IN VITRO CORRELATE OF *IN VIVO* ANTIVENOM NEUTRALIZATION POTENCY ASSAY FOR EFFICIENCY ASSESSMENT OF ANTIVENOM PURIFICATION STEPS

Maja Lang Balija[1], Tihana Kurtović[1], Monika Tunjić[1], Marija Brgles[1], Beata Halassy[1]

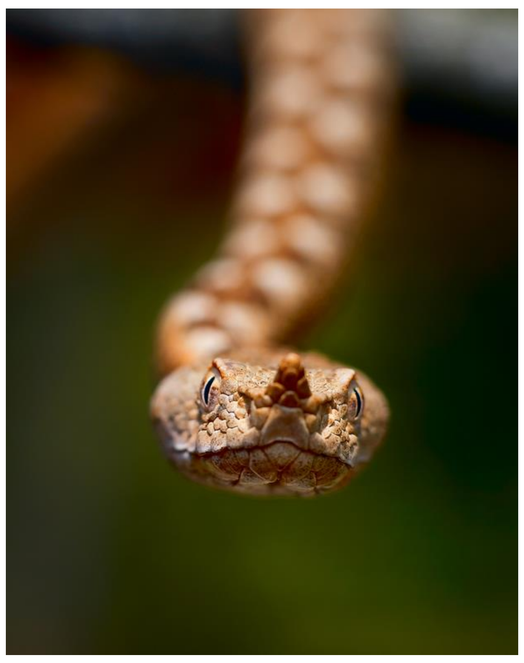
[1]University of Zagreb, Centre for Research and Knowledge Transfer in Biotechnology, Zagreb, Croatia

INTRODUCTION

Downstream processing of antivenom from hyperimmune plasma consists of series of purification steps. The whole process requires constant evaluation of antivenom yield in each purification step, especially during developmental phase. Preclinical evaluation of the efficacy of antivenoms in animals is required by international regulatory authorities, especially for product that will be used in human.

Determination of antivenom efficacy is based on two *in vivo* tests: (a) the venom lethal toxicity assay (determination of median lethal dose of venom or LD_{50}) and (b) test for determining the neutralization potency (effectiveness) of produced antivenom (determination of mean effective antivenom dose or ED_{50}). Large number of mice are injected with venom/antivenom mixtures and the number of surviving mice is statistically analysed to give an ED_{50} value reflecting the efficacy of that antivenom.

Both these tests, which cause suffering, pain and death of the experimental animals and also require a large number of animals, were identified by ECVAM (European Centre for Validation of Alternative Methods) as assays that are necessary to be replaced with alternative methods. Therefore, each laboratory that evaluates antivenom efficacy *in vivo* should aim to develop an *in vitro* alternative in accordance with 3R principles.



Long-nosed viper
(*Vipera ammodytes ammodytes*)

AIM

To monitor the efficiency of each purification step during development of an antivenom production process, we have developed ELISA for quantification of venom-specific antibodies, in which antibodies isolated from hyperimmune horse plasma by protein A affinity chromatography have been used as a standard. Knowing that protein A does not bind all horse IgG classes with an equal affinity, affinity purified antibodies might have different venom neutralization potency in comparison to original population of polyclonal antibodies in the starting hyperimmune plasma. Consequently, this might lead to imprecise determination of venom-specific antibodies in samples from different process steps. From that reason, we have investigated the *in vivo* neutralisation potency of hyperimmune plasma, its affinity purified horse IgGs and horse IgGs purified by a novel purification procedure and compared them to venom-specific IgG quantities determined *in vitro* by ELISA.

MATERIALS AND METHODS

Crude venom of *Vipera ammodytes ammodytes L.* (*Vaa* venom) was collected by milking snakes kept at the Institute of Immunology Inc., Croatia, air dried at ambient temperature and stored in the dark at 4 °C until use. *Vaa* venom-specific hyperimmune horse plasma was obtained from the Institute of Immunology Inc., Croatia. Mice, both sex 18-20 g, used for *in vivo* assays were bred at the Institute of Immunology. Procedures, handling and animal work were in accordance to the Croatian Law on Animal Welfare (2006) which complies with EC Directive 2010/63/EU.

