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Short communication

A new ELISA for determination of potency in snake antivenoms

A. Rial*, V. Morais, S. Rossi, H. Massaldi

Department of Biotechnology Development, Instituto de Higiene, Universidad de la República, Av. A. Navarro 3051, 11600 Montevideo, Uruguay

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Abstract

A competitive ELISA for potency determination of bothropic equine antivenom was developed and compared to the conventional *in vivo* ED_{50} assay, with the aim of partially substituting the *in vivo* assay in the monitoring of antivenom immunoglobulin levels. On this purpose, blood samples were taken at different times during and after the immunization protocol of the lot of horses used for production of snake antivenom at the Instituto de Higiene, Uruguay. Both the competitive ELISA and the ED_{50} assay were performed on those samples. In addition, a group of five commercial pepsin-digested antivenoms were tested by both methods. A significant ($P<0.001$) correlation (Pearson's $r = 0.957$) was found between the ELISA titres and the corresponding ED_{50} values, indicating that the *in vitro* test can estimate the neutralizing antibody capacity of the sera as well as the *in vivo* assay. By means of this new ELISA, it was found that the immunized animals maintained good venom antibody titres, in the order of 20–50% of the maximum achieved, even 10 month after the end of the immunization schedule. The main advantage of our ELISA design is its ability to correctly estimate the neutralization capacity of crude hyperimmune plasma and antivenom sera independently of their antibody composition in terms of whole IgG or $F(ab')_2$ fragment.

Keywords: Antivenom; ELISA; ED_{50}; *Bothrops* antivenom; *In vitro* assay

The neutralization ability of snake antivenoms is still assessed by the traditional *in vivo* lethality assay (minimum effective dose: ED_{50}), comparable to those used for bacterial antitoxins, usually performed in mice (World Health Organization (WHO), 1981). Besides its inherent aggressiveness to the animals, this procedure is expensive, cumbersome, and time consuming. Reproducibility is quite difficult to achieve, and it is strongly dependent on qualified and trained personnel. In the last decades, the interest in developing *in vitro* assays that may replace, at least in part, the use of *in vivo* assays has grown rapidly. *In vitro* assays offer several advantages: they are easier to perform, more reproducible, take much less time and mainly, they contribute to reduce the number and suffering of animals (Roush, 1996). In this sense, several international organizations such as WHO, FDA (USA) and the European Centre for the Validation of Alternatives Methods (ECVAM, European Union) have recently recommended the reduction of animal testing and emphasized the importance of diminishing the pain inflicted to them (Balls and Straugham, 1996; Theakston et al., 2003; Sells, 2003). For these reasons, *in vitro* techniques should be applied

*Corresponding author.
E-mail address: arial@higiene.edu.uy (A. Rial).

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whenever possible and convenient, keeping in mind that the final method of reference is, undoubtedly, the *in vivo* assay.

Neutralization assessment by alternative *in vivo* (Sells et al., 1997, 2000) and *in vitro* assays, including ELISA (Theakston and Reid, 1979; Barbosa et al., 1995; Maria et al., 1998), and haemolysis (Gutiérrez et al., 1988; Alape-Girón et al., 1997) has been attempted. Initially, the ELISA was developed for identification of the snake species involved in human accidents in Africa, in order to improve medical treatment and for epidemiological studies (Theakston et al., 1977). Later, Theakston and Reid (1979) extended the application to estimate the potency of the antivenoms used. Since then, various ELISA tests have been proposed for medical diagnosis and as an alternative for the *in vivo* neutralization assay of antivenom potency in other species. Rungsiwongse and Ratanabanangkoon (1991) reported a significant correlation between their ELISA and the antivenom potency tested *in vivo* against *Naja Naja* venom. Barbosa et al. (1995) also found a very good correlation for *Crotalus durissus terrificus* venom but not so for *Bothrops* venom. On the other hand, Heneine et al. (1998) have shown a good correlation between ELISA and the *in vivo* potency of bothropic antivenoms when crude *Bothrops* venom was fractionated and the purified fractions were used as the antigen for ELISA. It has been suggested that the multifactorial nature of the lethal activity of bothropic venoms, in which no single component is solely responsible for lethality, could be the reason why it has been difficult to establish a good

Table 1
Neutralizing *in vivo* potency and ELISA antibody titre of hyperimmune horses and commercial antivenoms

