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Antigenic cross-reactivity and species-specific identification of *Pseudocerastes persicus fieldi* snake venom

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ABSTRACT

In the present study, we recognized progressively high immunological cross-reactivity between *Pseudocerastes persicus fieldi* (Pf) venom and six other medically important Egyptian snake venoms belonging to families Viperidae and Elapidae. Antibodies with a range of bonding strengths were shown to be involved in such cross-reactivity. Two strategies have been tried to access specificity; (*i*) using affinity purified species-specific anti-Pf antivenom antibodies, (*ii*) conducting the assay in the presence of ammonium thiocyanate (NH4SCN). The discrimination power of the prepared species-specific antivenom was demonstrated by its ability to detect Pf venom over a range of Pf concentrations (2.5 ng–2.5 μg) in a variety of body fluids. The assay could distinguish circulating Pf antigens from other viper antigens in the whole blood of experimentally envenomed mice. What seems promising in our work is the use of the chaotrope, NH4SCN, which renders the reaction medium more favorable for the specific homologous antigen-antibody interactions, primarily via preventing lower avid antibodies to share and, to a bit lesser extent, by decreasing non-specific absorbance signals frequently encountered with ELISA assays. The ELISA described herein may be useful for clinicians for identification of snake bites inflicted by Pf snake species. Balancing between specificity and sensitivity has to be considered for best results.

1. Introduction

Pseudocerastes persicus fieldi (Pf), also known as false horned or field's horned viper, is a venomous subspecies little bigger than other vipers, endemic to the desert of the Middle East (Mallow et al., 2003). In Egypt, it inhabits rocky areas of Sinai Peninsula and the Eastern desert (Saleh, 1997). Unlike other viper species, Pf venom is transparent when fresh as it lacks the yellow flavin pigment characteristic of L-amino acid oxidase enzyme. It also lacks hemorrhagin and most of the high molecular weight proteins (Bdolah, 1986; Wahby et al., 2000). Moreover, Pf venom shows marked neurotoxic activity (Gitter et al., 1962) that cannot be neutralized by polyvalent viperid antisera (Mallow et al., 2003).

Haemorrhagic activity is generally a characteristic feature of venoms from family Viperadae, whereas elapid venoms are mainly neurotoxic (Theakston and Laing, 2014). Few exceptions, however, have been reported in some species as *Vipera xanthine palestinae* (Gitter et al., 1959), Pf (Gitter et al., 1962), and *Crotalus durissus terrificus* (Theakston and Laing, 2014) venoms, where strong neurotoxic manifestations predominate. Gitter et al. (1962) reported that mice injected with Pf venom showed no hematological abnormalities. Yet, strong neurotoxic signs manifested by muscle weakness, forced respiration, paresis and paralysis were observed.

In snake bites, it is often difficult for clinicians treating patients to accurately identify the species responsible for envenoming (Theakston and Laing, 2014). The presence of certain antigenic components in snake venoms appear to be common in several snake species, genera and families (Marshall and Hermann, 1984; Russell, 1988) and antivenoms cross-reacted along them (Dong et al., 2003; Wahby and Ibrahim, 2008; Gao et al., 2013). Consequently, species-diagnosis has best been achieved with affinity-purified species-specific antibodies. Sometimes, however, significant reactions were still present, where other alternative attempts were tackled (Amuy et al., 1997; Nakamura et al., 1993,Wahby and Ibrahim, 2008; Ibrahim et al., 2013) with different successes.

In the present report, we studied the cross-reactivity of Pf venom with other medically important snake species and used a modified ELISA for the detection of Pf venom in biological fluids and

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in the whole blood of mice inoculated by Pf venom in an attempt to establish a reliable assay to identify and/or confirm the identity of envenomation by Pf snake. We report on the use of NH_4SCN as a chaotropic agent that render the reaction medium more favorable for strong antigen-antibody interactions to take place and hence more specific conditions were created for detection and differentiation of Pf venom antigens from other highly cross-reactive snake species.

2. Materials and methods

2.1. Snake venoms

The venoms were obtained from five viperid snakes; *Pseudoserastes persicus fieldi* (Pf), *Cerastes cerastes* (Cc), *Cerastes vipera* (Cv), *Echis coloratus* (Eco) and *Echis carinatus* (Eca) (n = 30–36 except for *P. fieldi* n = 2) and two elapid snakes; *Naja haje* (Nh) (n = 3), *and Walterinnessia aegyptia* (Wa) (n = 1). The animals were collected by experts from their natural habitat and kept in captivity. The venom of each species was milked by trained individuals by manual compression of the venom glands, pooled, centrifuged and the supernatant was lyophilized and stored at −20 °C until used.