Protein concentration estimation

The protein concentrations in hyperimmune horse plasma and in samples from all purification steps were estimated spectrophotometrically (Ehresmann's method) using following equation: (A228.5 nm − A234.5 nm) × 0.3175 = [mg mL^{-1}]. Appropriate dilution of each sample was independently prepared three times to obtain the mean value of the measured concentrations for further calculation of yield and purity.

SEC HPLC (size - exclusion chromatography)

The molecular weight of the plasma proteins was determined by HPLC size exclusion chromatography using TSK-Gel G3000SWXL (7.8 x 300 mm) column. Analyses were run at a flow rate of 0.5 mL min^{-1} at room temperature and the absorbance was monitored at 280 nm. The running buffer was 0.1 M phosphate sulphate buffer, pH 6.6. Standard proteins used for molecular weight determination were tyroglobulin (Mr 669000), γ-globulin (Mr 150000), ovalbumin (Mr 43000), and ribonuclease A (Mr 13700).

ELISA

ELISA for detection of venom - specific antibodies in samples from hyperimmune horse plasma processing was performed by coating microtiter plate with 100 µL/well of venom coating solution (1 µg/mL) in 50 mM carbonate buffer, pH 9.6, and left overnight at room temperature (RT). After blocking with 0.5 % (w/V) BSA in PBS/T (0.05% (V/V) Tween 20 in PBS) buffer at 37 °C for 2 h, the plasma or samples from each purification step were added in two-fold serial dilutions in duplicates and left overnight at RT. The antibodies isolated from hyperimmune plasma by protein A affinity chromatography were used as a standard. In the subsequent steps, incubation with HRP-anti-horse $F(ab')_2$ (25 000-fold diluted) at 37 °C for 2 h occurred, followed by the addition of OPD (0.6 mg/mL solution) in citrate-phosphate buffer, pH 5.0. After 30 min of incubation in the dark, the enzymatic reaction was stopped with 1 M H_2SO_4 and the absorbance at 492 nm was measured.

