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Research Article

Development of an ELISA Assay for the Quantification of Venom Levels of Scorpions "*Androctonus mauretanicus*" and "*Buthus occitanus*" in Stung Patients

Khadija Daoudi[1,3], Salma Chakir[1,2], Fatima Chgoury[1], Imane Gourja[1,2], Abdelaziz Hmyene[2], Rachida Cadi[3] and Naoual Oukkache[1]*

[1]*Laboratory of Venoms and Toxins at Institut Pasteur of Morocco, Casablanca, Morocco*

[2]*Laboratory of Biochemistry, Health and Food Technology, Faculty of Sciences and Technologies of Mohammedia, Morocco*

[3]*Laboratory of Molecular Genetics, Physiopathology and Biotechnology, Faculty of Sciences of Ain-Chock, Casablanca, Morocco*

***Corresponding Author**: Naoual Oukkache, Laboratory of Venoms and Toxins at Institut Pasteur of Morocco, Casablanca, Morocco.

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Abstract

In Morocco, scorpion stings are a serious problem of public health, the majority of which are attributed to the black scorpion *Androctonus mauretanicus* (*Am*) and the yellow scorpion *Buthus occitanus* (*Bo*). The severity of envenomation depends mainly on the amount of venom injected by the scorpion. On this matter, it is primordial to develop routine assays capable of quantifying venom levels in order to orient the diagnosis, to improve the epidemiological studies and to assess the efficacy of commercialized antivenoms. In this work, we designed a performing bi-specific sandwich ELISA assay for the identification and quantification of the venoms of two Moroccan scorpions *Am* and *Bo* in sera of envenomed patients. The results showed that our assay is very sensitive and reproducible (detection limit LoD equal to 33.98 ng/mL and 33.72 ng/mL and quantification limit LoQ equal to 35.41 ng/mL and 34.71 ng/mL for *Am* and *Bo* venoms respectively) with a good correlation (Spearman coefficient close to 1) for venom concentrations in human sera comprised between 0.5 and 20 ng/ml.

Keywords: *Scorpion; Androctonus mauretanicus; Buthus occitanus; ELISA Test; Quantification of Scorpion Venom*

Introduction

Scorpion stings are a public health issue in several parts of the world that involve an at-risk population of 2.3 billion [1]. The annual number of scorpion stings exceeds 1.2 million leading to more than 3250 deaths per year [2]. Underdeveloped countries, especially those located in tropical and subtropical areas including North Africa, Latin America and the Middle East are the most affected regions due to their climate and environment propitious to scorpion development [3,4].

In Morocco, scorpionism is the first cause of envenomation after food poisoning with an average of 30 000 to 50 000 stung-patients recorded every year, accounting for approximately 30% of envenomation cases [5]. The highest mortality rate (up to 53%) of cases is recorded in the Central provinces of the country (region of Marrakech Tensift El Haouz). 3 deaths per 1000 stings are recorded each year representing approximately 100 deaths and most of them are children under the age of 15 [6].

The Moroccan scorpion fauna is known to be rich and diverse due to the climate and the topography of the country. Two of the most dangerous scorpions in Morocco *Androctonus mauretanicus (Am)* and *Buthus occitanus (Bo)* and are responsible for 83% and 14% of most severe accident cases [7,8]. *Am* and *Bo* scorpions belong to the *Buthidae* family and while the *Bo* species is present in the Mediterranean basin, the *Am* species is endemic to Morocco [9,10].

Scorpion venoms are a complex biochemical mixture used by these animals for defense and prey capture. Mucopolysaccharides, hyaluronidases, phospholipases, serotonin, histamine, enzyme inhibitors, and proteins essentially neurotoxic peptides constitute the venom. Toxic and lethal effect of scorpion venoms is mainly due to neurotoxins active on ion channel (Na^+, K^+, Ca^{2+}, Cl^-) of excitable cells [11]. These neurotoxins trigger a variety of envenomation symptoms including severe pain, hyperemia, myocarditis, pulmonary edema, autonomic central nervous system and muscle function disturbances [12].

Envenomation symptoms are divided into three major grades and are treated depending on their severity.

