One-stage vs. chromogenic assays in haemophilia A

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Abstract

Haemophilia A severity is closely correlated to the factor VIII (FVIII) activity, which can be measured in different ways. The original one-stage clotting assay is still the most widely used. The two-stage coagulation assay eliminated many of the drawbacks of the one-stage assay and was further developed into the chromogenic assay, a two-staged test with purified coagulation factors in the first stage, and a FXa-specific chromogenic substrate in the second stage. In many patients with mild or moderate haemophilia A, there is a discrepancy between the one-stage and the two-stage assays. If only the one-stage assay is used, some patients will have normal FVIII levels and not be diagnosed as having haemophilia or be considered to have a milder bleeding risk than is the case. Other patients who have normal FVIII activity will be diagnosed as haemophilia A. All haemophilia treatment centre laboratories should have access to both one-stage and chromogenic FVIII:C assays. Appropriate standards should be employed to enable accurate FVIII:C measurement. Different assays to measure inhibitor activity to infused FVIII have been developed since 1959. Inhibitor results based on the one-stage or chromogenic FVIII:C assays are well correlated, but the one-stage assay may be influenced by nonspecific inhibition.

Keywords: inhibitors; haemophilia; chromogenic assays; one-stage assay

Haemophilia A is caused by an absence or reduced amount of factor VIII (FVIII) in the blood or a dysfunctional FVIII protein with reduced procoagulant activity. The severity is closely correlated to the FVIII activity, which can be measured in different ways. FVIII is a coenzyme, which means that its activity must be measured indirectly through the action of its target enzyme, factor IX which in turn converts factor X to active factor X (FXa).

The one-stage clotting assay, which was based on the partial thromboplastin time, originally described in 1953 and later modified into the activated partial thromboplastin time (APTT), is still the most widely used. The principle is to measure by what extent a plasma sample corrects the prolonged coagulation time of FVIII-deficient plasma in an APTT-based assay. The result may be affected by any coagulation factor in the intrinsic coagulation system, lupus anticoagulant, heparins, etc. The two-stage coagulation assay, where FXa is produced in the first incubation stage with FVIII activity being rate limiting and the amount of FXa is estimated in a second clotting stage, eliminated many of the drawbacks of the one-stage assay and was further developed into the chromogenic assay, a two-staged test with purified

coagulation factors in the first stage, and a FXa-specific chromogenic substrate in the second stage.

One-stage assay

In the one-stage assay, FVIII-deficient plasma is added to test plasma and the APTT reagent, mixed and incubated for 3–5 min. This is the contact activation phase during which factor XI (FXI) is activated, but little happens to FVIII. The mixture is then recalcified, and the coagulation time is recorded. This takes approximately 40–140 s depending on the APTT assay and the FVIII content. To determine the FVIII:C, the clotting time is compared to a standard curve which is constructed by plotting the clotting times of serial dilutions of standard plasma (FVIII 0–200%) vs. factor VIII activity on logarithmic/linear scale graph paper. The World Federation of Haemophilia (WFH) recommends a parallel line analysis where identical dilutions of the test and standard plasma are compared [1]. The parallel line analysis decreases variability and increases precision. If non-parallel lines are obtained, this may indicate technical error or the presence of an inhibitor or lupus anticoagulant.

Two-stage clotting assay

The two-stage clotting assay, devised in 1955, is only performed in a few specialised laboratories today. FVIII is the rate-limiting factor (normal/excess of everything except FVIII) and in the first stage, diluted sample plasma, which has been treated with $Al(OH)_3$ to remove prothrombin, is mixed with a combined reagent (human serum, factor V, phospholipids and calcium). This leads to generation of FXa, and in the second stage, a subsample from the first stage is mixed with normal plasma, providing prothrombin and fibrinogen, and the clotting time is measured to deduce the amount of FVIII present. There is no need to use FVIII-deficient plasma.

