Mediterranean Journal of Hematology and Infectious Diseases

Original Article

Correlation to FVIII:C in Two Thrombin Generation Tests: TGA-CAT and INNOVANCE ETP

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Competing interests: The authors have declared that no competing interests exist.

Abstract. ***Introduction:*** **Several thrombin-generation tests are available, but few have been directly compared. Our primary aim was to investigate the correlation of two thrombin generation tests, thrombin generation assay-calibrated automated thrombogram (TGA-CAT) and INNOVANCE ETP, to factor VIII levels (FVIII:C) in a group of patients with hemophilia A. The secondary aim was to investigate inter-laboratory variation for the TGA-CAT method.**
Methods: **Blood samples were taken from 45 patients with mild, moderate and severe hemophilia A. The TGA-CAT method was performed at both centers while the INNOVANCE ETP was only performed at the Stockholm center. Correlation between parameters was evaluated using Spearman´s rank correlation test. For determination of the TGA-CAT inter-laboratory variability, Bland-Altman plots were used.**
Results: **The correlation for the INNOVANCE ETP and TGA-CAT methods with FVIII:C in persons with hemophilia (PWH) was r=0.701 and r=0.734 respectively.**
The correlation between the two methods was r=0.546.
When dividing the study material into disease severity groups (mild, moderate and severe) based on FVIII levels, both methods fail to discriminate between them.
The variability of the TGA-CAT results performed at the two centers was reduced after normalization; before normalization, 29% of values showed less than ±10% difference while after normalization the number increased to 41%.
Conclusions: **Both methods correlate in an equal manner to FVIII:C in PWH but show a poor correlation with each other. The level of agreement for the TGA-CAT method was poor though slightly improved after normalization of data. Further improvement of standardization of these methods is warranted.**

Keywords: Thrombin generation test, Factor VIII, Hemophilia.

Citation: Ljungkvist M., Berndtsson M., Holmström M., Mikovic D., Elezovic I., Antovic J.P., Zetterberg E., Berntorp E. Correlation to FVIII:C in two thrombin generation tests: TGA-CAT and INNOVANCE ETP. Mediterr J Hematol Infect Dis 2017, 9(1): e2017064, DOI: http://dx.doi.org/10.4084/MJHID.2017.064

Published: November 1, 2017 **Received: August 2, 2017** **Accepted: October 9, 2017**

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Introduction. Routine laboratory analyses used to diagnose and monitor drug administration in bleeding disorders are primarily based on one-stage clotting assays and more recently, chromogenic assays. The one-stage clotting assay (based on clot forming endpoint) ends when only 3% of the total amount of thrombin of the coagulation process has been generated.1 Thrombin is a key enzyme in the coagulation cascade, having both pro- and anti-coagulant abilities, so thrombin generation tests (TGTs) are considered to provide a more comprehensive picture of the patient's coagulation capability. The method has demonstrated utility in evaluating the overall hemostatic capacity both in bleeding and thrombotic disorders. There is at present reliable evidence that the method highly reflects the bleeding[2-4] or thrombotic[5-10] risk of patients with different coagulation disorders. It can also be used to predict the response to bypassing hemostatic agents administered to patients with hemophilia and inhibitors.[11,12]

There are several thrombin generation tests on the market, and most of them have not been compared to each other. The first aim of this study was to investigate which of two tests: the thrombin generation assay-calibrated automated thrombogram (TGA-CAT) or the INNOVANCE ETP, correlated best with the factor VIII level in a group of persons with hemophilia (PWH). TGA-CAT uses a fluorogenic substrate while the INNOVANCE ETP uses a chromophore for detection of thrombin generation. For the latter test, the plasma samples are defibrinated by its ETP reagent containing a fibrin aggregation inhibitor.[13] With the TGA-CAT method, this is not required.