LD_{50} and ED_{50} test

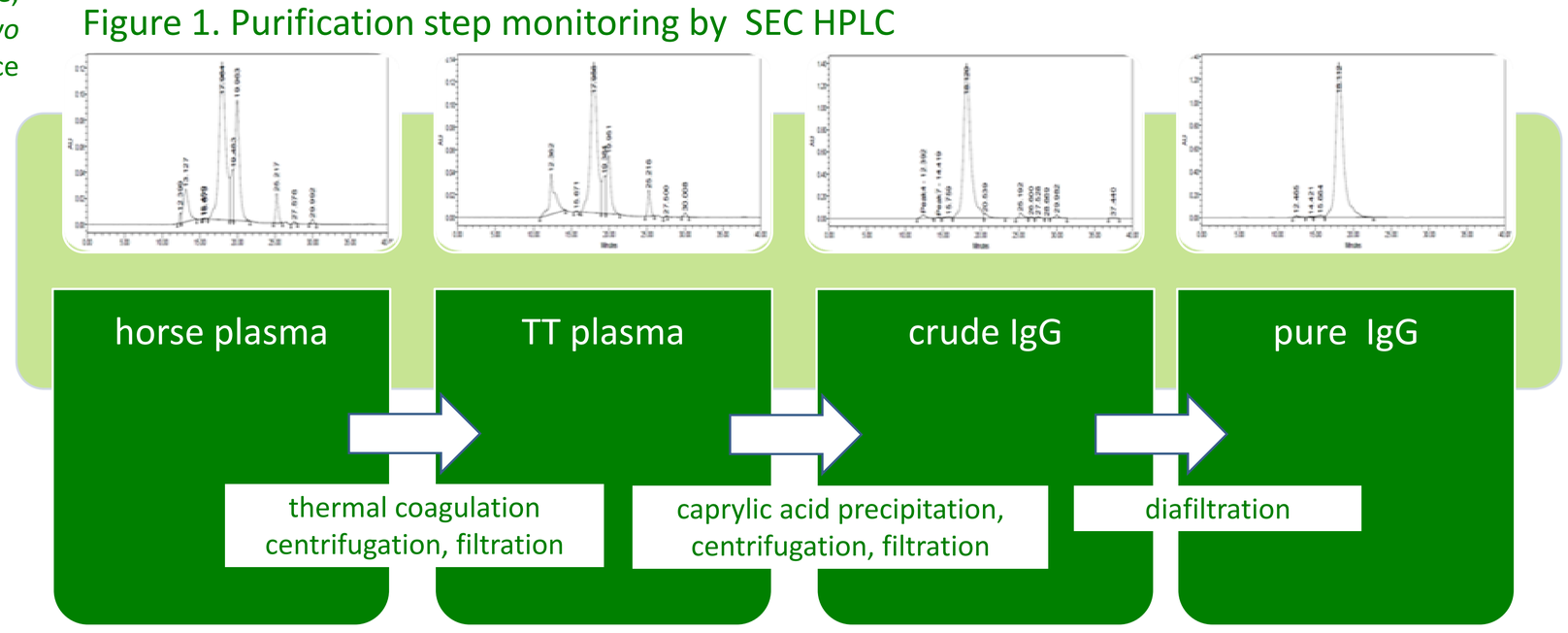
The potential of plasma and pure IgG samples to neutralise the venom's lethal toxicity was determined by lethal toxicity neutralisation assay in mice, as follows. Two-fold serial dilutions of samples were preincubated with equal amounts of the venom solution containing two median lethal doses (LD_{50}, the amount of dry venom (in µg) causing the death in half of the mice population used). The immunoprecipitates were removed by centrifugation and clear supernatants *i.v.* administered to groups of four mice. Deaths were recorded 48 hours later. For each sample the median effective dose (ED_{50}, the amount of undiluted serum capable of neutralising the venom's lethal effect in 50 % of the animals) was determined. The lethal toxicity neutralisation potency (R) was expressed as the number of LD_{50} venom doses that can be neutralised by 1 mL of undiluted sample and calculated by the equation R=(Tv-1)/ED_{50} where Tv represents the number of LD_{50} inoculated per mouse. R value was used as a measure of the protective efficacy of each sample.

CONCLUSION

We have successfully developed a precise and accurate *in vitro* method suitable for monitoring the efficiency of antivenom purification process that adequately replaces the *in vivo* neutralization assay in mice.

RESULTS

Figure 1. Purification step monitoring by SEC HPLC



Our process of immunoglobulin purification from hyperimmune horse plasma consists of three steps: thermal coagulation, caprylic acid precipitation and diafiltration, generating thermally treated plasma (TT plasma), crude IgG and pure IgG, respectively.

To properly evaluate purity and yield of IgGs obtained in each purification step, each sample was analysed for IgG content (by ELISA quantification of venom-specific antibodies), for total protein content, and for the purity (by SEC, Figure 1).

IgG quantity determined by ELISA in crude IgG and in pure IgG samples washigher than their total protein concentration (Table 1) indicating that ELISA gave inaccurate results. From that reason, ELISA results were corrected by correction factor. It was calculated from IgG in pure IgG sample determined by ELISA and IgG quantity calculated by multiplying IgG content determined by SEC with total protein concentration of the same sample. Corrected ELISA results were in accordance to the purity of each sample observed by SEC. Corrected ELISA results indicated that purification process has near 100% yield.

In vivo evaluation of neutralization potency (R) of starting plasma and final pure IgG proved that process has near 100% yield, indicating that developed ELISA can serve as reliable *in vitro* alternative.

