Development and evaluation of a novel FVIII domain-specific multiplex microsphere based immunoassay for characterization of anti-FVIII antibodies

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Introduction and Objective

FVIII neutralizing antibodies develop in response to replacement treatment in 30% of patients with severe hemophilia A (HA) (1-2). Such inhibitory antibodies represent a serious complication in the treatment of these patients. The aim of the present study was the development and evaluation of a microsphere-based immunoassay on the Luminex™ system for rapid detection and early characterization of anti-FVIII antibodies in patient plasma samples.

Materials and Methods

The FVIII protein was splitted into several single and multi-domain fragments and introduced to a baculovirus expression system. The domains were purified (>80%) and individually coupled to colored-coded magnetic microspheres. Commercially available full length (FL-FVIII) and B-domain deleted (BDD-FVIII) FVIII proteins were immobilized accordingly. The coupled target proteins were used to establish the Luminex™ based assay, termed **LumiTope.**

Results

Step 1: Preparation of the recombinant proteins

In the first step, fifteen different FVIII constructs were prepared and expressed in a baculovirus expression system (Fig. 1 A,B). All proteins were analyzed for their expression and size by western blotting (Fig. 1C).

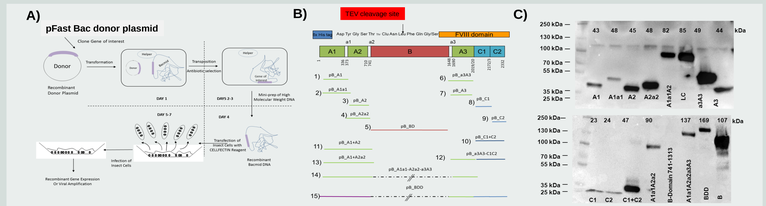

Figure 1: Methodology and cloning strategy of the domains. A) Generation of recombinant Baculovirus and the expression of gene of interest using the Bac-to-Bac® Baculovirus Expression System. B) Overview of the constructed recombinant proteins comprising one or several domains of FVIII protein along with the schematic presentation of the cloning strategy. C) Western blot analysis of the expressed recombinant FVIII domains using an anti-His antibody. The approximate size of the constructs is given in kDa. Only for one construct comprising 1/3 of B domain the protein production was not verifiable on western blot.

In the next step, eight constructs covering the complete FVIII protein were selected for high scale purification: A1a1, A2a2, A1a1A2, C1+C2, a3A3, the light chain (LC), C1 and C2. Subsequently, Sf9 cells were infected (1 L) and cells were harvested 48h after infection and lysed. The target protein was obtained by one-step purification using Ni column. Proteins were analyzed by SDS-PAGE and Western blot for molecular weight and purity (Fig. 2).

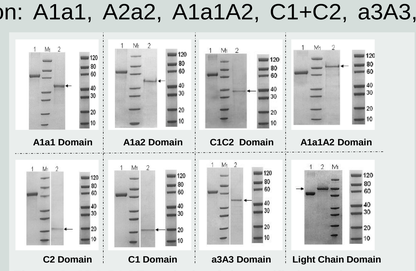

Figure 2. SDS-PAGE analysis of the purified proteins. Lane 1 is BSA (2 µg) in all gels and Lane 2 is the respective domain of purified FVIII domain.

Step 2: Establishing the domain specific assay on the xMAP Luminex™ technology

Subsequently, all purified products, along with commercially available recombinant full length- and BDD-FVIII-proteins, were coupled to different magnetic bead regions. The Luminex™ technology is a bead-based assay system using tiny, 6.4 micron superparamagnetic beads. Each bead contains its own distinct dye ratio which will generate a unique fluorescence pattern to enable individual bead identity. The beads are mixed with the plasma sample of the patients and later incubated with a phycoerythrin-(PE) conjugated antibody which produces a fluorescence signal proportional to the amount of the bound FVIII antibodies. The beads are individually passed through a small shaft where a light source excites the internal dye that is used to identify each bead (Fig. 3).

