Immunoreactivity and two-dimensional gel-electrophoresis characterization of Egyptian cobra venom proteome

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Abstract: The first and second (two) dimensional gel electrophoresis has a broad protein resolution power. It was used to separate and identify cobra venom proteome. The importance of characterizing venom proteins contents from the Egyptian elapidae, specifically neurotoxins, is based on the need to produce effective anti-venom. About 30-55distinct protein spots were identified on silver stained two-dimensional gels. Around two-thirds of the venom proteins displayed low a molecular weight and a migration into hydrophobic side. The venoms from *Naja haja* and *Naja nigricollus* showed 45-55 spots, while *Walternnesia aegyptia* had less (31-37) spots. The commercial prepared polyclonal antivenom had a strong signal for anionic and cationic venom protein spots with molecular weight 20-115 kDa. However, it showed weak or non immunoreactivity toward anionic low molecular weight spots (2.5-15kDa). These results suggest the need to change the immunization schedule to include low molecular weight toxin-proteomes as separate dose or sequester injection.

Keywords: Cobra venom, antivenom, immunoreactivity, 2-DE, proteome, analysis.

INTRODUCTION

Still, the snake envenomating is emergency crisis need immediate intensive medical care using effective anti-venom, although most patients are scattering over rural and/or villages' areas. The accurate global of snakebite cases and its associated mortality are difficult to estimated (Correa-Netto *et al.,* 2010). A recent study reported that the worldwide snakebite incidence was 20,000 deaths out of at least 4,210,000 envenomating cases annually (Calvete, 2009). In Egypt, snakes belonging to Elapidae such as *Naja haja*, *Naja nigricollus* and *Walternnesia aegyptia* are widely distributed in several rural and villages regions. All bites from Egyptian elapidae are potentially fatal for humans (Stein and Helmy, 1992). Biochemical analysis of the cobra venom indicated the presence of neurotoxin (NT) and other enzymes (Kamil 1974, Boquet, 1979, Redwan, 2002a). The venome protein content based on its dry weight will reach 90% (Nawarak *et al.,* 2003), while the neurotoxins (α-neurotoxin and short chain neurotoxin) represent approximately 20-65% by weight of the whole cobra venom, and are highly toxic (Le Goas *et al*., 1992, Lallem and, 1992, Nawarak *et al.,* 2003), which their biochemical, biological and protein characteristic are still wanted.

The anti-venome are produced commercially by more than 46 of different public and private laboratories worldwide (Theakson and Warrell, 1991, Pla *et al*., 2012). There are two major problems with the effectiveness of most antivenoms: i) approximately 30-84% of envenomating patients receiving anti-venom develop early (anaphylactic) and/or delayed serum sickness type reactions (Malasit *et al.,* 1986; Theakston and Smith, 1995; and Moran *et al*, 1998). ii) The anti-venoms are not effective against the low molecular weight toxins, specifically the NT (Boquet 1979; Li and Owenby 1992, and Sells *et al*, 1994). In addition to, the world has permanent anti-venom shortage specifically the poorer regions within developing countries (Gutierrez, 2012). Pharmaceutical antibody in general and anti-venom specifically have a significance development during the last 20 years (Redwan 2003, Royle *et al.*, 2003, Redwan 2006, 2007, 2002, 2009a, 2005). Animal-derived anti-venom remains the effective drug for this neglected and tropical pathology of snakebite envenoming (Redwan 2009b, Gutierrez, 2012). The anti-venom volume to be infused for each envenomated patient critically dependent on its ability to neutralize and/or reverse venom induced pathological signs. The low effectiveness anti-venom leads to increase the anti-venom volume to control the venom toxic effect, which mains subsequently increase of the adverse effects (Calvete *et al.,* 2009).

The inefficacy of anti-venom was shown by ELISA against purified NT, or SDS-PAGE/immunoblot of crude venom, and clinically by observation (Malasit *et al.,* 1986; Sells *et al.,* 1994). Two-dimensional gel electrophoresis (2DE) is one of the most powerful techniques for protein analysis, as it can separate and quantities thousands of individual proteins from complex biological samples (Pini *et al.,* 1998, Redwan, 2002a).

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While, recently the resolution power of 2-DE was equipped with mass spectroscopy, which has more molecular protein identification power (Calvete *et al.*, 2009). The use of 2DE can be extended if good quality affinity reagents (e.g. antibodies) can be produced against individual spots from gels. Unfortunately we do not have availability of this sophisticated equipment. The main aim was harnessed the 2DE technique for cobra venome proteome identification and anti-venom immunreactivity against different venome protein spots.

