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# Avidity based discrimination of venoms from two Egyptian *Echis* species

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ABSTRACT

Species-specific antibodies (Abs) to venoms from two species of the genus *Echis* were prepared by adsorption of monovalent and affinity purified anti-*Echis* Abs to the heterologous venom matrices (VMs). The avidity of the Abs to the homologous and the heterologous venoms is constantly ranked in the order; monovalent Ab > cross-reactive Ab > species-specific Ab. The avidity of the species-specific Abs to the homologous venoms of *Echis coloratus* (Eco) and *Echis carinatus pyramidum* (Epy) were $1.20 \pm 0.26$ and $1.10 \pm 0.29$, respectively. The avidity of the species-specific Abs to the heterologous venoms was too low to be detected. These results demonstrate for the first time that the avidity can be used to discriminate the immunologically high cross-reactive venoms. The discrimination of (14/14) venom samples of the two species on avidity bases confirmed the reliability and the specificity of the assay.



## 1. Introduction

Diagnosis of snake envenomation is based on the development of sensitive immunoassays, however, only few of these procedures were adapted for use by clinicians for detection of snake venoms in human victims (Selvanayagam and Gopalakrishnakone, 1999). The venoms of the various species are different in composition (Russell, 1988) and demonstrated a high immunological cross-reactivity. Accordingly complete specificity cannot be achieved with Abs to the whole venom or Abs to a single venom component (Barral-Netto and Sohsten, 1991; Guo et al., 1993; Tanigawa et al., 1994; Nakamura et al., 1992, 1995). Although, the cross-reactivity is significantly reduced using monoclonal Abs (Theakston, 1983; Pukrittayakamee et al., 1987), yet several disadvantages come about (Ho et al., 1986; Hoyer-Hansen et al., 2000). Consequently, immuno-affinity purification of species-specific Abs has become a key step in the development of most VDKs to defined groups of venoms in specific geographical areas (Chandler and Hurrell, 1982; Dhaliwal et al., 1983; Selvanayagam et al., 1999; Dong et al., 2003).

In Egypt, two main *Echis* species known as *Echis coloratus* (Eco) and *Echis carinatus pyramidum* (Epy) were identified (Mostafa, 1977). Species in the genus *Echis* represent one of the most complicated genera for taxonomic classification (Gasperetti, 1988; Wüster et al., 1997). Taxonomic classification of species in the genus *Echis* was mainly based on their morphological appearance and the analytical patterns of their serum (Ashe and Marx, 1988; Hermann et al., 1992; Wüster et al., 1997). More recently determination of the amino acid sequence of some venom components (Okuda et al., 2001) contributed to the phylogenetic and taxonomical classification of snakes of the genus *Echis*.

Most of the currently published procedures for discrimination of snake venoms are based on quantification of the venom reactivity with a group of homologous and heterologous species-specific Abs (Heneine and Catty, 1993; Chávez-Olórtegui et al., 1997; Selvanayagam et al., 1999; Dong et al., 2003) in Enzyme immunoassays. The specificity and the sensitivity of such immunodiagnostic assays are mainly determined by the magnitude of the specific antigen–antibody (Ag–Ab) interactions that were much

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improved by selecting the appropriate assay reagents and formats. Yet, no attention is paid for the power of avidity of the species-specific Abs rather than its activity in discrimination of self from cross-reactive antigens. In the present report, we demonstrate that the avidity based discrimination of venoms succeeds when quantification of the Abs titers failed due to the extensive immunological cross-reactivity.

## 2. Materials and methods

### 2.1. Snake venoms

Venom pools of Eco ($n = 30$), Epy ($n = 35$) and individual venom samples of Eco ($n = 4$) and Epy ($n = 10$) snakes were from snakes collected from their natural habitat. The venoms were lyophilized, stored at −20 °C and reconstituted in the appropriate buffer before being used.

### 2.2. Rabbit antivenoms

Monovalent antivenoms against Eco and Epy venoms were raised in New Zealand rabbits (weighing 2–3 kg) injected with 25 μg pooled venom/0.5 ml saline at two-week intervals. The first dose and the subsequent booster doses emulsified in Freund's complete and incomplete adjuvant, respectively, were delivered deep into the hind or fore leg. Seven days after two booster injections the rabbits were bled from the ear vein for preparation of the serum samples.

