Proteomic comparison of Hypnale hypnale (Hump-Nosed Pit-Viper) and Calloselasma rhodostoma (Malayan Pit-Viper) venoms

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ABSTRACT

Treatment of Hypnale hypnale bites with commercial antivenoms, even those raised against its sister taxon Calloselasma rhodostoma, has never been clinically successful. As these two genera have been separated for 20 million years, we tested to see whether significant variations in venom had accumulated during this long period of evolutionary divergence, and thus could be responsible for the failure of antivenom. Proteomic analyses of C. rhodostoma and H. hypnale venom were performed using 1D and 2D PAGE as well as 2D-DIGE. C. rhodostoma venom was diverse containing large amounts of Disintegrin, Kallikrein, L-amino acid oxidase, Lectin, phospholipase A\textsubscript{2} (acidic, basic and neutral) and Snake Venom Metalloprotease. In contrast, while H. hypnale also contained a wide range of toxin types, the venom was overwhelmingly dominated by two molecular weight forms of basic PLA\textsubscript{2}. 2D-DIGE (2-D Fluorescence Difference Gel Electrophoresis analysis) showed that even when a particular toxin class was shared between the two venoms, there were significant molecular weights or isoelectric point differences. This proteomic difference explains the past treatment failures with C. rhodostoma antivenom and highlights the need for a H. hypnale specific antivenom.

Biological significance
These results have direct implications for the treatment of envenomed patients in Sri Lanka. The unusual venom profile of Hypnale hypnale underscores the biodiscovery potential of novel snake venoms.

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1. Introduction
The Hump-Nosed Pit-Viper (Hypnale hypnale) inhabits Sri Lanka and southern India and is classified by the World Health Organisation (WHO) as a snake of the highest medical importance in Southern Asia \cite{1}. Contact between humans and H. hypnale is common due to its abundance and tolerance of disturbed habitat in both wet and dry deciduous zones. Its small
size (rarely exceeding 0.5 m) and effective camouflage make the snake difficult to detect, and bites most often result from victims accidentally stepping on coming into contact with an unseen snake [2]. H. hypnale has been estimated to be responsible for 35% of venomous snake bites in Sri Lanka and envenomation causes local necrosis, pain and haemorrhagic blisters in most patients [3]. While H. hypnale bites are rarely lethal (fatality rate 1.8%), up to 40% of patients experience systemic effects including haemostatic dysfunction, thrombocytopenia, spontaneous haemorrhage and acute kidney injury [2,3]. These effects have been replicated in mouse models, in which it has been demonstrated that H. hypnale venom has procoagulant, fibrinolytic, oedema-triggering and platelet aggregation activities [4,5]. Recently, thrombotic microangiopathy has also been described in a number of H. hypnale bites, which may contribute to tissue damage at the bite site and the development of renal complications [6].

Despite the medical importance of this snake, effective venom neutralisation has not been achieved in humans. Treatment with Bharat polyvalent antivenom (ASVS) and Haffkine polyvalent antivenom, both raised against the “big four” Asian venomous snakes (Naja naja, Bungarus caeruleus, Daboia russelli and Echis carinatus), has been found to be completely ineffective against H. hypnale envenomation both in rodent assays and clinical envenomations [7]. Mitochondrial DNA analysis suggests that H. hypnale and the Malayan Pit-Viper Calloselasma rhodostoma form a phylogenetic clade, although they have been diverging from one another for approximately 20 million years [8]. As their envenomations show similar clinical features, the efficacy of Thai Red Cross Malayan Pit Viper (MPV) monovalent antivenom in neutralising H. hypnale venom has also been investigated. There is controversy in the literature regarding the effectiveness of MPV in supressing the haemorrhagic, procoagulant and necrotic activities of H. hypnale venom in rodent models [9], and it has never been successful in a clinical setting [2]. The Hemato polyvalent antivenom (HPA) produced against C. rhodostoma and two other haemotoxic Thai snakes, Cryptelytrops albomarginatus and Dicrodon siamensis, has been demonstrated to abrogate the lethality of H. hypnale venom in rats, but has not yet been clinically trialled [9].

