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Harmonization and external quality assessment of antithrombin activity assays

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ABSTRACT

In the framework of a Dutch project named “Calibration 2000” harmonization of antithrombin activity assays was studied. The commutability of potential calibrators for antithrombin was assessed by means of a twin-study design, which is a multicentre, split-patient sample, between-field-methods protocol. The twin-study consisted of simultaneous analysis of fresh-frozen patient plasmas and three potential calibrators for antithrombin by 30 Dutch laboratories forming 15 couples. The state-of-the-art intralaboratory standard deviation (SD_{SA}) was used to assess the commutability of the potential calibrators. The regression line residuals for the potential calibrators were normalized by expressing them as multiples of SD_{SA} . All residuals of the potential calibrators were within the $3 \times SD_{SA}$ limit. One potential calibrator was used in an attempt to harmonize antithrombin assay results in a Dutch field study. The interlaboratory coefficient of variation (CV) of the antithrombin results for three test samples could be reduced from 6.9 – 13.2% (before harmonization) to 5.6 – 9.8% using the common calibrator.

Conclusion: The potential calibrators were commutable. Limited harmonization was achieved by using a common calibrator for all participants.

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Introduction

Antithrombin is a plasma protein critical to the regulation of coagulation [1]. Reduced plasma antithrombin may result from congenital deficiency or arise secondarily from a range of disorders such as liver dysfunction, premature infancy and sepsis, or as a result of interventions such as major surgery or cardiopulmonary bypass. The diagnosis of antithrombin deficiency is established by determination of the antithrombin activity in the patient's plasma [2].

Antithrombin activity is not directly traceable to SI units, but an international reference preparation for antithrombin has been made available since 1978 [3].

Many clinical laboratories measure antithrombin activity using chromogenic substrate assays [4]. External quality assessment (EQA) schemes have shown that the interlaboratory variation of antithrombin assays ranges between 4.5 and 27% CV [5–7]. For the majority of laboratories in a study of antithrombin variation, within-laboratory random error was the main component of the total error [5].

In the Netherlands national EQA scheme for coagulation assays, six surveys per year are provided, with three lyophilized plasma samples

per survey. In this scheme, antithrombin assays are performed by approximately 60 participants.

EQA providers recognised that some specimen materials used in the programmes are not commutable with authentic clinical specimens [8]. Commutability is the closeness of the agreement between the mathematical relationship of the measurement results obtained by two measurement procedures for a stated quantity in a given material, and the mathematical relationship obtained for the quantity in human samples [9].

The Dutch project “Calibration 2000” aimed to harmonize laboratory results via calibration by development of commutable, matrix-based, secondary reference materials [10,11]. As far as we know, the commutability of lyophilized materials for antithrombin activity assays has not been investigated.

The purpose of the present study was to assess the commutability of three potential calibrators for antithrombin assays. In a subsequent study, one of these was selected as a common calibrator for the Dutch laboratories to assess the effect on the interlaboratory variation of antithrombin activity assay results. A flowchart of the study is shown in Fig. 1.

Materials and methods

Assessment of the state-of-the-art standard deviation

The state-of-the-art standard deviation (SD_{SA}) was defined as the median intralaboratory SD of laboratories participating in the

Abbreviations: CV, coefficient of variation; EQA, external quality assessment; IU, international unit; SD, standard deviation; SD_{SA} , state-of-the-art standard deviation; SI, système international d'unités.

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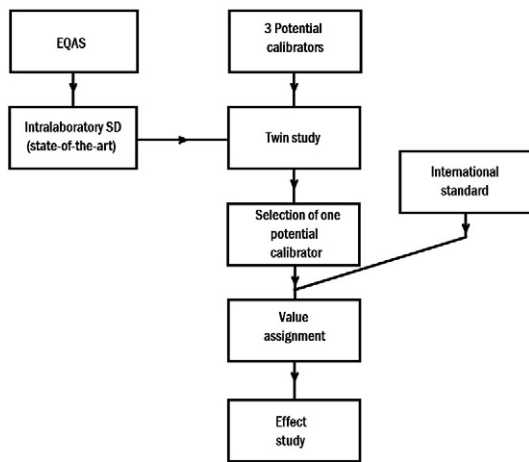


Fig. 1. Flowchart of the “Calibration 2000” antithrombin study. EQAS = External Quality Assessment Scheme.

