Biomacromol. J., Vol. 6, No. 2, 131-138, December 2020.

Biomacromolecular Journal
www.bmmj.org

Evolution of Specific Antibody Response of Horses Immunized with *Naja Naja Oxiana* Snake Venom

S. Khamehchian, M. Tebianian* and H. Zolfagharian

Department of Venomous Animals and Antivenom Production, Razi Vaccine and Serum Research Institute, Agricultural Research Education and Extension Organization (AREEO), Karaj, Iran

(Received 1 June 2021, Accepted 25 June 2021)

ABSTRACT

An indirect ELISA for potency assessment of *Naja naja oxiana* horse antivenom was developed, with the aim of monitoring of antivenom immunoglobulin levels to replace the well-established conventional *in vivo* neutralization assay. According to this purpose, blood sera were taken during immunization schedule of the nineteen horses used in snake antivenom manufacture at the Razi Vaccine and Serum Research Institute. In addition, a group of fifteen batches of monovalent and polyvalent anti-snake venom plasma was examined by both *in vitro* and *in vivo* assays. ELISA is based on the horse anti-*Naja* antibodies recognizing toxic fractions mixture, the main antigen that induces neutralizing antibody against *Naja naja oxiana* venom. Consistent results were found between the ELISA optical densities and corresponding neutralization potency values, demonstrating that the *in vitro* assay as well as *in vivo* assay can be used to estimate neutralizing antibody activity of the sera. It was found that venom antibodies reached a maximum level about 3rd week after immunization. The major advantage of designed ELISA is its ability to correctly separate poor or non-responder horses during initial steps of immunization which venom antibodies are very low in sera and antivenom estimation is difficult by *in vivo* neutralization test.

Keywords: *Naja naja oxiana*, Snake venom, Polyvalent antivenom, ELISA, Potency

INTRODUCTION

Snakebites are an occupational hazard and remain a major medical problem that imposes a high burden of morbidity and mortality in many parts of the world, especially in Asia and Africa [1,2]. The administration of snake antivenom is the only rational, effective and widely accepted treatment of snake envenomation [3]. In the last few years, studies on numerous research area are being carried out for improvement in the potency of antivenoms [4,5]. It has been proposed, due to individual variation in the immune response to snake venom [6], one important step to produce potent antisera is horses following up during immunization to monitor antivenom immunoglobulin levels through any stage of bleeding [7]. The immune response to snake venoms extremely depends on the structure, molecular mass and relative abundance of individual toxins in venoms, the quantity of administered venom, and the host factors such as administration route, and individual genetic nature of the immunized animal [8].

The most traditional way for assessment of antivenoms potency involves an *in vivo* neutralization assay, usually performed in mice [9]. Besides the killing of animals in a large number, this procedure is time-consuming, laborious and expensive [10-12]. It is hard to achieve reproducibility and heavily dependent on personnel knowledge and experience [12]. Therefore, despite the widespread use of this *in vivo* assay, it is always interested to develop *in vitro* alternative methods [10] including ELISA [13], cell line assay [14], and hemolysis [15]. *In vitro* assays offer numerous benefits: they are more reproducible, easier to perform, less time consuming and most importantly, they contribute to reduce the number of lab animals experiencing severe suffering [16]. Various *in vitro* ELISA-based assays have been developed as an alternative for in vivo potency assessment of antivenoms [12]. It was concluded that some of the proteins in snake venoms are less toxic but much more immunogenic than highly toxic peptides with low molecular weights. Therefore, it seems to be better venom toxic fractions used to estimate antivenom

*Corresponding author. E-mail: mtebianian@yahoo.com

potency rather than crude venom as an antigen in the ELISA [11].

Naja naja oxiana (NNO) is a species of poisonous snake in the *Elapidae* family that mostly found in the northeast of Iran. A number of studies have demonstrated that the snake venom comprises four main fractions named F1 to F4. Fractions 1 and 2 consist of non-toxic proteins with high molecular weight, whereas F3 and F4 composed of low molecular weight toxic components [17]. In the present study, an ELISA method, as a pre-screening test, has been developed for potency estimation of serum samples from horses immunized with *Naja naja oxiana* crude venom. The neutralizing activity of specific antibodies was considered by the binding ability of anti-*Naja naja oxiana* immunoglobulins to venom toxic fractions.

