THE ROAD TOWARDS *IN VITRO* ALTERNATIVE TO *IN VIVO* SNAKE VENOM TOXICITY AND ANTIVENOM POTENCY ASSAYS

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INTRODUCTION

Continuous quality control of venoms and antivenoms is necessary for successful production of viper antivenom. Control 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 antivenoms (determination of median effective antivenom dose or ED_{50}) (1). 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 (2).

Ammodytoxins (Atxs) and haemorrhagins (H) are the main constituents of *Vipera ammodytes ammodytes* (*Vaa*) venom whose involvement in the generation of human pathology has been considered to be highly probable. Since development of *in vitro* tests requires identification of the components that contribute to the venom's overall toxicity or specificity of antibodies that effectively neutralize them, the aim of our study was to investigate participation of anti-Atx and anti-H antibodies in neutralization of the whole *Vaa* venom lethal toxicity in mice. Also, two *„in vitro"* methods for determination of Atx content in different venom batches were developed and evaluated as possible screening tool for selection of the best antigen for immunisation and antivenom production.

MATERIAL AND METHODS

Vaa venom was extracted from snakes held at the Institute of Immunology Inc., Zagreb, Croatia (2 batches – KR95 and SL05). Atxs and total haemorrhagic fraction were prepared at Jožef Stefan Institute, Ljubljana, Slovenia.

SANDWICH ELISA

Microtiter plate was coated with rabbit anti-Atx IgG in 0.05 M carbonate buffer, pH 9.6 and left overnight at RT. After blocking with 0.5% (w/v) BSA in PBS/T (0.05% (w/v) Tween 20 in PBS buffer for 2 h at 37 °C, the investigated venom batches and the pure AtxA solution used as standard were added in five serial two-fold dilutions in duplicates and incubated overnight at RT. Plate was washed, guinea pig anti-Atx antibody was added and incubated for 2 h at 37 °C. After washing, HRP-anti-guinea pig IgG was added (10 000-fold diluted) and incubated for 2 h at 37 °C. Finally, plate was washed; OPD solution was added and incubated for half an hour at RT in the dark. The enzymatic reaction was stopped with 1 M H_2SO_4 and $A_{492\,nm}$ was measured. Quantitative determination of Atx content was done by parallel line assay comparing each venom batch to the standard. The Atx content is expressed as percentage of Atx weight in the total venom weight.

HPLC METHOD

Samples of venom batches were analyzed as follows. Briefly, solution of venom in Tris/HCl buffer, pH 9.0 was applied to the CIM CM disk. Bound material was eluted by stepwise NaCl gradient: 0.04 M (6th min), 0.08 M (12th min), 0.12 M (18th min), 0.2 M (24th min), and 1 M (30th min). Five elution fractions (EF1–EF5) were obtained and profiles of EF1 (containing AtxB and AtxC) and EF2 (containing AtxA) were compared for the analyzed venom batches.

PREPARATION OF VENOM COMPONENT-SPECIFIC ANTISERA

Anti-Atx sera were produced by immunizing rabbits with pure AtxA, anti-H sera by immunizing rabbits with total haemorrhagic fraction and sera specific for both Atxs and haemorrhagins (anti-Atx+H) by immunizing rabbits with total haemorrhagic fraction combined with AtxA or AtxC. The immunization protocol consisted of 3 *s.c.* immunizations at 28 days intervals, with antigen(s) formulated in ISA720^{PGM} (3). Sera were collected on day 14 after the third injection.

