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**Development of an individual
Anti-FVIII Inhibitors ELISA to improve Haemophiliacs
therapeutic follow up**

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Aim: Haemophiliacs A patients treated with FVIII concentrates from human or recombinant origin may develop inhibitors, the most frequent complication leading to increased mortality. It then requires an adapted characterization and therapeutic management. A new anti-FVIII IgG ELISA was developed as a diagnostic help for the detection and quantitation of these inhibitors in patients' plasma.

Method: Elisa strips is coated with highly purified recombinant FVIII (phospholipid free), identical to the one used for patients. A 1:100 dilution of the tested plasma is introduced into the well, followed by a 1 hour incubation at RT and a washing step. A goat anti h-IgG Ab coupled to peroxidase is then added, and reacts with the captured patients IgG, when present. After a new washing step, a peroxidase substrate (TMB) is used for revelation and the reaction stopped with H₂SO₄. The amount of color generated at 450nm is directly proportional to the inhibitors concentration present in the tested sample.

Results: A “humanized” chimera made of anti-VIII Ab coupled to (h)IgG was prepared to establish the calibration curve. This allows relative quantitation of inhibitors, when present, as high levels of inhibitors could correspond to the most severe cases. A negative control is also included and is a reference normal plasma. The kit was evaluated on normal plasmas. Normals, Haemophiliacs without or with inhibitors were tested and A₄₅₀ was respectively of 0.05 (SD 0.03), 0.06 (SD 0.04), and >1.0 . Results correlated with previously determined Bethesda units and another commercial Elisa for the same application, while presence of ACC antibodies does not interfere in the assay. The lower limit of quantitation was estimated at about 0.5 Bethesda Units.

Conclusion: This new assay could be proposed as an efficient and reliable standardized tool for managing patients in such critical states, for improved therapeutic follow up, and possibly for retrospective or prospective studies.

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Introduction

Haemophiliacs A patients treated with FVIII concentrates from human or recombinant origin may develop inhibitors, the most frequent complication leading to increased morbidity and mortality. These patients cannot be treated with usual FVIII concentrates, and bleeding episodes require the use of a bypassing coagulant therapy (such as Novoseven®). Identification and quantitation of those antibodies is important as these patients require an adapted medical care and therapeutic management. Using a solid phase Elisa is then a practical way for a rapid identification of presence of these antibodies.

Aim

A new individual anti-FVIII IgG ELISA was developed as a diagnostic help for the detection and quantitation of antibodies to FVIII in plasma from haemophiliacs A, who can develop FVIII inhibitors.

Materials and Methods

Assay: Solid phase Elisa as depicted in the "assay principle" and uses a recombinant human FVIII for antibody (Advate®) capture.

Positive control: A "humanized" chimera made with an anti-VIII Ab covalently coupled to human IgG is used as a positive control, and possibly for the calibration curve.

A reference normal plasma is included as **negative control**, already diluted.

Assayed specimen: Citrated normal plasmas, plasmas from patients with Circulating Anti-Coagulant (CAC) antibodies, and from Haemophiliacs without or with inhibitors or who developed inhibitors previously and became negative later (ex). Results were compared to previously determined Bethesda Units.

Normal plasmas are from healthy blood donors (EFS, France), Pathological plasmas are from haemophiliacs, untreated or treated, and kindly supplied by Dr TERNISIEN (CHU Nantes, France),

Functional FVIII inhibitors were assayed with the usual Bethesda method and expressed in Bethesda Units (BU).