The objective of this research was to prevent the production of the final batch with poor neutralizing capacity following mixing of samples with extremely different potencies.

MATERIALS AND METHODS

Immunization

The experimental animals [n = 19] consisted of mature adult horses aged 5-10 years old, were immunized with the prepared venom mixture. The lyophilized crude venom of *Naja naja oxiana* was provided by Razi Vaccine and Serum Research Institute and solubilized in saline. This solution was mixed with appropriate dilutions of the five other snake venoms including venoms from *Vipera lebetina*, *Vipera albicornuta*, *Echis carinatus*, *Pseudocerastes persicus*, and *Agkistroden halys*. Immunization of horses was performed as outlined by Latifi and Manhouri [18] with some modification. According to this procedure, the animals were immunized subcutaneously 3 times with gradually increasing venom doses on the neck at 1-week intervals. After 1 week of resting, 5 steps of additional injection were given to raise the antivenom level in the animal's sera. The venoms were formulated with Freund's complete adjuvant (FCA, Razi Institute) for the first immunization and, Freund's incomplete adjuvant (FIA) for the remaining boosters. The 5 ml of peripheral blood were collected from the jugular vein of each animal every 1 week after immunization. The samples were allowed to clot at room temperature. The clots were removed by centrifugation, and clear sera stored frozen at -20 °C for later assays.

Protein Determinations

The protein concentration of crude venom and its toxic fractions were measured according to the Lowry *et al.* [19] method using bovine serum albumin as standard.

Quantification of the Antivenom Antibodies by ELISA Method

An indirect ELISA was optimized through checkerboard assessment with some modifications [20]. Briefly, each well of the Maxisorp ELISA plates (NUNC, Denmark) was coated with 2 µg/100 µl antigen (toxic fractions mixture) in carbonate bicarbonate buffer (pH 9.6) and kept overnight at 4 °C. To remove the excess antigen, the wells were washed with phosphate buffer saline (PBS) containing 0.05% Tween 20 (PBST) and were blocked with 5% skim milk in PBST for 90 min at 37 °C. The wells were washed and appropriate dilution of horse sera was added and incubated for 70 min at 37 °C. After washing, binding of polyvalent antisnake venom antibodies was detected with anti-horse whole immunoglobulin horseradish peroxidase (HRP) conjugate (Abcam) diluted in PBS (1/10,000). After conjugate addition and incubation for 70 min at 37 °C, the wells were washed five times with PBST. The color reaction was developed by using 3,3',5,5'-tetramethylbenzidine (TMB) and terminated after 20-30 min with 1 N H_2SO_4. The colorimetric reaction was read at 450 nm in an ELISA plate reader. The same procedure was used to assess horse anti-*Naja naja oxiana* antibodies in the last bleeding samples using three other antigens (crude venom, F3 and F4 fractions).

Moreover, fifteen samples of *Naja naja oxiana* hyperimmune horse plasma batches (5 monovalent and 10 polyvalent) taken from venomous animals and antivenom production department of Razi Institute and used as controls for the ELISA assay against toxic fractions mixture. Horse immunization process to obtain all of these samples was according to the procedure above mentioned except that *Naja naja oxiana* venom toxic fractions used as an antigen for monovalent antivenom.

SDS-PAGE Analysis and Western Blotting

The electrophoresis pattern of *Naja naja oxiana* crude venom and toxic fractions (F3, F4, and fractions mixture) was determined by SDS-PAGE analysis, carried out on 20% vertical slab gel under non-reducing conditions. Gels were either submitted to coomassie blue staining or semi-dry blotting onto nitrocellulose membrane, as described by Laemmli [21]. The membrane was blocked with blocking buffer (PBST containing 5% skim milk) at room temperature for 1 h, and then incubated with an appropriate dilution of polyvalent anti-*Naja* serum from immunized horse (with the highest optical density against toxic fractions mixture in the last bleeding samples) diluted in dilution buffer (PBST containing 2% skim milk). After incubation at room temperature for 1 h, peroxidase-conjugated goat anti-horse IgG (Abcam) (1/5,000 in dilution buffer) was added, and the membrane was incubated for 1 h at room temperature. The blots were visualized using chloronaphthol and hydrogen peroxide in PBS. After each incubation step, the membrane was washed with PBST for four times.