QUANTIFICATION OF ANTI-ATX AND ANTI-H IgGs IN RABBIT SERA

Plates were coated with pure AtxA or haemorrhagin preparation (H) in 0.05 M carbonate buffer, pH 9.6 and left overnight at RT. After blocking with 0.5% (w/v) BSA in PBS/T (0.05% (v/v) Tween 20 in PBS) buffer for 2 h at 37 °C, the investigated rabbit sera and the respective "in house" standards (sera containing high levels of IgG specific for AtxA or for H, both were assigned 10 000 AU/mL) were added in five serial two-fold dilutions in duplicate and incubated overnight at RT. Plates were washed, HRP-anti rabbit IgG was added (10 000-fold diluted) and incubated for 2 h at 37 °C. After washing, the OPD solution with H_2O_2 was added and incubated for 30 min at RT in the dark. The enzymatic reaction was stopped with 1 M H_2SO_4 and $A_{492\,nm}$ was measured. Specific IgG was determined quantitatively by parallel line assay, comparing each serum to the respective "in house" standard.

ASSAY OF NEUTRALIZATION OF LETHAL TOXICITY

The potential of rabbit sera to neutralize the lethal toxicity of venom (protective efficacy) was determined in mice. The toxicity neutralization potency (R) of each serum was expressed as the number of LD_{50} venom doses that can be neutralized with 1 mL of undiluted serum and was calculated from the equation: $R = (Tv - 1)/ED_{50}$, where Tv represents the number of LD_{50} inoculated per mouse and ED_{50} the quantity of undiluted serum (in µL) that neutralizes the lethal toxicity of the applied venom dose in 50% of mice (the result of the assay).

RESULTS

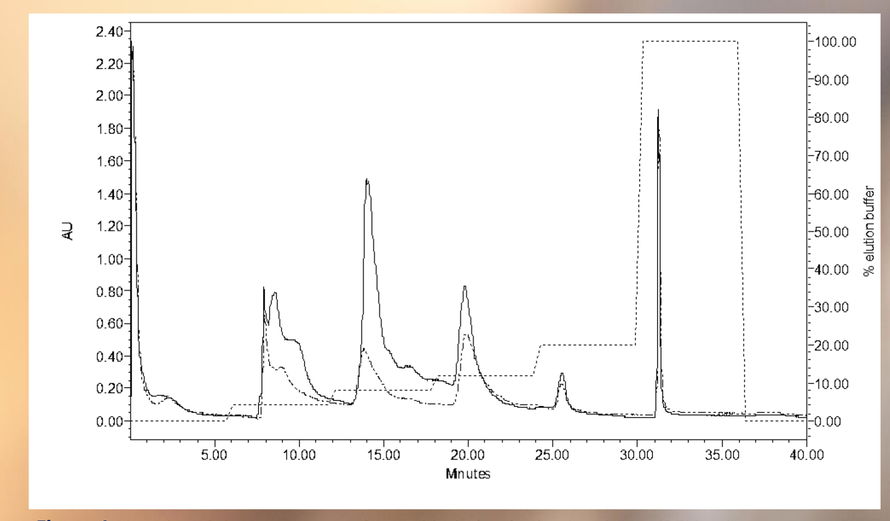

Figure 1. Overlayed chromatograms of KR95 (dash dot dot line) and SL05 (full line) Vaa venom sample after HPLC method for estimation of differences in Atx content. 4 mg of each venom in 50 mM Tris/HCl buffer, pH 9.0 was applied to the disk, and fractions (EF1-EF5) eluted with NaCl gradient steps: 0.04 M (6th min), 0.08 M (12th min), 0.12 M (18th min), 0,2 M (24th min) and 1M (30tmin). Absorbance was measured at 214 nm. Dashed line presents NaCl gradient steps.