Assay principle and protocol

OD measurement at 450nm

5 min. at RT

TMB/H₂O₂ substrate and reaction stopped with sulfuric acid

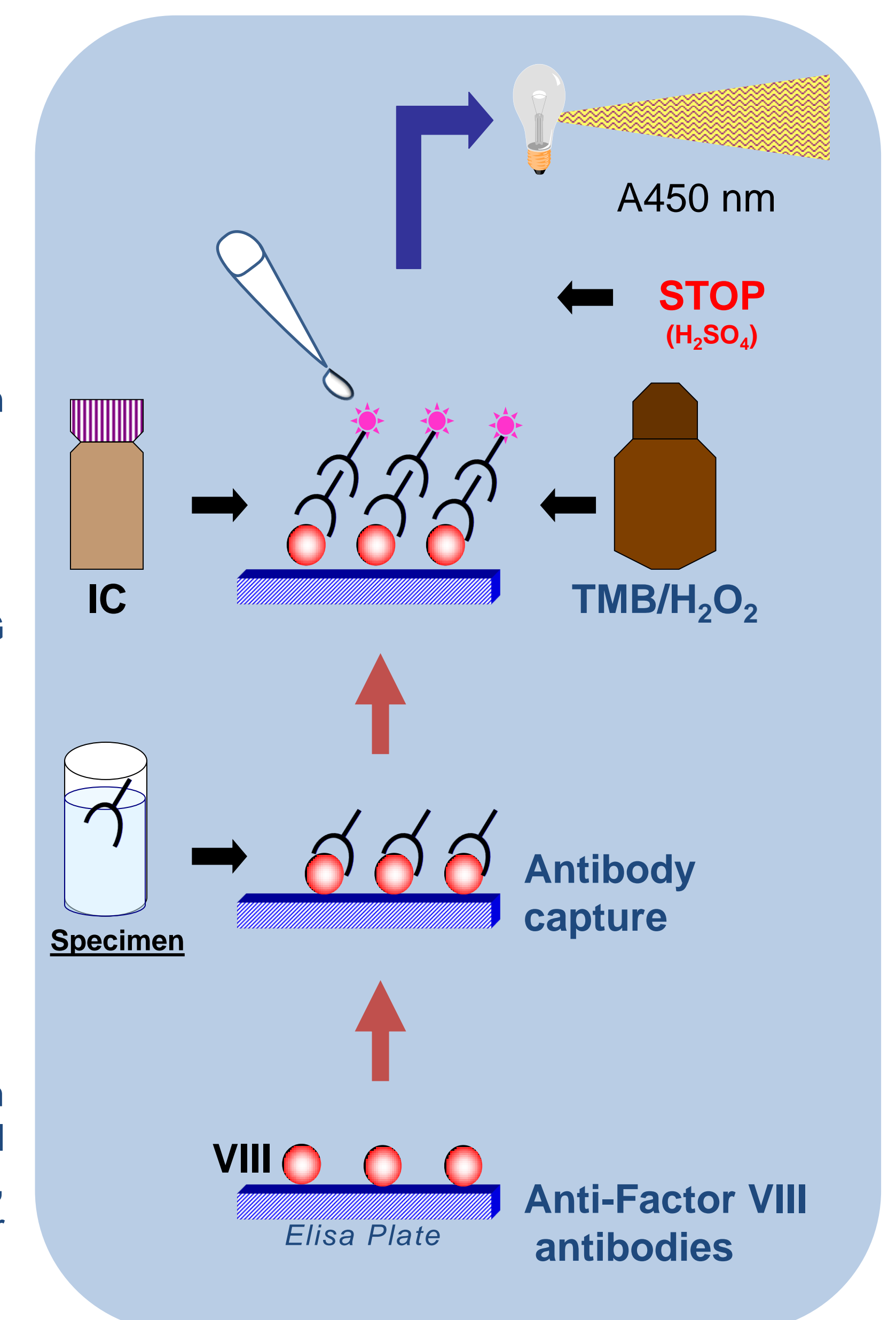
1 h at RT

Second antibody: goat anti human-IgG Antibody (peroxidase conjugated)