Chromogenic assay

This method has largely superseded the two-stage assay. The assay is similar to the two-stage FVIII assay, in that it involves an incubation step where FVIII activity is rate limiting to generate FXa and a second stage to determine the amount of FXa produced. However, in the chromogenic assay, a reagent containing purified coagulation factors (FIXa, FX and thrombin) in optimal concentrations is used in the first stage, which does not rely on the extrinsic or intrinsic initiation pathways as the thrombin activates FVIII. The amount of FXa generated in the first stage is measured by its action on a specific chromogenic substrate, which releases a chromophore upon cleavage that absorbs light of a certain wavelength. The colour intensity produced is directly proportional to the amount of FXa, which in turn is directly proportional to the FVIII activity in the sample [2].

Which assays are used?

Based on a survey from the ECAT Foundation, a European external quality assessment programme for laboratories in the field of haemostasis performed in 2013, it could be seen that 193 of 214 participants used the one-stage assay (90.2%) whereas 13 used the chromogenic assay (6.1%). Eight laboratories reported using the two-stage clotting assay (3.7%). Of the laboratories using the one-stage assay, many different methods were used, with 16 different APTT reagents, whereas only five different chromogenic kits were used.

Factor VIII

FVIII is a coenzyme of 2332 amino acid residues (300 kDa). It is comprised of five domains: A1, A2, B, C1 and C2 which in the secreted form are arranged as a heterodimer with a heavy chain and a light chain (Fig. 1) [3]. FVIII is tightly bound to von Willebrand factor (VWF) in the circulation by the C2 domain. VWF protects against degradation and inhibits the FVIII–phospholipid interaction. The C2 domain interacts with phospholipids after activation. After cleavage by thrombin, FVIII becomes activated and is in the form of a heterotrimer consisting of A2, A1 and A3–C1–C2 domains. The A2 loosely adheres to the A1 and A3 domains. The heterotrimer has a half-life of 2 min, the A2 domain is dissociated in a time-dependent manner leaving the inactive FVIII comprising the A1 and A3–C1–C2 domains. Activated protein C is a natural regulator of FVIIIa and acts by limited proteolysis at specific arginine residues that renders the molecule inactive (FVIIIi).
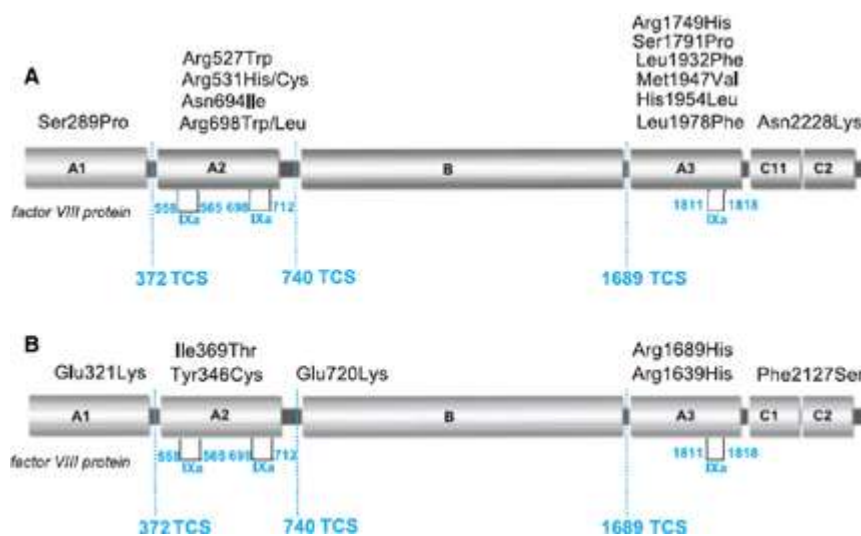

Figure 1. Localisation of missense mutations on the factor VIII protein (presented in its domain structure; numbers: amino acid position) contributing to higher FVIII:C measured by (A) one-stage assay gives higher results than chromogenic method: genetic defects clustered in the A1/A2/A3 domain interfaces. (B) chromogenic assay gives higher results than one-stage method: genetic defects clustered around thrombin cleavage sites (TCS) and factor IX binding sites (IXa: activated factor IX binding sites) (from 3)

Assay discrepancy

In a large proportion of patients with mild or moderate haemophilia A, there is a systematic discrepancy between the one-stage and the two-stage (including chromogenic) assays. The classic discrepancy was first reported in 1976 when a lower value for the two-stage assay compared to the one-stage was found [4]. Bleeding symptoms were consistent with the two-stage and chromogenic assays. In reverse discrepancy, which was first reported in 2002 [5], the one-stage gives lower results than the two-stage or chromogenic assays. These patients display few or no bleeding symptoms. No discrepancy has been found in the assays in patients with severe haemophilia.

