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Intra-specific variation in venom of the African Puff Adder (*Bitis arietans*): Differential expression and activity of snake venom metalloproteinases (SVMPs)

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ABSTRACT

Bitis arietans is considered one of the most medically significant snakes in Africa, primarily due to a combination of its extensive geographical distribution, common occurrence and highly potent haemorrhagic and cytotoxic venom. Our investigation has revealed a remarkable degree of intra-species variation between pooled venom samples from different geographical origins across sub-Saharan Africa and Arabia, and within a group of individual specimens from the same origin in Nigeria as determined by a combination of immunological, biochemical and proteomic assays. We demonstrate significant quantitative and qualitative differences between *B. arietans* venom in terms of protein expression, immunogenicity and activity of snake venom metalloproteinases (SVMPs); toxins with a primary role in the haemorrhagic and tissue-necrotic pathologies suffered by envenomed victims. Specifically, we have identified a processed PII SVMP that exhibits striking inter-specimen variability.

1. Introduction

The puff adder, *Bitis arietans*, occupies a diverse range of habitats throughout the savannah areas of sub-Saharan Africa and parts of the Arabian Peninsula, regions which are also densely populated with people and livestock. The venom of this species is highly potent and typically interferes with the haemostatic system to cause severe local and systemic effects such as swelling, haemorrhage and necrosis (Warrell et al., 1975). Without antivenom treatment, *B. arietans* envenoming can have fatal consequences (Warrell et al., 1975). In Africa, it is estimated that snakebite accounts for up to 32,000 deaths per year, with many more victims left with permanent disabilities and sequelae (Kasturiratne et al., 2008). *B. arietans*, the saw-scaled viper (*Echis ocellatus*) and the spitting cobra (*Naja nigricollis*) are responsible for the majority of these fatalities in Africa. The medical importance of *B. arietans* and it's unusually extensive pan-African distribution for a single species, makes it an ideal subject to investigate intra-species venom variation, not only from a biological and evolutionary standpoint, but also from the perspective of the possible consequences venom variation may have on the clinical symptoms of envenoming and patient response to antivenom.

Viper venom is a highly complex biologically active mixture typically containing numerous enzymatic and non-enzymatic components (Serrano et al., 2005), most of which are proteins and peptides, enabling the rapid immobilisation, killing and digestion of envenomed prey. Variation in venom protein composition has been well documented on several taxonomic levels and particularly within species occupying wide geographical distributions. Several studies investigating inter-species venom variation have attributed their

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findings to factors such as ontogenetic, dietary and gender associated changes. For example, ontogenetic shifts in respect of (i) coagulant activity of *Bothrops atrox* and *Crotalus atrox* venom (Lopez-Lozano et al., 2002; Reid and Theakston, 1978) (ii) PIII to PI SVMPs in *Bothrops asper* (Alape-Giron et al., 2008), and (iii) hemorrhagic and oedema-forming activities both in *B. atrox* and *B. asper* (Saldarriaga et al., 2003) have been demonstrated. Variation in venom protein composition between closely related subspecies of *Sistrurus* rattlesnakes (Sanz et al., 2006) and *Echis* species (Barlow et al., 2009) have also been correlated with the recruitment of different toxins into the proteome of species with predominantly mammalian or varied diets. Daltry et al. correlated variation in the protein composition and biological activity of *Calloselasma rhodostoma* venom to geographic location and phylogenetic relationships. Importantly, this study presented strong evidence for the association between variation in the venom of this species and its diet (Daltry et al., 1996). Finally, examples of gender-specific differences in *Bothrops jararaca* venom have also been demonstrated (Menezes et al., 2006) in respect of fibrinogenolytic and amidolytic properties, differential processing of bradykinin-potentiating peptides (Pimenta et al., 2007), and differential hyaluronidasic, hemorrhagic, coagulant, phospholipasic and myotoxic properties (Furtado et al., 2006).

