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

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Intra assay

CV: <3%

SD: 0.02

vielded

isotype)

when present, as high

A450

0.06

control

(laG

NORMALS

Introduction Ref. ARK039A

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).

Results

Positive control and dose response curve



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

- Mean A450 SD Mean + 2SD 0.032 0.06 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 preserve	d after reconstitu	tion, for at least 2

eks at 2-8°C

Accelerated ageing A450	Fresh	3 weeks (30°C)	Performances are maintained over time, in
C+	0.95	0.86	accelerated ageing (3 weeks at 30°C), and in
C-	0.05	0.06	real time follow-up at 2-8°C (data not shown).
Positive sample	0.98	0.97	
Normal Plasma	0.06	0.09	

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.

Assay principle and protocol



Performances on normal individuals and Haemophiliacs without

of with Anti-1 vin Infibitors, of who had infibitors 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



Comparison of FVIII antibody levels with Bethesda Units 4 Hemophiliacs with inhibitors Α B C D 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 Elisa 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.

Acknowledgments

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1HYPHEN BioMed, Research, Neuville sur Oise, France, 2HYPHEN BioMed, Neuville sur Oise, France 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-lgG Ab coupled to peroxidase is then added, and reacts with the captured patients lgG, 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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