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REVIEW ARTICLE

Geographical variation of Indian Russell's viper venom and neutralization of its coagulopathy by polyvalent antivenom

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Abstract

Indian Russell's viper venoms of four different geographical locations were found to vary in composition, coagulopathy and phospholipase A_2 (PLA_2) activity. Venom from Kerala showed highest procoagulant activity followed by Tamil Nadu, West Bengal and Karnataka whereas PLA_2 activity was highest in venom from West Bengal. The commercial polyvalent antivenom differentially neutralized the aforesaid activities of the crude venoms. Antivenomics study showed the presence of non-immunodepleted and partially immunodepleted proteins in the crude venoms. Thus, Indian Russell's viper venom from different region varies in composition and accentuates the need to design regiospecific antivenoms to confront the problem of envenomation more effectively.

Keywords

Antivenomics, coagulopathy, geographical variation, Indian Russell's viper, *in vitro* neutralization

History

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Introduction

Snake venom is a complex mixture of proteins and polypeptides which varies from species to species and also within the same species. This variation has been mainly attributed to difference in diet, gender, age, season and geographical locations of the snakes (Alape-Giron et al., 2008; Chippaux et al., 1991; Daltry et al., 1996a,b; Jayanthi & Gowda, 1988; Menezes et al., 2006; Minton & Weinstein, 1986; Williams & White, 1992). Clinical symptoms of envenomation like neurotoxicity, myotoxicity, hemotoxicity, anticoagulant, procoagulant, haemorraghic, necrosis, renal damage and muscular paralysis in prey/victims might also vary within the same species due to this variation in venom composition (Hung et al., 2002a; Markland, 1998; White, 2005). The haemostatic system of prey/victim is a common target of all the snakes for capture of prey. The anticoagulant components of venom cause defective coagulation of blood leading to excess blood loss from the bite site and also from gums and internal organs. This in turn causes hypovolemic shock to vital organs like brain, kidney and pituitary glands leading to death (White, 2005). The procoagulant components of venom proteins cause excess clot formation leading to thrombosis in the blood vessels. This is often followed by consumptive coagulopathy which leads to heavy bleeding at later stages and thus compromising the functioning of vital organs like kidney, heart and brain (White, 2005). Some of the venom protein families act as both procoagulant and anticoagulant. For example, snake venom phospholipase A_2 (PLA_2) enzymes hydrolyze the membrane phospholipids of platelets leading to the release of arachidonic acid and platelet aggregation factors which cause platelet aggregation during the process of primary haemostasis (Braud et al., 2000). Some of them act as inhibitors of secondary haemostasis by enzymatically hydrolyzing the membrane phospholipids that are required to form complexes like the prothrombinase, extrinsic tenase and intrinsic tenase (Kini & Evans, 1989). Further, some PLA_2 enzymes interact non-covalently with some clotting factors of these complexes and exhibits anticoagulant activity (Kini, 2006, 2011). Snake venom proteases like metalloproteases and serine protease have been reported to inhibit or activate the components of haemostasis. They cause severe vascular damage by interacting with the extracellular matrix (White, 2005). This in turn interfere the regulation of the coagulation cascade. A schematic representation of the various protein families of snake venom acting as agonist and antagonist to the secondary haemostatic system is shown in Figure 1(a and b).

Daboia russelii, commonly referred as Russell's viper, is one of the medically important snakes of the world (Warrell, 1989). In India, subspecies *Daboia russelii russelii* is found across the country and responsible for majority of the snakebites cases (Warrell, 1989). It is one of the members of the ''Big Four'' snakes of India (Simpson & Norris, 2007). Russell's viper envenomation mainly causes excess bleeding due to consumptive coagulopathy by haemostatically active

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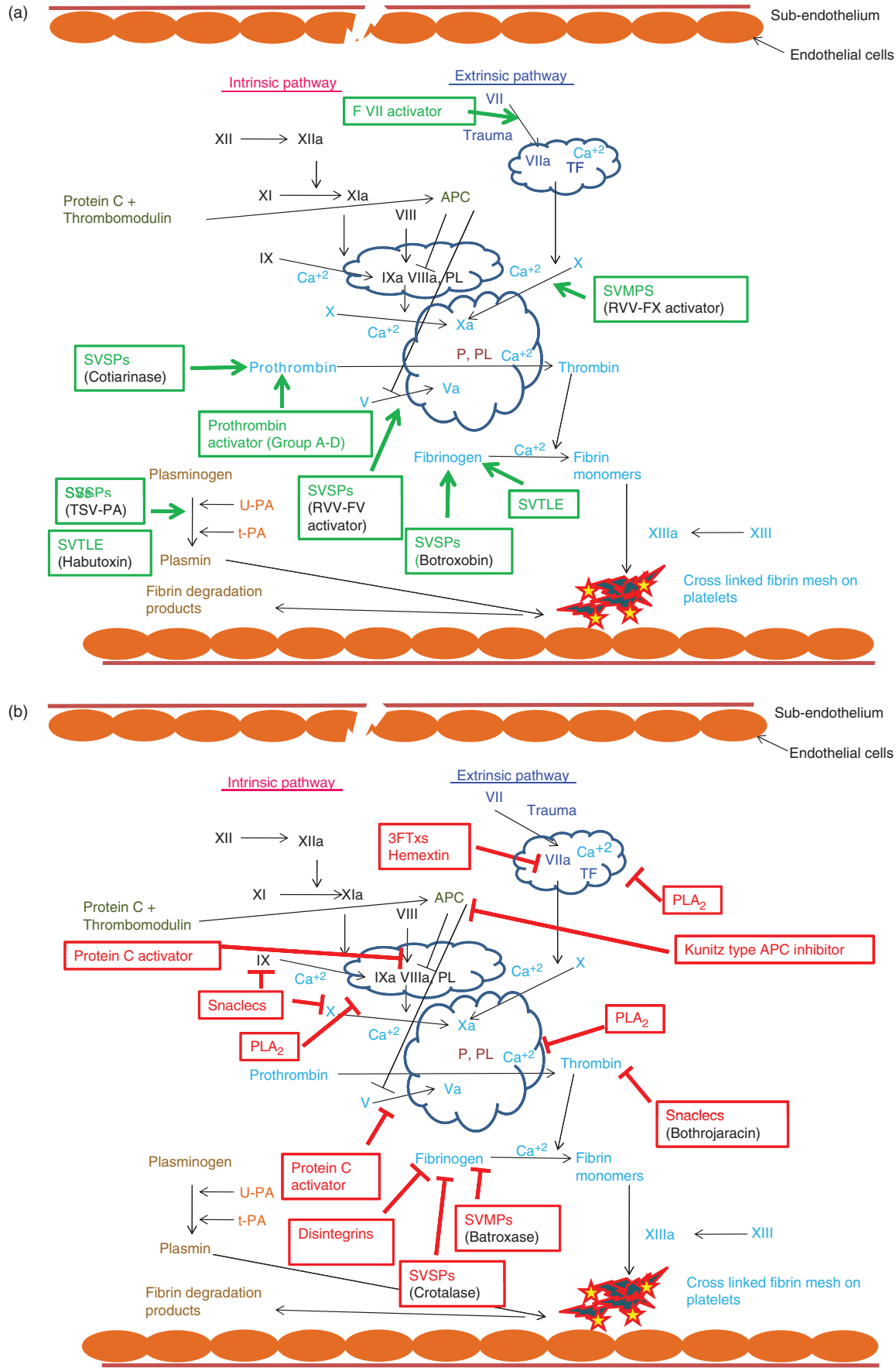

