombosis. Performance characteristics of clot-based and chromogenic protein C activity assays are different. The clot-based assay has limitations because of interference with coagulation inhibitors resulting in spuriously increased protein C levels or underestimation because of elevated levels of factor VIII and Factor V-Leiden mutation. The chromogenic assay is not influenced by such interferences but only detects functional defects of protein C that involve the active site rendering it insensitive to rare mutations. To compare two methods, we conducted a retrospective study from January 2015 to June 2017. Our results showed a good correlation between clot-based and chromogenic assay ($R = 0.94$ and $r^2 = 0.88$). The study of agreement between the two methods by the Bland–Altman method showed that chromogenic method on an average measures 7.8% more protein C than that of clot-based. The results also showed that the bias between the two methods is significant. The positive trend noted was contributed by the values of less than 20% of protein C. Both clot-based and chromogenic assays had high sensitivity; however, the chromogenic assay showed better specificity (97%) as compared with the clot-based assay (93%). In conclusion, we recommend the chromogenic method as the assay of choice, which is also recommended by the College of American Pathologist Consensus Study over activated partial thromboplastin time-based assay. We have shown here that despite a good correlation between the two techniques, there is a difference as highlighted by the difference plots. *Blood Coagul Fibrinolysis* 30:156–160**


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## Introduction

A natural anticoagulant, protein C is a vitamin K-dependent serine protease, which when acted upon by thrombin–thrombomodulin complex is converted into activated protein C (APC). Together with the cofactor protein S and in the presence of calcium ions and phospholipids, APC degrades Factor Va and Factor VIIIa. Both congenital and acquired causes of protein C deficiency are known. The congenital deficiency is inherited in an autosomal fashion with variable penetrance [1] and is resulted from heterogeneous mutations rendering molecular testing for its deficiency impractical [2]. Deficiency of this natural anticoagulant may result in recurrent venous thrombosis and pulmonary embolism with heterozygous deficient patients having a seven-fold increased risk of venous and arterial thrombosis [3,4]. The anticoagulant activity of APC requires three factors including activation of the zymogen, normal substrate recognition and proteolytic activity of the enzyme. Two types of protein C deficiencies have been reported; type I, which is common (75–80%), results in a quantitative decrease in the factor and qualitative-type II deficiency, which is seen in approximately one-third of the patients (2025%). It is recommended that the assay for qualitative defects should be used as an initial test in cases suspected to have protein C deficiency. Once the results from functional assay show decreased levels of protein C, then antigenic assay should be performed to differentiate between type I and type II diseases. Determination of functional protein C, which detects qualitative protein C deficiencies can be done by using clotting and chromogenic techniques [5,6]. The clot-based techniques used partial thromboplastin time (aPTT)-based or Russell viper venom (RVV) methods and the chromogenic (amidolytic assay) technique measures chromogenic substrate resulting in color generation proportional to the protein C concentration. Both of these techniques use activation of protein C by Protac.

The clot-based assay has limitations because of interference with coagulation inhibitors including heparin, direct thrombin inhibitors and lupus anticoagulant, which may result in spuriously increased protein C levels. On the other hand, elevated levels of factor VIII as seen in acute phase response and Factor V Leiden (FVL) mutation may result in underestimation of protein C levels. The chromogenic assay is not influenced by such interferences but only detects functional defects of protein C that involves the active site, rendering it insensitive to

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rare mutations affecting the ability of protein C to interact with thrombin, endothelium, phospholipids and protein S [7,8]. Protein C testing at Calgary Laboratory Services was done by using both clot-based and chromogenic assays. The choice of test selection was influenced by the patient's information. ProClot a clot-based method was used to screen most patients. However, the chromogenic method was used in situations where interference because of certain variables with clot-based assay was anticipated like the use of direct oral anticoagulants (DOACs). Following the standard protocols, protein C was not performed during a thromboembolic event or other medical, surgical or trauma-related illness. In these situations, physicians were advised to wait for 3 months before protein C testing. Similarly, if the test results were reported previously as normal or elevated, protein C testing was canceled. Moreover, repeat testing using different principal (chromogenic) was performed on individuals with abnormal results. This algorithm was helpful in avoiding missing rare cases of functional defects by chromogenic assay alone and in situations, where interference with clot-based assay was possible. Although the use of two different methods can be useful in rare cases of protein C deficiency, this strategy was not cost effective in terms of reagents, quality control-related procedures and staffing. Cooper *et al.* [9] reported that coagulometric assays are not equally sensitive to clinically critical functional defects of protein C and that multiple assays may be required to identify all variants. However, College of American Pathologists (CAP) consensus study recommended using chromogenic assay, given its better performance characteristics. To implement any changes in the process, we analyzed our data in cases where both the clot-based and chromogenic assays were performed.