MATERIALS AND METHODS

Venoms and anti-venoms

Venom was obtained by milking from *Naja haja*, *Naja nigricollus* and *Waltrnnesia aegyptia*. All venom collected was weighed, lyophilized and stored at -20^{o}c in the dark. Polyclonal anti-venom (produced against *Naja haja*, *Naja nigricollus*, *Echis carinatus* and *Cerastus cerastus*) was raised in horses and purified by caprylic acid methodology (VACSERA, Cairo, Egypt).

SDS-PAGE

Electrophoretic separation of venom proteins were performed under reducing and non-reducing conditions using 15% gels (Laemmli, 1970). Venom was dissolved in pyrogen free water (1mg/ml) then mixed with an equal volume of the corresponding sample buffer, and 100 Mg of sample was applied to each lane. Following SDS-PAGE, gels were transblotted or stained with Coomassie brilliant blue R-250 (BioRad, CA, USA).

Immunodiffusion

Horse anti-venom F $(ab)_2$ was tested by immunodiffusion on 1% agarose plates (Ouchterlony and Nilson 1978). Samples included 10 Ml of individual venom and horse anti-venom were loaded into separate wells. Plates were incubated for 72 hour at 4C, then extensively washed with saline buffer for 3 days and stained with Coomassie brilliant blue R-250.

Two dimensional gel electrophoresis (2DE)

2DE electrophoresis was performed according to the method of O'Farrell (1975). The first dimension; was carried out using 2% ampholines in the range of pH 3-10. Bio Rad colored IEF standards were run in parallel with each gel as IP markers. The gel was focused for 30 min at 150 constant volts then for 3 hours at 200 constant volt. The final pH gradient was measured by slicing the slab gel into 0.5cm strips/tube containing 1ml of 0.1M KCl. the second dimension, SDS-PAGE, was performed using 15% acryl amide gels. Following the electrophoresis, the gels were fixed overnight (10% acetic acid/50% methanol/40% water) then stained with silver nitrate kit (Bio Rad).

Western blotting

The venom proteins separated by SDS-PAGE or 2DE were transblotted onto nitrocellulose sheets. The blot was blocked for 60 min in 3% bovine serum albumin in 0.5% Tween 20-Tris buffer saline (TBS), then washed three times (5-10 min each) with TBS (0.5% BSA-0.1% Tween 20). The blot was incubated with polyclonal horse anti-venom F (ab) 2 (1:600) for 2 hours at room temperature (Redwan, 2002b). After washing, the blot was incubated for 60 min with goat anti-horse IgG alkaline phosphates (1:1000) (KPL, MA, USA). The blot was washed and the stained proteins were visualized with ready-made substrate (Promega, MD, USA).

RESULTS

The results demonstrated in fig. 1 venom proteins of the *W. aegyptia* (A;2 and B;7, 8), *N. haja* (A;3, 6 and B; 3, 5), *N. nigricollus* (A;4, 7 and B; 4, 6) were separated under reduced (A; 2,3, 4, and B; 3, 4, 8; lane 2 control horse serum) or non-reduced (A; 5, 6, 7 and B; 5, 6, 7) SDS-PAGE and either visualized by Coomassie blue staining (A) or transferred to nitrocellulose sheet and probed with horse anti-venom (1:600) (B). The acryl amide concentrations in stacking and separating gels were 4% and 15%, respectively. Each loaded sample contains 50 µg of snake crude venom. Lane 1 was molecular weights marker. While, fig. 1B illustrated the staining patterns by western blot of the Egyptian commercial anti-venom against three different crude venoms separated by SDS-PAGE under reduced (lane 3,4,8) and non-reduced (lane 5-7) conditions. All venoms and anti-venoms were used at the same concentration as indicated in fig. legend. The anti-venom has strong signals against most separated bands under reducing or non-reducing conditions except the very low molecular weight bands 3-15 kDa in both *Naja haja* and *Naja nigricollus*. In addition, the same anti-venom generated very weak signals against the reduced and non-reduced pattern of *W. aegyptia* (lanes 7, 8).