1 h at RT

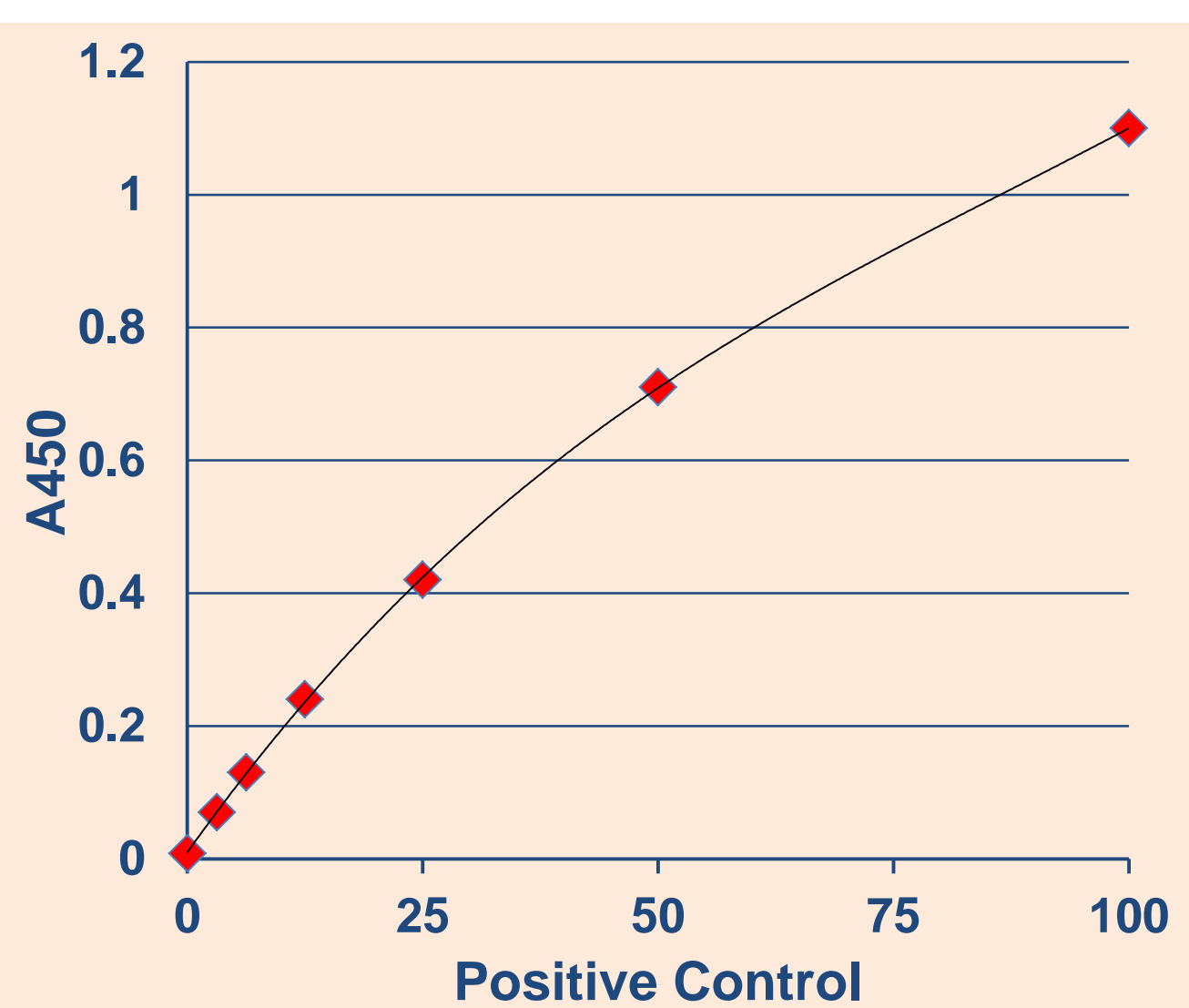
1:100 diluted test plasma

Individual Elisa strips coated with highly purified recombinant FVIII (Advate®, phospholipid free), identical to the product used for treating patients, then saturated.