Despite the advantages of TGTs, the TGA-CAT is used primarily in research laboratories due to the lack of standardization of the method and the large inter-center variability. However, in recent studies, a number of problems have been addressed, and promising improvements have been made in increasing the level of standardization for pre-analytical and analytical techniques.[14-16] Several investigations have also evaluated different standard reference plasmas' ability to reduce TGA-CAT inter-center variability.[14,15,17] The second aim of this study was to investigate the inter-laboratory variability of the TGA-CAT method between two centers.

Material and Methods

Research subjects. The study material was collected at two hemophilia care centers: The Hematology Center, Karolinska University Hospital, Stockholm, Sweden and the Hemophilia Centre, Belgrade, Serbia. Subjects had severe (<0.01 IU/ml), moderate (0.01-0.05 IU/ml) and mild (>0.05-<0.40 IU/ml) FVIII:C deficiency.[18] The samples from Stockholm were taken from 23 subjects (10 with mild, five with moderate and eight with severe hemophilia A). Patients with severe hemophilia from the Stockholm center were on prophylactic treatment. Time from the last dose of clotting factor was not standardized as samples were taken as part of routine visits. The samples from Belgrade were taken from 17 patients with hemophilia A (six with mild, five with moderate and six with severe disease) all of whom were on on-demand treatment.

Written informed consent was obtained from all subjects prior to the study. The study was approved by the Ethics Committee, Stockholm (Dnr 01-0003; 2006/778-32; 2013/263-32).

Blood samples and plasma preparation. Peripheral venous blood was collected into BD Vacutainer® plastic tubes (Becton Dickinson, Franklin Lakes, NJ, USA) with anticoagulant trisodium citrate (0.129 M, pH 7.4) (one part trisodium citrate and nine parts blood). Plasma was prepared within 60 minutes from venipuncture by centrifugation at 2000xG for 20 minutes at room temperature (RT), then divided into aliquots and stored at -70° C. Frozen samples on dry ice were transported from Belgrade to Stockholm and then from Stockholm to Malmö, and were still frozen and in good condition upon arrival.

Methods. TGA-CAT was measured according to the method described by Hemker et al.[19,20] Briefly, twenty microliters of PPP-reagent LOW (1 pM Tissue Factor (TF) and 4 µM phospholipids (Pl)(TS31.00) and twenty microliters of Thrombin Calibrator (TS20.00) were manually pipetted into the wells of a round-bottom 96 well-microtiter plate (Immulon 2HB, Thermo Scientific, Rochester, NY, USA). All three reagents were

manufactured by Thrombinoscope BV, Maastricht, The Netherlands. Eighty microliters of plasma were added to each PPP-reagent well and its corresponding Thrombin Calibrator well.

The plate was then placed in a Fluoroscan Ascent reader (Thermo Labsystems, Shanghai, China) for a 10 minute, 37°C incubation. Following the incubation twenty microliters of the starting reagent, FluCa-kit (TS50.00), was automatically dispensed into each well by the fluorometer. The wavelengths of 390 nm (excitation) and 460 nm (emission) were used to detect the fluorescence intensity. Thrombin generation curves were calculated by a dedicated software program, Thrombinoscope (Thrombinoscope BV, Maastricht, The Netherlands) version: V5.0.0.742. The TGA-CAT setup, instrumentation, software version, method and reagents, were identical for both laboratories. The intra-assay and inter-assay coefficients of variation (CV) for endogenous thrombin potential (ETP) with the TGA-CAT, Malmö, are 2.3% (n=12) and 9.5% (n=5), respectively.

The second thrombin generation test, INNOVANCE ETP, a chromogenic ETP assay (Siemens Healthcare Diagnostics, Marburg, Germany), was performed in Stockholm on a BCS XP system using C-settings according to the manufacturer´s instructions and as previously described.[21] Dilutions of Innovin, 1:555 and actin, 1:20 are mixed in the proportion 1:2 and this mixture is used as an activator. The intra-assay and inter-assay CV for the area under the curve (AUC) with the INNOVANCE ETP method (C-setting) are 4,7% (n=5) and 5,7%(n=4) respectively.