Netherlands EQA scheme. For the assessment of SD_{SA} six different lyophilized test plasmas were included in 7–12 surveys. Lyophilized test plasmas for the Netherlands EQA scheme were prepared from pooled normal plasmas or pooled patient plasmas (either treated with vitamin K antagonists, or FVIII deficient). Three antithrombin deficient test plasmas were prepared from pooled normal plasmas by affinity chromatography on heparin-sepharose (AT-deficient). All EQA test plasmas were buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid as described by Zucker et al. [12]. Lyophilization was performed by the MCA laboratory (Winterswijk, Netherlands).

For each participant laboratory, the intralaboratory SD was determined for each test plasma, if results from five or more surveys were available. For the six test plasmas, a regression line of the median intralaboratory SD on the mean antithrombin activity was calculated. Using the regression line, the SD_{SA} could be estimated for each antithrombin level.

Although all participants reported antithrombin results in % activity, we transformed these to IU/L assuming that 1000 IU/L corresponds to 100% activity.

Twin-study

Three potential calibrators for antithrombin were purchased from commercial manufacturers: Normal Plasma Control 1 from DiaMed Benelux NV (Turnhout, Belgium), referred to as potential calibrator no. 1; Verify LCA control plasma from Organon Teknika Nederland BV (by this time Kordia, Leiden, Netherlands), referred to as potential calibrator no. 2; Cryo Check Gold Standard Abnormal Reference Plasma 1 (deep-frozen) from Precision Biologic (Dartmouth, NH, Canada), with a stated antithrombin activity of 290–380 IU/L, referred to as potential calibrator no. 3.

The twin-study consisted of the simultaneous analysis of patient plasmas and potential calibrator materials for antithrombin. Thirty laboratories were included and 15 couples were formed. The laboratories acting as partners were selected on the basis of a modest geographic distance between them. Each laboratory couple was asked to select 30 fresh patient plasmas, preferably spanning the relevant activity interval for antithrombin. The clinical conditions of the patients and types of defect were not known to us, but most defects would have been acquired deficiencies. The selected patient samples were considered to represent the routine clinical population. If possible, equal numbers of patients' samples should be collected in the intervals 0–300 IU/L, 300–600 IU/L, 600–1000 IU/L, and >1000 IU/L activity. After these samples were split into two portions and frozen at -20°C , one portion from each sample was transported to the partner laboratory. The three potential calibrators were sent beforehand to each participant. The

interchanged fresh frozen samples and the three potential calibrators were then analysed by both partner laboratories in three runs.

The statistical analysis of the data was performed essentially as described by Baadenhuijsen et al. [10]. The regression residuals of the potential calibrators were expressed as the absolute values of the perpendicular distances of each potential calibrator to the respective patient regression line and were normalised by expressing them as multiples of the state-of-the-art intra-laboratory SD (SD_{SA}). An antithrombin activity-dependent correction of the SD_{SA} was carried out by use of a linear approximation of the precision profile of the intra-laboratory variation. The decision limit for accepting a potential calibrator as commutable was set at three SD_{SA} .

Value assessment

The 2nd international standard for antithrombin in plasma was obtained from the National Institute for Biological Standards and Control (Potters Bar, UK). This standard had an assigned potency of 0.85 IU/ampoule.

Six laboratories were invited to participate in the value assignment of a selected potential calibrator for antithrombin. Each laboratory used the same design for the antithrombin assay, but used their local reagents and instrument. Four laboratories used an assay based on inhibition of factor Xa by antithrombin and measurement of the remaining factor Xa with a chromogenic substrate. Two laboratories used an assay based on inhibition of thrombin by antithrombin and measurement of the remaining thrombin with a chromogenic substrate. Five vials of the potential calibrator and one ampoule of the international standard were reconstituted with water. Three dilutions of each of the five vials of potential calibrator and five dilutions of the international standard in the local dilution buffer were made. The assays of the diluted samples were performed in the following order: international standard, five vials of potential calibrator, international standard. The international standard dilutions were analyzed at the start and at the end of the series to compensate for any drifting. The activity of the potential calibrator was calculated using a calibration line made with the international standard.

Effect of harmonization

One of the potential calibrators (no. 1) was used to determine the effect of harmonization on the antithrombin assay. For this part of the study, three other test plasmas were purchased from commercial manufacturers: Abnormal Control Plasma P from Dade Behring BV (by this time Siemens Healthcare Diagnostics BV, Breda, Netherlands) referred to as test plasma A, Abnormal control plasma Preciplot Plus II from Roche Diagnostics Nederland BV (Almere, Netherlands) referred to as test plasma B, and Normal Plasma Control 1 from DiaMed Benelux NV (Turnhout, Belgium) referred to as test plasma C.