Results

Positive control and dose response curve



	A450	Intra assay
Positive Control (N=5)	1.12	CV: <3%
Negative Control (N=4)	0.06	SD: 0.02

- The positive control (IgG isotype) yielded homogeneous A450>1.0, and can possibly be used to establish a calibration curve. This could help in quantitation of inhibitors, when present, as high levels of inhibitors could correspond to the most severe cases.
- A negative control, as reference normal plasma, yielded A450<0.10.

- Quantitative results expressed in AU/ml (arbitrary units), established respectively to the mean absorbance in normals and standard deviation.

NORMALS		
Mean A450	SD	Mean + 2SD
0.06	0.032	0.125 (i.e. 10 AU)

- By definition, the concentration of the chimera antibody is defined respectively to the A450 mean value for normals (N=113) + 2 SD, which corresponds to 10 Arbitrary Units (10 AU), and is the cut-off for normal range. Using this definition, the positive control is found at 133 AU/ml.

Reagent Stability

After reconstitution	Fresh	6 days (2-8°C)	2 weeks (2-8°C)
A450 for C+	0.95	0.94	0.99

Performances are well preserved after reconstitution, for at least 2 weeks at 2-8°C.

Accelerated ageing A450	Fresh	3 weeks (30°C)
C+	0.95	0.86
C-	0.05	0.06
Positive sample	0.98	0.97
Normal Plasma	0.06	0.09

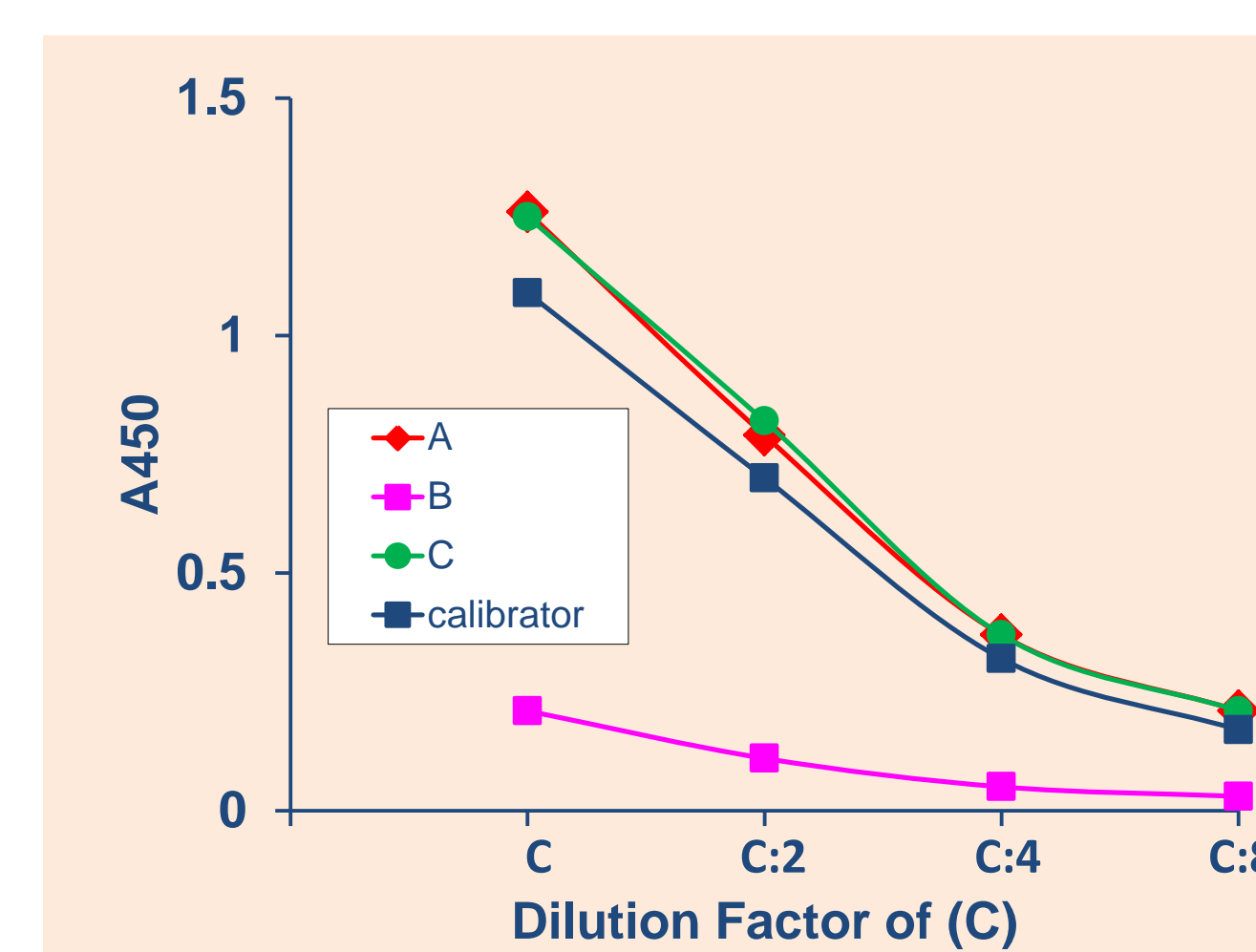
Performances are maintained over time, in accelerated ageing (3 weeks at 30°C), and in real time follow-up at 2-8°C (data not shown).