Figure 1. Schematic representation of snake venom proteins acting on haemostatic system. Various snake venom proteins families acting as (a) agonist (green box) and (b) antagonist (red box) of the secondary haemostatic system. Green arrow indicates proteins acting as agonist while proteins acting as antagonist are indicated by ''T'' shape bars. Examples of proteins belonging to the families are shown in brackets.

proteins which acts immediately (Cheng et al., 2012; Hung et al., 2002b; Phillips et al., 1988; Than et al., 1988). This often leads to hypovolemic shock to the vital organs of the prey/victim leading to severe clinical manifestations. Therefore, immediate medical attention is required in Russell's viper envenomation. In India, Russell's viper victims are treated with polyvalent antivenom and in many instances, adverse effect of antivenom therapy is being reported. This might be due to the variation in the venom composition between the venom used for raising the antibodies and venom present in the victim's body.

In India, Gowda and others studied the Russell's viper venom variation and toxicity from different regions, namely: western, northern, southern and eastern region (Jayanthi & Gowda, 1988; Prasad et al., 1996; Shashidharamurthy et al., 2002). Although variation in venom composition has been carried out but variation in venom composition with respect to coagulopathy has not been assessed. Hence, we reinvestigated the venom variation from four different geographical locations of India using proteomic approaches. Neutralization of coagulopathy of the crude venoms by polyvalent antivenom was also investigated using *in vitro* inhibition studies and antivenomics approach. In the present study we have selected geographically close regions as well as a far-off region to understand the variation in Indian Russell's viper venom composition.

Compositional analysis of Indian Russell's viper venom

Snake venom collection

Crude venom was obtained from four different states of India with typical climatic and geographical variations. The three geographically close states, namely Tamil Nadu, a land of coastal area; Karnataka, a land of hills and plateaus; Kerala, with typical humid equatorial climate surrounded by hills and coastal areas; and West Bengal, with completely distinct conditions of mixed climatic conditions comprising of hills, plateaus and delta.

The crude venom obtained from Tamil Nadu is designated as RvTN (Russell's viper Tamil Nadu), this venom was procured from Irula Snake catchers Society, Tamil Nadu; venom from West Bengal is designated as RvWB (Russell's viper West Bengal), which was obtained from Kolkata Snake Park; venom from Kerala is designated as RvKE (Russell's viper Kerala), which was milked from the snake kept in Agadantantra snake park, Ayurveda Medical college, Thiruvananthapuram, and venom from Karnataka is designated as RvKA (Russell's viper Karnataka), it was collected from Hassan, Western Ghats (Milked Personally).

SDS–PAGE analysis of crude venom

Crude venoms (15 μg) after reduction with β-mercaptoethanol were loaded on 12.5% SDS–PAGE along with standard protein markers (Thermoscientific; Lafayette, CO) and performed according to the method of Laemmli (1970). The gels were stained with 0.25% (w/v) coomassie brilliant blue and destained till the bands appeared. All the four crude venoms have shown protein bands in the molecular mass range of 170–10 kDa with varying intensity of protein bands (Figure 2). The SDS–PAGE profile of RvTN has shown the presence of 9 protein bands with two very high-intensity protein bands at 70 and 15 kDa. RvKA has shown the presence of 13 protein bands with major bands at 15 and 10 kDa, while no prominent band was observed at 70 kDa. On the other hand, RvKE has revealed the presence of 11 protein bands with very intense bands at 100, 25 and 15 kDa. The RvWB has shown the presence of 12 protein bands with major bands at 70, 35, 25 and 15 kDa. The analysis has revealed that all the four crude venoms consist of a major protein band at 15 kDa, but with different intensities. Thus, SDS–PAGE analysis of the four venoms revealed that they differ in composition and expression level of venom proteins in the glands.

Gel filtration chromatography analysis

Crude venoms were subjected to gel filtration chromatography on a Shodex column pre-equilibrated with 20 mM Tris–Cl pH 7.4. Elution was carried out with the same buffer at a flow rate of 0.5 ml min^{-1} using a Waters HPLC system (Milford, MA) and monitored at 215 and 280 nm. All the four geographically isolated crude venoms had different elution profiles. The crude venom of RvWB has 10 peaks of which the 1st and the 5th peaks were found to be prominent. The crude venom of RvKE has only 6 protein peaks with the 1st and the 6th peak as the major peaks. RvKA has 13 peaks of which the 1st, 4th, 7th, 9th and the 10th peaks were found to be prominent. RvTN has 10 peaks of which peak 1st, 3rd and 5th are the prominent peaks. The gel filtration profile documents the variation in venom composition with respect to protein families as well as its expression level (Figure 2).

Reverse phase high-performance liquid chromatography analysis

To further understand the variation in the venom composition, 200 μg of each crude venom was loaded on a Jupiter C_{18} columns pre-equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA). The proteins were eluted by a linear gradient of 80% (v/v) acetonitrile (AcCN) containing 0.1% (v/v) TFA at a flow rate of 0.8 ml min^{-1} over 180 min. The elution was monitored at 280 and 215 nm. The Rp-HPLC profiles of the four venoms were different (Figure 3).

