

The Art of Detecting Antibodies against Factor VIII

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Abstract

Antibodies against factor VIII (FVIII) can be detected based on their ability to neutralize the procoagulant activity of FVIII (neutralizing antibodies, inhibitors), or based on their specific binding capacity to FVIII protein. This article reviews the available assays and their clinical interpretation in patients with congenital and acquired hemophilia.

Keywords

- ▶ coagulation
- ▶ assay
- ▶ inhibitor
- ▶ autoantibodies
- ▶ Bethesda assay

Zusammenfassung

Antikörper gegen Faktor VIII (FVIII) können anhand ihrer neutralisierenden Funktion gegenüber der prokoagulatorischen FVIII Aktivität oder anhand ihrer Bindung an das FVIII-Protein nachgewiesen werden. Dieser Artikel gibt einen Überblick über die verfügbaren Laborteste und ihre klinische Interpretation bei Patienten mit angeborener oder erworbener Hämophilie.

Schlüsselwörter

- ▶ Gerinnung
- ▶ Laborteste
- ▶ Hemmkörper
- ▶ Autoantikörper
- ▶ Bethesda-Test

Introduction

Antibodies against coagulation factor VIII (FVIII) develop in patients with congenital and acquired hemophilia A. They are called inhibitors, if anti-FVIII antibodies neutralize the procoagulant capacity of FVIII. Anti-FVIII antibodies can be alloantibodies (in patients with congenital hemophilia A after exposure to therapeutic FVIII protein) or autoantibodies (in patients with acquired hemophilia A).

The detection of anti-FVIII antibodies is important to diagnose acquired hemophilia and to detect neutralizing inhibitors in patients with congenital hemophilia A under treatment with FVIII products. Several methods exist to detect neutralizing antibodies based on their functional capacities, and to detect FVIII-binding antibodies (FVIII:Abs) that may or may not neutralize FVIII activity and can interfere with its pharmacokinetics.

This article provides an overview on the methods to detect antibodies against FVIII, reviews potential pitfalls, and supports the clinical interpretation of results.

Nomenclature of Assays for FVIII Activity and Antigen

The International Society on Thrombosis and Haemostasis endorsed a nomenclature in 1985, denoting the FVIII coagulant activity as “FVIII:C.”¹ At that time, FVIII activity was predominantly measured by clotting assays based on the activated partial thromboplastin time (APTT). Today, different types of chromogenic substrate assays of FVIII activity have become popular, even for inhibitor assays. To avoid confusion, the designation and abbreviation of assays discussed in this article are provided in **▶ Table 1**.

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Table 1 Nomenclature of assays of FVIII activity and inhibitor detection as used in this article

Abbreviation	Definition	Notes
FVIII:C	FVIII coagulant activity	Generic term for any FVIII activity assay
FVIII:CBA	FVIII coagulant activity in one-stage clot-based assay (CBA)	Traditional standard for diagnosis and monitoring of hemophilia Artificially high results in samples with emicizumab
FVIII:CSB	FVIII coagulant activity in chromogenic substrate (CS) assay with bovine (B) components	Insensitive to emicizumab: useful for measuring human FVIII activity in samples with emicizumab Porcine recombinant FVIII activity will be underestimated
FVIII:CSH	FVIII coagulant activity in CS assay with human (H) components	Useful for monitoring emicizumab concentration
NBA	Nijmegen-modified Bethesda assay using clot-based assay	Gold standard for FVIII inhibitors, but cannot be used in samples with emicizumab
NBA:CSB	Nijmegen-modified Bethesda assay using CS assay with bovine (B) components	Useful for detecting inhibitors in samples with emicizumab
NBA:POR	Nijmegen-modified Bethesda assay using recombinant porcine (POR) FVIII as a substrate	Useful to measure cross-reactivity of anti-human FVIII antibodies with susoctocog alfa FVIII:CBA should be used for detection.
FVIII:Ab	Immunoassay for FVIII binding antibodies.	Useful for confirming anti-FVIII antibodies in doubtful inhibitor assays. Isotype/subclass should be reported.

Abbreviation: FVIII, factor VIII.