The selected calibrator and the three lyophilized test plasmas (A, B, and C) were mailed to 55 participants of the Dutch EQA scheme who had agreed to participate in the study. Each participant analysed the three test plasmas using the routine calibration line of the antithrombin assay system. In addition, each participant prepared a new calibration curve using the selected calibrator. The antithrombin activities of the three test plasmas were determined by each participant using the new calibration curve. The participants were requested to report their routine and new calibration curves.

Student's *t*-test was used to assess differences in antithrombin levels between methods. A two-tailed *p*-value of less than 0.05 indicated significance. Differences in coefficient of variation (CV) were tested with Snedecor's variance ratio test (*F*-test), as described by Moroney [13]. A graphic system devised by Skendzel and Youden was used to portray the results of the three test plasmas [14].

Results

Assessment of the state-of-the-art SD

Six lyophilized plasmas were analyzed in multiple surveys of the Dutch EQA scheme (Table 1). There was a wide range of intralaboratory variation. The median of the intralaboratory SD was 32 IU/L in two samples with median activities of 408–410 IU/L. Three samples with higher antithrombin activities (880–940 IU/L) had median intralaboratory SD of 45–54 IU/L. There was a significant correlation ($P < 0.01$) between the median SD_{SA} and median antithrombin activity (Pearson's correlation coefficient 0.93). We assumed that there was a linear relationship between median SD_{SA} and median antithrombin activity (AT). A linear regression line was calculated for the six samples and the resulting formula $SD_{SA} = 19 + 0.035 \times AT$ was used for analysis of the data of the twin study.

Twin-study

Results were obtained from 30 laboratories forming 15 pairs. There were 4 pairs of laboratories using the same brand of reagent (Coamatic, based on factor X_a). The other pairs used heterogeneous combinations of reagents. The following median antithrombin activities were reported by the participants for the potential calibrators no. 1, no. 2, and no. 3: 860 IU/L, 670 IU/L, and 290 IU/L, respectively. Although there was a good spread of the patients' antithrombin activities in the majority of laboratory pairs, there were two pairs in which patients' activities were all higher than 600 IU/L. In the latter two pairs, the patients' regression lines had to be extrapolated for the assessment of the perpendicular distance of potential calibrator no. 3 to the regression line. Normalized residuals of the potential calibrators are shown in Fig. 2. Normalized residuals were less than 2 for potential calibrators no. 1 and 2 and less than 3 for potential calibrator no. 3.

It was decided to select potential calibrator no. 1 for the value assignment and the study of the effect of harmonization.

Value assignment

Six laboratories participated in the value assignment of the selected potential calibrator no. 1 for antithrombin. The results of one laboratory were not consistent and could not be used. The other five laboratories provided consistent results. Five dilutions of the international standard were used to construct a calibration line by plotting the change of optical density per minute against international units per ml. A linear regression line was calculated and the activity of the potential calibrator was read from this line. There was good agreement between the values obtained for three different dilutions of the potential calibrator, indicating that the value assignment was valid. The antithrombin activity values of calibrator no. 1 determined by the five laboratories were 870, 850, 800, 880 and 860 IU/L respectively. The mean value (850 IU/L) was used for the assessment of the effect of harmonization.

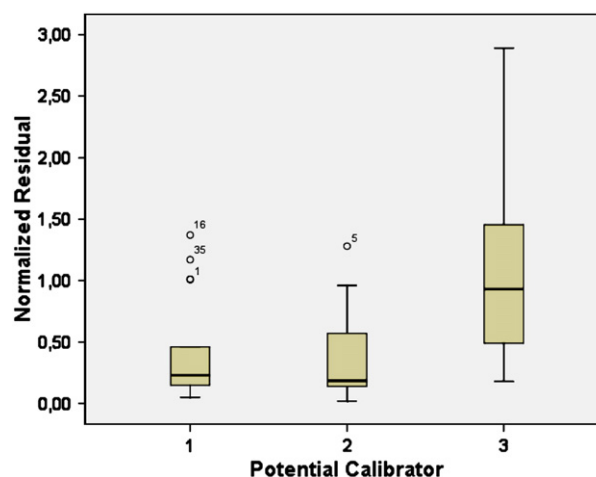


Fig. 2. Box plots of normalized regression residuals for the three potential calibrators to the patients' regression line, as derived from the twin study. Normalized residuals were calculated as multiples of SD_{SA} . Laboratory pairs with outlying residuals are identified by numbers.