PLA_2 activity

Phospholipase A_2s are hydrolytic enzymes ubiquitously present in all snake venoms that induce multiple pharmacological effects (Harris, 1985; Kini, 1997). This group of enzymes exhibit anticoagulant activity either through enzymatic or non-enzymatic mechanisms (Kini, 2006). PLA_2 activity of the four crude venoms of Russell's viper was measured using egg yolk as substrate by the method of Joubert & Taljaard (1980). The analysis revealed that 1 μg of RvWB has PLA_2 activity of $\sim 58.18 \pm 0.02$ U while the same amount of RvKA, RvKE and RvTN, has only 18.7 ± 0.5, 7.12 ± 0.011 and 5.0 ± 0.18 U, respectively. Hence, venom from the Eastern region has the most hydrolytically active PLA_2 enzymes followed by venom from Karnataka, Kerala and Tamil Nadu.

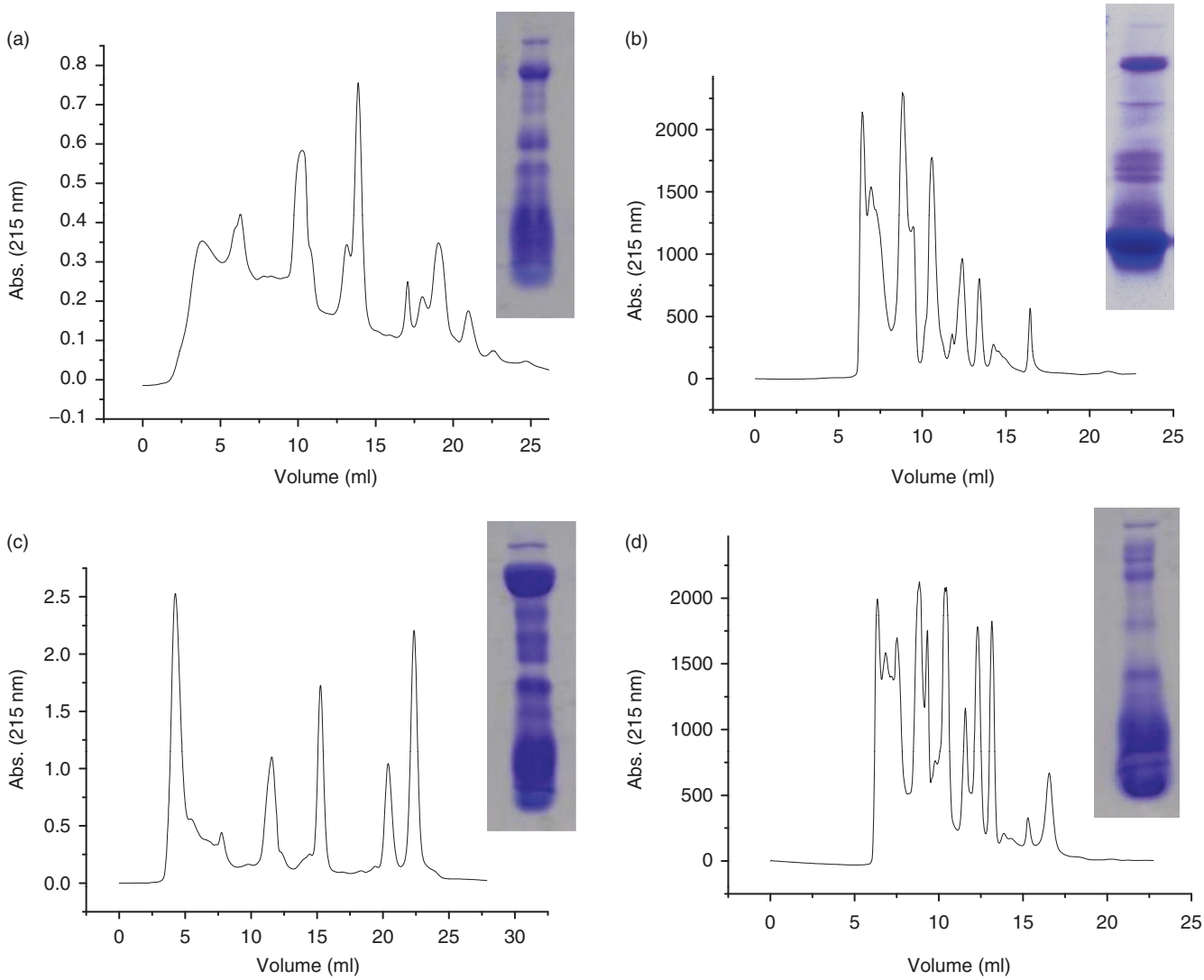

Figure 2. Gel filtration chromatography profile of crude venoms of Russell's viper on a shodex column (Waters HPLC System). The column was pre-equilibrated with 20 mM Tris–Cl pH 7.4 and 50 µg of sample was loaded. Elution was carried out at flow rate of 0.5 ml min^{-1} and monitored at 215 nm. (a) Elution profile of RvWB, inset 12.5% SDS–PAGE. (b) RvTN, (c) RvKE, (d) RvKA.