Both MPV and HPA are substantially more effective in neutralising C. rhodostoma venom than that of H. hypnale, suggesting that some toxins present in the latter venom may not be neutralised [9]. However, immunological profiling of H. hypnale venom using indirect ELISA with antisera revealed 90% cross-reactivity with C. rhodostoma venom [10]. C. rhodostoma venom toxins have been comprehensively described and include the metalloproteases kistomin and rhodostoxin [11]; multiple phospholipase A₂ isoforms (PLA₂) [12]; L-amino oxidases [13]; C-type lectins [14] and serine proteases such as Ancrod, which is being trialled as a clinical anticoagulant (under Dynamic). For three dyes and the resultant images were overlaid and laser scanner (GE Healthcare)™. After electrophoresis, gels were scanned on a Typhoon™ laser scanner (GE Healthcare) for three dyes and the resultant images were overlaid and analysed using the Progenesis SameSpots software (Nonlinear Dynamics).

3. Results and discussion

In this study we undertook the first detailed proteomic characterisation of H. hypnale venom and provided a comparison with the sister-taxon C. rhodostoma venom using 1D and
The combined proteomics approaches revealed a diverse composition of venom components (Figs. 1–4, summarised in Table 1, full-details in Supplementary Tables 1–6). Horizontal trains of spots were common on the 2D gels from these species, indicating extensive isoform and glycoform variation. All three techniques revealed considerable differences between the two venoms in terms of protein composition and expression levels.

C. rhodostoma venom was particularly diverse containing disintegrin, kallikrein, L-amino acid oxidase, Lectin, phospholipase A2 (acidic, basic and neutral) and Snake Venom Metalloprotease. The comparison of our C. rhodostoma sample with previously annotated gels of C. rhodostoma [18–21], Crotalus durissus terrificus [22] and Daboia. russelii siamensis [20,23] venoms showed a generally similar patterns of basic PLA2, C-type lectins, serine proteases and metalloproteases but with significant differences in the specific relative composition. This is congruent with previous work demonstrating that C. rhodostoma venom shows significant differences between populations [24].

In contrast, H. hypnale venom was dominated by PLA2, with two molecular weight isoforms of basic PLA2 being particularly abundant. The low expression levels of high molecular weight proteins in the H. hypnale sample were unexpected as previous studies on the enzymatic activity of H. hypnale venom had reported comparable protease, thrombin-like enzyme, hemorrhagin and hyaluronidase activities to C. rhodostoma venom [25]. While there is no evidence yet of individual or

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**Fig. 1** – 12% SDS-PAGE analysis of Calloselasma rhodostoma (CR) and Hypnale hypnale (HH) venoms carried out under dissociating (reducing, R) and dissociating and denaturing (non-reducing, NR) conditions. Gel was stain in 0.2% colloidal coomassie brilliant blue G250. M = molecular mass standard markers.

**Fig. 2** – 2-dimension gel of the Calloselasma rhodostoma crude venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown.
Fig. 3 – 2-dimension gel of the Hypnale hypnale crude venom stained with colloidal coomassie brilliant blue G250. First dimension: isoelectric focusing (pH3-10 non-linear gradient); second dimension: 12% SDS PAGE. The pH gradient and the molecular weight marker positions are shown. The marked spots were analysed by MS2 and the positive results are summarised in Supplementary Table 4.

Fig. 4 – 2D-DIGE comparison of Calloselasma rhodostoma and Hypnale hypnale venoms.
Table 1 - Homologues of toxin types identified in Calloselasma and Hypnale venoms.