## Methods

### Study design

To compare these two methods, we conducted a retrospective study from January 2015 to June 2017 and analyzed 344 cases in which both clot-based and chromogenic protein C studies were performed.

### Human samples

The study included samples, which were sent to Diagnostic and Scientific Center, Calgary Laboratory Services, Canada. The samples were collected in trisodium citrate tubes with nine parts of freshly drawn venous blood and one part of trisodium citrate. The samples were processed according to Clinical & Laboratory Standards Institute (CLSI) guidelines [10].

### Protein C activity: chromogenic assay

The chromogenic assay was performed according to the manufacturer's protocol. Briefly, the Instrumentation Laboratory (IL) Protein C kit (HemosIL) (Instrumentation Laboratory Company, Bedford, Massachusetts, USA) is a protein C assay based on a synthetic chromogenic substrate. The protein C level in patient plasma is measured automatically on the ACL TOP (ACL TOP 700, Instrumentation Laboratory Company) in two stages. First, the plasma is incubated with protein C activator (a protein fraction derived from the venom of the copperhead snake *Agkistrodon contortrix contortrix*). In the second step, quantification of activated protein C with a synthetic chromogenic substrate is done. The paronitoaniline released is monitored kinetically at 405 nm and is directly proportional to the protein C in the plasma sample.

The reference ranges for ACL ADVANCE Protein C chromogenic were established using a statistical determination of the central distribution of 52 healthy individuals' results and validated for the ACL TOP using 43 normal individuals' results. The normal ranges are between 0.70 and 1.26 U/ml.

### Protein C activity: clot-based

The IL Test ProClot kit (HemosIL) (Instrumentation Laboratory Company) was used in a functional test to measure protein C activity according to the manufacturer's protocol using ACL TOP instrument (ACL TOP 700, Instrumentation Laboratory Company). Briefly, the test was based on the prolongation of an aPTT assay in the presence of activated protein C. The aPTT (Instrumentation Laboratory Company)-based test is sensitive to Factor V and Factor VIII levels. Activated protein C in plasma samples is generated by Protac, a rapid in-vitro protein C activator (a protein fraction derived from the venom of the copperhead snake *A. contortrix contortrix*). Activated protein C generated in the assay inhibits factors V and VIII, and thus prolongs the aPTT.

The reference ranges were established using a statistical determination of the central distribution of 34 normal subject results and re-established/validated for ACL TOP using 38 normal subject results. The normal ranges are between 0.67 and 1.26 U/ml.

### Calibration

Normal reference plasma and pooled normal plasma were obtained from Precision BioLogic (Dartmouth, Nova Scotia, Canada). CRYOcheck normal reference plasma consists of normal citrated human plasma collected from a minimum of 20 carefully screened donors, which exhibits hemostatic parameters that are representative of a healthy population. The plasma was buffered with 0.01 M HEPES buffer, aliquoted (1.0 ml) and rapidly frozen. The plasma was thawed for 4 min in 37 °C water bath. It was allowed to acclimatize to room temperature and vortex before use. Reference plasma was used to plot calibration curves. Calibration was done on the analyzer ACL TOP with every change in the regent lot number. Calibration allows the conversion of a measured value to a value expressing units of concentration or activity of the analyte. In brief, diluted reference plasma of known factor activity is used to construct a reference curve that

was used to convert values of the test plasma to units of activity. CryoCheck abnormal one reference control for protein C was obtained from Precision BioLogic.

The linearity of both ProClot and the Chromogenic assay was 0.10–1.50 U/ml. A chromogenic assay is not affected by heparin (UF or LMW heparin) up to 2 U/ml, hemoglobin up to 5 g/l, triglycerides up to 10.0 mmol/l and bilirubin up to 360 μmol/l. The ProClot assay is not affected by UF heparin up to 1.5 U/ml, LMW heparin up to 0.70 U/ml, hemoglobin up to 2 g/l, bilirubin up to 428 μmol/l, and triglycerides up to 6.8 mmol/l.