Horse no.	Weeks after priming[a]	ED50 (μg/mL) Mean (conf. interval)	ELISA titre Mean (SD)
1	0	207 (172–313)	147 (40)
3	0	260 (200–310)	515 (108)
7	0	296 (246–346)	280 (46)
9	0	237 (165–309)	278 (23)
1	8[b]	2176 (1348–2735)	2269 (106)
2	8[b]	2864 (2412–3828)	3039 (110)
3	8[b]	3601 (3016–4454)	3938 (194)
4	8[b]	2098 (1435–2475)	2234 (137)
5	8[b]	2632 (1696–5819)	3428 (140)
6	8[b]	2136 (1800–2447)	2296 (165)
7	8[b]	2578 (2272–3000)	3511 (92)
8	8[b]	2937 (2513–3945)	3023 (122)
9	8[b]	2136 (1800–2447)	2125 (77)
1	28	1232 (1032-1432)	1356 (75)
2	28	960 (530–1165)	974 (75)
3	28	1668 (1388–6260)	2542 (116)
4	28	1098 (970–1230)	1190 (36)
5	28	1383 (1091–3538)	1632 (117)
6	28	1730 (1544–2126)	1953 (136)
7	28	1597 (1250–2760)	2264 (74)
8	28	1342 (1115–1565)	2066 (141)
9	28	1518 (1312–1810)	1325 (15)
1	50	964 (840–1105)	982 (27)
2	50	907 (789–1126)	704 (58)
3	50	858 (716–1079)	870 (15)
Antivenom	BIOL (Argentina)	1445 (1288–1783)	1530 (56)
Antivenom	I Malbrán (Argentina)	3842 (3460–4307)	3754 (164)
Antivenom	I de H vet (Uruguay)	2600 (2300–3010)	2422 (112)
Antivenom	I de H 6/2 (Uruguay)	2105 (1600–2300)	2072 (51)
Antivenom	I de H 8 (Uruguay)	2518 (2300–2800)	2885 (53)

[a]Animals were immunized at weeks 0 (priming), 2 and 5. The basal value of ED_{50} at week 0 reflects the fact that the equine lot has been immunized previously.
[b]Final, production bleeding.

correlation between *in vivo* neutralization and *in vitro* assays (Maria et al., 1998).

The present work describes a competitive ELISA that estimates the potency of *Bothrops alternatus* venom antibodies in hyperimmune equine blood samples and commercial, pepsin-digested, liquid antivenoms. The horses were immunized with a 50% mixture of *B. alternatus* and *B. pubescens* venoms, the two main prevalent species in Uruguay. Both venoms were obtained from a pool of adult snakes kept at the Instituto de Higiene serpentarium. At days 0, 14 and 35 (weeks 0, 2 and 5, respectively) each horse was inoculated subcutaneously with 3 mg of the venom mixture, emulsified with Freund's complete adjuvant. Final bleeding of horses was carried out on day 56 (week 8). All commercial antivenoms tested were presented in liquid form and raised against the same snake species, except that one from Argentine (BIOL) that was also active against *Crotalus durissus terrificus*, and was presented as a dry powder. *B. alternatus* venom was preferentially used in the development of the new ELISA because it is the one used as the reference in our laboratory. Hyperimmune blood samples were obtained from the lot of horses at regular intervals during the immunization period and also up to 10 months after the final bleeding (week 50). The corresponding sera were kept at −20 °C, as well as the vials of commercial antivenoms used.

The competitive ELISA we developed required the preparation of a labelled antibody—the conjugate—as the first step. To this end, the immunoglobulin whole fraction from pooled antivenom horse sera was obtained by caprylic acid precipitation (Rojas et al., 1994), and labelled with horseradish peroxidase (HRP) according to Tijssen (1985). The appropriate dilution of the conjugate was determined as the concentration belonging to the linear dose–response range that gave an optical density about 1.0. To this purpose, a direct ELISA was carried out as follows. Polystyrene 96-well microplates (Maxisorp, Nunc, USA) were coated with 1 μg *B. alternatus* venom and blocked with 1% bovine serum albumin (BSA, Sigma, USA) as described below. Several conjugate dilutions in PBS with 1% BSA and 0.05% Tween 20 (PBS-T-BSA) were incubated 2 h at room temperature and after several washings, a solution of H_2O_2 plus *o*-phenylendiamine dihydrochloride (OPD, Sigma, USA) was added (Crowther, 2001). The reaction was stopped after 15 min with H_2SO_4 3 M, and colour development was measured at 490 nm.

To perform the competitive ELISA, polystyrene 96-well microplates were coated with 1 μg *B. alternatus* venom diluted in 0.1 M carbonate buffer, pH 9.6 (50 μL/well, 18 h at 4 °C). After coating, remaining active sites were blocked with 1% BSA for 1 h at room temperature (100 μL/well) and plates were washed three times with PBS-T. Subsequently, several dilutions of the test sample (1/50–1/10 000) were prepared in PBS-T-BSA containing peroxidase-labelled IgG antivenom (2 μg/mL). These mixtures were incubated (50 μL/well) for 2 h at room temperature. Peroxidase-labelled IgG antivenom alone, diluted in PBS-T-BSA (2 μg/mL), was also included (50 μL/well), as the reference value. Then, plates were washed 5 times with PBS-T, and colour development was performed as described above. The titre of each sample was defined as the sample dilution that reduced the signal to one half the absorbance shown by the conjugate alone (50% inhibition).