In Morocco, the adopted treatment is painkillers for grade 1 cases (sting without envenomation) and antiemetics combined to antispasmodics to neutralize symptoms of grade 2 envenomations (hypo/hyperthermia, abdominal pain, fever). For grade 3 accidents (cardiac and respiratory failure, neurological disorders) the treatment does not include antivenom and consists in the administration of diazepam, dobutamine and artificial ventilation. Numerous studies clearly established a strong correlation between scorpion venom levels in serum of envenomed patients and the severity of the observed symptoms [2,13-17].

Venom dosage has been done in the past using a wide range of techniques including radioimmunoassay, immunodiffusion or electrophoresis. These methods displayed multiple disadvantages: high cost, low sensitivity threshold and very instable reagents.

It is, therefore, necessary to elaborate detection and quantification routine methods that will help orient the diagnosis and treatment of envenomed patients while also being proficient and highly performing.

In this work, we developed a bi-specific sandwich ELISA assay for the identification and quantification of the two most dangerous scorpions in Morocco; *Androctonus mauretanicus* (*Am)* and *Buthus occitanus (Bo)* in a pool of sera collected from several healthy donors. The developed ELISA is highly sensitive and able to detect circulating venom in stung patients and will provide data for a more objective treatment, a better management of envenomed patients, and the establishment of efficacy of commercialized scorpion antivenoms.

Material and Methods

Scorpion venoms

The *Androctonus mauretanicus* and *Buthus occitanus* scorpions were gathered from the region of Morocco with the highest incidence of stings (Regions of Safi and Essaouira). Crude venoms were collected by electrical stimulation of the scorpions kept in captivity at the Animal Facility of Institut Pasteur of Morocco. The extracted venoms were pooled and centrifuged at 10.000g for 15 minutes; supernatants were then lyophilized and stored at -20°C [7].

Quantification of the proteins (280 nm)

Protein concentration was determined using a spectrophotometer with an extinction coefficient (ε) of 1 for a 1 mg/mL protein concentration at 280 nm and 1.4 mg/mL to determine immunoglobulin concentration, both using a 1 cm light path length tank [18].

Production and purification of antibodies

Horse polyvalent $F(ab)'_2$ antibodies anti *Am* and anti *Bo:* Bi-specific polyclonal antibodies anti *Am* and anti *Bo* were produced at the Animal Facility of Institut Pasteur of Morocco. Hyperimmunized horses were injected with a mixture of *Am* and *Bo* crude venoms. The polyclonal IgG were subjected to pepsin cleavage in order to obtain $F(ab')_2$ anti *Am* and *Bo* that were purified, lyophilized and stored at -20°C in individual dose vials until use [19,20].

Preparation of rabbit monovalent IgG antivenoms anti *Am* and anti *Bo:* Adult male New Zealand rabbits (2 - 3 kg) were used for the production of monospecific polyclonal antibodies anti-*Am* and anti-*Bo*. These animals were randomly divided into 2 groups of 4 animals each. One of the groups was immunized with crude *Androctonus mauretanicus* venom and the second with crude *Buthus occitanus* venom over a 40-days period. Lyophilized crude *Am* and *Bo* venoms (5 mg/ml in physiological saline) were filtered through 0.22 µm sterile filters. The venom (20 µg/rabbit) was mixed with 1 ml of Freund's complete adjuvant (v/v) and 0.25 mL of the emulsion was injected intradermally into six sites along the back of each animal. The booster doses were made in IFA (Incomplete Freund Adjuvant) repeated three times at 10 day-intervals with increasing doses of venom. Rabbits received a second inoculation of venom subcutaneously into three sites. Blood samples were collected via the marginal vein after 42 days of booster injections and were immediately centrifuged at 3000 rpm for 10 minutes; the serum was recovered and stored at -20°C until use [19,20].