Generally a twofold difference between the methods has been used to define discrepancy.

Several genetic mutations have been described to be associated with these discrepant observations. The mutations result in a dysfunctional protein, but the FVIII antigen levels may be normal or even high (CRM+ mutations).

In discrepancies where the one-stage assay gives higher results than the two-stage or chromogenic method, genetic defects are clustered in the A1–A2–A3 domain interfaces (Fig. 1A) [3]. The mutations in the A1–A2–A3 domain interface cause reduced stability of the FVIIIa heterotrimer and increased A2 dissociation. This does not affect the one-stage assay as much as the result of the two-stage assay, probably because of the longer reaction time of the two-stage assay.

In discrepancies where the two-stage or chromogenic assays give higher results than the one-stage assay, seven genetic defects have been described that are clustered around thrombin cleavage sites (TCS) and factor IXa binding sites (IXa: activated factor IX binding sites), causing an impaired FVIII activation by thrombin or an impaired binding of FVIII to FIXa (Fig. 1B) [3]. In the chromogenic assay, these effects are overcome by the supraphysiological concentrations of thrombin and FIX as well as the longer reaction time, compared to the one-stage assay.

Prevalence of assay discrepancy

The prevalence of assay discrepancies depends on the definition, populations and screening methods. Various authors have reported on this phenomenon. In a study of 133 patients with haemophilia (PWH) by Parquet-Gernez *et al*. in 1988 [6], the authors found a clear discrepancy between the procoagulant activity levels obtained with the one- and two-stage assays in 11/73 patients (15%) with mild or moderate haemophilia. In 1994, Duncan *et al*. [7] carried out a study of 95 patients with haemophilia A. They found that results were equivalent in all 21 patients with severe haemophilia (16 families) and in 45 of the patients with mild or moderate haemophilia (18 families). However, the results were discrepant (FVIII:C by the one-stage assay was two- to sevenfold higher than by the two-stage assay) in the other 29 patients (39%) with mild or moderate haemophilia (12 other families). They concluded that in some families with haemophilia A, the gene defect leads to a discrepancy between the one-stage and two-stage assay results and may be more widespread than previously recognised. Cid and co-workers, in a study of 163 patients with mild haemophilia A, in 2008 [8], found discrepancies in 20% of the patients, most of whom had higher levels of FVIII:C with the one-stage assay. In 2009, Poulsen *et al*. [9] carried out a study of 109 patients with mild haemophilia A, among whom 92 were eligible to enter the study. They reported that an assay discrepancy pattern was found quite frequently among their mild haemophilia A families with 36% showing a lower two-stage assay compared to the one-stage assay. However, when they selected a cut-off level for the FVIII:C chromogenic/FVIII:C clot ratios at 0.7, 0.6 and 0.5, respectively, they found that 38 (72%), 27 (51%) and 19 (36%) of families, respectively, displayed this assay discrepancy. Bowyer *et al.,* in 2013 [10], in a study of the incidence of assay discrepancy in their centre in 84 patients with mild haemophilia A, found assay discrepancy in 31% of individuals: 12% with lower activity in the two-stage assay and 19% with lower activity in the one-stage assay.

Implications of assay discrepancies

If only the one-stage assay is used for screening of patients with mild haemophilia A, some patients will seem to have normal FVIII activity levels and not be diagnosed as having haemophilia or else will be assigned to a milder bleeding risk than is the case and risk bleeding complications in a subsequent surgery without proper haemostatic treatment. Some patients with moderate haemophilia will be diagnosed as having the mild form of the disease. Other patients who have normal FVIII activity *in vivo* will be diagnosed as having

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haemophilia A, risking cancellation or delay of necessary surgery or exposure to unnecessary factor concentrates or blood products.