The FVIII:C was determined with a FVIII assay, Coamatic (Chromogenix, Instrumentation Laboratory SpA, Milano, Italy) on a BCS XP Instrument (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) in Stockholm. In the test of agreement, the TGA-CAT parameters were normalized[17] by dividing the value obtained when analyzing each patient's plasma with that obtained when analyzing CryoCheck pooled normal plasma (Precision BioLogic, Dartmouth, Canada).

Statistical analysis. The associations between parameters were evaluated using Spearman´s (non-parametric) rank correlation test. For the statistical presentation and evaluation of the TGA-CAT inter-laboratory variability, Bland-Altman plots, 45-degree lines, and frequency plots were used.

Results

Correlations. The correlation between the TGA-CAT parameter ETP and the corresponding parameter for INNOVANCE ETP AUC was r=0.546.

Both methods (ETP and AUC) showed a similar correlation to FVIII:C (r=0.734 and r=0.701). The FVIII:C values were divided according to disease severity (severe, moderate and mild) and the results for FVIII:C to ETP and AUC at the group level are shown in **Table 1**. Results grouped by severity showed lower associations compared to the total sample.

Table 1. Correlation coefficient (r) for ETP and AUC to FVIII:C in all patients and grouped by disease severity.

	FVIII:C			
	Severe	Moderate	Mild	All patients
TGA-CAT ETP	0.240	0.172	0.458	0.734
INNOVANCE ETP AUC	0.185	0.305	0.238	0.701

To determine the precision in terms of severity, FVIII levels were grouped as severe, moderate and mild (laboratory severity) and plotted against ETP values (**Figure 1**). Substantial overlap in the groups was observed with both assays, indicating that neither of the methods was considerably better in discriminating among categories of disease severity.

Test of Agreement: TGA-CAT Malmö vs. Stockholm. When performing inter-laboratory tests of agreement, a difference of ±10% is considered to be an acceptable level by most laboratories. In most cases, 95% of the observations need to be within the ±10% acceptance level.[22] The variability between the TGA-CAT results performed in Malmö and Stockholm was rather extensive, but after normalization it was reduced. The results for the ETP parameter are presented in Bland-Altman plots, before and after normalization (**Figure 2**). Before normalization, 29% of the samples were within the ±10% cone of acceptance. Normalization
of the data improved results to 41%, still far from the 95% acceptance level. The results for peak, lag time (LT) and time to peak (ttpeak) are inferior to that of the ETP parameter (**Table 2**).

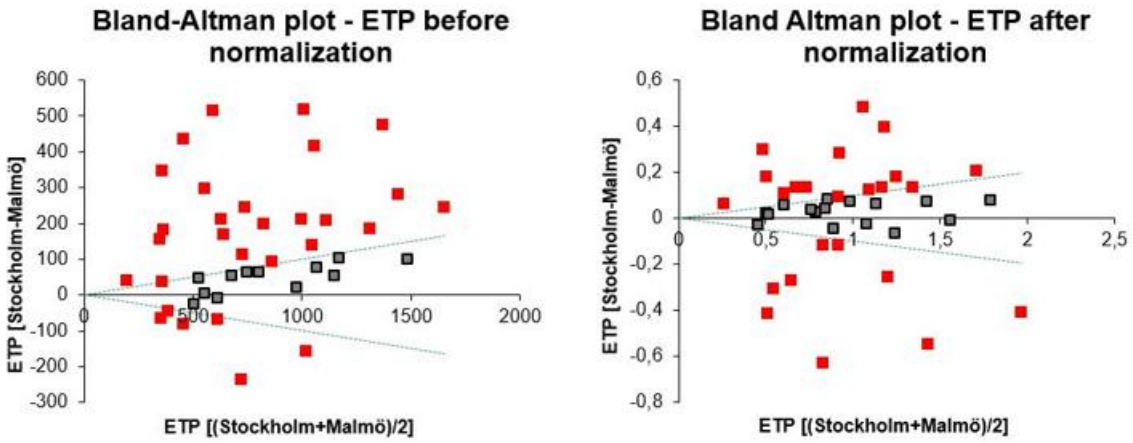

Figure 1. Patients grouped by severity of hemophilia A and plotted against ETP for both methods, TGA-CAT Malmö and ETP INNOVANCE (AUC).