Using the above-mentioned method, 5 monovalent and 10 polyvalent hyperimmune plasma samples were analyzed for the presence of specific antibodies against toxic fractions mixture antigen using western blotting and for location and intensity of the specific reaction between antigens and antibodies.

Neutralizing Activity of Horse Antivenoms (*In Vivo* Potency Assay)

Neutralizing activity of fifteen horse monovalent and polyvalent hyperimmune plasma samples assayed by the ELISA was carried out as described by Tan *et al.* [22]. Briefly, $5LD_{50}$'s of crude *Naja naja oxiana* venom were mixed with different dilutions of antivenoms prepared in distilled water. The venom-antiserum mixture was incubated for 1 h at 37 °C. Then aliquots (0.5 ml) of each mixture were injected intravenously in the tail of different groups of mice (18-20 g, 4 per group). Animal survival or death was registered after 96 h and the potency was estimated. Neutralization potency of the antivenoms was expressed in terms of LD_{50}. The LD_{50} of the *Naja naja oxiana* venom was reported as 7.8 µg/mouse (18-20 g) [17]. This should be neutralized by a specific amount of the polyvalent or monovalent antivenom serum. The potency of antivenom neutralization was expressed as the largest quantity of venom neutralized per milliliter antivenom.

Statistical Analysis

All analyses and graphics were done in GraphPad Prism (GraphPad Software, version 8.0). All experiments were repeated at least twice and data were expressed as mean ± SD. The statistically significant difference between median antibody levels in ELISA has been considered with one-way independent ANOVA test, Kruskal-Wallis test (non-parametric).

RESULTS

Estimation of Anti-*Naja* Antibody Levels

The levels of anti-*Naja* immunoglobulins from nineteen horses against toxic fractions mixture antigen at various bleeding stages are shown in Fig. 1. For all except two horses, when Freund's complete was used as an adjuvant, *Naja*-specific antibody rapidly rose to a peak at about the 3rd immunization. In contrast, when Freund's incomplete was used in the remaining five immunizations, anti-*Naja* ELISA levels rose very slowly with some fluctuation and then remained at the plateau. Antibodies in the two poor responders reached satisfactory levels at around the 2nd week of the secondary immunization schedule.

The anti-*Naja* antibodies level in the last bleeding samples were assessed simultaneously in reaction to crude venom, F3 and F4 fractions moreover to fractions mixture antigen. The median antibody levels against different antigens of venom were similar; however, the levels of those against fractions mixture antigen were very slightly greater ($p > 0.05$, Fig. 2 and Supplementary materials Fig. 1). In addition, the ELISA system was used for measuring antibody reactive to fractions mixture antigen in the sets of monovalent and polyvalent *Naja*-hyperimmune plasma samples (Fig. 3a). As expected, the antibody response of horses that received fractions mixture antigen was comparable and higher than those in horses immunized with crude venom.

Western Blot Analysis

Different antigens of *Naja naja oxiana* snake venom (crude, F3, F4, and fractions mixture) were electrophoresed