Effect of harmonization

The selected potential calibrator no. 1 and three test plasmas A, B, and C were sent to 55 laboratories. Results were received from 51 laboratories. The results of three laboratories were not included in the statistical analysis because of obvious outliers. The results obtained with each participant's routine calibration line were compared to the values obtained with the line constructed with calibrator no. 1 (Table 2). At first, results obtained with thrombin-based assays and with X_a -based assays were evaluated separately. In both groups the interlaboratory variation was reduced by using the common calibrator for all test plasmas. For the combined groups, the interlaboratory coefficients of variation for test samples A and B were significantly lower with the common calibrator ($P < 0.05$). Interestingly, the mean values of the antithrombin activities did not change significantly by using the common calibrator.

Youden plots were used to assess systematic differences between laboratories. Pearson correlation coefficients were determined for antithrombin assay results obtained with different test samples (Table 3). In many cases, the correlation was significant.

Discussion

The present study consisted of three parts, i.e. assessment of the within-laboratory variation of antithrombin assay results, a twin-study to assess the commutability of three potential calibrator materials for antithrombin assays, and a multi-center study to assess the effect of a common calibrator on the harmonization of antithrombin assays (Fig. 1).

Intralaboratory variation, i.e. between-day imprecision, of antithrombin assays was assessed using six freeze-dried plasma samples

Table 1
Antithrombin activity and intralaboratory SD for lyophilized plasmas in regular Netherlands EQA surveys.

Type of test plasma	Number of surveys	Number of laboratories	Mean antithrombin activity per laboratory (IU/L)		Intralaboratory SD (IU/L)		Intralaboratory CV (%)
			Median	Range	Median	Range	Median
AT-deficient #2	8	68	637	570 – 740	46	11 – 100	7.4
AT-deficient #3	7	49	410	330 – 500	32	19 – 86	8.1
AT-deficient #5	8	65	408	320 – 480	32	14 – 77	8.0
Normal	16	65	886	810 – 950	45	18 – 85	5.1
FVIII-deficient	9	52	913	810 – 1010	54	19 – 110	5.9
Coumarin	12	56	945	850 – 1060	52	16 – 114	5.6

Table 2
Antithrombin activity and interlaboratory coefficient of variation in three lyophilized test plasma samples obtained by laboratories using their routine and common calibrator calibration curves. *n* is the number of laboratories. Significant differences in coefficient of variation between routine and calibrator methods are identified (**P*<0.05).

Plasma	Mean antithrombin activity (IU/L)						Inter-laboratory coefficient of variation (%)					
	Thrombin-based (<i>n</i> = 31)		X _a -based (<i>n</i> = 17)		All methods (<i>n</i> = 48)		Thrombin-based (<i>n</i> = 31)		X _a -based (<i>n</i> = 17)		All methods (<i>n</i> = 48)	
	Routine	Calibrator	Routine	Calibr.	Routine	Calibr.	Routine	Calibr.	Routine	Calibr.	Routine	Calibr.
A	326	324	331	315	328	321	13.0	7.9*	13.9	12.8	13.2	9.8 *
B	409	407	413	399	410	404	13.0	7.1*	11.0	7.2	12.2	7.1 *
C	909	892	909	878	910	887	7.9	6.1	5.1	4.7	6.9	5.6

in Dutch EQA surveys. The between-day imprecision varied among the participants (Table 1). It is not clear which factors have a major influence on test performance over time, but the following have been suggested: preparation of reagents and calibrators, pipetting, maintenance of pipettes and equipment [15]. The CV of between-day imprecision depends on the antithrombin activity: the median CV is increasing with decreasing antithrombin activity (Table 1). A similar trend of the between-day CV observed in the present multi-center study was also seen in a single-center evaluation of an automated coagulation analyzer for antithrombin assays [16]. Meijer et al. determined the long-term analytical CV of the antithrombin assay and found median values of 7.2% and 7.6% in two studies [5,15]. These values are in good agreement with the median intralaboratory CV values determined in our study (Table 1).