Performances on normal individuals and Haemophiliacs without or with Anti-FVIII Inhibitors, or who had inhibitors previously (ex)

Groups	N	A450	SD	Extreme values A450	[C] AU/ml	Bethesda BU/ml
Normals	113	0.06	0.03	0.01-0.14	4.2	ND
Plasmas with ACC+	4	0.05	0.04	0.03-0.09	4	<0.5
Haemophiliac without inhibitors	46	0.08	0.11	0.02-0.74*	6.6	<0.5
Haemophiliacs with ex Inhibitors	9	0.05	0.015	0.04-0.07**	3.1	<0.5
Haemophiliacs with Inhibitors	4	>2.60	>2.60	17.9-140,000	35,088	0.7 -2,200

* 1 plasma without inhibitors presents non neutralizing anti-FVIII antibodies
** All of the 9 plasmas from patients with ex-inhibitors are negative

Dose response curves generated with the positive control and with serial dilutions of haemophiliac plasmas (4) with inhibitors



Comparison of FVIII antibody levels with Bethesda Units

	4 Hemophiliacs with inhibitors			
	A	B	C	D
BU/ml	9.1	0.7	22	2,200
AU/BU	17.47	25.57	8.1	62

Correspondence between FVIII antibodies measured by Elisa and Bethesda Units: R² = 0.999

- The Elisa values matched those measured with the Bethesda method.
- Normals, Haemophiliacs without or with inhibitors were tested and mean A450 were respectively of 0.05 (SD 0.03), 0.06 (SD 0.04), and >1.0.
- Results correlated with previously determined Bethesda Units and another commercial Elisa for the same application, while presence of ACC antibodies does not interfere in the assay.
- The clinical detection threshold for this assay was estimated at about 0.5 Bethesda Units.

Conclusions

- This new individual assay could be proposed as an efficient and reliable standardized tool for characterizing Haemophiliacs A patients in critical states, developing or not anti-FVIII IgG antibodies, for adapting an improved therapeutic follow-up.
- It could provide a useful tool for retrospective or prospective studies and an easy approach for identification of patients with anti-FVIII antibodies.
- This assay measures all anti-FVIII antibodies, possibly including those which are non neutralizing. A complementary investigation with the Bethesda method, allows characterizing those with inhibitory activity.

Acknowledgments

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