Moreover, neutralization potency per mg of IgG calculated for both samples was 1.39 for plasma and 1.38 for pure IgG indicating that our process does not lead either to the change in the ratio of venom-specific and nonspecific IgGs or to the loss of some IgG subtypes. In contrast, protein A affinity purification leads to the loss of neutralization power of IgGs (0.85 LD_{50}/mg), most probably due to the loss of some IgG isotypes that bind weakly to the protein A. The use of affinity purified IgG, having different neutralisation potency, from samples in which we quantify IgG (plasma, TT plasma, crude IgG, pure IgG) is the most probable cause for the inaccurate ELISA results. However, these can be easily corrected as we clearly demonstrated here.

Table 1. Monitoring of purification process by ELISA

	γ(IgG)[1]/ mg mL^{-1}	γ(prot.)[2]/ mg mL^{-1}	IgG content[3]/ %	purity[4]/ %	yield[5]/ %
plasma	47.7±5,97 (*n=15*)	75.6±2.00 (*n=14*)	63.06		
TT plasma	47.9±4.65 (*n=20*)	72.9±2.00 (*n=22*)	65.67		100.49
crude IgG	47.6±5.42 (*n=23*)	39.1±1.23 (*n=23*)	121.66	92.2±0.78 (*n=16*)	99.83
pure IgG	46.2±10.96 (*n=11*)	35.1±2.55 (*n=12*)	131.76	97.7±1.16 (*n=10*)	96.87

[1] *determined by ELISA or ELISA with correction of results*
[2] *determined by Ehresmann's method*
[3] *calculated as γ(IgG)/γ(protein)*100*
[4] *determined by SEC*
[5] *calculated as γ(IgG) of the sample/γ(IgG)of the plasma * 100*

Table 3. Comparison of *in vivo* neutralisation potency and γ(IgG) determined *in vitro*

	plasma	eIgG	pure IgG
[1]R / LD_{50} mL^{-1}	48.0±11.09 (*n=3*)	48.9±7.5 (*n=5*)	48.9±2.74 (*n=3*)
[2]γ (IgG) / mg mL^{-1}	34.4±4.49 (*n=5*)	57.2	35.3±2.21 (*n=5*)
[3]LD_{50} / mg IgG	1.39	0.85	1.38

[1] *determined by in vivo neutralisation potency assay*
[2] *determined by ELISA with correction of results*
[3] *calculated as R / γ (IgG)*

Table 2. Improvement of purification process efficiency monitoring after correction of ELISA results for γ(IgG) determination

	Original ELISA					ELISA with correction of results				
	γ(IgG)[1]/ mg mL^{-1}	γ(protein)[2] / mg mL^{-1}	IgG content[3]/ %	purity[4]/ %	yield[5]/ %	γ(IgG)[1]/ mg mL^{-1}	γ(protein)[2] / mg mL^{-1}	IgG content[3]/ %	purity[4]/ %	yield[5]/ %
plasma	58.0±16.58 (*n=5*)	75.6±2.00 (*n=14*)	76.77			34.4±4.37 (*n=5*)	75.6±2.00 (*n=14*)	45.47		
TT plasma	56.2±9.57 (*n=7*)	72.9±2.00 (*n=22*)	76.99		96.77	34.2±3.46 (*n=5*)	72.9±2.00 (*n=22*)	46.92		99.57
crude IgG	61.3±10.40 (*n=6*)	39.1±1.23 (*n=23*)	156.6	92.2±0.78 (*n=16*)	105.61	36.2±0.91 (*n=5*)	39.1±1.23 (*n=23*)	92.61	92.2±0.78 (*n=16*)	105.40
pure IgG	60.7±14.95 (*n=5*)	35.1±2.55 (*n=12*)	173.19	97.7±1.16 (*n=10*)	104.59	35.3±2.21 (*n=5*)	35.1±2.55 (*n=12*)	100.82	97.7±1.16 (*n=10*)	102.80

[1] *determined by ELISA or ELISA with correction of results*
[2] *determined by Ehresmann's method*
[3] *calculated as γ(IgG)/γ(protein)*100*
[4] *determined by SEC*
[5] *calculated as γ(IgG) of the sample/γ(IgG)of the plasma * 100*