Inhibitor assays

Development of neutralising inhibitors to infused factor VIII is the most significant complication of haemophilia treatment today. Reliable measurement of inhibitor titres remains an important goal. The basic principle of FVIII inhibitor assays comprises accurate measurement of the decrease in FVIII activity in a mixture of external FVIII (normal pooled plasma) and the test plasma vs. FVIII-deficient plasma devoid of inhibitor.

The first assay to measure FVIII inhibitor activity (Oxford method) was introduced in 1959 by Biggs and Bidwell using bovine FVIII concentrate [11]. This was followed in 1975 by the introduction of the Bethesda assay in which normal pooled plasma as the FVIII source is mixed 1 : 1 with patient plasma and imidazole buffer is used as control [12]. By buffering the normal pooled plasma and replacing the imidazole buffer in the control mixture by FVIII-deficient plasma (Nijmegen modification), the specificity and sensitivity were further enhanced in that the assay became more reliable to detect the low-level inhibitors by reducing false negatives [13]. The Nijmegen modified Bethesda assay was validated in the haemophilia population of Canada and is currently recommended by the International Society on Thrombosis and Haemostasis (ISTH) Factor VIII/IX Scientific and Standardisation Committee (SSC) [14]. Despite this recommendation, a recent report by the Royal College of Pathologists of Australia Quality Assurance Programme (RCPAQAP) Haematology showed that only approximately 30% of participants in external quality assurance programmes used the Nijmegen modified assay as it was originally described [15]. In an attempt to make the Nijmegen assay more affordable, it was later suggested that the test could be modified by replacing the FVIII-deficient plasma with 4% bovine albumin without jeopardising results appreciably at low inhibitor titres [16]. In 2011, the Osaka modification of the assay was proposed [17]. The authors used buffered plasma that was made by the addition of 1 volume of 1 mol/L HEPES buffer at pH 7.35 to nine volumes of plasma to form the test samples. The inhibitor titre was calculated by the remaining rate of FVIII coagulation activity (FVIII:C), using the ratio of actual value to the theoretical value. By this method, the FVIII inhibitor titre of type I inhibitor-positive samples was higher than using the Nijmegen method, and for type II inhibitor-positive samples, the titre was similar. However, the assay is more difficult to perform than the Nijmegen method.

The commonly used Nijmegen and Bethesda FVIII inhibitor assays exhibit a poor sensitivity for low-titre inhibitors. Recently, a low-titre inhibitor assay that is 20 times more sensitive has been devised (cut-off value as low as ~0.03 BU/mL) (18). With the assay, the authors demonstrated that low-titre inhibitors are still present in the early postimmune tolerance induction phase in people with haemophilia who have been treated for FVIII inhibitors. These low-titre inhibitors decrease the half-life and the recovery of infused FVIII products.

Issues with inhibitor testing

Accurate measurement of inhibitory antibodies in patients remains difficult and wide interlaboratory variability has consistently been reported. A need exists to standardise methods for measurement of inhibitors (cut-off, FVIII:C assay protocol, ± heat inactivation, etc.). Various authors have made suggestions in this regard such as Verbruggen *et al*. who advocated incorporation of the use of buffered normal pooled plasma as the substrate and

FVIII-deficient plasma as the reference sample in the local procedures of coagulation laboratories (19). In a study to investigate the interlaboratory variability of factor inhibitor assay results, Raut looked at whether candidate reference materials might influence variability in the results (20). Fifteen laboratories determined FVIII inhibitor activity on each of six samples: three patient samples (previous inhibitor level <20 BU/mL) and three

Table 1. Results of assays showing interlaboratory variability (20)