Bethesda Assay

Principle

This assay detects neutralizing antibodies (inhibitors) against FVIII:C.² The gold standard is the Nijmegen-modified Bethesda assay (NBA). Patient plasma, or serial dilutions thereof, is mixed with FVIII-containing standard human plasma (SHP) and incubated at 37°C for 2 hours. The incubation is important because inhibitors against FVIII:C are temperature- and time-sensitive. After incubation, the FVIII:C residual activity (RA) will be determined and compared with that of a control

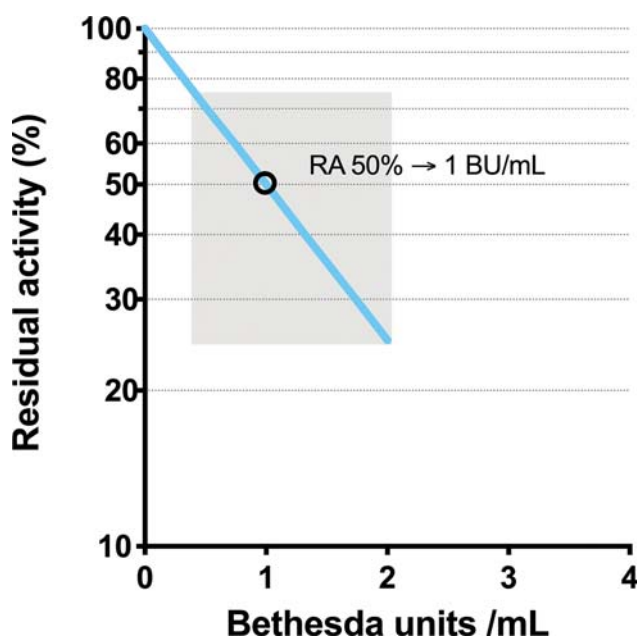


Fig. 1 Nomogram for inhibitor quantification.

mixture of SHP with inhibitor-free FVIII-deficient plasma. 1 Bethesda unit (BU) is defined as the amount of inhibitor that will neutralize 50% of FVIII:C in SHP. A window defined by the RA range of 25 to 75% is recommended to determine inhibitor strength (►Fig. 1). Outside this window, samples will be diluted, and the dilution that gives an RA nearest to 50% (and within the range of 25–75%) will be used to determine the inhibitor strength.

Method

An overview on the method is provided in ►Table 2. In the classical version of the Bethesda assay, patient samples had been diluted in buffer and unbuffered normal plasma was used as a source of FVIII.³ The Nijmegen modification, introduced with the NBA to improve specificity at low inhibitor titers, contained the following:⁴

- Sample diluent: FVIII-deficient plasma instead of diluent buffer.
- FVIII source: buffered SHP (e.g., 0.1 M imidazole, pH 7.4) instead of unbuffered plasma.

Instead of FVIII-deficient plasma, 4% albumin has been suggested.⁵

Type 1 versus Type 2 Inhibitors

The principle of inhibitor quantification in diluted samples, later on multiplied by the dilution factor, was originally developed for the so-called type 1 inhibitors that typically occur in patients with congenital hemophilia A. Type 1 inhibitors are characterized by a log-linear relationship between dilution and RA, whereas type 2 inhibitors are not⁶ (►Fig. 2). The latter can be quantified very roughly only. Sometimes, several dilutions are very close to RA 50% in type 2 inhibitors, yielding very unreliable results. Heat inactivation does not

Table 2 Nijmegen-modified Bethesda assay

<i>Sample dilution</i>
Prepare serial dilution of patient plasma in FVIII-deficient plasma
• Dilution factor 1 (undiluted): patient plasma (0.2 mL)
• Dilution factor 2: patient plasma (0.2 mL) + FVIII-deficient plasma (0.2 mL)
• Dilution factor 4: previous dilution (0.2 mL) + FVIII-deficient plasma (0.2 mL)
• Etc.
Control sample: FVIII-deficient plasma (0.2 mL)
<i>Mixing and incubation</i>
Add 0.2 mL SHP to each dilution and to the control sample
Cap tubes, mix, and incubate at 37°C for 2 hours
<i>FVIII:C assay</i>
Measure FVIII:C in each sample including the control sample
• FVIII:CBA in original NBA
• FVIII:CSB in NBA:CSB (insensitive to emicizumab)
Perform assay immediately
Consider using a chromogenic assay with bovine components, if patient on emicizumab
<i>Results and interpretation</i>
Express RA by dividing the FVIII:C of each sample by FVIII:C of control
Select dilution that gives RA nearest to 50% and within the range 25–75%
Determine inhibitor units from nomogram (→ Fig. 1) or formula: $c_{inh} [BU/mL] = (2 - \log_{10} RA) / \log_{10} 2$
Multiply by dilution factor to give final result

Abbreviations: FVIII, factor VIII; NBA, Nijmegen-modified Bethesda assay; RA, residual activity; SHP, standard human plasma.