We assumed that a linear relation exists between the median intralaboratory SD_{SA} and the median of the antithrombin activity. Other authors assumed a linear relationship between analytical variance and concentration, but no evidence was given to support the assumption [17]. In our study, the number of lyophilized test samples was limited to 6 and the median activities of the samples were limited to an interval of 408 to 945 IU/L (Table 1). Furthermore, there was considerable scattering of the points about the linear regression line (correlation coefficient: 0.93). In spite of these limitations, a linear regression line was used to estimate SD_{SA} for any antithrombin activity observed in the twin-study. The error in the estimated SD_{SA} is determined mainly by the random scatter of the points and much less by the type of the assumed mathematical relationship. We believe that a linear relationship is a reasonable assumption for the purpose of the present study.

In the twin-study, there were several combinations of a laboratory using a thrombin-based method linked to a laboratory using a factor X_a-based method. Despite these heterogeneous field method combinations, the normalized residuals for the three potential calibrators were all below the limit of $3 \times SD_{SA}$ (Fig. 2) demonstrating that these materials were commutable. Two of the potential calibrators were freeze-dried (no. 1 and no. 2) and one was deep-frozen (no. 3).

It is common practice for clinical laboratories to construct an antithrombin assay calibration line using dilutions of a single calibrator rather than multiple calibrators. For this reason we selected potential calibrator no. 1 for further studies. Potential calibrator no. 1, which had a higher antithrombin activity than the other two potential calibrators, was assayed against the international standard for

antithrombin. As a result, the assigned mean activity was 850 IU/L. Calibrator no. 1 was then used to study the effect of harmonization. Potential calibrators no. 2 and 3 were not studied further.

For the study of the effect of harmonization, potential calibrator 1 and three lyophilized test plasmas were used. It should be realized that all participants reported antithrombin results routinely in % activity. To compare the routine results with the calibrator results we transformed the activity in % to IU/L. The mean antithrombin activities of the three test plasmas expressed in IU/L did not change significantly by using a common calibrator with an assigned value in IU/L (Table 2). This indicated that the routine calibration lines used by the participants were, on average, in good agreement with the certified international unit value of potential calibrator no. 1. Two test plasmas (A and B) showed a significant reduction of the inter-laboratory variation by using the common calibrator, suggesting that the routine calibration is associated with calibrator bias. On the other hand, the reduction of interlaboratory variation for test plasma C was relatively small and was not significant.

The correlation of antithrombin assay results obtained with different test samples in Youden plots was significant in many cases (Table 3), as a result of systematic differences between participants. Although the systematic differences became smaller after calibration with the common calibrator no. 1, they did not disappear completely. It may be possible that there are systematic differences between laboratories resulting from the reconstitution of the samples or the construction of the calibration line, e.g. differences caused by the dilution series of the calibrator. Further studies are needed to assess the precision of the calibration procedure.

Our study showed that it is possible to reduce the inter-laboratory variation of antithrombin activity by using a common commutable freeze-dried calibrator, especially at low antithrombin activity levels of the test samples. Dutch clinical laboratories may use available calibrators at will. There is no law or official guideline compelling Dutch laboratories to use a single common calibrator. On the other hand, the Dutch Foundation for Quality Assessment in Clinical Laboratories (SKML) is promoting harmonization of laboratory tests by providing common commutable calibrators. SKML may advise clinical laboratories to use available common calibrators and improve the conditions of reporting harmonized results of laboratory tests.

Conflict of interest statement

None (for all co-authors).

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Table 3
Pearson correlation coefficients for antithrombin assay results obtained with test samples A, B, and C in Youden plots. *n* is the number of laboratories. Significant correlations between test samples are identified (**P*<0.05; ***P*<0.01).

Test samples	Thrombin-based (<i>n</i> = 31)		X _a -based (<i>n</i> = 17)		All methods (<i>n</i> = 48)	
	Routine	Calibrator	Routine	Calibrator	Routine	Calibrator
A/B	0.782**	0.700**	0.773**	0.680**	0.775**	0.678**
A/C	0.682**	0.386*	0.246	0.429	0.551**	0.390**
B/C	0.768**	0.555**	0.277	0.560*	0.655**	0.559**

supervising the antithrombin assays for value assignment: Mrs. T. Teeuwen (Laurentius ziekenhuis, Roermond), Mr. J. Smeets (Atrium Medisch Centrum, Heerlen), W. Kluitenberg (Deventer Ziekenhuis, Deventer), Dr. F. Van der Graaf (Maxima Medisch Centrum, Veldhoven), Mr. M.A. Kariman (Jeroen Bosch Medisch Centrum, 's-Hertogenbosch), Dr. S.C. Eindhoven (Slingeland ziekenhuis, Doetinchem). Last but not least, the participants of the twin-study and the effect-study are acknowledged for their work.

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