2.2. Anti-Pf antivenoms

Polyclonal antivenoms rabbit anti-Pf antivenoms (antivenoms I & III[1]) were raised in New Zealand rabbits (2–3 kg) injected with 25 μg venom/0.5 ml saline at two week intervals as previously described (Wahby and Ibrahim, 2008). Two serum samples from different animals collected at different time intervals were arbitrary chosen and designated as antivenom I & III. Whereas horse polyclonal anti-Pf antivenoms (antivenoms II & IV) were monospecific, raised in different horses against Pf venom at the Egyptian organization for biological products and vaccines (VACSERA) and were kindly offered by the VACSERA authorities. All collected antisera were stored at −20 °C until used.

Species-specific anti-Pf antivenom was prepared from rabbit anti-Pf antivenom I following the manufacturer's instructions as previously mentioned in details (Ibrahim et al., 2014). Sequential immune-affinity purification scheme on four different viper venom (Cc, Cv, Eco & Eca) matrices was applied and the cross-reactive antibodies that bind to the heterologous venom matrices were discarded while the unbound antibodies depleted from cross-reactive proteins were collected as species-specific anti- Pf antivenom.

2.3. ELISA test

Principally, the indirect and double-sandwich ELISA were performed according to Theakston et al. (1977) with the modifications of Pullen et al. (1986).

For the indirect ELISA, the microtiter plates (Dynatech) were coated overnight at 4 °C with 100 μl of a constant concentration (5 μg/ml) of each venom in the coating buffer (5 mM carbonate buffer pH 9.6). The plates were washed at least 3 times with PBS-T buffer (100 mM PBS pH 7.5 containing 0.05% Tween 20) and incubated for 1 h at 37 °C with 150 μl/well of the blocking buffer (PBS containing 2% gelatin). After wash, a defined concentration of the antivenoms under test (100 μl, dilution 1:3000 for polyvalent antivenoms or 2 μg/ml for the species-specific antivenoms) was dispensed into at least twelve similar wells and binding was allowed to proceed for 1 h at 37 °C. After another washing cycle, different concentrations of NH4SCN (0-5μ, 100 μl) in PBS pH 6 was added to duplicate wells and incubated for 15 min at room temperature. After another washing cycle, the appropriate horseradish peroxidase (HRPO) conjugate (either rabbit anti-horse HRPO or goat anti-rabbit HRPO, dilution 1:3000) was added (100 μl) and incubation was allowed at 37 °C for 1 h. The plates were washed before allowing them to react with Ortho-phenylene diamine (OPD) in phosphate citrate buffer, pH 5.2 (100 μl/well), kept in the dark for 15 min till the color is developed, the reaction was stopped by the addition of 2 N sulfuric acid (25 μl), and the optical densities (ODs) were recorded at 490 nm in a Micro ELISA Reader Photometer. The NH4SCN concentration that develops 50% of the binding in the NH4SCN-free sample was considered as the relative avidity index of the tested antivenom. The percentages of high and low avid antibodies were calculated as previously reported (Ibrahim et al., 2016).

$$\text{The percentage of high avid antibodies} = \frac{\text{OD in presence of 3}\mu\text{ NH4SCN}}{\text{OD in absence of NH4SCN}} \times 100$$

$$\text{The percentage of low avid antibodies} = \frac{\text{OD in absence of NH4SCN} - \text{OD in presence of 1}\mu\text{ NH4SCN}}{\text{OD in absence of NH4SCN}} \times 100$$

The double-sandwich ELISA was performed as follows: the plates were coated with horse anti Pf antivenom II (dilution 1:1000), followed by PBS-T wash and incubation for 10 min at room temperature with 3μ NH4SCN in PBS pH 6. After another wash, different concentrations of venom in saline and various biological

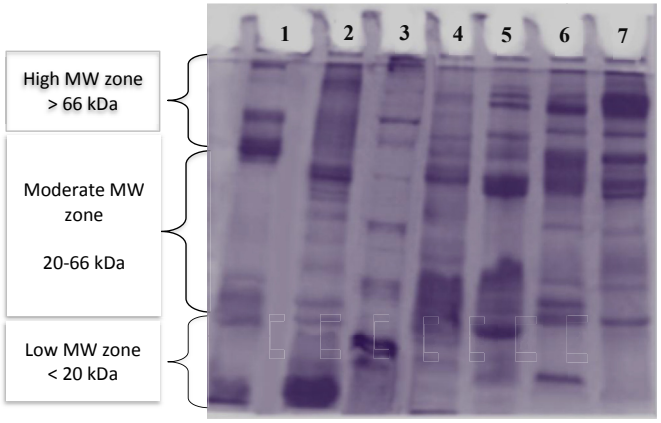

Fig. 1. 12% SDS-PAGE of crude venoms (30 μg) from snakes, Wa (1), Nh (2), Pf (3), Eco (4), Eca (5), Cc (6) and Cv (7).

[1] The rabbit, from which antivenom III was obtained, received once erroneously a Cc venom booster dose instead of Pf venom dose during the immunization program that lasts ~ three months with 1 primary injection and 5 boosters.