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Harmonisation of factor VIII:C assay results: study within the framework of the Dutch project 'Calibration 2000'

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Summary

In a Dutch project for harmonisation of factor VIII coagulant activity (FVIII:C) assays, the commutability of potential calibrators for FVIII:C was assessed by means of a 'twin-study design', which is in essence a multi-centre, split-patient sample, between-field-methods protocol. Commutability was defined as the degree to which a material yielded the same numerical relationships between results of measurements by a given set of measurement procedures as those between the expectations of the relationships for the same procedures applied to those types of material for which the procedures were intended. The study consisted of the simultaneous analysis of fresh frozen patient plasmas and three potential calibrators for FVIII:C by 16 Dutch laboratories forming eight couples. The state-of-the-art intra-laboratory standard deviation was used to assess the commutability of the potential calibrators. One potential calibrator was used to harmonise FVIII:C assay results in a Dutch field study. The inter-laboratory coefficient of variation of two test samples could be reduced significantly, but no significant effect was observed with three other test samples. We recommend that at least three different sample dilutions be used in each FVIII:C assay, in agreement with previous recommendations.

Keywords: factor VIII:C assay, calibration, commutability, harmonisation, external quality assessment.

Factor VIII coagulant activity (FVIII:C) assays are used mainly for the diagnosis and treatment of haemophilia A and in the evaluation of von Willebrand disease. Many studies have shown considerable inter-laboratory variation in FVIII:C assay results (Arkin *et al*, 1992; Preston, 1998). An important contributor to inter-laboratory differences is the lack of a uniform calibrator among laboratories (Arkin *et al*, 1992; Preston & Kitchen, 1998).

In the Netherlands national external quality assessment scheme (EQAS) for coagulation assays, six surveys per year are provided, with three lyophilised plasma samples in each survey. In this scheme, FVIII:C assays are performed by approximately 35 participants. The inter-laboratory variation depends on the FVIII:C activity in the test samples, but the lower limit is approximately 11% coefficient of variation.

External quality assessment providers recognised that some specimen materials used in the programmes are not commutable with authentic clinical specimens (Miller, 2003). Commutability is defined as the degree to which a material yields the same numerical relationships between results of measurements by a given set of measurement procedures, purporting to measure the same quantity, as those between the expectations of the relationships for the same procedures applied to those types of material for which the procedures are intended.

The Dutch project 'Calibration 2000' aimed to harmonise laboratory results via calibration by development of commutable, matrix-based, secondary reference materials (Baadenhuijsen *et al*, 2002). As far as we know, the commutability of lyophilised materials for FVIII:C assays has not been investigated.

The purpose of the present study was to assess the commutability of three potential calibrators for FVIII:C assays. Subsequently, one of these was selected as a common calibrator for the Dutch laboratories in an attempt to harmonise FVIII:C results.

Materials and Methods

The 4th international standard for FVIII and von Willebrand factor in plasma was obtained from the National Institute

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for Biological Standards and Control (Potters Bar, UK). This standard had an established activity of 0·57 IU/ampoule for FVIII:C activity (Hubbard *et al*, 2001). Potential calibrators for factor VIII:C were purchased from commercial manufacturers: Control Plasma N lot no. 502719B from Dade Behring (Marburg, Germany), referred to as potential calibrator no. 1; STA Preciclot Plus II lot no. 601562 from Roche Diagnostics (Mannheim, Germany), referred to as potential calibrator no. 2; Cryo Check Gold Standard Abnormal II Reference Check lot no. 9020 (deep-frozen) from Precision Biologic (Dartmouth, NH, Canada), referred to as potential calibrator no. 3.

Lyophilised test plasmas were prepared from pooled normal plasmas or pooled patient plasmas (either treated with oral anticoagulants, or FVIII deficient). These plasmas were buffered with *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulphonic acid as described by Zucker *et al* (1970). One lyophilised test plasma, J.T. Baker Coagulation Control Plasma level III lot no. 129, was purchased from Mallinckrodt Baker B.V. (Deventer, the Netherlands).

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

The state-of-the-art standard deviation (SD_{SA}) was defined as the median intra-laboratory SD of all laboratories participating in the Netherlands EQAS. Eight lyophilised test plasmas were included in four to nine surveys. The test plasmas were coded differently in each survey. Although all participants reported FVIII:C results in % activity, we transformed these to U/ml assuming that 1 U/ml corresponds to 100% activity. For each participant, the SD was calculated for each test plasma if results from three or more surveys were available. The SD_{SA} was determined for each test plasma separately and could be fitted to the empirical formula

$$SD_{SA} = a + b(FVIII : C)^{1/2},$$

in which FVIII:C is the activity in U/ml.