While variation in venom composition has been described for members of the *Bitis* genus of less medical significance, (*Bitis gabonica gabonica, Bitis gabonica rhinoceros*, *Bitis nasicornis* and *Bitis caudalis*) (Calvete et al., 2007a,b), there have been no such studies on *B. arietans*, the most clinically important member of the genus. A previous investigation of *B. arietans* venom demonstrated that the proteome may be less complicated than originally predicted, containing proteins belonging to only a few major toxin families, the majority of which are snake venom metalloproteinases (SVMPs) and to a lesser extent, serine proteases, disintegrins and C-type lectins (Juárez et al., 2006). The latter proteomic studies, in conjunction with our own venom gland transcriptomic results (Wagstaff and Harrison, unpublished) indicate that the composition of *Bitis* venom exhibits extensive isoform diversity, potentially within one of the most toxic groups of venom proteins, the SVMPs. The aim of this study was to explore the *B. arietans* venom proteome and determine the extent of variation between specimens from different or the same geographical origins, particularly within the major toxin groups responsible for the most significant clinical pathology. Much of the pathology of viper envenoming is caused by enzymes and venom is the immunogen used for antivenom preparation. We therefore elected to use biochemical assays of enzyme function and immunological assays, in addition to the standard proteomic techniques used to characterise venom proteome diversity, to produce a more comprehensive understanding of variation in *B. arietans* venom.

2. Materials and methods

2.1. Venom samples

Individual venom samples extracted from 12 Nigerian and 6 Ghanaian wild-caught adult specimens were analysed. Individual snakes were maintained in the Herpetarium at the Liverpool School of Tropical Medicine under identical climatic conditions where they were fed the same diet and the technique and frequency of venom extraction was identical for all animals. Following manual extraction, venom was frozen, lyophilised and stored at 4 °C until analysis. Pooled venom samples from *B. arietans* specimens originating from six different geographical origins (Ghana, Nigeria, Saudi Arabia, Zimbabwe, Malawi and Tanzania) typically comprise multiple extractions from larger pools (usually tens) of individual specimens.

2.2. One-dimensional SDS-PAGE

Lyophilised venom samples were reconstituted at 10 mg/ml in phosphate-buffered saline (PBS) and diluted to 1 mg/ml in reducing or non-reducing SDS-PAGE sample buffer. Samples were separated on 1 mm 15% SDS-PAGE gels according to the manufacturer's recommendations (BioRad) and stained overnight using Coomassie Blue R-250 or silver stained.

2.3. Western blotting

Gels were electro-blotted to 0.45 μm nitrocellulose membranes using the manufacturer's protocols (BioRad) under reducing conditions. Following transfer and visualisation by Ponceau S, membranes were incubated overnight in blocking buffer (5% non-fat dried milk in PBS), followed by washing in TBST (10 mM Tris-Cl, pH 8.5, 150 mM NaCl, 0.1% Tween 20) and incubated for 5 hours-overnight with the following primary antibodies at 1/200 dilution in blocking buffer (unless otherwise stated); EchiTAb-Plus-ICP (an equine IgG polyspecific antivenom prepared against *E. ocellatus, B. arietans* and *N. nigricollis* venoms); Instituto Clodomiro, University of Costa Rica (Gutierrez et al., 2005) (1/1000 dilution), rabbits immunised with recombinant *E. ocellatus* snake venom metalloproteinase (SVMP) epitope string [Eo-SVMP string] (Wagstaff et al., 2006) or sera from mice immunised with a DNA-encoding expression plasmid pVaxSec (Wagstaff et al., 2006) containing *E. ocellatus* vascular endothelial growth factor (VEGF) clone Eo_venom_02G03 (Accn no: DW360875) [Eo-VEGF]. Although the antibodies used were specific to *E. ocellatus* venom, previous investigation has demonstrated varying degrees of cross-reactivity with venoms of other viperid species, including *B. arietans* (Wagstaff et al., 2006). Blots were washed with 6 washes of TBST over 1 h and incubated for 2 h with appropriate secondary antibody (1/1000 dilution) coupled to horseradish peroxidise before a final wash with TBST and the results visualised after addition of the DAB peroxidise substrate (Sigma, UK).

2.4. Substrate zymography

Zymography was carried out using gelatin and fibrinogen substrates to visualise the degradative activity of the enzymes present in viper venoms. 0.75 mm polyacrylamide gels (2.5 ml ddH_20, 2.5 ml 1.5 M Tris pH 8.8, 2.5 ml 40% Bis-Acrylamide, 50 μl 10% SDS, 150 μl 10% ammonium