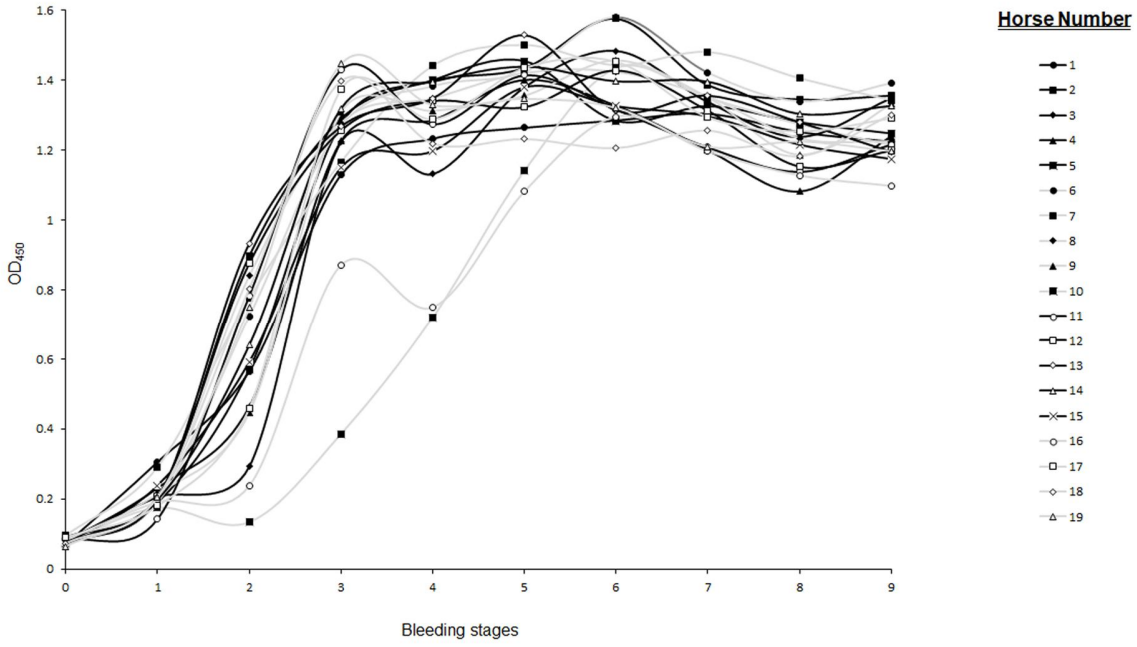


Fig. 1. Antibody response of horses against *Naja naja oxiana* cobra venom. Horses were immunized at weekly Intervals with a mixture of the *Naja naja oxiana* and five viper