Naja haja and *Naja nigricollus* venom proteins exhibited intense low molecular weight bands around 3-15 kDa in both reduced and non-reduced gels (fig 1A). *W. aegyptia* venom showed 9 intense plus 4 faint bands in reduced gel. fig. 1A shows the reduced (lane 1-3) and non-reduced (lane 4-6) of SDS-PAGE patterns of the *W.aegyptia* (1,4) *Naja haja* (3,6) and *Naja nigricollus* (2,5) venom. The bands of molecular weight 34, 31, 10, 7 and 3 kDa were completely absent in non-reduced gel, which contained a heavy band of molecular weight 20-22 kDa. These changes were not clearly apparent in *Naja haja* and *Naja nigricollus*, which showed very faint bands above molecular weight 22 kDa. The result in fig. 2 shows the quantitative immunoreactivity of anti-venom against native crude venoms. The anti-venom formed about 5-6 precipitin lines with venom of *Naja haja* (a), 4 precipitin

lines with *Naja nigricollus*, and 2 precipitin lines with *W. aegyptia* (c). fig. 2 Ouchterlony-Nilson immunodiffusion of snake venom against antibody. Central well contained horse anti-venom (100 µg), a, b, c wells contained *N. haja* (50 µg), *N. nigricollus* (50 µg), *W. aegyptia* (50 µg), respectively.

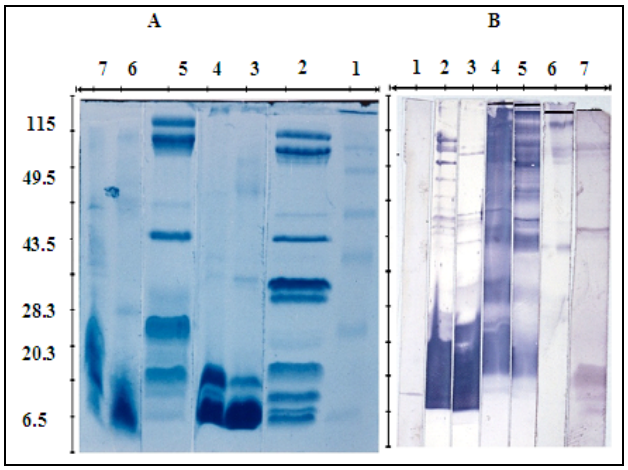

Fig. 1: Snake venom (50µg/well) fractionated on 15% SDS-PAGE then either visualized with Coomassie blue staining (A) or transferred to nitrocellulose sheet and probed with horse anti-venom (1:600) (B). venom proteome of *W aegyptia* (lane 2A and 7,8B), *N haja* (3,6A and 3,5B), *N. nigricollus* (lane 4,7A and 4,6B) were separated under reduced (lane 2,3,4A and 3,4,8B) or under non-reduced (5,6,7A and 5,6,7B) conditions. Lane1A and 1B were represent the prestain protein molecular weight marker (KDa) and normal horse serum, respectively.

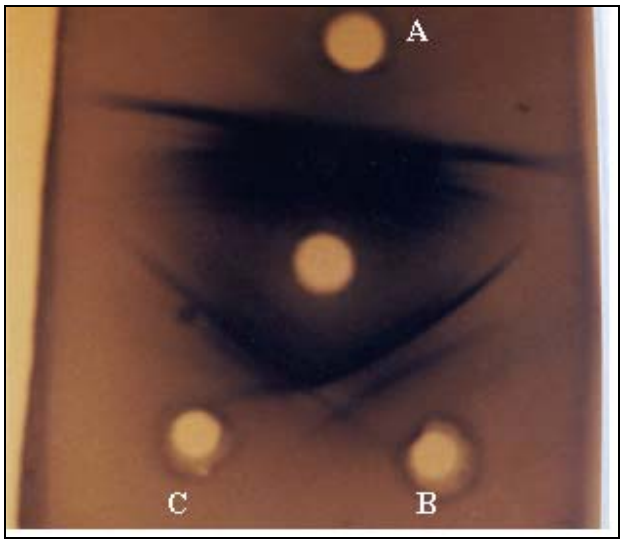

Fig. 2: Ouchterlony-Nilson immunodiffusion of snake venom against antibody. Central well contained horse anti-venom (100µg), A, B, C wells contained *Naja haja*, *Naja nigricollus*, *Wlaternesia aegyptia* (100 µg/each), respectively.

A gel of fresh venom from *Naja haja* is shown in fig. 3A. Several compact small protein spots are seen at the basic side around molecular weight 15-115 kDa, while below these spots, a heavy spot of molecular weight 3-13 kDa could be seen. Generally, these spot patterns are similar to *Naja nigricollus* venom protein spots pattern (fig. 4A). Also, fig. 4A shows the isoelectric points of 5 spots with molecular weights 115, 80, 75.5, 60.5, 49.5 kDa at acidic side, which exhibited very intense staining fig. 3. *Naja haja* crude venoms (10 µg) were analyzed using two-dimensional gel electrophoresis, then either stained with silver stain (A) or transferred to nitrocellulose sheet (B). Western blot was developed using VACSERA anti-venom (diluted 1:600) and goat anti-horse alkaline phosphates (1:1000). The fig. shows that the VACSERA anti-venom detects the low molecular weight spots visualized by silver staining.