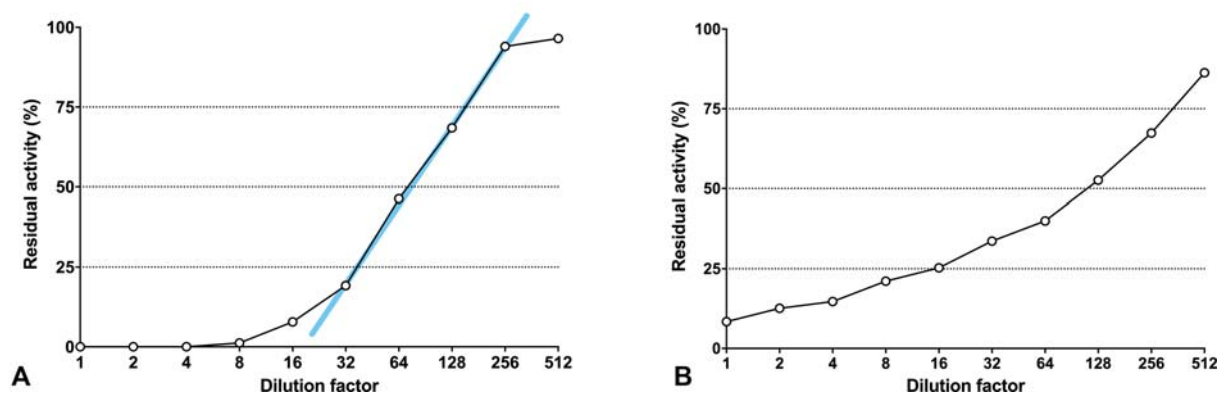


Fig. 2 Type 1 versus type 2 inhibitors. (A) Typical features of type 1 inhibitors are absent residual activity (RA) at low dilution factors and a log-linear relationship between dilution and RA as indicated by the *blue line*. (B) Typical features of type 2 inhibitors are measurable RA at low dilution and lack of a log-linear relationship between dilution factor and RA.

reliably solve the problem of type 2 inhibitors (personal experience), and so it is very important to qualify the test result by denoting a type 2 inhibitor as such.

Residual FVIII:C in Sample

If the test plasma contains significant amounts of FVIII:C (typically more than 5 IU/dL or 5%), the inhibitor will be underestimated by the NBA. Both in vivo absorption of inhibitors and intra-assay underestimation due to FVIII:C in the sample can occur. The latter can be solved in part by

heat inactivation at 56°C for 90 minutes.^{7,8} Large amounts of FVIII protein will, however, still neutralize some of the inhibitor capacity in the sample by absorption, and so samples for inhibitor quantification should ideally be drawn after washout.

Emicizumab in Sample

Emicizumab is a bispecific antibody that can replace the role of activated FVIII in the coagulation cascade by positioning factors IXa and X against each other, so that IXa can activate

X.⁹ Unlike natural FVIII, emicizumab does not require activation by thrombin, and therefore:

- APTT is artificially shortened.¹⁰
- APTT-based factor assays including FVIII:CBA give false-high results.¹⁰
- The FVIII:CBA-based NBA is false-negative.

A guideline for using coagulation tests in emicizumab-treated patients has been issued by the United Kingdom Haemophilia Centre Doctors' Organization.¹¹ NBA:CSB can be used if samples contain emicizumab because FVIII:CSB contains bovine proteins and is insensitive to the drug.¹² Alternatively, anti-idiotypic antibodies can be used to neutralize emicizumab in the sample.¹³

Other Conditions that Interfere with the Bethesda Assay

Pharmacological anticoagulants that prolong the APTT can cause false-positive results in the NBA and should be avoided at the time of sampling.¹⁴ More difficult to assess is the lupus anticoagulant (LA) that can sometimes interfere with the Bethesda assay. A negative dilute Russell's viper venom time (DRVVT) or DRVVT-based LA ratio helps to exclude LA in most cases. However, 22% of patients with congenital hemophilia A also had an increased LA ratio in a DRVVT-based assay.¹⁵ Therefore, LA does not exclude a FVIII inhibitor.

Modified Bethesda Assay with Chromogenic FVIII:C Detection

NBA:CSB is a modification of the NBA using FVIII:CSB to measure RA after incubation (► [Table 1](#)). NBA:CSB showed an overall good correlation with results from the NBA, could eliminate some low-titer, false-positive results,¹⁶ is expected to be less sensitive to LA,¹⁷ and is insensitive to the effects of emicizumab.¹² Imidazole-buffered bovine serum albumin as a sample diluent may improve specificity as compared with FVIII-deficient plasma.¹⁸

Inhibitors against Recombinant Porcine FVIII (Susoctocog Alfa)

Susoctocog alfa is a recombinant porcine FVIII (rpFVIII) molecule that is used to treat bleeds in acquired hemophilia A and is also under investigation for patients with congenital hemophilia A and inhibitors. Antibodies against human FVIII usually react much less with rpFVIII. However, 44% of patients with acquired hemophilia show at least some degree of cross-reactivity,¹⁹ and some patients will develop de novo cross-reactivity during treatment.²⁰

NBA:POR is a modified NBA using rpFVIII laboratory standard (provided by the manufacturer of susoctocog alfa) instead of SHP as a FVIII source.¹⁹ The rpFVIII substrate is usually supplied in high concentrations (e.g., 11 U/mL) and should be diluted in FVIII deficient plasma. FVIII:CBA is the preferred test for RA determination because chromogenic assays can underestimate the activity of rpFVIII.