<table>
<thead>
<tr>
<th>Toxin type</th>
<th>UniProt accession of representative</th>
<th>Characterised bioactivities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disintegrin-domain of SVMP</td>
<td>P0cb14</td>
<td>Anticosuglant</td>
</tr>
<tr>
<td>Type II phospholipase A2</td>
<td>Q9PFV4; Q9PFV0</td>
<td>Anticosuglant, oedema-inducing and myotoxic</td>
</tr>
<tr>
<td>Lectin</td>
<td>Q9PSM4 (hommeric); P81398 (heteromic)</td>
<td>Anticosuglant</td>
</tr>
<tr>
<td>Kallikrein type S1 protease</td>
<td>Q91053</td>
<td>Fibrinolytic and also release of kinin from kininogen</td>
</tr>
<tr>
<td>SVMP</td>
<td>O57413</td>
<td>Nectrotic and also anticosuglant</td>
</tr>
<tr>
<td>LAAO</td>
<td>P81382</td>
<td>Apoptotic and anticosuglant</td>
</tr>
</tbody>
</table>

SVMP = snake venom metalloprotease; LAAO = L- amino acid oxidase.

geographic variation in H. hypnale venom, as noted above significant sexual and regional differences have been described in C. rhodostoma venom. As intraspecific variation in venom toxins is clinically significant, future studies exploring this aspect of H. hypnale venom are needed prior to any production of a H. hypnale specific antivenom.

DIGE revealed that even where protein types were shared between the two venoms, they existed in subtly different isoforms (with variations in both molecular weight and charge) between the two venoms, which could have considerable effects on antigenicity. This latter result demonstrates the value of DIGE analysis, the sensitivity of which uncovers variations in venom profiles which may be too subtle to be detected by standard SDS-PAGE techniques. The dominance of H. hypnale venom by PLA2 isoforms obscured the presence of other toxins. 1D gel showed that these toxins are indeed present, but at very low quantities. The sensitivity of DIGE allowed for more accurate relative quantitation. Qualitatively higher expressions of serine proteases, metalloproteases with disintegrin domains and PLA2, along with l-amino oxidases were observed in the C. rhodostoma sample, while H. hypnale venom was clearly dominated by PLA2.

The regional variation of C. rhodostoma venom not only has significant implications for the relative neutralisation of C. rhodostoma antivenom for treatment of envenomation of different populations of this species, but also for any potential cross-reactivity with H. hypnale venom. Such extreme variation of C. rhodostoma venom is likely responsible for the conflicting reports regarding the relative usefulness of different C. rhodostoma antivenins in neutralising H. hypnale envenomations [2,9]. Compounding this are suggestions by our results that the venom of H. hypnale may also vary significantly across its range.

In addition to regional variation within C. rhodostoma venoms leading to different antivenom stoichiometry, whereby antivenoms raised against different populations will have differential composition in regards to antibodies targeting specific toxin types, the differential toxin expression between C. rhodostoma and H. hypnale also contributes to described failures of C. rhodostoma antivenom in neutralising H. hypnale venom [2]. As antibodies are more readily generated against large antigens [26], C. rhodostoma antivenin is expected to contain disproportionately more antibodies against medium to large proteins in C. rhodostoma venom. Conspicuously such large enzymatic toxins are only in trace expression levels in H. hypnale venom. Instead, H. hypnale venom is dominated by PLA2 isoforms that differ significantly from those found in C. rhodostoma venom. It is perhaps unsurprising, therefore, that this antivenom is less successful in neutralising the toxic effects of H. hypnale venom, particularly those resulting from phospholipase activity such as platelet aggregation inhibition and possibly myotoxicity. This finding has real medical significance and provides a compelling argument for the development of a H. hypnale specific antivenom.

Our proteomics results show that H. hypnale differs significantly in toxin composition and expression levels from that of its sister taxon, C. rhodostoma. Our studies showed that while both venoms contained PLA2 and that this toxin type dominates H. hypnale venom, the H. hypnale forms were either higher or lower molecular weight than the C. rhodostoma type, with these size variations having implications for antigenic recognition and also potentially indicating differential bioactivity. Such differences may account for the previous failures of Thai Red Cross MPV monovalent antivenom to neutralise H. hypnale venom in a clinical setting. While the presence of many high molecular mass enzymes in the C. rhodostoma venom sample supports previous findings, our results bore little visual resemblance to previously published 2D studies and our results also suggest that H. hypnale venom may also vary considerably across its range. As the distribution of H. hypnale ranges from Sri Lanka to southern India and across a wide range of habitats, intraspecific variation of H. hypnale venom may be clinically relevant and is an important avenue of future research.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.07.020.

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