Value assignment

Five laboratories participated in the value assignment of the selected potential calibrator for FVIII. Each laboratory used the same design for the FVIII:C assay, but used their local activated partial thromboplastin time (aPTT) reagent and instrument. 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 calibrator and five dilutions of the international standard in the local dilution buffer were made. The aPTT values of the diluted samples were determined in the following order: international standard, five vials of calibrator, international standard. The international standard dilutions were tested at the start and the end of the measurements to detect drifting. The logarithms of the clotting times were plotted against the logarithms of the dilutions. The activity of the calibrator was calculated against the established activity of the international standard as described by Kirkwood and Snape (1980).

Twin-study

The twin-study consisted of the simultaneous analysis of patient plasmas and potential calibrator materials for FVIII:C. Sixteen laboratories were included and eight 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 concentration interval for FVIII:C. 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 on dry ice. The interchanged 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* (2002). 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}). A FVIII:C activity-dependent correction of the SD_{SA} was carried out by use of a square root 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}.

Effect of harmonisation

One of the potential calibrators (no. 1) was used to determine the effect of harmonisation on the FVIII:C assay. The selected calibrator and five lyophilised test plasmas were mailed to 36 participants of the Dutch EQAS. Each participant analysed the five test plasmas using the routine FVIII:C assay system and calibration curve.

Differences between FVIII:C activity were tested with Student's *t*-test on paired observations. Differences in coefficient of variation (CV) were tested with Snedecor's variance ratio test (F-test), as described by Moroney (1968).

Results

Assessment of the state-of-the-art SD

Eight lyophilised plasmas were analysed by participants of the Dutch EQAS, in multiple surveys. For example, one plasma sample with a median FVIII:C activity of 0·08 U/ml was analysed by 30 participants in four or five separate surveys. The intra-laboratory SD of the FVIII:C activity ranged from 0 to 0·066 U/ml. The median of the intra-laboratory SD for this sample was 0·014 U/ml. The median SD for the eight plasmas was plotted against the square root of the median FVIII:C

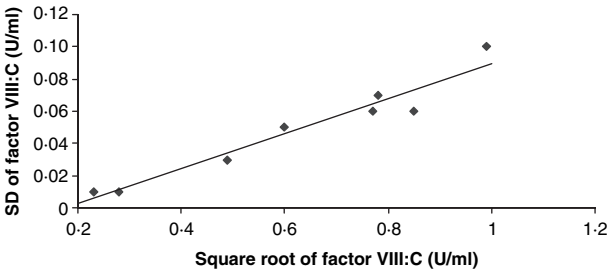

Fig 1. Median intra-laboratory standard deviation of FVIII:C (U/ml) as a function of the square root of the median FVIII:C activity (U/ml) for lyophilised test plasmas in the Netherlands external quality assessment scheme.

activity (Fig. 1). A linear regression line was calculated and the resulting formula $SD_{SA} = -0{\cdot}019 + 0{\cdot}109(FVIII)^{1/2}$ was used for analysis of the twin-study.

Twin-study

Results were obtained from 16 laboratories forming eight pairs. The following median FVIII:C activities were reported by the participants for the potential calibrators no. 1, no. 2 and no. 3: 0·96, 0·46 and 0·08 U/ml respectively. Examples of regression lines through patient results together with the three potential calibrators are shown in Fig. 2. The regression equations are given in Table I. The normalised regression residuals of the three potential calibrators are shown in Fig. 3. The normalised residuals for potential calibrators no. 1 and no. 2 were all <3 SD_{SA}. In contrast, four residuals for potential calibrator no. 3 were >5 SD_{SA}.

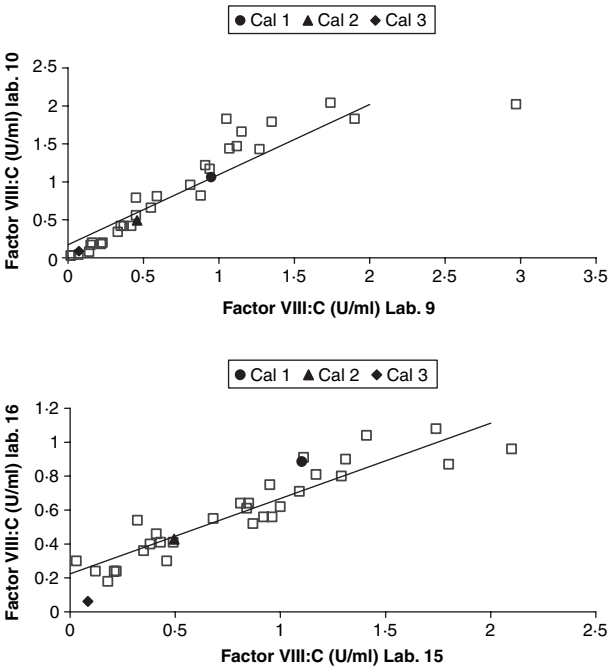

Fig 2. Regression line through patient results together with the potential calibrators' results of two laboratory couples.