venoms emulsified with FCA and then with FIA. Blood was taken before immunization, at rest (4th bleeding), and weekly intervals.

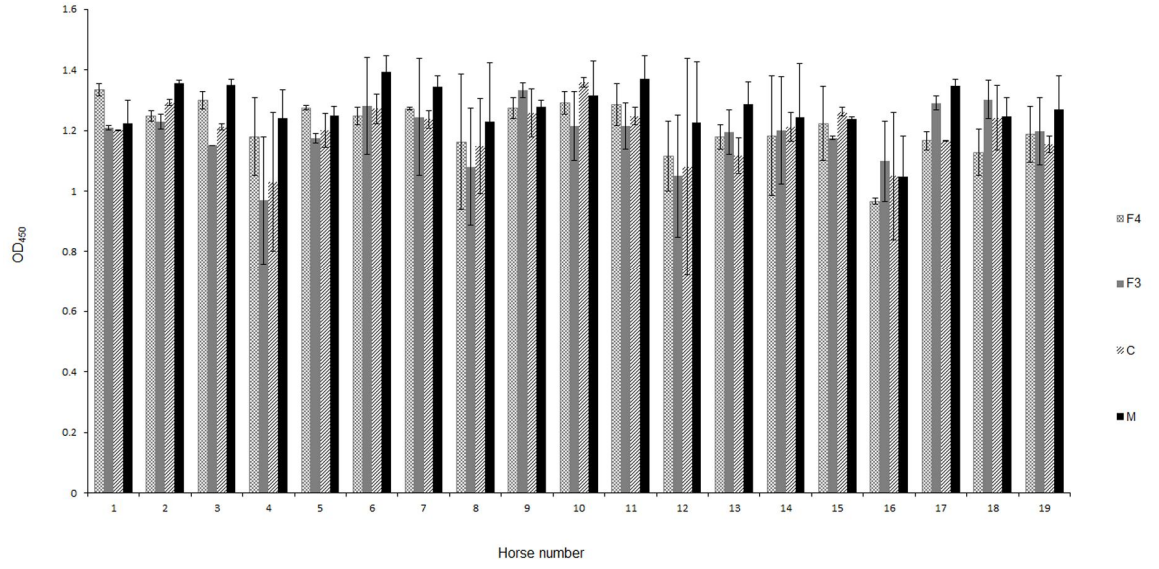
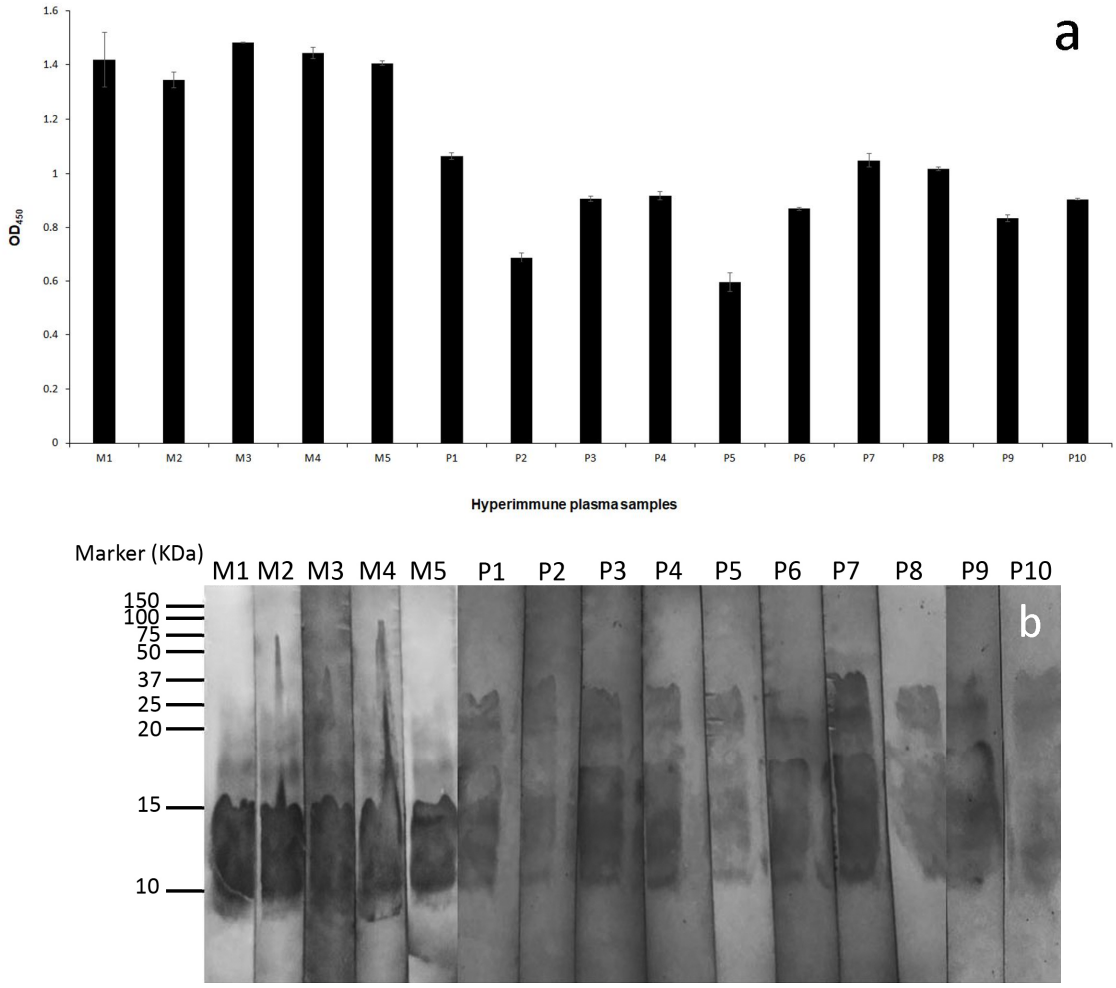