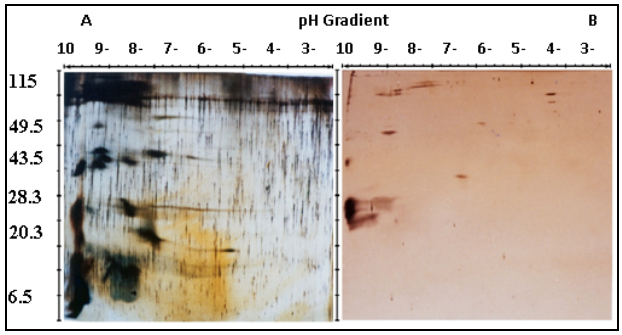

Fig. 3: *Naja haja* crude venom (10µg) were analyzed using two-dimensional gel electrophoresis then either stained with silver nitrate (A) or transferred to nitrocellulose sheet (B). Western blot was developed using VACSERA anti-venom (diluted 1:600) and goat anti-horse alkaline phosphates (1:1000). The figure shows that the VACSERA anti-venom weakly immunodetect the low molecular weight spots visualized by silver staining.

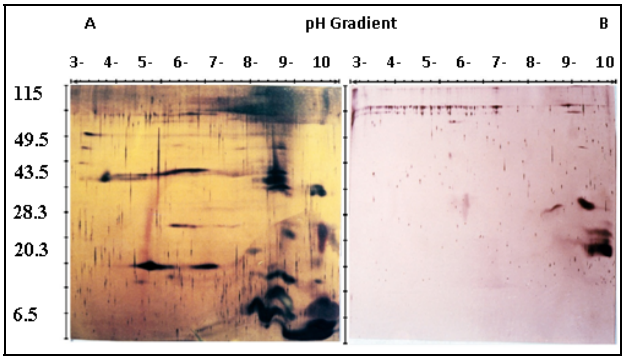

Fig. 4: *Naja nigricollus* crude venom (10µg) were analyzed using two-dimensional gel electrophoresis then either stained with silver nitrate (A) or transferred to nitrocellulose sheet (B). Western blot was developed using VACSERA anti-venom (diluted 1:600) and goat anti-horse alkaline phosphates (1:1000). The fig shows that the VACSERA anti-venom weakly immunodetect the low molecular weight spots visualized by silver staining.

The venom from *W. aegyptia* (fig. 5A) gave 30-35 spots corresponding to fig. 1A (lane 2). Most intense spots were of molecular weights 115-110, 50, 25 and 3-8 kDa with relatively anionic migration. Immunoblots of the same 2DE (fig. 3B, 4B, 5B) were performed. Under the 2DE conditions, the horse anti-venom recognized 19 out of 51 spots (67%) and 29 out of 55 spots (49.3%) of *Naja haja* and *Naja nigricollus*, respectively (fig. 3B, 4B). However, it failed to recognize any characterized spots of *W. aegyptia* venom (fig. 5B). Most of these signals (fig. 3B, 4B, 5B) were directed to the spots in the basic side above the molecular weight 20 kDa. Identical results were obtained in three separate experiments.

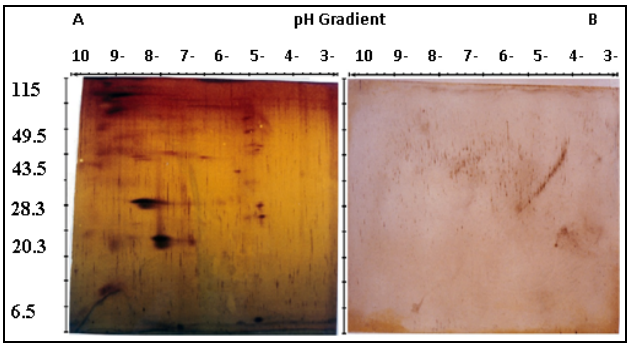

Fig. 5: *Walternesia aegyptia* crude venom (15µg) were analyzed using two-dimensional gel electrophoresis then either stained with silver nitrate (A) or transferred to nitrocellulose sheet (B). Western blot was developed using VACSERA anti-venom (diluted 1:600) and goat anti-horse alkaline phosphates (1:1000). The fig shows that the VACSERA anti-venom weakly immunodetect the low molecular weight spots visualized by silver staining.

