Quantitation of venom Antigens from Moroccan vipers in serum by using an Enzyme-Linked Immunosorbent Assay (ELISA) toward improving health vigilance systems

Fatima Ezzahra Khaddach [1,2,3], Brahim Benaji [1,2], Mohamed Azougagh [1], and Noreddine Ghalim [3]*

[1]Groupe of Biomedical Engineering and Pharmaceuticals Sciences - National Graduate School of Arts and Crafts (ENSAM)-Mohammed V University Rabat, Morocco.

[2]Department of Biology, Pharmacology and Toxicology Unity, Microbiology Pharmacology Biotechnology and Environment Laboratory - Faculty of Sciences Ain Chock, Hassan II University. Casablanca- Morocco.

[3]Laboratory of venoms and toxins, Pasteur Institute of Morocco -Casablanca, Morocco.

Abstract. In the present study an ELISA assay was developed and validated for detection and determination of the concentration of snakes venom in biological samples. Individual component of each venom (Cerastes cerastes and Macrovipera mauretanica) used as immunogen to raise specific rabbit IgGs in order to set up a sandwich-type ELISA. Lower detection limit, linearity, accuracy, precision, reproducibility, and reference intervals were determined. The method proved to be simple, specific, reproducible, sensitive (detection limit = 0.5 ng/ml) and the calibration plot was based on linear regression analysis (r = 0.980) between 0.9 and 1000 ng/mL of venom concentration, with a lower limit of quantification of 1.58 ng/mL. The intra- and interassay coefficient of variation ranged from 2,02 to 4.62% and 5.29 to 7.40%, respectively. The specificity of the assay was tested using vipers, cobra and scorpion venom. This method detected venom from all viper species tested without significant cross reactivity with other venoms in the concentration range of 0.9–1000 ng/mL. This ELISA described is sufficiently validated for clinical evaluation. The method is adaptable to other venoms. This is potentially useful for clinical diagnosis of snakebite, to monitor antivenom dose, and consequently to improve the national health monitoring systems.

1 Introduction

Snake envenomation is an important medical problem in many parts of the world particularly in tropical and subtropical regions [1,2]. In Morocco, Snakebites are well-known as a medical emergencies [3,4] especially in rural areas where agricultural workers and children are the most affected in 70% of cases and who are considered to be a population of risk [5]. The species involved can be either non-poisonous snakes, or poisonous snakes represented mainly by vipers [6]. During the study period, 873 snakebite cases were reported to the Center Anti Poison et de Pharmacovigilence du Maroc (CAPM), an average incidence of 2.65 cases per 100,000 inhabitants with 218 cases each year [7]. Cerastes cerastes (Saharan horned viper) and Macrovipera mauretanica are classified among the potentially incriminated in the most envenomation accidents cases reported in Morocco [7] and they are one of the common causes of snake envenomation and is a category 1 medically important viperin north of Africa [8].The choice of an appropriate treatment has to be made at an early stage after the bite [9]. It should not be based on clinical signs to avoid the gradual and progressive development of symptoms. That is why a rapid determination of the concentration of venom in body fluids should therefore be of clinical interest in order to assess the severity of envenomation, also it allows to monitor the evolution of envenomation and the monitoring of the treatment. and, consequently, to improve health vigilance systems and the management of patient.

Several immunological techniques have been developed in order to quantitate venom levels in serum after snakebites like immumodiffusion, [10], passive hemagglutination [11],immuno-electrophoresis [12], radioimmunoassay [13], and enzyme-linked immunosorbent assay (ELISA) [14-15]. This latter method seems to be the most widely used technique for the immunodiagnosis of snakebite because it is simple to perform, reliable, and easily adaptable for clinical use. It can also be performed rapidly enough to be of

*Corresponding author: khaddach.fatimazahra@gmail.com

medical relevance in the diagnosis of viper envenomations [16-17] and could be useful improve the national health monitoring systems. The double sandwich ELISA method is usually used for detecting snake venom in tissues and body fluids [18], while indirect ELISA is mainly used for detecting antibodies to venom resulting from previous envenoming. Specificity of ELISA for detecting venom and venom antibody is one of the major considerations in evaluating the applicability of the method for retrospective immunodiagnosis of snakebite.