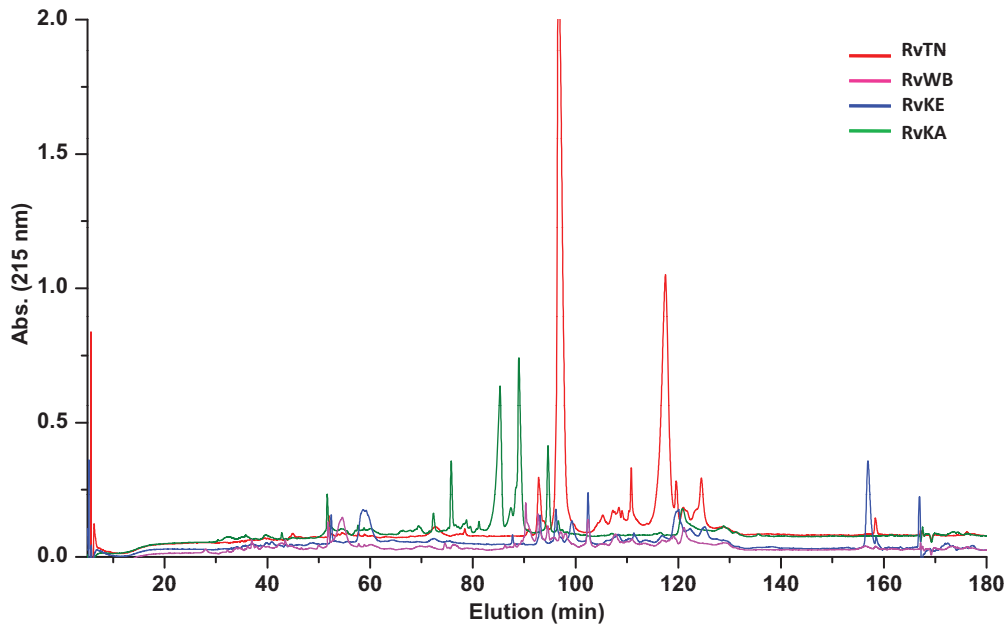

Figure 3. Comparative analysis of RvWB, RvTN, RvKE and RvKA on Rp-HPLC. Rp-HPLC was performed on a Jupiter C_{18} column (Phenomenex) pre-equilibrated with 0.1% (v/v) TFA. About 200 µg of crude venom was loaded on the column and eluted with a linear gradient of 80% (v/v) AcCN containing 0.1% (v/v) TFA over 180 min at a flow rate of 0.8 ml min^{-1}. The elution was monitored at 215 nm.

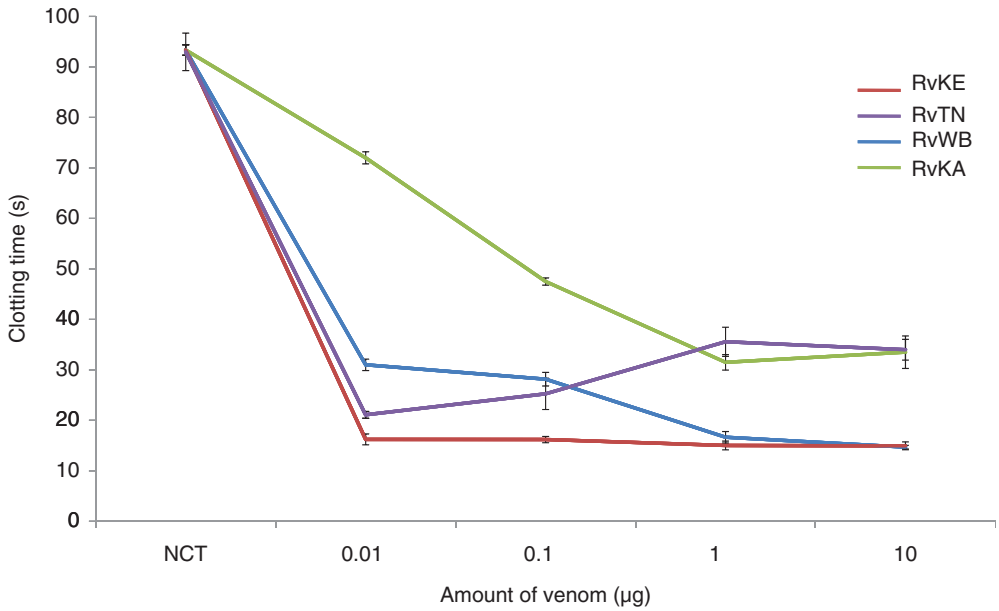

Figure 4. Comparative analysis of the recalcification time of RvTN, RvWB, RvKE and RvKA. About 150 µl of goat plasma was pre-incubated with various amount of crude venom in 50 µl of 20 mM Tris–Cl pH 7.4 at 37 °C. About 100 µl of 50 mM of $CaCl_2$ was added to initiate the clot formation and the time taken to form the clot was recorded on a Coastat-1 coagulation analyser (Tulip Diagnostics; Goa, India). About 20 mM Tris–Cl pH 7.4 instead of crude venom was used to determine the control clotting time. Experiments were repeated thrice and the means values were used to plot the graph.

Recalcification time test

The effect of crude Russell's viper venoms on recalcification time was analyzed using goat plasma in a Coagstat-1 (Tulip Diagnostics) coagulometer. All the crude venoms exhibited procoagulant activity compared to the control clotting time of 93 s. The venom from Tamil Nadu and Kerala reduced the clotting time to 21 and 16.23 s at 0.01 µg ml^{-1}. With increase in concentration there was no change in the clotting time of Kerala venom however, there was a slight increase in clotting time of Tamil Nadu venom when tested up to 10 µg. The venom from Karnataka and West Bengal showed procoagulant activity in a dose dependent manner and at 10 µg the clotting time was 33.5 and 14.7 s, respectively. The increase in clotting time of Tamil Nadu venom might be due to the presence of higher concentrations of anticoagulant components in its venom (Figure 4).

Neutralization by polyvalent antivenom

Indian polyvalent antivenom (Bharat serums and vaccines limited, B. No. A5310028) raised against viz: *Daboia russelii* (Russell's viper), *Naja naja* (Spectacled cobra), *Bungarus caeruleus* (Common krait), *Echis carinatus* (Saw-scaled viper) commonly referred to as ''Big four'' was used for the neutralization study.

Various concentrations of polyvalent antivenom were pre-incubated with venom at 37 °C for 1 h. The percentage neutralization of activity was calculated considering the activity of the crude venom in the absence of polyvalent antivenom as 100%. About 100 µg of the polyvalent antivenom could neutralize the PLA_2 activity of RvWB upto 94%, RvKE upto 67%, RvTN upto 55% and RvKA upto 24% (Figure 5). For complete neutralization of the PLA_2 activity of 1 µg of RvWB 250 µg of polyvalent antivenom is required whereas for RvTN and RvKE, 500 µg is required and for RvKA, 1000 µg of polyvalent antivenom is required. Similarly, to neutralize the procoagulant effect of the crude Russell's viper venoms higher amounts of polyvalent antivenom is required. About 100 µg of polyvalent antivenom could neutralize the procoagulant effect on goat plasma by ~76% (RvTN) and ~66% (RvKA), respectively. However, at the same concentration the antivenom could not neutralize the procoagulant effect of RvWB and RvKE (Figure 6). This shows the absence of specific antibodies for the epitopes of the RvWB and RvKE venom procoagulant proteins in the polyvalent antivenom. This in turn reflects the inefficacy of the available antivenom to combat the envenomation effects of Russell's viper in West Bengal or Kerala due to difference in geographical variation.