Purification of homologous venom antibodies

Monovalent and polyvalent antibodies anti *Am* and *Bo* scorpion venoms were purified by immuno-affinity chromatography on specific venom columns. Venom columns were made by coupling *Am* and *Bo* venoms onto CNBr-activated Sepharose 4B beads (Amersham Pharmacia, Uppsala, Sweden) following manufacturer's instructions. In brief, 0.5g CNBr-activated Sepharose 4B was swollen in HCl 1.0 mM, pH 3.0 for about 15 minutes and centrifuged. Sepharose beads were incubated with 3.3 mg of each venom (*Am* or *Bo*) dissolved in coupling buffer (0.1M $NaHCO_3$, pH 8.3) overnight at 4°C on a round rotator in order to couple venom components onto Sepharose beads. After washing with washing buffer (100 mM Tris-HCl, 0.5 M NaCl pH.8), the active sites on the beads were blocked with blocking buffer (1M Tris-HCl pH.8) 1 hour at 4°C on a rotator. The beads were then washed and equilibrated with washing buffer then packed into the column and stored at 4°C until use. 1 mL of $F(ab)'_2$ anti *Am* and *Bo* and 2 mL of IgG anti *Am* and anti *Bo* were added to the column and incubated 1 hour at room temperature on a rotator. Unbound antibodies were washed thoroughly with washing buffer and bound antibodies were then eluted with acetic acid pH 2.1. As soon as eluted, antibodies were neutralized with 500 µL of 1M Tris-HCl pH.8 [4,19,20].

Optimization of the ELISA test

Maxisorp plates were coated overnight with 100 µL $F(ab)'_2$ antibody diluted at different concentrations (2, 3, 5, 10, 20 and 30 µg/ml) in 6 ml of 0.1M carbonate/bicarbonate buffer, pH 9.5 and incubated at 4°C. The plates were washed 4 times with a washing buffer (50 mM Tris-HCl pH.8, 150 mM NaCl, 0.05% Tween 20). The remaining binding sites were blocked with 200 µL 50 mM Tris-HCl pH.8, 5 mg/mL gelatin, 0.2% Tween 20 for 2 hrs at 37°C, and then the plates were washed 4 times with the washing buffer. 100 µL/well of whole venom (*Am* or *Bo*) were added with a concentration ranging from 0 to 1000 ng/mL and dissolved in a vehicle buffer (50 mM Tris-HCl pH.8, 0.5 M NaCl, 1 mg/mL gelatin, 0.05% Tween 20). Plates were then incubated 2 hrs at 37°C, and washed 4 times with washing buffer. Plates were incubated again 1h at 37°C with 100 µl/well of anti-rabbit IgG anti-*Am* or anti-*Bo* (primary antibodies) at different concentrations 5, 10, 15 and 20 µg/ mL diluted in the vehicle buffer. The plates were washed (4 cycles) and incubated 1h at 37°C with 100 µl/well of secondary antibody conjugated with peroxidase (Goat anti-rabbit IgG, Promega,) diluted at 1:1000 in the vehicle buffer followed by 4 cycles of washing. 10 mL of the revelation solution were prepared using of Citric Acid (50 mM, pH 4) mixed with 50 µL of 40 mM ABTS (2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) and 40 µL of H_2O_2. 100 µL/well of the revelation solution were added and allowed to react for 15 - 30 minutes, the reaction was then stopped with 25 µL/well of SDS 20% for 5 minutes. The plate was then read at 405 nm using an ELISA plate reader. The statistical analysis was carried out with the GraphPad Prism software [4].

The objective of the ELISA test here is to dose scorpion venom in the plasma of envenomed patients. Since we have optimized the steps of the assay, we repeated this experiment but this time we prepared a venom range (*Am* or *Bo*) at different dilutions (1/5, 1/10 and 1/20) of sera collected from healthy donors in order to determine serum concentration giving the closest result to venom diluted in the buffer.

Calibration and quality control of the ELISA

The ELISA assay was validated using different parameters affecting the performance of the test such as specificity, sensitivity, linearity and correlation of the test. We evaluated the shape of the curve (dose-response sigmoid pace curve), the specificity and sensitivity of the test through the determination of the limit of detection (LoD) and the limit of quantification (LoQ). The correlation of the assay was assessed via the Spearman and finally the linearity of the test through the coefficient of variation.

Results

Purification of $F(ab)'_2$ anti *Am* and *Bo* and IgG anti *Am* and anti *Bo* venoms

The capability of the Moroccan polyspecific *Androctonus mauretanicus* (*Am)* and *Buthus occitanus* (*Bo*) $F(ab')_2$ and IgG to immunocapture proteins from the venoms of *Am and Bo* was assessed by affinity chromatography.

Figure 1 shows that we obtained two fractions for each immuno-affinity chromatography. The peaks with the highest absorbances correspond to antibodies with the highest affinity to the venom (F1 for $F(ab')_2$ anti *Am* and *Bo* and F2 for IgG anti *Am* and IgG anti *Bo*). The tubes containing these fractions were pooled, aliquoted and kept at -20°C until use.

