



The conserved structure of snake venom toxins confers extensive immunological cross-reactivity to toxin-specific antibody

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Abstract

We have demonstrated previously that antisera from mice immunised with DNA encoding the carboxy-terminal domain (JD9) of a potent haemorrhagic metalloproteinase, jararhagin, neutralised over 70% of the haemorrhagic activity of the whole *Bothrops jararaca* venom. Here, we demonstrate that the JD9-specific antibody possesses extensive immunological reactivity to venom components in snakes of distinct species and genera. The polyspecific immunological reactivity of the antibody showed a correlation with amino acid sequence identity and with predicted antigenic domains of JD9-analogues in venoms of snakes with closest phylogenetic links to *B. jararaca*. This study further promotes the potential of DNA immunisation to generate toxin-specific antibodies with polyspecific cover. An analysis of the reactivity of the JD9-specific antisera to *B. atrox* complex venoms that exhibited intraspecific variation in the venom proteome revealed, however, that the toxin-specific approach to antivenom development requires a more in-depth knowledge of the target molecules than is required for conventional antivenoms.

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1. Introduction

Viper venoms consist of numerous toxic components that cause haemorrhagic and coagulopathic, and occasionally neurotoxic, pathologies to the envenomed patient. Because of the inter- and intra-specific variation in the toxin composition of viper venoms, treatment of envenoming is best achieved by the administration of polyspecific antivenom. Monospecific antivenoms are used preferentially if the envenomed patient identifies the snake responsible for the symptoms that are characteristic within a region (e.g. monospecific *Echis* viper antivenom in West Africa). Polyspecific antivenoms are prepared from the sera of horses or sheep immunised with increasing doses of whole venoms from the most medically important snake species

within a defined region. Antivenoms therefore contain antibodies to the majority of venom components, irrespective of their toxicity. While this comprehensive responsiveness satisfies the polyspecific requirements of antivenoms, it also means that antivenoms contain numerous redundant antibodies specific to non-toxic venom antigens that dilute the efficacy of the toxin-specific antibodies (Theakston and Reid, 1983). This deficiency is exacerbated by the observation that the most toxic venom components are not necessarily the most antigenic, particularly those of low molecular weight (Mandelbaum and Assakura, 1998; Schöttler, 1951; Theakston, 1983).

The development of toxin-specific antivenoms to address these issues was initially encouraged by the production of monoclonal antibodies with venom-neutralising potential (Boulain et al., 1982; Iddon et al., 1988; Perez et al., 1984; Pukrittayakamee et al., 1983). However, the prospect of monoclonal antivenoms never materialised because of the logistic problems of identifying and purifying, in sufficient

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quantity, toxins from whole venoms and the perception that their epitope specificity would restrict their polyspecific venom-neutralising efficacy. Recent technical advances in the molecular and biochemical disciplines have made the identification of the venom proteome an achievable objective (Fox et al., 2002). We propose to exploit this increased understanding of the identity and function of snake venom toxins to generate specific antibodies to neutralise toxin function and ultimately to develop antivenom that consists solely of antibodies that target venom toxins in snakes of a defined geographic region. Rather than rely upon the purification of very limited amounts of protein from whole venom for immunising material, we have elected the more expedient approach of immunisation with DNA encoding venom toxins. We have demonstrated that epidermal immunisation with DNA encoding the disintegrin and cysteine-rich domain (hereafter termed JD9) of jararhagin, a potent haemorrhagic zinc metalloproteinase in the venom of *B. jararaca*, generated an antibody that neutralised over 70% of the haemorrhagic activity of the whole venom (Harrison et al., 2000). The efficacy of the epitopically restricted JD9-specific antibody was attributed to its reactivity to other molecules of the same molecular weight, but distinct isoelectric points, as jararhagin that were presumed to also contribute to venom haemorrhagic activity.

Here we explore the phylogenetic range of the cross-reactive nature of this JD9 antibody and tentatively identify the protein configurations underpinning the polyspecific immunoreactivity of such toxin-specific antibodies.

2. Materials and methods

2.1. Venoms

The 13 venom samples from different snake species used in Fig. 1 were obtained from lyophilised pools of venoms extracted from live animals maintained in the herpetarium of the Alistair Reid Venom Research Unit at the Liverpool School of Tropical Medicine. The 11 venom samples from snakes of the *Bothrops atrox* complex used in Fig. 4 were collected from a pool of venoms extracted from individual snakes, of mixed sex and age, resident in different locations across Central and South America.

2.2. Antivenom and antibody

The equine antivenom used to treat envenoming by *Bothrops* snakes (Sôro Antibotrópico, Instituto Butantan, São Paulo, Brazil) was an F(ab')₂ preparation of IgG purified from sera of hyper-immunised horses. The pooled murine JD9 antisera were from Balb/c mice immunised by the GeneGun with a plasmid encoding JD9 (Harrison et al., 2000).