While, *Naja nigricollus* crude venoms (10ug) were analyzed (fig. 4) using two-dimensional gel electrophoresis, then either stained with silver stain (A) or transferred to nitrocellulose sheet (B). Western blot was developed using VACSERA anti-venom (diluted 1:600) and goat anti-horse alkaline phosphatase (1:1000). The fig. shows that the VACSERA anti-venom detects the low molecular weight spots visualized by silver staining. *W. aegyptia* crude venoms (15µg) were analyzed using two-dimensional gel electrophoresis (fig. 5), then either stained with silver stain (A) or transferred to nitrocellulose sheet (B). Western blot was developed using VACSERA anti-venom (diluted 1:600) and goat anti-horse alkaline phosphatase (1:1000). The fig. shows that the VACSERA anti-venom detects the low molecular weight spots visualized by silver staining.

DISCUSSION

The successful anti-venom should have three characteristics: first, should be safe and cannot elicit high incidence of both early and late reactions. Second, affordability the anti-venom should be available by its intend market. Third, the product should be active with minimal dose volume. Using immunodiffusion, our results indicated that, the anti-venom-venom interaction is strong and gave quantitively clear precipitin lines. When venom proteins of three cobra species were separated by SDS-PAGE under reducing and non-reducing conditions, could observed that *N. haja* and *N. nigricollus* had similar staining patterns characterized by intense bands of low molecular weights 3-15kDa, while the *W. aegyptia* had a distinctive pattern. On the other hand, the 2DE of the three venoms gave similar results, which the *W. aegyptia* had distinct protein spots, and *Naja haja* and *N. nigricollus* had a conserved intense and highly basic protein spots pattern of low molecular weight. These results are consistent with previous comparisons of the chromatographic and SDS-PAGE profiles (Arid and Da Silva, 1991; Tan and Ponnudurai, 1992, Nawarak *et al.*, 2003). Most venome proteome spots of the cobras toxin are scattered in lower molecular mass region of the hydrophobic migration, which gave a unique features for cobra venome 2DE. It was documented that the hydrophobic proteome has historical 2DE separation and identification difficulties (Cordwell *et al*, 2001).

The immunore activity analysis of the cobra venom using 2DE indicates that the commercial anti-venom had strong signals against anionic and cationic venom proteins of molecular weight 20-115 kDa and very weak or no signal to low molecular weight (3-15 kDa) venom proteome of *N. haja*, *N. nigricollus* and *W. aegyptia*. The weak or no signal may be due to one of the following reasons: a) the anti-venom recognizes only the conformational epitopes of the venom proteins. However, venom proteins are usually extremely stable compounds, resistant in particular to various enzymatic attacks, high temperature, or even chemical modification (Boquet, 1979; Menez *et al*, 1980; Maillere *et al*, 1995). b) The anti-venom did not contain specific antibodies against these low molecular weight proteins. This suggestion may support the antigenic competition between high and low molecular weight antigens of snake venom during the immune response (Li and Owenby, 1992). It was observed that the rabbit immunized with γ-globulin (160kDa) and albumin (66kDa) produced antibodies against γ-globulin, but not against the albumin, although both proteins were present in the inoculums (Kelin and Horejsi, 2000). In addition, the study of Sells *et al* (1994) showed that out of fifty patients envenomed by Thai cobra *Naja kouthia* and tested for the prevalence of antibodies against the purified neurotoxin, only 16% were positive for the antibodies against neurotoxins, while 76% were positive for antibodies against whole venom. However, the purified low molecular weight proteins (neurotoxins) have been shown to be highly antigenic in mice when injected with Freunds Complete Adjuvant (Sells *et al*, 1994, Espino-Solis, 2009). Also, in considering the availability of a neurotoxin as an antigen ii *in vivo* as a result of natural

envenoming, its low molecular weight may reduce its exposure to the immune system through rapid excretion and may form antigenic competition with other venom components (Kochwa *et al*, 1959). Another characteristic of the neurotoxin, which may affect its availability as an antigen, is its basic *pI* (9-10) which can lead to tissue binding (Sells *et al*, 1994, Nawarak *et al*., 2003).

In summary, 2DE analysis of cobra venom provides us with a new analytical tool. The results presented here do not differ significantly from previous report (Redwan, 2002a) therefore, it reinforced that the available VACSERA antivenom has a very weak or no reactivity against low molecular weight venom proteins and crude venom of *N. haja*, *N. nigricollus* and *W. aegyptia*, respectively. As previously mention (Redwan, 2002a) our studies recommend that the venom of *W. aegyptia* and the purified low molecular weight proteins (neurotoxins) of *N. haja* and *N. nigricollus* to be included in the production immunization regime as separate dose or sequester injection. The alternative and may be more accurate avenue is the generation of protein/peptide library of cobra species proteome species using more advance proteomics tools prelude to produce effective, specific and distinguish pharmaceutical anti-venom.

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