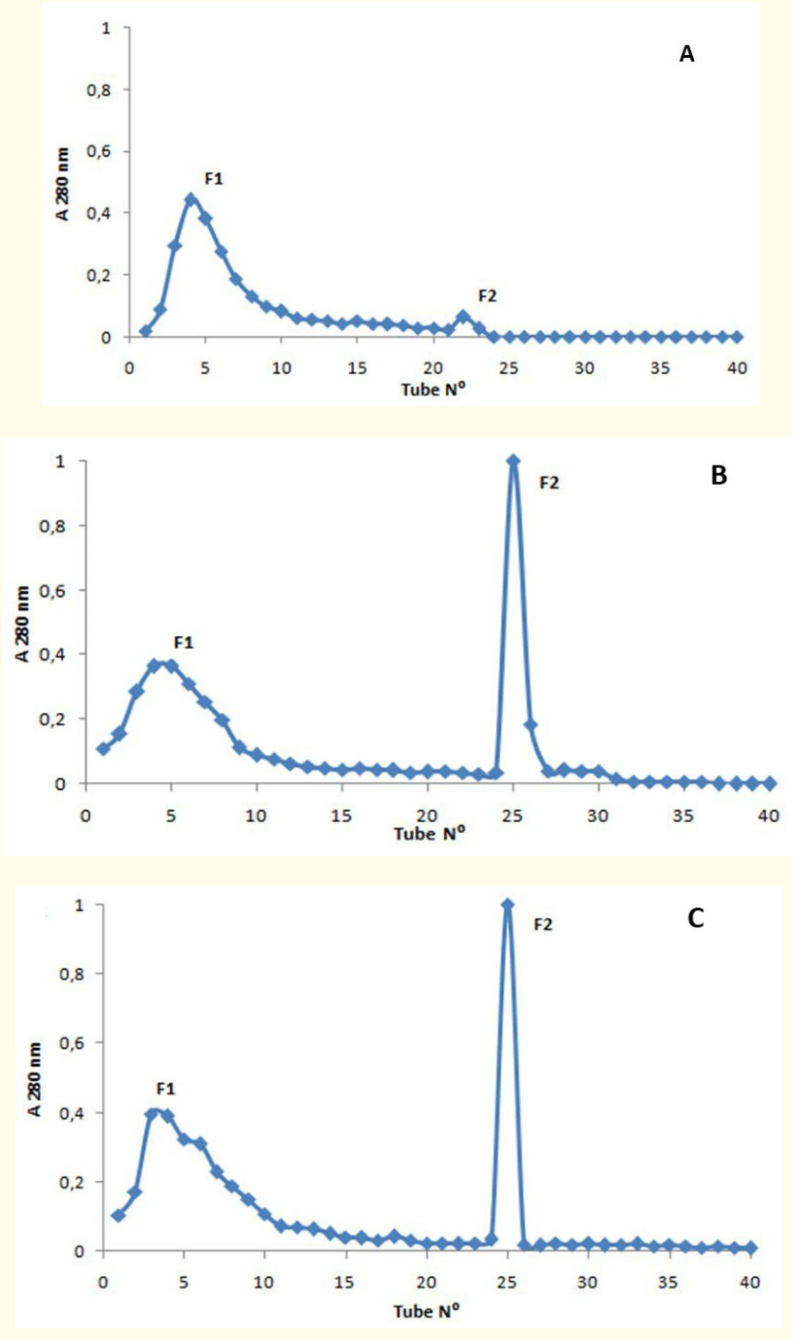

Figure 1: *Panel A represent immuno-affinity chromatogram of F(ab')2anti Am and Bo venoms. Panels B and C display respectively immuno-affinity chromatograms of IgG anti Am and IgG anti Bo. All chromatograms showed two distinct fractions, F1 being the fraction containing antibodies with the highest affinity to venom for F(ab')2 antibodies and F2 for IgG anti Am and IgG anti Bo.*

and 1/10 for Bo venom.