Immunodepletion of venom proteins

Antivenomics is a new generation proteomics approach to analyze the immunoreactivity of antivenoms with venom proteins both qualitatively and quantitatively (Calvete et al., 2009; Pla et al., 2012). To check the immunoreactivity of commercially available polyvalent antivenom, it was immobilized on CNBr-activated support (Sigma). The resin was washed several times to remove any unbound antibodies. Crude venom was passed through the column, the flow-through (non-retained fraction) was reloaded onto the column several times before loading on to a Jupiter C_{18} Rp-HPLC column pre-equilibrated with 0.1% (v/v) TFA. The fractions were eluted by a linear gradient of 80% (v/v) AcCN and 0.1% (v/v) TFA at a flow rate of 0.8 ml min^{-1}. The separation was monitored at 215 and 280 nm (Figure 7). The elution profile of the flow-through fraction was compared with the crude venom profile to identify the depleted, partially depleted and non-depleted venom proteins. The flow-through fraction of RvTN showed the presence of five peaks while one partially

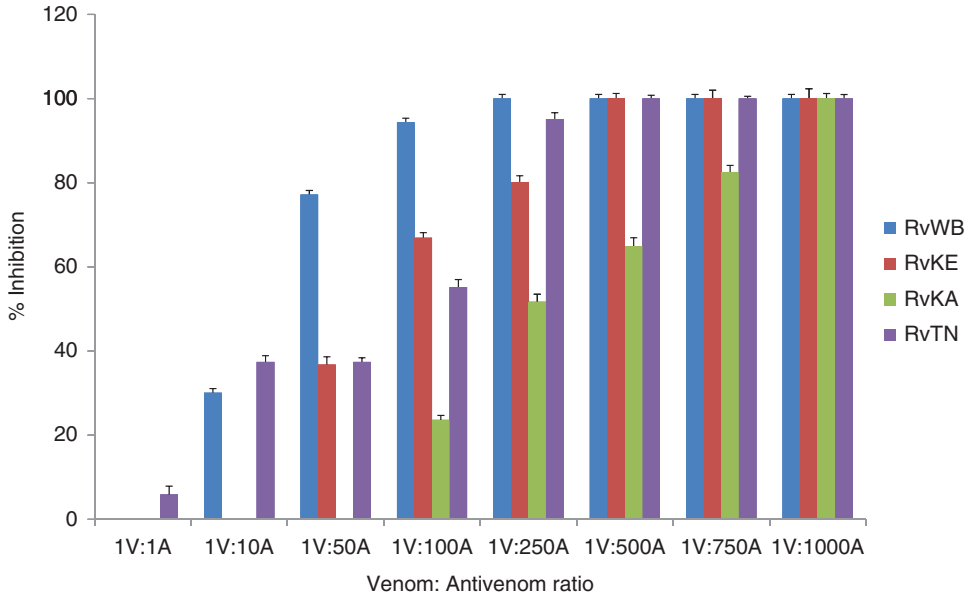

Figure 5. Neutralization of phospholipase A_2 activity of crude venoms of RvTN, RvWB, RvKE and RvKA by commercially available polyvalent antivenom. With different amount of antivenom, 1 µg of crude venoms of Russell's viper were incubated at 37 °C for 1 h. To this, 200 µl of egg yolk adjusted to 1 O.D. were added. The absorbance of the reaction was monitored at 740 nm for 10 min in a Thermo Scientific spectrophotometer. One unit of PLA_2 activity is defined as the amount of protein which produces a decrease in 0.01 absorbance units in 10 min at 740 nm. Experiments were repeated thrice and the means values were used to plot the graph.

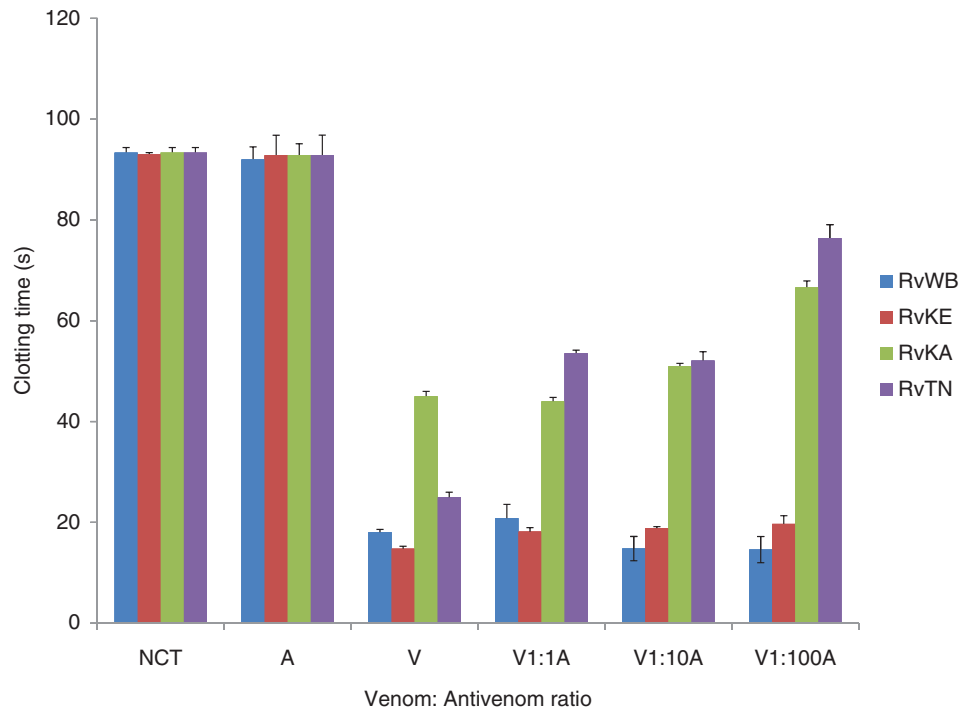

Figure 6. Neutralization of recalcification time of RvTN, RvWB, RvKE and RvKA by commercially available polyvalent antivenom. Various amount of polyvalent antivenom were incubated with 1 µg of crude venoms at 37 °C for 1 h. The pre-incubated mixture was added to 150 µl of goat plasma and incubated at 37 °C for 2 min. The clot formation was initiated by adding 100 µl of 50 mM of $CaCl_2$ and the time taken to form the clot was recorded on a Coastat-1 coagulation analyser. About 20 mM of Tris–Cl pH 7.4 without crude venom and antivenom was used to determine the control clotting time. Experiments were repeated thrice and the means values were used to plot the graph.

depleted peak was observed at 97.5 min. This protein exhibited anticoagulant activity and required higher amount of polyvalent antivenom to completely neutralize its activity when studied under *in vitro* conditions (data not shown). On the other hand, the elution profile of flow-through fraction of RvKA, RvWB and RvKE contained four different non-depleted protein peaks with no partially depleted proteins.