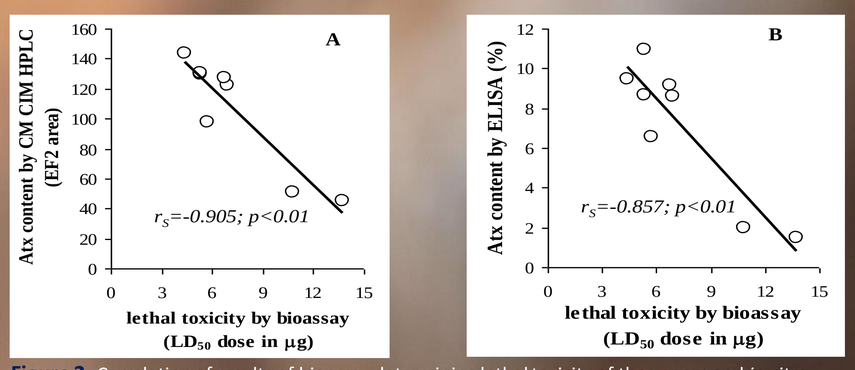

Figure 2. Correlation of results of bioassay determining lethal toxicity of the venom and *in vitro* assays estimating Atx content: CIM CM HPLC (A) and ELISA (B). Spearman's Rank Test of correlation was used to evaluate correlation

Table 1. Quality attributes of KR95 and SL05 samples of *Vaa* venom used for immunogenicity comparison. Results are given as mean ± 95% CI from indicated (in bracelets) number of measurements. All tested quality attributes of the two venoms were significantly different at indicated *p*-values.

Vaa venom sample	Lethal toxicity (LD_{50} / µg)	AtxA content (%)	CM-HPLC of basic venom components	
			EF1 area (x 10^6)	**EF2 area (x 10^6)**
KR95	82,14 ± 12,31 (5)	1,39 ± 0,236 (4)	60,9 ± 3,75 (6)	**58,5 ± 3,64 (6)**
SL05	159,68 ± 13,95 (5)	5,99 ± 0,757 (4)	100,4 ± 6,96 (6)	**147,3 ± 8,06 (6)**
p-value	**<10^{-5}**	**<10^{-5}**	**<10^{-6}**	**<10^{-6}**

Table 2. The quantities of anti-Atx IgG and anti-H IgG determined by ELISA, and the protective efficacies (R) against the venom's lethal toxicity, of the sera of rabbits immunized with Atxs, total haemorrhagic fraction (H) or their combination. Serum of the rabbit immunized with whole venom served as positive control. Results are given as mean values ± SE from a minimum of three independent experiments.

Immunisation antigen	Serum	Anti-Atx IgG (AU[a]/ml)	Anti-H IgG (AU/ml)	Anti-V IgG (AU/ml)	Protective efficacy (R^b)
ammodytoxins	1	72459 ± 3420	0	25996 ± 477	**14.45 ± 2.51**
	2	64843 ± 1324	0	19221 ± 104	**13.65 ± 2.14**
	3	35282 ± 841	0	12688 ± 332	**12.06 ± 2.55**
	4	73115 ± 1953	0	19090 ± 54	**14.87 ± 3.23**
haemorrhagins	5	0	9703 ± 78	98865 ± 1249	**0**
	6	0	10239 ± 296	88658 ± 2092	**0**
	7	0	10220 ± 115	90540 ± 340	**0**
	8	0	6289 ± 211	54858 ± 475	**0**
ammodytoxins + haemorrhagins	9	3743 ± 32	2573 ± 73	13961 ± 77	**21.96 ± 6.47**
	10	10269 ± 287	5695 ± 68	59987 ± 1011	**14.09 ± 1.16**
	11	30879 ± 549	11055 ± 571	112449 ± 3123	**16.38 ± 3.59**
	12	31380 ± 313	5244 ± 171	62076 ± 2381	**9.88 ± 2.25**
venom	**13**	**29965 ± 378**	**5086 ± 61**	**127999 ± 1628**	**>60**

[a]Arbitrary units.
[b]R expresses the number of LD_{50} venom doses that can be neutralised by 1 mL of undiluted serum

CONCLUSION

- **The results showed that functional anti-Atx antibodies were only partially involved in the neutralization of the venom toxicity.**
- **Functional anti-H antibodies did not provide protection at all.**
- **Atx content determination in different venom batches could serve as a good screening method in the selection of the best antigen for immunisation (3,4).**
- **Developed methods for anti-Atx or anti-H determination, cannot be a substitute for *in vivo* antivenom potency determination. Deeper insight into snake venom composition at the molecular level and the participation of the particular components in the venom toxicity has yet to be done before solving this task (5).**

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