ELISA characteristics and performance reference curve and linearity

Several *Am* and *Bo* concentrations (0 to 1 µg/ml) were prepared freshly, and incubated in every microtitration plate. The standard dose response curve showed a good linearity between human sera comprised between 0.5 and 15 ng/ml. The linear regression analysis of the proportionality between measured and expected *Am* and *Bo* concentrations showed a strong correlation (coefficient of Spearman of r^s = 0, 9879 P < 0,001 for both *Am* and *Bo* venom) which allowed an accurate determination of the venom level by interpolation.

Sensitivity and detection limit

The sensitivity of the ELISA was determined by measuring the absorbance at 405 nm of 20 serum samples from healthy non-envenomed donors.

The curve scorpion venom concentration in human plasma obtained from healthy donors at different dilutions (1/5, 1/10 and 1/20) *vs.* optical densities for each venom was plotted (Figure 2). The correlation of optical density with concentration was well linearized and the showed a good correlation for venom concentrations tested (Tables). The limit of detection (LoD) expressed in ng/mL correspond to the concentration of scorpion venom equal to the mean times 3 standard deviations and is usually defined as the lower quantity or concentration of a component that can be reliably detected with a given analytical method with an acceptable level of accuracy and precision. The quantification limit (LoQ) also expressed in ng/mL correspond to the lowest concentration venom quantifiable, with acceptable uncertainty, under the conditions described in the method. In our experiment, the LoD equals to 33, 98 ng/mL (for *Am* venom) and 33.72 ng/mL (for *Bo* venom), and LoQ of 35.41 ng/mL for *Am* and 34.71ng/mL *Bo* venoms respectively.

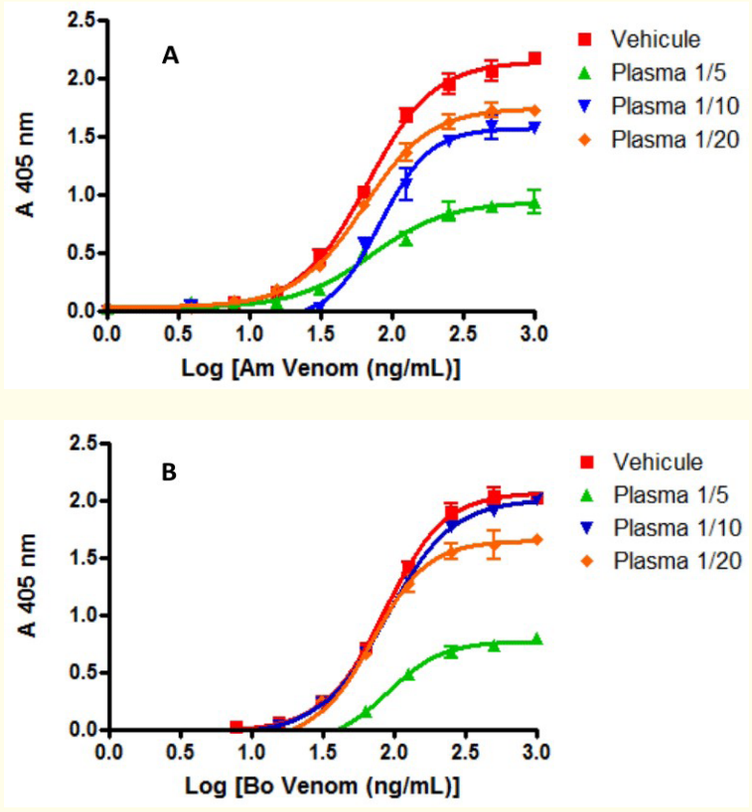

Figure 2: *Panel A and B show the titration curve standard range of Am and Bo venoms respectively prepared with the optimum conditions with the vehicle buffer and the venoms diluted in different concentrations of plasma (1/5, 1/10 and 1/20) from healthy donors.*

Assay precision was evaluated using ELISA diluent controls diluted to all points on the standard curve. Every experiment was repeated in duplicate and the standard deviation were calculated and plotted on each curve. Accuracy, linearity and reproducibility of the assay were assessed through the calculation of coefficients of variation (C_v usually less than 10%). These coefficient ranged from 1,696 to 1,787 for *Am* venom corresponding to C_v = 9.1% and 1,981 to 2,036 which correspond to C_v = 5.5% for *Bo* venom (Data shown in tables 1-3).