Discussion

Variation in the snake venom composition based on geographical location is well documented in the literature.

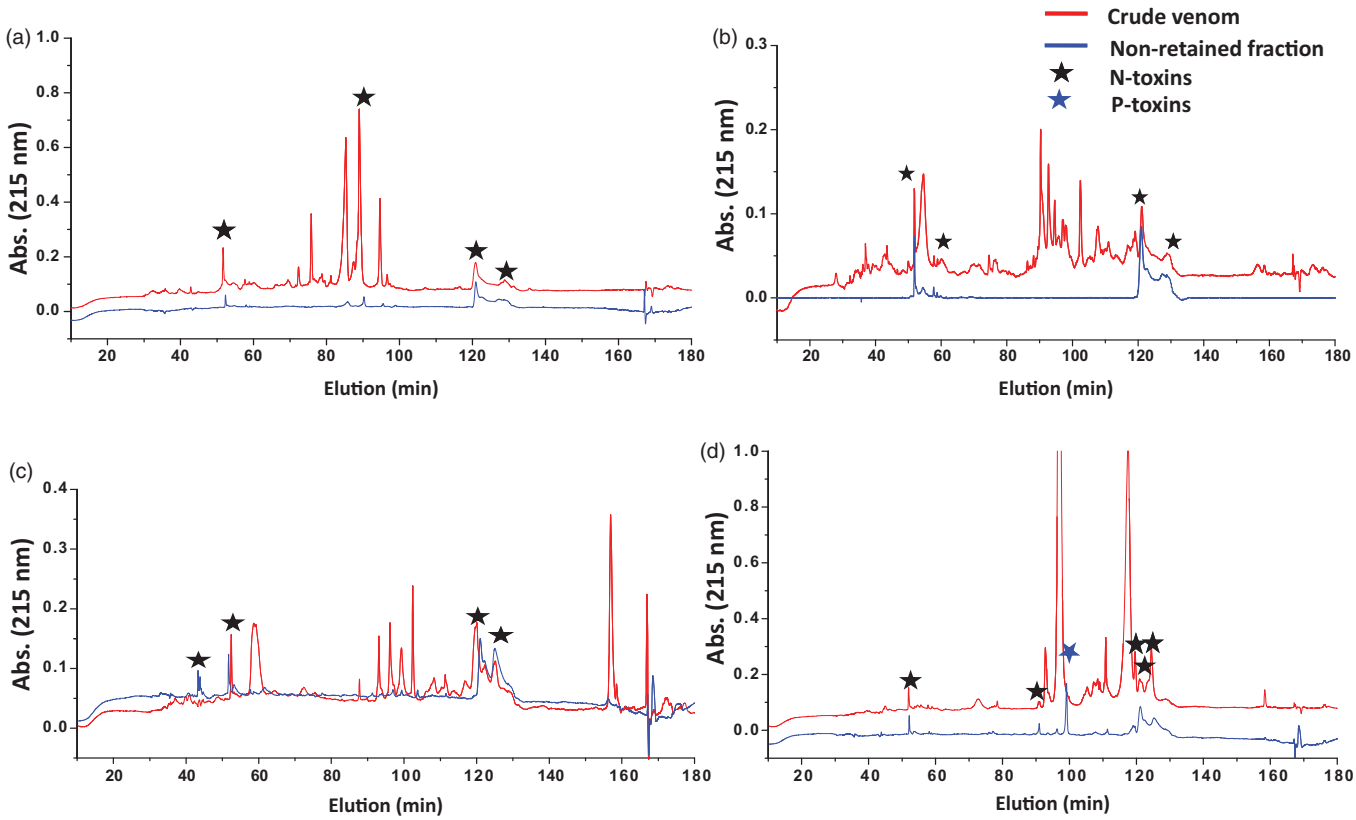

Figure 7. Rp-HPLC profile of non-retained fraction of crude Russell's viper venoms. (a) RvKA, (b) RvWB, (c) RvKE and (d) RvTN. Rp-HPLC was performed on a Jupiter C_{18} column (Phenomenex, Torrance, CA) pre-equilibrated with 0.1% (v/v) TFA. The non-retained fractions of crude venom was loaded on the column and eluted with a linear gradient of 80% (v/v) AcCN containing 0.1% (v/v) TFA over 180 min at a flow rate of 0.8 ml min^{-1}. The elution was monitored at 215 nm.

Indian Russell's viper venom from northern, southern, western and eastern region has been found to differ significantly (Prasad et al., 1999). Basic PLA_2 activity was found to be more prominent in venom of northern and southern regions while acidic proteins are abundant in eastern and western origin venoms. Proteolytic activity and trypsin inhibitory activity were more pronounced in the venom of western and northern regions compared to other region venoms. Moreover, eastern Russell's viper was the most lethal compared to the venoms of the other three regions (Jayanthi & Gowda, 1988; Prasad et al., 1999). Comparison of Russell's viper venom from Indian and Myanmar origin showed variation in the composition (Tsai et al., 2007). PLA_2 enzymes (two acidic and two basic) isolated from *Daboia siamensis* and *Daboia russelii* differs in toxicity and lethality on model animals (Tsai et al., 2007). This suggests variation in the biological activities of the similar proteins in venom of same species but geographically different origin. Moreover, envenomation by *Daboia russelii siamensis* from Taiwan exhibits both neurotoxic and haemotoxic effects on victims leading to systemic thrombosis and neuromuscular blocking (Hung et al., 2002a) whereas *Daboia russelii* envenomation in India shows symptoms of bleeding, renal failure, nausea, convulsions, hypotension, myonecrosis and edema with no neurotoxic effects (Mukherjee et al., 2000).