In the case of Moroccan vipers, the amount of injected venom is expected to be lower, and consequently requires a more sensitive ELISA to quantitate antigens. Current ELISA systems involving snake venoms have low specificity, and most cannot reliably differentiatevenoms of related snakes. Up to now, no report describes an ELISA capable of quantifing moroccan viper venom antigens in rabbit blood. This assay was, however, performed with immunoglobulins which are produced and purified from rabbit blood and tested to be specific of each snake venom evaluated.

In the present report, we describe an ELISA using specific Fab'$_2$ for the measurement of venom antigens of the most common cause of snake venom poisoning in Morocco [7] *Cerastes cerastes*, and *Macrovipera mauretanica* in plasma. The relevance of this assay for monitoring health vigilance system**s** has been assessed by measuring the concentration of venom in the plasma of bitten patients after the envenomation and the evolution of the venom concentration during the following hours.

2 Material and methods

2.1 Materials

2.1.1 Snake venom and antivenom

Venoms were milked from adult specimens snakes of C. cerastes kept in captivity at the Serpentarium of Pasteur Institute of Morocco at Tit Mellil, Morocco. Venoms were obtained by manual compression of the venom glands. After extraction, pooled venoms were centrifuged, filtered, lyophilized and stored at -20 °Cuntil use.
Monospecific experimental antivenoms anti-*Cerastescerastes* (Anti-Cc) produced by horse immunization were suppliedby Pasteur Institute of Morocco.

*2.1.2 Apparatus and reagent***s**

Ninety-six-well flat bottom microtiter plates (Nunc-Maxisorp®,Denmark), Goat antirabbit IgG- horseradish peroxidase (HRP) was purchased from Bio-Rad Laboratories (USA), PBS-Tween20 (Sigma, USA), ortho-phenylenediamine (OPD) (Sigma,U SA). All other chemicals and reagents utilizedwere of analytical grade (Sigma Chemical Company or Merck).

2.1.3 Animals

Male New Zealand white rabbits (1.96 - 2.37 kg) were obtained from Pasteur Institute Supplier and housed in the LaboratoryAnimal Centre of the institution under standard conditions.

2.2 Methods

2.2.1 Preparation of antiserum to snake venom

Antiserum against the viper venoms (*C. Cerastes* and *M.mauretanica*) were prepared as follows: the venom (25µg) in 0.1 ml physiological saline solution of PBS (pH 7.2) , pH 7.4) was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously (s.c) into multiple sites along theback of the rabbits (3-3.5 kg) at biweekly intervals. The rabbits were bled 9 days after the subsequent booster immunization. The sera obtained after clotting were stored frozen until used.
The immunoglobulin G (IgG) purification from the antiserum was carried out with ammonium sulfate precipitation then cleaved with pepsin with slight modification [19-20].

2.2.2 Determination of protein concentration

Protein concentration was determined according to the Lowry's method [21].