Fig. 2. Reactivity of anti-*Naja* antibodies against Crude (C), toxic fractions mixture (M), F3 and F4 antigens from *Naja naja oxiana* snake venom in the ELISA system. The mean optical density was approximately the same and equal to 1.2 across all antigens.



Figs. 3a and b. Immunoreactivity of polyvalent (P) and monovalent (M) hyperimmune plasma samples to toxic fractions mixture antigen of *Naja naja oxiana* snake venom in (a) ELISA assay and (b) western blot test. The ELISA results showed good agreement with those in western blot positive samples when were compared.

in 20% SDS-polyacrylamid gel under non-reducing conditions. Samples were either stained with coomassie blue (Supplementary materials Fig. 2) or transferred onto nitrocellulose blot transfer membrane for recognition with the *Naja*-polyvalent antiserum (eighth immunization) (Supplementary materials Fig. 3). SDS-PAGE of the crude *Naja naja oxiana* and the relevant toxic fractions showed the protein bands of the size of 10-15 and 15-20 kDa corresponding to the antigen used for the ELISA and western blot analysis.

The additional 15 monovalent and polyvalent hyperimmune plasma samples were investigated for interaction with toxic fractions mixture antigen (Fig. 3b). Western blot analysis showed that antibodies from hyperimmune polyvalent samples recognized most of the components in fractions mixture antigen with similar intensity in molecular masses of 10-15 and 15-20 kDa. However, antibodies from monovalent antivenoms were more strongly reactive with antigen components below 15 kDa than the larger proteins (15-20 kDa). Moreover, the reaction intensity of the monovalent plasma samples toward venom proteins in the mass range of 10-15 kDa was

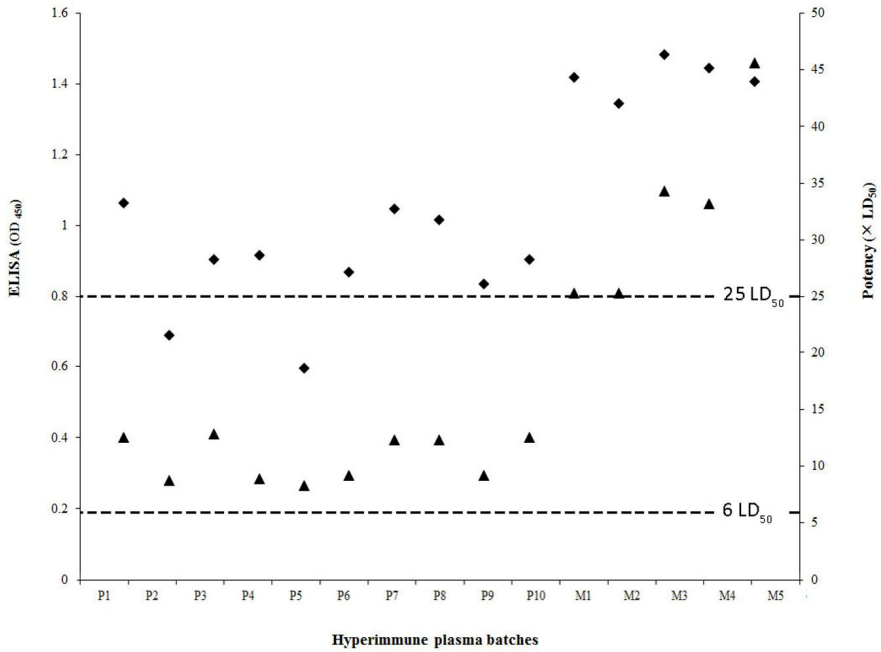


Fig. 4. Comparative analysis of different batches of hyperimmune monovalent and polyvalent plasma before starting the antivenom purification process. The horizontal bar corresponds to the threshold for conformity. Triangle and right-hand y-axis: *In vivo* neutralization test; Square and left-hand y-axis: ELISA assay; P: Polyvalent hyperimmune plasma batches; M: Monovalent hyperimmune plasma batches.

significantly higher than that of polyvalent antivenoms.

Agreement of Anti-*Naja* ELISA Estimates with Mouse Potency Tests

We evaluated the relevance between the ELISA and mouse potency tests. In the current study, for all of the fifteen monovalent and polyvalent of *Naja*-hyperimmune plasma samples that were found to be in accordance with the minimum potency specification (6 LD_{50} and 25 LD_{50} for hyperimmune polyvalent and monovalent antivenoms respectively), the results of the ELISA and neutralizing capacity of antivenom were consistent (Fig. 4). When crude venom was used as antigen (polyvalent antivenoms), the plasma potency was low (8.3-12.8 LD_{50}) and ELISA optical density values varying between 0.595 and 1.064. In contrast, plasma of the horses in which toxic fractions mixture antigen was used (monovalent antivenoms), 1.97 to 3.56 fold increases in neutralizing activity than the highest potency value in the polyvalent plasma samples were observed. A higher potency value indicates a better ability of venom neutralization by the monovalent antivenoms.

DISCUSSION

Snakebites are an occupational hazard and remain a major medical problem in many parts of the world, especially in tropical developing countries [23]. Administration of snake antivenom is the only effective, rational and widely accepted treatment of snake envenomation to date [24]. Currently, the quality testing of antivenoms is a great challenge and has not quite evolved [25]. Although several attempts have been made to resolve this problem, preclinical efficacy assessment of antivenoms still relies only on the neutralization of venom-induced lethality or potency test [26]. Even though it is difficult to replace the *in vivo* potency test in estimation of antivenom efficacy, surrogate *in vitro* assays can greatly decrease the number of mice used for monitoring the evolution of antibody levels in horse, or help in attaining an elementary yes or no decision on about whether an antivenom will be effective against a venom [25,26]. This view was

incorporated into the second edition of the WHO guidelines for antivenoms, in which alternative immunochemical tests such as antivenomics or ELISAs are used as pre-screening tests to examine the efficacy of antivenoms [3]. Likewise, *in vitro* assays offer high specificity and sensitivity are easier to perform, take much less time, and more reproducible [7].