The SDS–PAGE, gel filtration and Rp-HPLC profile of the crude venoms from four different geographical locations of India showed significant variation in the venom composition. The differences in intensity of bands and peaks reveal that expression of venom proteins is different in all the venoms. Venoms from Kerala and Karnataka showed the presence of more low molecular mass proteins compared to Tamil Nadu and West Bengal venoms. Gel filtration elution profile of the venoms from Tamil Nadu, Kerala and Karnataka were similar whereas the venom from West Bengal was different. The former origins are geographically close and the snakes are likely to have similar diet which might be responsible for the similarity in venom protein composition to some extent. However, the Rp-HPLC elution patterns of the venoms showed different retention time of venom proteins. Two distinct proteins peaks were observed in venom of Tamil Nadu, which is absent in all other venoms demonstrating the variation in venom composition though they belong to the same species. This difference in the venom composition would contribute to differences in clinical symptoms during envenomation which would require different strategies to neutralize. PLA_2 activity and recalcification time of the crude venoms further demonstrates that these geographically close snakes of the same species are different in venom composition and expression level. The recalcification time of all the venom analyzed in the present study showed procoagulant activity

however they differ in their mode of action. The venoms of Tamil Nadu and Kerala showed the presence of fast acting procoagulant proteins whereas the other two venoms were slow to exhibit their activity.

Antivenom therapy is the only available therapy for the treatment of snakebite patients. However on many occasions, it has been observed that polyvalent antivenom failed to neutralize the toxic effect of venom (Isbister et al., 2008; Phillips et al., 1988). During antivenom therapy, about 10–20 vials of polyvalent antivenom is administered (Saravu et al., 2012) which might lead to anaphylactic reactions in patients (Caron et al., 2009; Stone et al., 2013). This is due to the unwanted antibodies present in the commercially available Indian polyvalent antivenom as it contains only ~25% of the antibodies to a particular snake (Simpson & Norris, 2007). It has been observed that polyclonal antibodies raised against southern Russell's viper showed significant difference in diffusion and cross-reactivity pattern with other geographical regions. Moreover, these antibodies were incapable in providing any sort of protection against the lethality by venoms of other regions (Prasad et al., 1999). Further, it has been observed that antibodies raised against the same species of *Naja naja* were also ineffective in neutralizing the toxic effect of venom from other geographical locations (Shashidharamurthy & Kemparaju, 2007). This failure is most likely associated with venom variation due to the geographic location and also due to the presence of unique toxins. Hence, regiospecific antivenom needs to be raised for treatment of snakebite patients from a particular geographical location. In the present study the comparative analysis of Rp-HPLC profile of crude venom and non-retained fraction after immunodepletion demonstrated that the polyvalent antivenom is ineffective in complete depletion of the venom components. This is due to the variation in venom composition, as venom from a particular geographical location is used for the production of antivenom. Expression of venom proteins differs greatly among the species as well as within the same species, which might also be responsible for non-immunodepletion (Lomonte & Carmona, 1992; Rodrigues et al., 1998; Saad et al., 2012). Apart from this the non-immunogenicity might be also responsible for the presence of these proteins in the flow-through fraction. Interestingly, highly hydrophobic proteins in all the venoms were not depleted by the polyvalent antivenom. Thus, the variation and non-immunogenecity of venom proteins need to be considered while producing antivenoms.

Conclusion and future prospective

The availability of food differs with different geographical locations for a snake. In order to adapt itself for survival, it has to induce various patho-physiological symptoms in prey for immediate immobilization and subjugation. Evolutionarily venom protein families have undergone accelerated evolution followed by point mutation to incorporate wide array of toxins with diverse biological functions to target their prey (Chijiwa et al., 2000; Nakashima et al., 1995; Ogawa et al., 1996; Zupunski et al., 2003). This evolutionary mechanism has resulted in variation in the venom composition among the snake species and also within the same species. The effective neutralization of these pharmacological effects in victims would be successful only when the detailed composition of venom proteins is understood and specific antivenoms are raised. The present study documents the variation in venom composition within the same species of Russell's viper which are from different geographically locations.

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Declaration of interest

The authors declare no conflicts of interest

References

Alape-Giron A, Sanz L, Escolano J, et al. (2008). Snake venomics of the lancehead pitviper *Bothrops asper*: geographic, individual, and ontogenetic variations. J Proteome Res 7:3556–71.

Braud S, Bon C, Wisner A. (2000). Snake venom proteins acting on hemostasis. Biochimie 82:851–9.

Calvete JJ, Sanz L, Angulo Y, et al. (2009). Venoms, venomics, antivenomics. FEBS Lett 583:1736–43.

Caron EJ, Manock SR, Maudlin J, et al. (2009). Apparent marked reduction in early antivenom reactions compared to historical controls: was it prophylaxis or method of administration? Toxicon 54:779–83

Cheng AC, Wu HL, Shi GY, Tsai IH. (2012). A novel heparin-dependent inhibitor of activated protein C that potentiates consumptive coagulopathy in Russell's viper envenomation. J Biol Chem 287: 15739–48.

Chijiwa T, Deshimaru M, Nobuhisa I, et al. (2000). Regional evolution of venom-gland phospholipase A2 isoenzymes of *Trimeresurus flavoviridis* snakes in the southwestern islands of Japan. Biochem J 347:491–9.

Chippaux JP, Williams V, White J. (1991). Snake venom variability: methods of study, results and interpretation. Toxicon 29:1279–303.

Daltry JC, Ponnudurai G, Shin CK, et al. (1996a). Electrophoretic profiles and biological activities: intraspecific variation in the venom of the Malayan pit viper (*Calloselasma rhodostoma*). Toxicon 34: 67–79.

Daltry JC, Wuster W, Thorpe RS. (1996b). Diet and snake venom evolution. Nature 379:537–40.

Harris JB. (1985). Phospholipases in snake venoms and their effects on nerve and muscle. Pharmacol Ther 31:79–102.

Hung DZ, Wu ML, Deng JF, Lin-Shiau SY. (2002a). Russell's viper snakebite in Taiwan: differences from other Asian countries. Toxicon 40:1291–8.

Hung DZ, Wu ML, Deng JF, et al. (2002b). Multiple thrombotic occlusions of vessels after Russell's viper envenoming. Pharmacol Toxicol 91:106–10.