Sample	Assay method	Number of estimates	Mean FVIII inhibitor activity (Bethesda unit)	95% limits	Inter-laboratory variability (CV%)
Inhibitor 1	Chromogenic	5	9.72	5.07–14.37	38.5
	One-stage	11	15.93	13.17–18.68	25.8
	Two-stage	1	4.10	–	–
	All	17	13.41	10.68–16.13	39.5
Inhibitor 2	Chromogenic	5	4.40	3.55–5.25	15.6
	One-stage	11	5.62	3.54–7.70	55.1
	Two-stage	1	2.10	–	–
	All	17	5.05	3.69–6.41	52.4
Inhibitor 3	Chromogenic	5	10.44	7.45–13.43	23.0
	One-stage	11	14.84	12.08–17.59	27.7
	Two-stage	1	6.10	–	–
	All	17	13.03	10.77–15.29	33.7
Rabbit polyclonal	Chromogenic	5	24.56	19.40–29.72	16.9
	One-stage	11	35.22	30.11–40.33	21.6
	Two-stage	1	24.80	–	–
	All	17	31.47	27.23–35.71	26.2
Human monoclonal type II	Chromogenic	5	32.74	13.57–51.91	47.2

Table 1. Results of assays showing interlaboratory variability (20)

Sample	Assay method	Number of estimates	Mean FVIII inhibitor activity (Bethesda unit)	95% limits	Inter-laboratory variability (CV%)
	One-stage	11	32.33	27.81–36.84	20.8
	Two-stage	1	31.80	–	–
	All	17	32.42	27.60–37.24	28.9
Human monoclonal type I	Chromogenic	5	35.66	18.11–53.21	39.6
	One-stage	11	36.05	29.97–42.12	25.1
	Two-stage	1	22.30	–	–
	All	17	35.12	29.68–40.56	30.1

candidate reference materials formulated with human albumin. The latter comprised rabbit polyclonal antibody (~30–75 BU/mL), human monoclonal antibody type I directed to the C2 domain of the FVIII molecule (~25–40 BU/mL) and human monoclonal antibody type II directed to the C1 domain of the FVIII molecule (~25–40 BU/mL). Thirteen laboratories performed the Nijmegen modified Bethesda assay. One laboratory used a two-stage assay while 11 and five laboratories used one-stage and chromogenic assays, respectively. Results showed a large variation in interlaboratory variability (Table 1) (20) ranging from 26% to 52%. The two-stage assay gave the lowest values for patient specimens but not for the reference material. Results from the chromogenic assay were consistently lower than those obtained from the one-stage assay. When the investigators looked at the correlation between different patient samples and the candidate reference materials, they found that the rabbit polyclonal antibody showed the best correlation and proposed that it could be considered in a move towards a standardised assay although variability was still high.

Do clot-based or chromogenic assays influence inhibitor testing?

To our knowledge, very little has been published on this subject. In a comparative study of clot-based, chromogenic and fluorescence assays for measurement of FVIII inhibitors in the USA, Miller *et al*. performed the modified Nijmegen–Bethesda clotting assay and a chromogenic Bethesda assay on 1005 specimens from 17 haemophilia treatment centres (21). Inhibitor measurements were performed centrally and demographic data, previous inhibitor history and treatment product exposures were also recorded. The dilute Russell viper venom time (DRVVT) and heparin were quantitated. The chromogenic Bethesda assay was negative for 880/883 specimens (99.7%) with Nijmegen–Bethesda units <0.5 and positive on 42/42 specimens (100%) with Nijmegen–Bethesda units ≥2.0 and 43/80 specimens (53.8%) with Nijmegen–Bethesda units 0.5–1.9. Above ≥2 BU, there was excellent correlation between the Nijmegen–Bethesda assay and the chromogenic Bethesda assay ($r = 0.98$, $P < 0.0001$). The authors concluded that FVIII specificity could not be demonstrated by the chromogenic

Bethesda assay or fluorescence immunoassay for 26% of inhibitors of 0.5–1.9 Nijmegen–Bethesda units; such results must be interpreted with caution. They recommended that low-titre inhibitors detected in clot-based assays should always be repeated, with consideration given to evaluating their reactivity with FVIII using more specific assays.