The present study describes an indirect ELISA that estimates the potency of *Naja naja oxiana* venom antibodies. Replacement of the in vivo neutralization assay by the ELISA have advocated in previous studies to produce satisfactory results for the detection of antibodies in serum against various snake venoms [12] however, the purpose of this research was to prepare information important to the future of manufacturing of horse antivenom through conducting detailed analysis of the antibody responses of horses to venom immunization. Taking into consideration of the ELISA results, all except two horses responded well to the immunization with a high optical density at the 3rd week and reached a plateau at about the 4th-5th week post-immunization (Fig. 1). Immune response towards snake venom is influenced by many factors such as the genetic nature of the animal being immunized [8]. Because of this, slowly rise of antivenom levels in the two horses of the group is not surprising. We also estimated the *Naja*-venom antibodies levels in fifteen batches of hyperimmune monovalent and polyvalent antivenoms. The obtained results showed that the reactivity of polyvalent antivenoms generated against crude *Naja naja oxiana* snake venom, with the toxic fractions mixture antigen is the lower than of monovalent antivenoms (Fig. 3a).

The western immunoblot analysis of monovalent and polyvalent plasma samples showed that multiple venom immunization induced a broader spectrum of venom antibodies specificities than individual venom (Fig. 3b), maybe suggesting that cross-reactive proteins induced a synergetic immunological effect [27]. Also, when the whole venom is used to immunize animals, it is to be noted that all toxins in the venom are not present at the same concentration [28]. Moreover, differences in the molecular mass of toxins cause that with a similar weight, the higher number of small molecules are injected in comparison with large molecular components. Thus, due to actual variations in toxin concentration and differences in molecular mass, administered dose of each toxin and the nature of immune response would be different [6].

We next sought to compare the ELISA results obtained for hyperimmune samples with those obtained using an in vivo neutralization assay (Fig. 4). According to these results, ELISA did not appear to correlate well with *in vivo* neutralization. The reason for this matter is not clear at the moment. The relative amount of lethal component in toxic fraction proteins maybe involved in this correlation [29]. On the other hand, the ELISA results were consistent well with neutralizing activity results. Department of venomous animals and antivenom production of Razi institute defined potency confidence limit of 6 LD_{50} and 25 LD_{50} for each batch of hyperimmune polyvalent and monovalent plasma respectively which indicates that the ELISA test is appropriate for analysis of batch to batch venom antibodies levels. The great precision of the ELISA makes it possible to use it for antivenom assessment as a release test or for the in-process monitoring.

The aim of the study was application of ELISA specially to separate of poor or non-responder in the early stage of immunization; with ELISA technology it is possible to detect very slight amount of antibodies which is hard to quantify during initial stages by *in vivo* neutralization assay. This is very important in terms of ethics that poor responder animal not to unnecessarily induct into production process and continue unfavorable immunization for a long term. It will greatly reduce time and cost factor relevant to animal issue.

ACKNOWLEDGEMENTS

We thank the personnel and academic staff at department of venomous animals and antivenom production, Razi Vaccine and Serum Research Institute, for preparing the snake venom fractions and assisting in collection of data, and horse blood samples.

Conflict of Interest

The authors declare that there is no conflict of interest.

Funding

No funding was received for this work.

Ethical Approval

The horse's care and blood collection was performed according to WHO Guidelines for the production control and regulation of snake antivenom immunoglobulins (WHO/BS/2016.2300). Hence, there are no specific ethical considerations.

Abbreviations

FCA, Freund's complete adjuvant; ICA, Freund's incomplete adjuvant; PBS, phosphate-buffer saline; PBST, phosphate-buffer saline containing tween 20; HRP, horseradish peroxidase; TMB, 3,3',5,5'-tetramethylbenzidine; LD_{50}, Median lethal dose; *Naja naja oxiana* (NNO).

REFERENCES

[1] F.Z. Khaddach, B. Benaji, F. Laraba-Djebari, B. Faragher, D.G. Lalloo, J. Chem. Pharm. Res. 6 (2014) 446.