Antivenom potency was determined by the ED_{50}, *in vivo* method (WHO, 1981), from the equine blood samples taken on days 0, 56 (final bleeding), 196 and 350 (weeks 0, 8, 28 and 50, respectively) listed in Table 1, and from the five commercial antivenoms also listed. Briefly, neutralization of lethality was carried out by incubating mixtures containing different venom/antivenom ratios at 37 °C for 60 min. Then 0.5 mL of each mixture was intravenously injected in the tail of 5 groups of CD1 mice (18–20 g, 6 per group), and animal death was

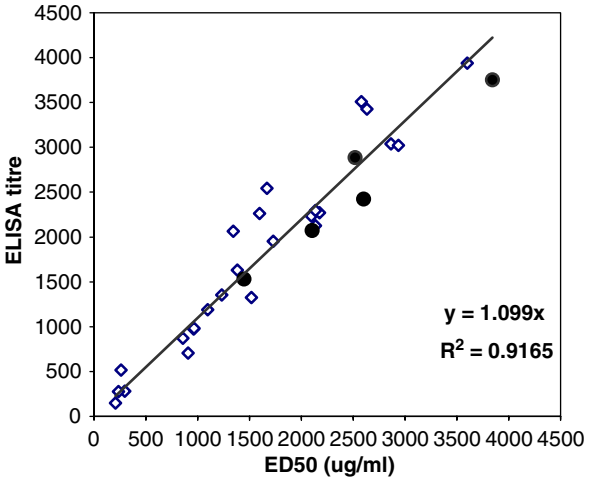

Fig. 1. Correlation between ELISA titres and *in vivo* neutralizing potency of hyperimmune horse sera and antivenoms against *B. alternatus* venom. Twenty-five hyperimmune serum samples (squares) and five different antivenoms were assayed. All data points are means of two separate experiments.

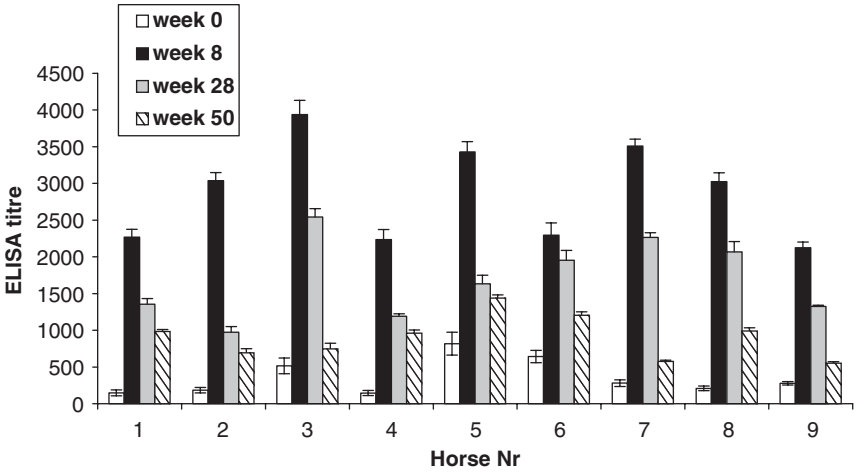

Fig. 2. Time evolution of anti-bothrops antibodies measured by competitive ELISA during immunization experiment with lot of horses at Instituto de Higiene.

registered for 48 h. Results were analysed by Probit analysis, and neutralizing ability was expressed as the 50% effective dose (venom/antivenom ratio that protects 50% of the population). A correlation analysis was performed to evaluate the ability of the antivenom ELISA titres to estimate the ED_{50} values obtained by the *in vivo* potency assay. The corresponding values are listed in Table 1, and the graphical relationship is presented in Fig. 1, where a highly significant correlation ($P<0.001$) is apparent, with a Pearson's coefficient $r=0.957$ ($r^2=0.916$, $n=30$). Also included in the correlation are the five commercial antivenoms, consisting of $F(ab')_2$ fragment, three of which are from previous production batches at the Instituto de Higiene. Fig. 2 compares the evolution of ELISA titres during and after the immunization period for each horse in the lot. It shows that as a whole, rather high titres were reached at the end of the immunization schedule, which decreased progressively thereafter. As expected, individual variability is also present in several horses which showed lower maximum titres at bleeding, but similar remnant values after the subsequent seven or ten months. Regarding the antivenom production process, we have previously used the competitive ELISA (Morais and Massaldi, 2005) to monitor activity loss during pepsin digestion of immunoglobulins purified from equine plasma. We were able to do so because the results obtained with this ELISA depend mainly on the specificity and avidity of antibodies rather than on the composition of the sample in terms of whole or fragmented IgG's ($F(ab')_2$). The results obtained with the $F(ab')_2$ antivenoms in the correlation—the black circles in Fig. 1—confirmed this statement.

In conclusion, our results indicate that this kind of ELISA is adequate to follow antibody titres during immunization procedures and also to determine antibody activity during production of enzyme-digested antivenom. More generally, this *in vitro* test can be satisfactorily used for monitoring activities and to carry out determinations at intermediate steps of manufacturing processes, where the application of the *in vivo* assay is restricted due to practical and ethical considerations. In this way, the *in vivo* assay is left as the reference for the critical steps, thereby softening the ethical conflict and providing the additional benefits of reduced cost and time.

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