Table I. Linear regression equations for factor VIII coagulant activity results (U/ml) obtained by partner laboratories.

Laboratory number				
x-axis	*y*-axis	Slope	Intercept	Correlation coefficient (R^2)
3	4	0·893	0·064	0·909
5	6	0·819	0·075	0·660
7	8	1·038	0·033	0·987
9	10	0·923	0·173	0·824
11	12	1·104	−0·020	0·879
13	14	0·588	0·199	0·887
15	16	0·444	0·224	0·851
37	38	1·855	−0·346	0·771

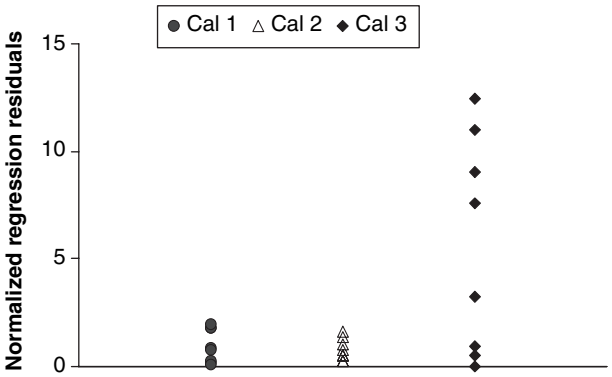

Fig 3. Normalised regression residuals for the potential calibrators to the patient regression line, as derived from a twin study including eight Dutch laboratory pairs. Normalised residuals were calculated as multiples of SD_{SA}.

It was decided to select potential calibrator no. 1 for the value assignment and the study of the effect of harmonisation. This choice was based also on the fact that all the laboratories used dilutions of a single calibrator instead of multiple calibrators to construct a calibration line.

Value assignment

For each laboratory, the logarithms of the mean aPTT values were plotted against the logarithms of the dilutions. The lines for the standard and the selected potential calibrator no. 1 were parallel and the horizontal displacement between them (log R) was calculated. The activity of calibrator no. 1 was calculated as the product of R and the activity of the international standard. The calculated activities of calibrator no. 1 by the five laboratories were 0·84, 0·84, 0·99, 0·83 and 0·92 IU/ml respectively. The mean value (0·88 IU/ml) was used for the assessment of the effect of harmonisation.

Effect of harmonisation

Results were obtained from 35 laboratories participating in the Dutch EQAS. Fifteen of these had analysed the lyophilised test

samples in three dilutions (group 1), as recommended by the organisers of the scheme. The remaining 20 laboratories (group 2) had analysed the test samples in only one or two dilutions. The results of the two groups were analysed separately and all together. The results obtained with each participant's routine calibration curve were compared with the values obtained with the curve constructed with calibrator no. 1. There was no significant difference between the FVIII:C activities obtained with the routine and calibrator no. 1 calibration curve (Table II), but there was a trend to slightly lower FVIII:C activities obtained with the calibrator no. 1 curve. The inter-laboratory coefficients of variation are shown in Table II. In group 1, the inter-laboratory CV was significantly lower with the calibrator no. 1 curves than with the routine calibration curves for test sample A only. In group 2, the CV was significantly lower with the calibrator no. 1 curves for test samples A and D. The reduction of the CV for test sample A was mainly because of one participant whose FVIII:C activity rose from 0·20 U/ml with the routine calibration curve to 0·32 U/ml with the calibrator no. 1 curve. The fall in CV for test sample D was mainly because of another participant who reported 0·23 U/ml with the routine calibration line and 0·08 U/ml with the calibrator no. 1 curve. Although the result of 0·23 U/ml could be classified as outlying, we included all results for calculation of the CV. When the results of groups 1 and 2 were analysed together, the CVs with the calibrator no. 1 curves were significantly lower for test samples A and D. When comparing the CVs for groups 1 and 2, we found that the CVs for test plasmas A and C were significantly lower for group 1 (Table II).

Discussion

The inter-laboratory variation of FVIII:C assay results is considerable. In several studies the CV range was between 20% and 30% or even higher (Arkin *et al*, 1992; Preston, 1998). A number of variables that may contribute to inter-laboratory variation have been suggested, such as lack of calibrated reference material, assay design, factor-deficient substrate plasma, and selection of reagent and instrument. The present study investigated the utility of a common calibrator plasma.