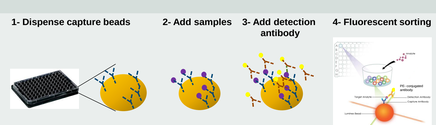

Figure 3: Schematic representation of a immunoassay sandwich-based assay workflow. First the beads are added to a 96-well plate and incubated with the plasma sample of the patients. Later a PE-conjugated anti-human IgG detection antibody is added. Finally the samples are subjected to the Luminex™ machine.

Based on the dye ratios, each bead will exhibit a unique fluorescence that can be identified with a unique bead region. A second wavelength is used to determine the magnitude of the phycoerythrin signal. The software analyzes the images to determine the bead identity and phycoerythrin signal. The Luminex™ xPONENT® software program will provide a real-time readout of signal measured as median fluorescent intensity (MFI). Since each antibody-conjugated bead contains a different fluorescent label, FVIII domains can be added to the same sample for multiplexing. The readout from the phycoerythrin is proportional to the amount of anti-FVIII antibodies captured.

Step 3: Testing LumiTope on plasma samples of patients

All coupled beads were tested in singleplex and multiplex assay formats on Luminex™ and were tested against commercially available anti-FVIII antibodies (data not shown). Later LumiTope was applied on plasma samples from controls, patients with inhibitor and without inhibitor. The Net MFI from the patient samples for each domain was subtracted from the Net MFI obtained for the respective domain of the control sample. Our preliminary results show that LumiTope is a sensitive test for detection of anti-FVIII antibodies (Table 1). The analysis of three HA patients with inhibitor (titres 1,1-8,6 BU/ml) revealed the presence of anti-FVIII antibodies against the A2a2 and C2 domain for patients P1 and P2, respectively. For P3 antibodies against all domains, except the C1 domain were detected. In patients with a history of inhibitors and successful immune tolerance therapy (ITI) (P4 and P5,) as well as the HA patient with no history of inhibitor (P6), no anti-FVIII antibodies were detected.

Table 1. LumiTope analysis of plasma samples of HA patients. The analysis on three HA patients with inhibitor revealed anti-FVIII antibodies against the A2a2 and C2 domain for patients P1 and P2, respectively. For P3 antibodies against all domains except C1 domain were detected. For P4-P6 no anti-FVIII antibodies were detected.

Patient		P1	P2	P3	P4	P5	P6
Antibody titer (BU/ml)		2,5	1,1	8,6	0	0	0,29
FVIII Antibody ELISA (Immucor™)		Pos.	Pos.	Pos.	Neg.	Neg.	Neg.
ITI status					successful	successful	no inhibitor
Domain	A1+A2	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
	A1a1	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
	A2a2	Pos.	Neg.	Pos.	Neg.	Neg.	Neg.
	a3A3	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
	LC	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
	C1	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.
	C1+C2	Neg.	Neg.	Pos.	Neg.	Neg.	Neg.
	C2	Neg.	Pos.	Pos.	Neg.	Neg.	Neg.
	FL-FVIII	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.
	BDD-FVIII	Pos.	Pos.	Pos.	Neg.	Neg.	Neg.

Conclusion

Our new immunoassay, LumiTope, provides a sensitive and fast method for early characterization of inhibitory anti-FVIII-antibodies in hemophilia A patients. The characterization of the binding regions of these antibodies may provide the basis for better understanding of inhibitory mechanisms and help for the eradication of FVIII inhibitors.

»References

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2) Oldenburg J, Schroder J, Brackmann HH, Muller-Reible C, Schwaab R, Tuddenham E. Environmental and genetic factors influencing inhibitor development. Semin Hematol. 2004;41(1 Suppl 1):82-8.

Acknowledgment
This work was supported by a BONFOR grant and a Bayer young investigator award to BP and a Biotest research grant to JO.