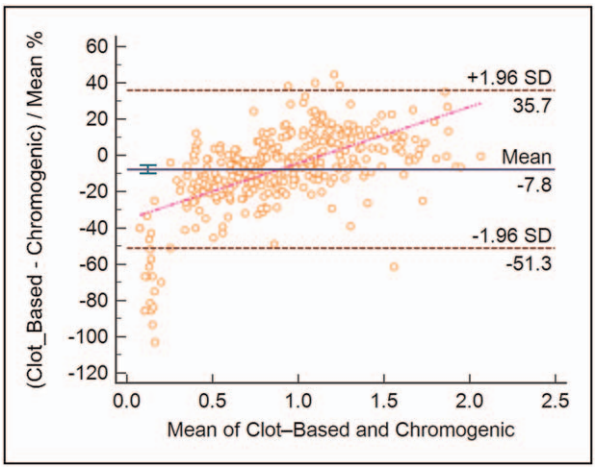
### Data analysis

The data was entered and analyzed using Microsoft Excel 2010 (Microsoft, Redmond, Washington, USA). The graphs were made using MedCalc Software's (VAT registration number is BE 0809 344 640).

## Results

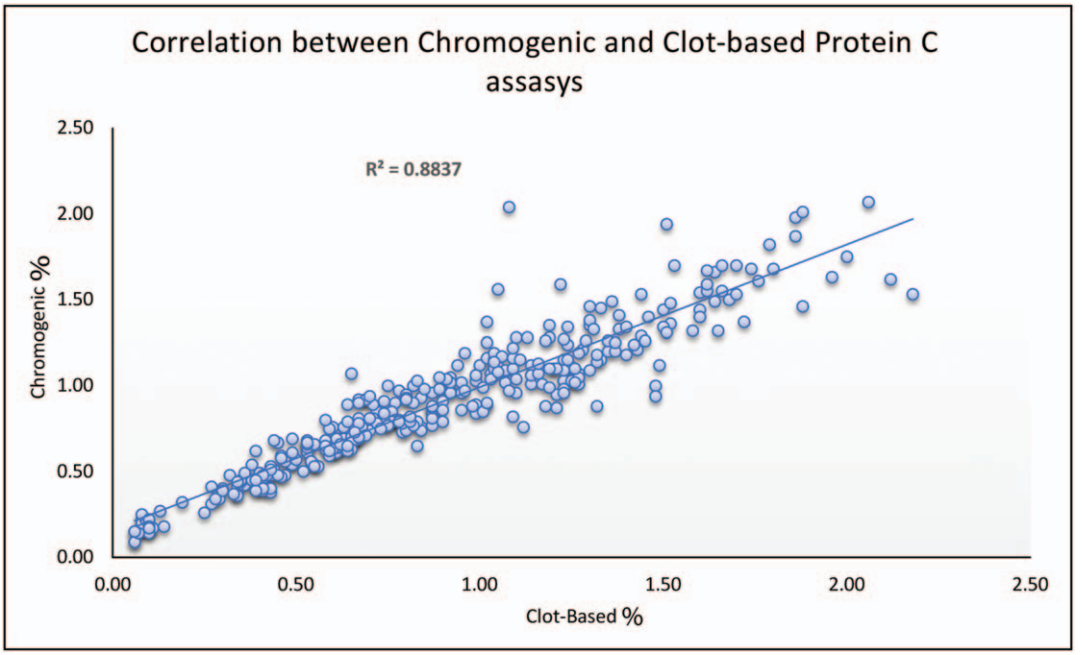
Our results showed good correlation between clot-based and chromogenic assay ($R = 0.94$ and $r^2 = 0.88$) with 95% confidence interval of 0.016 and $P < 0.001$ (Fig. 1). We understand that the correlation studies are used to see the relationship between two variables and do not address the difference between the two methods. As recommended, we use the Bland–Altman method to study the agreement between the values gained by two methods [11]. Difference plot showed that there was an average difference of negative 7.8%, indicating that chromogenic method on an average measure more PC as compared with clot-based method (Fig. 2). The data analysis also showed a positive trend, and that was contributed by the values of less than 20% of the factor or values above 150% of the protein C levels. For concentrations between 20 and 150%, the data is closely comparable with each other by two methods. The bias between the two methods is significant as the line of equality is not within the confidence interval of the mean. The agreement limits are

**Fig. 2**




Bland–Altman graph of a clot-based and chromogenic assay.

**Fig. 1**




Correlation studies of clot-based and chromogenic assay showing good correlation between the two assays.

from −51.3 to 35.7%. A clot-based assay can result in underestimation of protein C activity in Factor V Leiden mutation. However, in our hands, the two of the heterozygous patients for Factor V Leiden mutation had borderline normal clot-based assay results (0.67) whereas chromogenic assays as expected showed normal levels of protein C.

Chromogenic assays are appealing methods because of their insensitivity to aPTT values. In our study as expected, patients with prolonged aPTT had higher values of protein C by the clot-based method. Both clot-based and chromogenic assays had high sensitivity; however, the chromogenic assay showed better specificity (97%) as compared with the clot-based assay (93%).