The state-of-the-art intra-laboratory SD was estimated from repeated measurements in freeze-dried plasmas. We assumed that the freeze-dried plasmas were appropriate for the purpose. In the following phase of the study, the commutability of three potential calibrator plasmas was assessed by means of a 'twin-study' design, which is in essence a multicentre, split-patient sample, inter-field-methods protocol (Baadenhuijsen *et al*, 2002).

The present study showed that two lyophilised calibrators were commutable according to the criteria defined previously (Baadenhuijsen *et al*, 2002). The lack of commutability of potential calibrator no. 3 may be due to its low FVIII:C activity and the fact that the regression line for the patient samples was determined mainly by samples with high FVIII:C activities. It cannot be excluded that potential calibrator no. 3 would have been found commutable if the regression line had been determined mainly with low FVIII:C patients' samples. This is a limitation of the present study.

It is common practice for clinical laboratories to construct a FVIII:C 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 the higher FVIII:C activity, was assayed against the international standard for FVIII:C. As a result, the assigned mean activity was 0·88 IU/ml. Calibrator no. 1 was then used to study the effect of harmonisation. Five lyophilised test plasmas were included. It should be realised that all participants reported FVIII:C results routinely in % activity. To compare the routine results with the calibrator results we transformed the activity in % to U/ml. The mean FVIII:C activities of the plasmas expressed as U/ml did not change significantly by using a uniform calibrator with a certified value in IU/ml. This indicated that the routine calibration lines used by the Dutch laboratories were in good

Table II. Mean factor VIII coagulant activities (FVIII:C) (U/ml) and inter-laboratory coefficient of variation (%) of five lyophilised plasma samples obtained by two groups of laboratories, each using both routine and calibrator no. 1 (cal. no. 1) calibration lines. Group 1 laboratories analysed the samples in three dilutions. Group 2 laboratories analysed the samples in one or two dilutions.

	Mean factor VIII:C activity (U/ml)						Inter-laboratory coefficient of variation (%)					
	Group 1 ($n = 15$)		Group 2 ($n = 20$)		All ($n = 35$)		Group 1 ($n = 15$)		Group 2 ($n = 20$)		All ($n = 35$)	
Plasma	Routine	Cal. no. 1	Routine	Cal. no. 1	Routine	Cal. no. 1	Routine	Cal. no. 1	Routine	Cal. no. 1	Routine	Cal. no. 1
A	0.34	0.33	0.33	0.33	0.34	0.33	10.9	6.5*	19.4†	13.3*	16.0	10.8*
B	0.87	0.87	0.87	0.84	0.87	0.85	10.8	11.3	11.6	10.3	11.1	10.7
C	0.54	0.53	0.55	0.54	0.54	0.54	7.0	6.6	14.4‡	12.6	11.8	10.4
D	0.08	0.08	0.09	0.08	0.09	0.08	35.5	29.1	44.5	22.3**	40.9	25.2**
E	0.32	0.33	0.34	0.34	0.33	0.33	9.4	9.3	13.1	12.9	11.9	11.4

n, number of laboratories.

Differences in coefficient of variation were tested using Snedecor's *F*-test (*, significant difference at 5% level; **, significant difference at 1% level).

†Significant difference in coefficient of variation between groups 1 and 2, at 5% level.

‡Significant difference in coefficient of variation between groups 1 and 2, at 1% level.

agreement with the certified international unit value of potential calibrator no. 1. Two test plasmas (A and D) showed a significant reduction of the inter-laboratory variation. The two test samples with the significant reduction of the CV had relatively low FVIII:C activities. We did not assess the commutability of the five lyophilised test plasmas. It cannot be excluded that the absence of effect of harmonisation with three test plasmas (i.e. B, C and E) is because of non-commutability. On the contrary, the CV for B, C, and E were already relatively low with the routine calibration lines. It is possible that, for these test plasmas, the major part of the inter-laboratory variation was not because of calibrator bias, but differences in reagents, instruments, and design of the assay. In this respect, it is worth noting that the CV in group 2 was significantly greater than in group 1 for test plasmas A and C. It is inferred that an assay design in which three sample dilutions are tested produces more uniform results than a design in which only one or two dilutions are tested. It is recommended to use at least three different sample dilutions in each FVIII:C assay, supporting previous recommendations (Over, 1984). We conclude that the use of a commutable lyophilised FVIII:C calibrator resulted in a limited reduction of the inter-laboratory variation assessed with lyophilised test plasmas.

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