	Vehicule	Plasma 1/5	Plasma 1/10	Plasma 1/20
Sigmoidal dose-response (Variable slope)				
Best-fit values				
Bottom	0.01276	0.02289	-0.09246	0.03211
Top	2.144	0.9345	1.569	1.741
Logec 50	1.815	1.853	1.905	1.792
Hill Slope	1.815	1.624	2.405	1.843
EC 50	65.27	71.27	80.29	61.93
Std. Error				
Bottom	0.02688	0.02530	0.04193	0.01732
Top	0.03473	0.03577	0.05752	0.02157
Logec 50	0.01864	0.04436	0.03499	0.01464
Hill Slope	0.1283	0.2473	0.4038	0.1038
95% Confidence Intervals				
Bottom	-0.04423 to 0.06976	-0.03074 to 0.07652	-0.1813 to -0.003563	-0.004606 to 0.06883
Top	2.071 to 2.218	0.8587 to 1.010	1.447 to 1.691	1.696 to 1.787
Logec 50	1.775 to 1.854	1.759 to 1.947	1.830 to 1.979	1.761 to 1.823
Hill Slope	1.543 to 2.087	1.100 to 2.149	1.549 to 3.261	1.623 to 2.063
EC 50	59.60 to 71.49	57.40 to 88.51	67.68 to 95.24	57.66 to 66.52
Goodness of Fit				
Degree of Freedom	16	16	16	16
R^2	0.9961	0.9818	0.9803	0.9975
Absolute Sum of Squares	0.05985	0.04853	0.2029	0.02461
Sy.x	0.06116	0.05507	0.1126	0.03922
Data				
Number of X values	12	12	12	12
Number of Y replicates	2	2	2	2
Total number of values	20	20	20	20
Number of missing values	4	4	4	4

Table 1: *Results for the statistical analysis of the titration curve of the Am venom diluted in the vehicle buffer and in different concentrations of human healthy plasma.*

	Vehicule	Plasma 1/5	Plasma 1/10	Plasma 1/20
Sigmoidal dose-response (variable slope)				
Best-fit values				
Bottom	-0.01542	-0.1234	-0.04336	-0.1032
Top	2.069	0.7685	2.008	1.648
Logec 50	1.925	1.954	1.940	1.839
Hill slope	2.039	2.254	1.868	2.190
EC 50	84.21	89.90	87.06	68.96
Std. Error				
Bottom	0.01778	0.01728	0.008459	0.04236
Top	0.02612	0.02558	0.01307	0.05438
Logec 50	0.01295	0.02850	0.006616	0.03346
Hill slope	0.1101	0.2912	0.04759	0.3296
95% Confidence Intervals				
Bottom	-0.05312 to 0.02228	-0.1600 to -0.08675	-0.06129 to -0.02543	-0.1930to -0.01341
Top	2.014 to 2.125	0.7143 to 0.8228	1.981 to 2.036	1.533 to 1.763
logec 50	1.898 to 1.953	1.893 to 2.014	1.926 to 1.954	1.768 to 1.91O
Logec 50				
Hill slope	1.806 to 2.272	1.637 to 2.871	1.767 to 1.969	1.491 to 2.888
EC 50	79.05 to 89.70	78.22 to 103.3	84.29 to 89.92	58.57 to 81.20
Goodness of Fit				
Degrees of Freedom	16	16	16	16
R2	0.9978	0.9878	0.9995	0.9838
Absolute Sum of Squares	0.03284	0.03438	0.006948	0.1814
Sy.x	0.04530	0.04635	0.02084	0.1065
Data				
Number of X values	12	12	12	12
Number of Y replicates	2	2	2	2
Total number of values	20	20	20	20
Number of rrissing values	4	4	4	4

***Table 2**: Results of the statistical analysis of the titration curve of Bo venom diluted in the vehicle buffer and in different concentrations of human healthy plasma.*

A	Parameter	Vehicule	Plasma 1/5	Plasma 1/ 10	Plasma 1/20
	Number of XY Pairs	10	10	10	10
	Spearman r	0.9758	0.9273	0.9879	0.9152
	P value (two-tajled)	P < 0.0001	0.0003	P < 0.0001	0.0005
	P value summary	***	***	***	***
	Exact or approximate P value? Is the correlation significant? (alpha=0.05)	Exact Yes	Exact Yes	Exact Yes	Exact Yes
B	**Parameter**	**Vehicule**	**Plasma 1/5**	**Plasma 1/ 10**	**Plasma 1/20**
	Number of XY Pairs	10	10	10	10
	Spearman r	1.000	0.9636	0.8424	0.9879
	P value (two-tailed)	P < 0.0001	P < 0.0001	0.0037	P < 0.0001
	P value summary	***	***	**	***
	Exact or approximate P value? Is the correlation significant? (alpha=0.05)	Exact Yes	Exact Yes	Exact Yes	Exact Yes

***Table 3**: Correlation table (A) for Am and (B) for Bo venom.*

We can also conclude from all this data that the best dilution for human serum is 1/20 for *Am* venom.