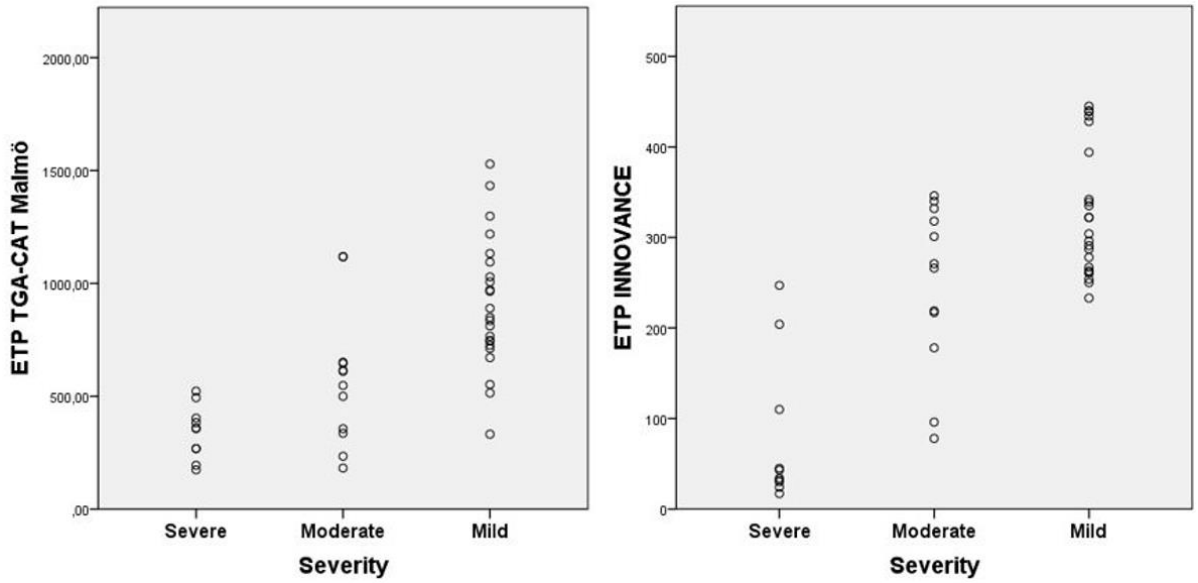

Figure 2. Before normalization: Observations within the green dashed cone represent a ±10% or less difference between ETP values of Stockholm and Malmö results. 29% of the observations lie in the ±10% cone of acceptance. After normalization: Observations within the green dashed cone represent ±10% or less difference between normalized ETP values of Stockholm and Malmö results. 41% of the observations lie in the ±10% cone of acceptance.

Table 2. Level of agreement between Malmö and Stockholm TGA-CAT parameters, before and after normalization.

	ETP	peak	lagtime	ttpeak
Before normalization	29%	15%	0%	20%
After normalization	41%	32%	30%	39%

Discussion. In this study, two thrombin generation methods (TGA-CAT and INNOVANCE ETP) were compared. In our comparison, we choose to focus on ETP and AUC (ETP). Our evaluation showed poor correlation for ETP between the two TGTs. Another study comparing the two reported a good correlation;[23] however, this result was obtained with a higher TF concentration, 10 pM. By inhibiting the intrinsic coagulation with an anti-factor VIII antibody, Devreese et al. showed that the INNOVANCE ETP detected extrinsic coagulation exclusively for all TF concentrations tested (1-300 pM), while in TGA-CAT the amplification of the intrinsic pathway was measured at low TF concentrations (1 and 2.5 pM).[13] In our comparison, we used 1 pM TF in the fluorogenic method (CAT) and a low TF concentration in the chromogenic method (INNOVANCE).

Since we do not know the exact TF concentration for the INNOVANCE ETP method, we cannot know if the difference in TF concentration had a part in the lack of correlation between the methods.