Isbister GK, Brown SG, MacDonald E, et al. (2008). Current use of Australian snake antivenoms and frequency of immediate-type hypersensitivity reactions and anaphylaxis. Med J Aust 188:473–6.

Jayanthi GP, Gowda TV. (1988). Geographical variation in India in the composition and lethal potency of Russell's viper (*Vipera russelli*) venom. Toxicon 26:257–64.

Joubert FJ, Taljaard N. (1980). Purification, some properties and amino-acid sequences of two phospholipases A (CM-II and CM-III) from *Naja naja* kaouthia venom. Eur J Biochem 112:493–9.

Kini RM. (1997). Phospholipase A2 a complex multifunctional protein puzzle. In: Kini RM, ed. Venom phospholipase A2 enzymes: structure, function and mechanism. Chichester: John Wiley & Sons, 1–28.

Kini RM. (2006). Anticoagulant proteins from snake venoms: structure, function and mechanism. Biochem J 397:377–87.

Kini RM. (2011). Toxins in thrombosis and haemostasis: potential beyond imagination. J Thromb Haemost 9 Suppl 1:195–208.

Kini RM, Evans HJ. (1989). A model to explain the pharmacological effects of snake venom phospholipases A2. Toxicon 27:613–35.

Laemmli UK. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–5.

Lomonte B, Carmona E. (1992). Individual expression patterns of myotoxin isoforms in the venom of the snake *Bothrops asper*. Comp Biochem Physiol B 102:325–9.

Markland FS. (1998). Snake venoms and the hemostatic system. Toxicon 36:1749–800.

Menezes MC, Furtado MF, Travaglia-Cardoso SR, et al. (2006). Sex-based individual variation of snake venom proteome among eighteen *Bothrops jararaca* siblings. Toxicon 47:304–12.

Minton SA, Weinstein SA. (1986). Geographic and ontogenic variation in venom of the western diamondback rattlesnake (*Crotalus atrox*). Toxicon 24:71–80.

Mukherjee AK, Ghosal SK, Maity CR. (2000). Some biochemical properties of Russell's viper (*Daboia russelli*) venom from Eastern India: correlation with clinico-pathological manifestation in Russell's viper bite. Toxicon 38:163–75.

Nakashima K, Nobuhisa I, Deshimaru M, et al. (1995). Accelerated evolution in the protein-coding regions is universal in crotalinae snake venom gland phospholipase A2 isozyme genes. Proc Natl Acad Sci USA 92:5605–9.

Ogawa T, Nakashima K, Nobuhisa I, et al. (1996). Accelerated evolution of snake venom phospholipase A2 isozymes for acquisition of diverse physiological functions. Toxicon 34:1229–36.

Phillips RE, Theakston RD, Warrell DA, et al. (1988). Paralysis, rhabdomyolysis and haemolysis caused by bites of Russell's viper (*Vipera russelli pulchella*) in Sri Lanka: failure of Indian (Haffkine) antivenom. Q J Med 68:691–715.

Pla D, Gutierrez JM, Calvete JJ. (2012). Second generation snake antivenomics: comparing immunoaffinity and immunodepletion protocols. Toxicon 60:688–99.

Prasad BN, Kemparaju K, Bhatt KG, Gowda TV. (1996). A platelet aggregation inhibitor phospholipase A2 from Russell's viper (*Vipera russelli*) venom: isolation and characterization. Toxicon 34:1173–85.

Prasad NB, Uma B, Bhatt SK, Gowda VT. (1999). Comparative characterisation of Russell's viper (*Daboia*/*Vipera russell*i) venoms from different regions of the Indian peninsula. Biochim Biophys Acta 1428:121–36.

Rodrigues VM, Soares AM, Mancin AC, et al. (1998). Geographic variations in the composition of myotoxins from *Bothrops neuwiedi* snake venoms: biochemical characterization and biological activity. Comp Biochem Physiol A Mol Integr Physiol 121:215–22.

Saad E, Curtolo BL, Biscola N, et al. (2012). Intraspecific variation of biological activities in venoms from wild and captive *Bothrops jararaca*. J Toxicol Environ Health A 75:1081–90.

Saravu K, Somavarapu V, Shastry AB, Kumar R. (2012). Clinical profile, species-specific severity grading, and outcome determinants of snake envenomation: an Indian tertiary care hospital-based prospective study. Indian J Crit Care Med 16:187–92.

Shashidharamurthy R, Jagadeesha DK, Girish KS, Kemparaju K. (2002). Variations in biochemical and pharmacological properties of Indian cobra (*Naja naja naja*) venom due to geographical distribution. Mol Cell Biochem 229:93–101.

Shashidharamurthy R, Kemparaju K. (2007). Region-specific neutralization of Indian cobra (*Naja naja*) venom by polyclonal antibody raised against the eastern regional venom: a comparative study of the venoms from three different geographical distributions. Int Immunopharmacol 7:61–9.

Simpson ID, Norris RL. (2007). Snakes of medical importance in India: is the concept of the ''Big 4'' still relevant and useful? Wilderness. Environ Med 18:2–9.

Stone SF, Isbister GK, Shahmy S, et al. (2013). Immune response to snake envenoming and treatment with antivenom; complement activation, cytokine production and mast cell degranulation. PLoS Negl Trop Dis 7:e2326.

Than T, Hutton RA, Myint L, et al. (1988). Haemostatic disturbances in patients bitten by Russell's viper (*Vipera russelli siamensis*) in Burma. Br J Haematol 69:513–20.

Tsai IH, Tsai HY, Wang YM, et al. (2007). Venom phospholipases of Russell's vipers from Myanmar and eastern India – cloning, characterization and phylogeographic analysis. Biochim Biophys Acta 1774: 1020–8.

Warrell DA. (1989). Snake venoms in science and clinical medicine. 1. Russell's viper: biology, venom and treatment of bites. Trans R Soc Trop Med Hyg 83:732–40.

White J. (2005). Snake venoms and coagulopathy. Toxicon 45:951–67.

Williams V, White J. (1992). Variation in the composition of the venom from a single specimen of *Pseudonaja textilis* (common brown snake) over one year. Toxicon 30:202–6.

Zupunski V, Kordis D, Gubensek F. (2003). Adaptive evolution in the snake venom Kunitz/BPTI protein family. FEBS Lett 547: 131–6.