In a Swedish study, the influence of assay type on the inhibitor titre was investigated by the use of purified IgG from two different inhibitor patients that were spiked into pooled normal plasma to achieve low-titre inhibitor levels (0–2 BU/mL) (22). Low levels of recombinant factor VIII (rFVIII; up to 20 IU/dL) were added. Assays were performed with and without a heat-inactivation step (58°C, 90 min) to neutralise residual FVIII. Mixing was according to the Nijmegen–Bethesda assay. All samples were assayed for FVIII:C content using a one-stage and a chromogenic assay. For non-heat-inactivated samples, results for the one-stage assay varied between 0.4 and 1.8 BU/mL and for the chromogenic assay from 0.4 to 1.9 BU/mL. For heat-inactivated samples, results for the one-stage assay and chromogenic assays varied between 0.4–2.0 BU/mL and 0.5–1.9 BU/mL, respectively. Most importantly, there was no significant difference (paired *t*-test statistics) between the one-stage and chromogenic assays in the calculated Bethesda inhibitor titres.

In vivo recovery: assays and standards

Accuracy and reproducibility of laboratory measurements are important in the diagnosis and treatment of bleeding disorders. The assessment of *in vivo* recovery of FVIII as part of the preoperative preparation for surgery or to determine the success of management is no exception. However, as eloquently discussed by Barrowcliffe *et al*., the assay of FVIII concentrates against plasma standards has been problematic, predominantly due to the variability of assay methods (23). When assayed against plasma, potencies of concentrates were higher by the two-stage/chromogenic methods than by the one-stage assay (24). One reason for this may be the extensive processing of both plasma-derived factor VIII (pdFVIII) and rFVIII concentrates which could lead to differences in their rates of activation and inactivation (23). Assay discrepancy was greater for rFVIII than pdFVIII (23). Using a chromogenic method, potencies were found to be 17–25% greater than by one-stage assay following infusion of pdFVIII (25). Chromogenic assays may measure 40–50% higher than one-stage assays when measuring full-length rFVIII:C in plasma (26). In an attempt to remedy this phenomenon that arose from comparing dissimilar materials, the first international standards were established for factor concentrate and plasma in 1971 and 1981, respectively (27, 28). The availability of the international standards allowed postinfusion plasma samples to be assayed against 'like' material which abolished the discrepancy. There is a special problem with B-domain deleted (BDD) rFVIII as one-stage assays give much lower results than with the chromogenic assay – a discrepancy of approximately 30% was observed between one-stage clotting and chromogenic potencies when the in-house plasma standard was used as the calibrator (29). Users of the one-stage assay may use a product-specific standard; this has been shown to produce accurate and precise FVIII:C results (29). However, all BDD rFVIII are not similar, and it was postulated that the length of the remaining B-domain linker may influence the one-stage clotting/chromogenic potency ratio (23). Another contributing factor may be the presence of artificial phospholipids in the reagents used (30). Variations in the phospholipid concentration were not found to affect the chromogenic assay, except at very low levels where the apparent activity increased. In contrast, dilution of the phospholipid reagent had a substantial influence on the activity measured by one-stage assays, especially in the case of rFVIII SQ (30). The one-stage activity of rFVIII SQ even exceeded that of the chromogenic assay at low levels of

phospholipid. It is anticipated that novel modified rFVIII products may cause novel assay problems.

Various suggestions have been made to help overcome some of the problems with assay discrepancies. One is to assay patients' postinfusion samples, which essentially consist of concentrates 'diluted' in a patient's haemophilic plasma, against a concentrate standard. The nature of the concentrate standard needs to be carefully considered – it should be as similar as possible to the injected product and should probably be restricted to recombinant and very high-purity plasma-derived products (23).

Conclusions

FVIII assay discrepancy is common in mild haemophilia A. In general, clinical phenotype corresponds better to the two-stage/chromogenic assays than to the one-stage assay. All haemophilia treatment centre coagulation laboratories should use both one-stage and chromogenic FVIII activity assays, especially when screening for possible haemophilia A. There is usually good correlation between inhibitor results based on the one-stage or chromogenic FVIII:C assays (both low and high titre), although there is a risk of false-positive inhibitors of low-titre with the one-stage FVIII:C assay. Appropriate concentrate standards or product standards should be used when possible for FVIII:C assays.

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