To achieve the desired number of samples for study, specimens from PWH in both Belgrade and Stockholm were used. All plasma samples were single centrifuged, thus, the chance of small amounts of platelets remaining in the plasma cannot be excluded. According to a study by Loeffen, et al.[16] TGA-CAT results were only affected by double centrifugation when the TF concentration was 1 pM or lower. Since our TF concentrations for the TGA-CAT method were 1 pM and 0.5 pM we cannot rule out the possibility that results may have been affected by the single centrifugation.

We did not, however, see a correlation between platelet counts and ETP values (results not shown) which indicates that single centrifugation, instead of the recommended double, did not have a significant impact on the results obtained. There are numerous reports describing contact activation as a reason for the poor reproducibility of TGA-CAT results[16,24,25] and it has been proposed that CTI (corn trypsin inhibitor) should be used for blood sampling. CTI was not used in this study, given the report by Spronk, et al.[26] stating that the addition of CTI, preventing the contact activation pathway, can only be motivated when TF concentrations are 0.5 pM or lower.

Further, we investigated the level of agreement when the same TGA method (TGA-CAT) was performed at two centers (Stockholm and Malmö). The inter-laboratory variation was decreased for all four parameters after normalization with pooled normal plasma, where the ETP results showed the highest concordance, 29% without normalization and 41% with normalization (**Table 2**). Even after normalization more than half of the samples did not reach the level of acceptance. The choice of centrifugation method, blood sampling tubes, and some other pre-analytical factors are of no concern when conducting agreement studies. Of crucial importance is that the characteristics of the plasma are identical for all samples at the start of the analysis. That said, some pre-analytical factors are influential, such as transportation, thawing, resuspension of reagents, pipetting, and time scheme from the end of thawing to start of analysis. In our investigation, thawing was performed identically, 37ºC for 10 minutes. However, we did not have full control of the other factors that may have contributed to the low level of agreement. Interestingly enough, the factor that may have had the greatest impact on the results is one that is out of the control of the lab technician, that is, the analyzing temperature of the measuring equipment. In a report by De Smedt, et al.[27] the importance of pre-heating was shown, leading to a ten-minute 37ºC incubation step before the start of measurement in the latest software version for the method (version: V5.0.0.742). A post-study service the Fluoroscan Ascent reader in Malmö showed a temperature deviation of almost three degrees below the intended and displayed 37°C. Identical measurements were performed at two other Swedish laboratories by the same service engineer using the same measuring equipment. Measurements were approximately 1ºC above and 1ºC below ours. No temperature data from Stockholm was available, but deviation from the intended assay temperature is one possible reason for our large inter-laboratory variability. These divergent measurements indicate the need for temperature calibration in laboratories participating in multicenter studies.

The choice of using 95% of the observations within ±10% as a quality standard for the whole measurement range of the TGA-CAT method could be argued. In several routine coagulation assays, a wider acceptance range is used for measurements in the outskirts of the methods measuring capacity, with acceptance ranges of up to ±15-20% in its high and/or low measurement ranges. It might be justified to use a similar approach for the TGA-CAT method.

The main study limitation is the relatively small number of samples. That might explain the poor discrimination between the disease severity groups.

To conclude, both methods correlate in an equal manner to FVIII:C in PWH but show a poor correlation with each other. When dividing the study material into disease severity groups, both methods fail to discriminate between them. The inter-center variability for TGA-CAT method showed a low level of agreement.

Earlier studies have shown that through enhanced standardization of the assay and pre-analytical factors, the inter-laboratory variability can be reduced to acceptable levels and therefore open up the possibility of conducting multi-center clinical studies.[13,14] Still, further improvement of standardization is warranted for this method.

Acknowledgements. This study was supported by funds from Region Skåne and Lund University

(Regional funds and ALF). Nida Mahmoud Hourani Soutari analyzed the Innovance ETP samples.

Grant Support. J. P. Antovic has granted unrestricted grant from Baxter and has received lecture honoraria from Stago, Siemens, Sysmex, Roche, Baxter and NovoNordisk. None of the other authors declare any conflict of interest.

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