[2] J.P. Chippaux, Bull. World Health Organ 76 (1998) 515.

[3] World Health Organization, Geneva: WHO. 2nd ed, 2017.

[4] D.J. Williams, J.M. Gutiérrez, J.J. Calvete, W. Wüster, K. Atanabanangkoon, O. Paiva, N.I. Brown, N.R. Casewell, R.A. Harrison, P.D. Rowley, M. O'Shea, S.D. Jensen, K.D. Winkel, D.A. Warrell, J. Proteomics 74 (2011) 1735.

[5] R.A. Harrison, D.A. Cook, C. Renjifo, N.R. Casewell, R.B. Currier, S.C. Wagstaff, J. Proteomics 74 (2011) 1768.

[6] G. León, L. Sánchez, A. Hernández, M. Villalta, M. Herrera, A. Segura, R. Estrada, J.M. Gutiérrez, Inflamm. Allergy Drug Targets 10 (2011) 381.

[7] S. Kumar, S. Kumar, Y. Behl, R.K. Gupta, Am. J. Biomed. Sci. 6 (2014) 20.

[8] M. Schunk, G. Macallum, ILAR J. 46 (2005) 241.

[9] World Health Organization. Geneva: Offset Publication, WHO, 1981.

[10] W.S. Maria, M.O. Cambuy, J.O. Costa, D.T. Velarde, C. Chávez-Olórtegui, Toxicon 36 (1998) 1433.

[11] J. Rungsiwongse, K. Ratanabanangkoon, J. Immunol. Methods 136 (1991) 37.

[12] A. Rial, V. Morais, S. Rossi, H. Massaldi, Toxicon 48 (2006) 462.

[13] R.D.G. Theakston, H.A. Reid. Toxicon 17 (1979) 511.

[14] S. Kumar, S. Kanwar, V. Bansal, R. Sehgal, Biologicals 37 (2009) 297.

[15] J.M. Gutiérrez, C. Avila, E. Rojas, L. Cerdas, Toxicon 26 (1998) 411.

[16] W. Roush, Science 274 (1996)168.

[17] A. Akbari, H. Rabiei, A. Hedayat, N. Mohammadpour, H. Zolfagharian, S.H. Teymourzadeh, Arch. Razi Inst. 65 (2010) 33.

[18] M. Latifi, H. Manhouri, Mem. Inst. Butantan 33 (1966) 893.

[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265.

[20] N.C. Salvi, R.L. Deopurkar, A.B. Waghmare, M.V. Khadilkara, M.Y. Kalolikara, S.K. Gadea, L.S. Mohite, Procedia Vaccinol. 2 (2010) 3.

[21] U.K. Laemmli. Nature 227 (1970) 680.

[22] C.H. Tan, P.K. Leong, S.Y. Fung, S.M. Sim, G. Ponnudurai, C. Ariaratnam, S. Khomvilai, V. Sitprija, N.H. Tan, Acta Trop. 117 (2011) 119.

[23] R.A. Harrison, A. Hargreaves, S.C. Wagstaff, B. Faragher, D.G. Lalloo, PLOS Negl. Trop. Dis. 3 (2009) e569.

[24] R. Raweerith, K. Ratanabanangkoon, Toxicon 45 (2005) 369.

[25] J.M. Gutiérrez, G. Solano, D. Pla, M. Herrer, Á. Segura, M. Vargas, M. Villalta, A. Sánchez, L. Sanz, B. Lomonte, G. León, J.J. Calvete, Toxins 9 (2017) e163.

[26] F. Chacón, A. Oviedo, T. Escalante, G. Solano, A. Rucavado, J.M. Gutiérrez, Toxicon 93 (2015) 41.

[27] Y. Angulo, R. Estrada, J.M. Gutiérrez. Toxicon 35 (1997) 81.

[28] J.J. Calvete, L. Sanz, Y. Angulo, B. Lomonte, J.M. Gutiérrez. FEBS Lett. 583 (2009) 1736.

[29] K. Ratanabanangkoon, K.Y. Tan, S. Eursakun, C.H. Tan, P. Simsiriwong, T. Pamornsakda, W. Wiriyarat, C. Klinpayom, N.H. Tan, PLOS Negl. Trop. Dis. 10 (2016) e0004565.