Quality Assurance

Significant interlaboratory variation is observed with inhibitor assays, as reflected by coefficients of variation that are

often greater than 30%, and some laboratories fail to detect low-level inhibitors of approximately 1.0 BU/mL.²¹ The NBA showed a better performance compared with the classical Bethesda assay. Interlaboratory comparisons of NBA:CSB have not been reported.

If interlaboratory quality assurance results show strong deviations from expected results, the local troubleshooting may include the following:

- Compliance with the NBA assay conditions (FVIII-deficient plasma for dilution, incubation time and temperature, and quality of assay for FVIII:C detection).
- Determination of cut point for assessing RA.
- Recognition and interpretation of type 2 inhibitors.
- FVIII:C in control mixture (may affect assay sensitivity).

Detection of FVIII-Binding Antibodies

Methods

FVIII:Abs can be detected by the enzyme-linked immunosorbent assay (ELISA). Recombinant FVIII is immobilized to microwells, and diluted plasma (1:100) or diluent control (blank) is incubated for 60 minutes at room temperature. If FVIII:Abs are present, these will bind to the immobilized FVIII. They are detected, after several washing steps, with anti-immunoglobulin G (IgG; Fcγ) conjugated to horse radish peroxidase and tetramethylbenzidine substrate.²²

The assay can be performed at a single dilution with reporting:

- Qualitative results based on optical density at 450 nm (OD₄₅₀): "positive" (OD₄₅₀ > 0.30), "grey zone" (OD₄₅₀ 0.15–0.30), and "negative" (OD₄₅₀ < 0.15).
- Quantitative results by using a known anti-FVIII antibody as a calibrator in arbitrary units (AU): "positive" (>24 AU/mL), "grey zone" (12–24 AU/mL), and "negative" (<12 AU/mL).

Both assay variants have become commercially available. For research purposes, a three-step methodology has been used consisting of:

- Screening (at dilution of 1:20, with cut-offs determined from negative samples).
- Determination of antibody titer (in 1:2 dilution steps), reporting the highest titer above the cut-off.
- Confirmation with a competition assay.^{23,24}

This assay has been used with isotype- or subclass-specific detection reagents for dedicated research questions. Other assays for research use include epitope mapping,²⁵ including the mapping of antibodies to functional sites such as the von Willebrand factor-binding site, and affinity measurement.²⁶

Interpretation and Limitations

Low-positive or "grey zone" results are sometimes seen in healthy individuals.²⁷ In the absence of reduced FVIII:C, this finding does not establish a diagnosis of hemophilia.

Positive results typically occur in patients with congenital hemophilia A and inhibitors. However, not all FVIII:Abs detected by ELISA are inhibitors. Nonneutralizing FVIII:Abs have been reported in patients just before inhibitors occurred,²⁶

and also in patients with impaired FVIII pharmacokinetics.²⁷ Cut points were calculated to optimize sensitivity (88%) and specificity (99%) for IgG1 antibodies with one specific assay but cannot be extrapolated to other assay variants.²⁶

Positive results are also expected in patients with acquired hemophilia. In the GTH-AH 01/2010 study, just one out of 102 patients was negative for FVIII:Ab (sensitivity 99%), and this patient happened to be positive for anti-FVIII IgM.²² The specificity, tested in a group of age-matched hospital patients using a cut point of 15 AU/mL, was also 99%. However, given the rarity of acquired hemophilia, the derived positive predictive value (<1%) is clinically useless. To make a diagnosis of acquired hemophilia A, a positive result must be combined low FVIII:C results.

Assays allowing for isotype and subclass differentiation have been developed for research use. It was reported that IgG4 antibodies are mainly associated with persistent high-titer inhibitors²⁸; IgG1 and IgG3 antibodies are predominantly seen in patients with congenital hemophilia A without inhibitors and can modulate the pharmacokinetics of FVIII²⁷; IgA antibodies are associated with relapse and poor prognosis in acquired hemophilia.²⁴

Conclusions

Antibodies against FVIII can be detected in functional assays, based on their ability to neutralize FVIII:C, and in immunoassays, based on binding to FVIII protein. The NBA based on FVIII:CBA is still the gold standard for functional inhibitor detection. However, several pitfalls exist in special patient populations, such as those with LA or under treatment with emicizumab. NBA:CSB is a modification using FVIII:CSB for detection of RA that becomes more popular and may solve some of the preanalytical problems. FVIII:Ab immunoassays can also provide valuable information but have not entered most routine clinical laboratories until now.

Disclosures

None.

Conflict of Interest

The authors declare, that they have no conflict of interest.

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