Discussion and Conclusion

Treatment of scorpion stings cases with specific and potent antivenom is the only and effective therapy available till date even though the current polemic about the use of antivenom as a treatment against envenomation. However, the production and quality testing of efficient antivenom is a complex procedure. Although extensive work has been done to address this problem, the determination of antivenom efficacy still relies mainly on *in vivo* neutralization test only [21].

Development of performing and robust assays capable of dosing scorpion venom in the blood of envenomed patients and to evaluate the correlation between venom levels and the gravity of the observed symptoms is a challenge. These assays will be also used to improve the clinical and epidemiological studies and to estimate the potency of available antivenoms [22].

In fact, classic methods to evaluate antivenom efficacy rest on conventional *in vivo* tests such as the determination of the ED_{50}. These tests usually involve the use of a lot of animals [23,24]. The main objective of the present ELISA assay is to propose an alternative to these tests and reduce the suffering of experimental animals. In addition to that, *in vitro* tests such as our ELISA display a lot of advantages; essentially a high sensitivity and specificity and are easy to use and also very cheap.

In the present study, we designed and standardized a bispecific ELISA assay to measure the concentration of *Am* and *Bo* venoms in human serum. Plus, the assay was validated on the basis of different variables such as specificity, sensitivity, linearity and correlation of the test.

The ELISA assay has already proved its efficacy in the detection of venoms and antivenoms sera of envenomed patients [21-25], but this is the first time that this assay was set up to identify and quantify venom levels of the two most dangerous Moroccan scorpions.

In this work, we were able to precisely give the best conditions for the use of this ELISA in a routine practice. The next step of this project is to collect sera of envenomed patients and attempt to identify incriminated species and to also quantify venom levels. The identification and quantification of venom levels in the blood of stung patients will help orient the doctor's diagnosis and help them administrate the right antivenom at the right dosage.

Our results show that our test is highly specific, sensitive with a good linearity and correlation and has been statistically validated. This ELISA assay will not only be used for the diagnosis and the treatment of envenomation, it will also be a valuable tool to study the efficacy of available commercialized antivenoms. The test will also be used to evaluate the correlation of venom levels and the severity of clinical symptoms suggesting that treatment should be readjusted depending on the envenomation grade. This ELISA assay can also be used for the comparison of the results with traditional *in vivo* assays.

In the present study, ELISA method was used for the identification and quantification of *Am* and *Bo* venom in plasma of healthy human donors. Titration curve adopted for the determination of optimal venom range concentration (0 - 1 µg/mL), coating antibody [$F(ab)'_2$ anti *Am* and *Bo*] = 3 µg/mL), primary antibody [IgG anti *Am* and IgG anti *Bo*] = 5 µg/mL and secondary antibody coupled to the horseradish peroxidase [Goat anti-rabbit IgG] = 1:1000.

Furthermore, the developed ELISA assay was validated using a very strict statistical analysis with a very competent software (Graph-Pad Prism) evaluating a wide range of parameters such as specificity, sensitivity, linearity and correlation through the calculation of LoD, LoQ, coefficient of Spearman and coefficient of variation.

Antivenom production is a very laborious process that involves multiple stages of horses immunization followed by multiple purification steps followed by enzymatic digestion (with pepsin) to obtain $F(ab)'_2$ fragments.

This ELISA assay will be a great alternative to animal use in *in vivo* neutralization assays and can also be used as a diagnosis test. It can also be opted as a pre-screen to test the efficacy of available antibodies at multiple stages of production, giving more accurate results and displaying a higher ethic by reducing animal burden.

Ethics Committee Approval

All the procedures involving animals were in accordance with the ethical principles in animal research adopted by the World Health Organization.

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