### 2.3. Species-specific antivenom Abs

In brief, two affinity VMs were prepared by coupling 10 mg of the Eco or Epy venom to 1 g of CNBr-activated Sepharose according to the manufacturer's instructions (Sigma). The residual reactive sites were blocked with 100 mM glycine in the coupling buffer (100 mM sodium carbonate, 0.5 M NaC1, pH 8.2). Excess uncoupled venom components were removed by washing the gel in four cycles; each consisted of a wash at pH 8.3 (100 mM $NaHCO_3$ buffer, 0.5 M NaC1) and at pH 4.0 (0.1 M acetate buffer, 0.5 M NaCl). The VMs were then suspended in 0.05 M PBS pH 7 in the presence of 0.02% thiomersal as a bacteriostatic agent and stored at 4–8 °C until being used. For preparation of the species-specific Abs, the monovalent antivenom was directly absorbed on the heterologous VM and the non-bound Abs were collected (one step). Otherwise, the bound Abs to the homologous VM were eluted and re-chromatographed using the heterologous VMs and the non-bound Abs were collected as species-specific Abs (two step) For affinity purification of Abs; each VM was packed in a standard column (4 × 1 cm) and equilibrated with about 10 times its volume 0.2 M borate buffer, pH 8.0. A known amount of the Abs (10 mg) dissolved in the same buffer was applied to the appropriate column and recycled overnight through the VMs to ensure complete binding. The VMs were washed with the borate buffer until the absorbency of the effluent (at 280 nm) returned to baseline. The bound proteins were eluted with glycine–HC1 buffer pH 2.9 at a constant flow rate of 1 ml/5 min. One-ml fractions were collected in tubes containing 100 μl 1 M Tris–HCl buffer, pH 8.0. The reactivity of the eluted Ab fractions against the two venoms was monitored by the indirect ELISA technique. The Abs under each peak were pooled, extensively dialyzed against distilled water, freeze-dried, reconstituted in the least volume of the appropriate buffer (PBS, pH 7.4) and stored at −20 °C.

### 2.4. Immunoblotting

SDS-PAGE was performed by the method of Laemmli (1970) using 12% separating gel. Transfer of venom proteins from SDS-PAGE to nitrocellulose sheets was performed by the method of Towbin et al. (1979), using the Semi-dry LKBBROMMA-21117-250 NOVABLOT Electrophoresis Transfer Cell, at 100 mA for 60 min. Immobilized venoms were then probed with suitably diluted anti-*Echis* rabbit Abs, and the patterns were developed using goat anti-rabbit horseradish peroxidase conjugate (dilution 1–4000) and 4-CIN substrate (Sigma).

### 2.5. ELISA

The indirect ELISA was performed according to Theakston et al. (1977) with minor modifications. In brief, microtiter plates (Dynatech) were coated overnight at 4 °C with 100 μl of 2.5 μg solution of either venom in coating buffer (0.05 M carbonate buffer pH 9.6). The plates were washed three times with the PBS-T buffer (100 mM PBS pH 7.5 containing 0.05% Tween 20) and incubated for 1 h at 37 °C with 200 μl/well of the blocking buffer (PBS containing 2% gelatin). Dilutions of the test Abs in the PBS-T were dispensed into duplicate wells coated with the different venoms and incubated for 1 h at 37 °C. After wash, goat anti-rabbit IgG-peroxidase conjugate diluted in PBS was added (100 μl/well) and incubated for 1 h at 37 °C. The plates were washed thoroughly for 3–5 times with PBS-T buffer before allowing them to react with Sigma fast OPD tablets dissolved in 20 ml distilled water (100 μl/well). The reaction was allowed to proceed for 15 min at room temperature in the dark before the addition of 50 μl 2 N sulfuric acid. The developed OD was measured at 490 nm in a Micro-ELISA Reader Photometer.

### 2.6. Ab avidity tests

Avidity was determined according to Pullen et al. (1986) with little modifications. Briefly, the dilution of each anti-*Echis* Abs that gives at least 0.8 OD with the homologous venom was determined in the standard ELISA. The defined dilution (concentration) of each Abs was then allowed to bind the homologous and heterologous venoms in the standard ELISA as above. Twelve similar wells of Ab-venom complexes were used to determine an avidity index, five concentrations of $NH_4SCN$ ranging from 3 M to 0.25 M in PBS pH 6 were added to duplicate wells (100 μl/well) for exactly 15 min, and two wells filled with the dilution buffer were used as control. The plates were washed with PBS-T and processed as in the standard ELISA, a plot of % binding ([OD in presense of $NH_4SCN$/OD in absence of $NH_4SCN$] × 100) versus the $NH_4SCN$ concentration was established and