2.2.3 Double-sandwich ELISA for venom detection andquantitation

Double-sandwich ELISA was used to monitor the serum venom antigen levels following experimental envenomation in rabbits. In Brief, the ELISA microtiter plates (Nunc-Maxisorp®) were coated with 100 µL of immunoglobulin fragments F (ab')$_2$ horse anti-*C.Cerastes or* anti- *M. Mauretanica*(20µg/mL) dissolved in carbonate- bicarbonate buffer, pH 9.6 overnight at 4°C. The solution was discarded and the wells were rinsed with PBS, pH 7.4, containing 0.05% Tween-20 with an automatic Mindray microplate washer (MW-12A),The unoccupied sites were blocked by incubating the wells with 5% skimmed milk (diluted in PBS, pH 7.4 containing 0.05% Tween 20) for 1 h at 37°C. The plates were incubated with 100 µLof appropriately diluted serum samples (1:10). The wells were rinsed and incubated with rabbit anti-*C.Cerastes or M. Mauretanica* venom F(ab')$_2$ (30 µg/mL) for 1 h at 37°C. Following another washing cycle,goat anti-rabbit IgG- horseradish peroxidase (HRP) conjugate freshly diluted (1:800, 100 µL) was then added into each microtiter well and incubated for 1 h 37 °C, followed by the addition of 100 µL of the substrate (2 mg/mL OPD and 0.006% H_2O_2 in 0.01M potassium phosphate buffer, pH 7.3). The enzymatic reaction was terminated 10min, at room temperature in the dark, later by adding 50 µL sulfuric acid (12.5%), and the absorbance

was measured at 492 nm with MindrayMicroplate Reader (MR- A96).

2.2.4 Analytical method validation

The specificity of the assay was demonstrated by its capacity to identify correctly the circulating antigen in rabbits experimentally poisoned with either of the two venoms. Lower detection limit, linearity, accuracy, precision, reproducibility, and reference intervals were determined.

3Results and discussion

the study of linear regression and the calculation of theregression coefficient between the values of the expected concentrations and the concentration values measured experimentally by the sandwich ELISA method made it possible to assess the linearity of the test. These results were confirmed with the regression curves where the correlation coefficient seems satisfactory (R2 = 0.996; 0.994; respectively for the venom of C.c, and M.m) (Figure 1). Linearity is observed between 0.9 and 100 ng / mL of venom concentration (Figure 1).

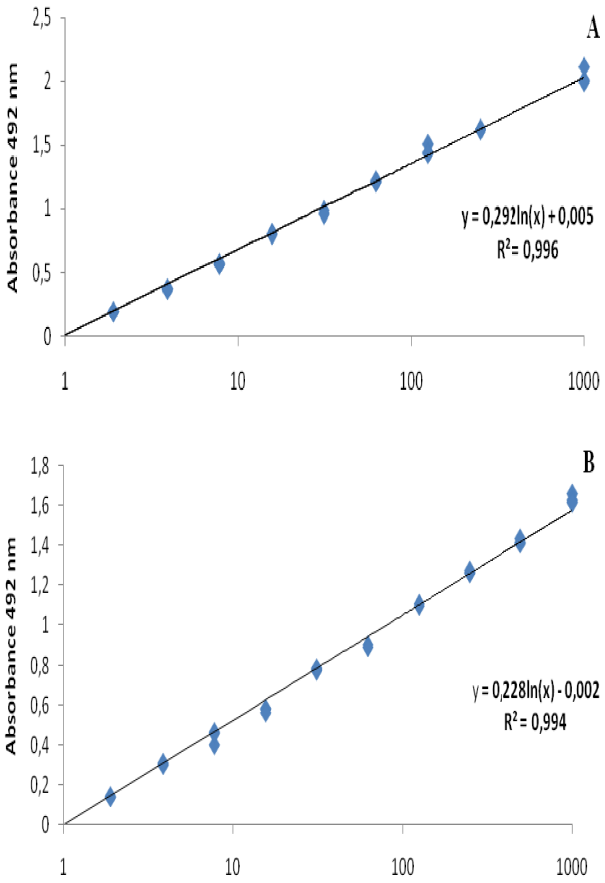

Fig. 1. Snake venom calibration curve by sandwich ELISAA: Cerastes cerastes B: Macrovipera mauretanica

The specificity of the method was checked against different venoms of snakes (Cc, Mm) and scorpions (Androctonus mauretanicus mauretanicus A. mm) Thus, this test makes it possible to detect and measure the venoms of vipers Cc, and Mm On the other hand, no response was observed in scorpion venom (Figure 2).