## Discussion

Protein C deficiency can be because of genetic mutations or acquired conditions. The acquired causes can be physiologic (newborn infants and children) or pathological conditions including but not limited to hepatic disorders, oral anticoagulants, vitamin K deficiency, disseminated intravascular coagulation, acute thrombosis, adult respiratory distress syndrome, plasma exchange, renal insufficiency, some types of chemotherapy and postoperative states [8]. It is well known that the laboratory phenotype does not always predict the potential for VTE formation (clinical phenotype) [12]. The first recommended assay for the screening of protein C deficiency is functional assay as protein C antigenic assays determine the level of protein C and cannot distinguish nonfunctional protein C from a functional molecule. The antigenic assays are recommended once the functional assays are abnormal and used to differentiate between Type I and Type II deficiencies [13]. Functional deficiency of protein C can result from defects in one of the five functional domains of the protein. Type I, quantitative defects are the most common defects and are easy to diagnose while qualitative defects can be active site defect (Type IIa) or cofactor binding defect (Type IIb) [14]. The diagnosis of Type IIb defects is problematic and requires functional assay, which can detect these defects. The two types of functional assays (clot-based and chromogenic) have their pros and cons. There are reports of overestimation of protein C by chromogenic assay because of lack of specificity to the substrate, whereas samples with significant hemolysis, lipemia, and icteric samples can also hinder incorrect estimation of the protein C [9,15]. On the other hand, clot-based assays can result in underestimation of protein C because of elevated levels of Factor VIII and Factor V Leiden mutation. Increased levels of protein S can also result in underestimation of protein C determined by clot-based assays [9]. Because of the effect of therapeutic heparin levels and other anticoagulants on aPTT-based assays; the protein C levels will not be accurate if determined by clot-based assay. Clot-based assays are also subject to interference by lupus anticoagulant, which when present can result in falsely normalized or even increased protein C. On the contrary chromogenic assay may fail to detect type II protein C deficiency where there is a defect in G1a domain (Type IIb), which is essential for the membrane-bound thrombin–thrombomodulin activation of protein C [7]. The recent increase in the use of DOACs in the different clinical situations has also posed a challenge to the coagulation laboratories. It is important for the laboratory personal to be aware of the type of the DOACs in use with the concentration and the time of the last dose. It is reported that Dabigatran results in increase in protein C estimation by clot-based assay. Similarly, anti-Xa, Rivaroxaban also results in elevated protein C levels determined by clot-based assay. These DOACs have no effect on protein C determination by chromogenic assay [16,17].

In any analyte measurement, it is required to establish that the two methods have a good agreement and this is true for a new method, which should have good agreement with an established method to replace the old one. It is noted that often this is inappropriately analyzed by using correlation studies and reported as correlation coefficients. It is a well understood fact that the two methods can have good correlation, but still, there is no agreement between the two methods [11]. This does not undermine the importance of good correlation but enforces that in addition to a good correlation between the two tests, there should be a good agreement as well. Keeping this in mind, we ran the difference analysis of our measurements in addition to correlation studies and showed that despite good correlation, the clot-based assay is at least not in good agreement with the chromogenic assay when factor levels are either less than 20% and above 150%. Although chromogenic assay has better specificity, for a good screening test, sensitivity is what matters the most, and both assays have very high sensitivity. Therefore, both tests can be used as a screening tool. Baron *et al.* [18] in their study concluded that there is a small difference in accuracy and precision of protein C assays and the laboratories can choose different methods entirely based on cost or convenience of any particular method [18]. Here we debate that clinical laboratories can use chromogenic assay as an initial test to screen for a qualitative defect of the protein C. The use of clot-based assay has its inherent interferences because of underlying patient factors and clinical/analytical factors, which can result in either over or underestimation of protein C activity. We also concluded that both the chromogenic and clot-based assays are better at diagnosing or excluding protein C deficiency in patients with extreme protein C levels than in those with borderline levels. Although there is increasing difference in the values obtained by both methods at lower levels of proteins as highlighted by difference plot, the differences in the values are of no

clinical significance as values above 50% of the protein levels are closely comparable.

In conclusion, the chromogenic assay is the assay of choice and is also recommended by CAP Consensus Study over aPTT-based assays because of the reasons mentioned above. Recently, with the increasing use of DOACs, and their effects on special coagulation testing including clot-based assays, supports the use of chromogenic assay [19]. Therefore, we also recommend using a chromogenic assay as the first screening test for protein C deficiency.

## Acknowledgements

### Conflicts of interest

There are no conflicts of interest.

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