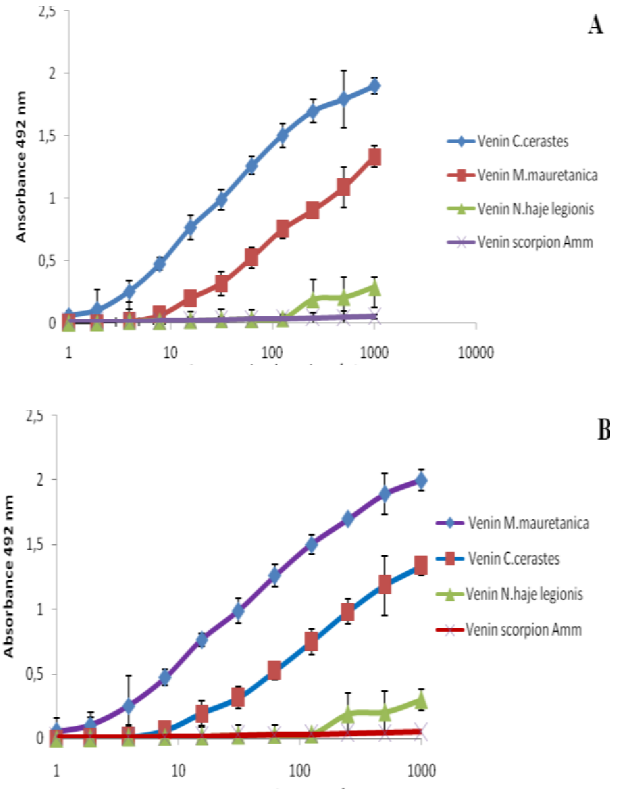

Fig. 2. Evaluation of the specificity of the sandwich ELISA test. A: Cerastes cerastes B: Macrovipera mauretanicaThe results are presented as mean ± E.S (n = 3).

Based on the accuracy test data (Table1), the confidence interval of this method for both venoms appears to be less than 120% and greater than 80%. This means that the confidence interval has a 95% chance of containing the mean percentage recovery (m), so the method is correct for the determination of the venoms of C. cerastes and M.mauretanica.

Table 1:accuracy test data of *C.cerastes* and *M.mauretanica*

Confidence interval	***C.cerastes venom***	***M.mauretanica venom***
m + t*S/√N	101,18	102,08
m - t*S/√N	100,17	99,37

Intra-assay and inter-assay repeatability evaluation were determined for 3 levels of venom concentration. In fact, the intra-assay repeatability was obtained by analysis of 6 assays, carried out under the same conditions (same plate,

same sample and same reagents). Inter-assay repeatability was obtained by analyzing 6 trials for 3 consecutive days.

The repeatability data obtained show that the intra-assay and inter-assay coefficients of variation (CV) are less than 15% for the three concentration levels. From these results it is concluded that the difference between the three means is not significant and therefore the intermediate repeatability is verified for the protein assay method, so there is no dispersion of the measurements during the three days of the study. The sandwich ELISA method developed is thus validated and reliable for the determination and quantification of venoms in biological fluids. After its development and validation, the ELISA test was then applied in order to quantify the venom of the venoms Cerastes cerastes and *Macrovipera mauretanica* after envenomation of rabbits with a sublethal dose.

4 General discussion

The medical interest of this ELISA developed and validated for the first time in Morocco has been demonstrated by monitoring the venom level in the serum of bitten patients in order to identify to identify the species incriminated in the envenomation. The procedure described here will be very useful for such studies. The procedure should also be helpful for the diagnosis and the quantitation of venom in body fluids of bitten patients, allowing an early and accurate biological measurement of the severity of envenomations and, thus, better therapeutic management and improving the health vigilance system. This assay is simple and can easily be performed in less than two hours. Further investigations are in progress to adapt it to field use, shorten the time required, and adapt the method to other snake venoms.

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