2.3. SDS-PAGE

Venom samples were rehydrated in phosphate buffered saline (PBS), reduced in SDS-PAGE loading buffer (2% SDS, 5% β-mercaptoethanol in 62 mM Tris-HCl, pH 6.8) and boiled for 5 min. Samples (12.5 μg) of these venom

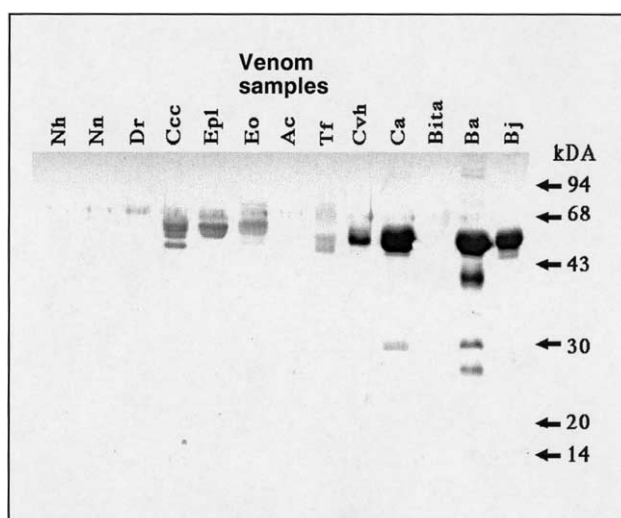


Fig. 1. Cross-reactivity of the JD9 antibody with venoms from snakes of distinct species and genera. Venoms from *B. jararaca* (Bj), *B. atrox* (Ba), *Bitis arietans* (Bit), *C. atrox* (Ca), *C. viridis helleri* (Cvh), *Protobothrops* (formerly *Trimeresurus*) *flavoviridis* (Tf), *Agkistrodon contortrix* (Ac), *Echis ocellatus* (Eo), *E. pyramidum leakeyi* (Epl), *Cerastes cerastes cerastes* (Ccc), *Daboia russelli* (Dr), *Naja nigricollis* (Nn) and *N. haje* (Nh) were fractionated by SDS-PAGE, blotted onto nitrocellulose and incubated with antisera from mice immunised with JD9 DNA (diluted 1:2000).

preparations and molecular weight markers (low molecular weight mix of 97, 66, 45, 31, 21, 14 kDa proteins, BioRad, Hercules, California, USA) were fractionated on a 15% SDS–PAGE gel using Minin-PROTEAN 3 electrophoresis cells (BioRad) and stained with Coomassie blue. The key to venoms used is given in the respective figure legends.

2.4. Immunoblotting

After electrophoresis the proteins were transferred to nitrocellulose using a Mini Trans-Blot cell (BioRad) and transfer confirmed by reversible staining with Ponceau S. The filters were blocked with 5% non-fat milk (Carnation, UK) overnight at 4 °C, washed with TBST (0.01 M Tris, pH 8.5, 0.15 M NaCl and 0.1% Tween 20) and incubated in antivenom or murine JD9 antibody (diluted 1:10,000 and 1:2000 in 5% milk, respectively) for 3 h at room temperature. The filters were washed in several changes of TBST and incubated with the appropriate horseradish peroxidase-conjugated goat anti-horse or anti-mouse IgG, (H&L, 1:2000, Nordica) for 2 h at room temperature. After washing off unbound secondary antibody with TBST, the immunoblots were visualised with substrate buffer (50 mg 3,3-diaminobenzidine, 100 ml PBS, 20 µl hydrogen peroxidase).

2.5. Amino acid sequence analysis

The amino acid sequences of the following molecules were selected on the basis of their similarity (BLAST search) to the JD9 (Bj-JD9) domain of Jararhagin (X68251; Paine et al., 1992) and the representation of the venom in the immunoblot of Fig. 1, and aligned to JD9 using CLUSTAL W program (Thompson et al., 1994) with PAM 250 residue weight matrix, (DNASTAR, Madison, USA): jararhagin C (Bj-Jc, JC2245, Usami et al., 1994) and bothrospasin (Bj-bsp, AAC61986, Assakura et al., direct submission) from *Bothrops jararaca* venom; catrocollastatin (Ca-st, AAC59672, Zhou et al., 1995) from *Crotalus atrox* venom; a metalloproteinase (Ac-d, AAC18911, Selistre de Araujo and Ownby, 1995) from *Agkistrodon contortrix laticinctus* venom; cobra (Nn-cob, AAF00693, Bambaï et al., direct submission) from *Naja naja* venom; ecarin (Epl-ec, A55796, Nishida et al., 1995) and ECH1 (Epl-E1, S48160, Paine et al., 1994) from *Echis pyramidum leakeyi* venom; cotiarin (Bc-co, P31988,) from *Bothrops cotiara* venom; cerebrin (Cv-cer, P31985) from *Crotalus viridis cerberus* venom (Scarborough et al., 1993); bitistatin (Bita-st, P17497, Dennis et al., 1990) from *Bitis arietans* venom; batroxostatin (Ba-st, P18618, Rucinski et al., 1990) from *B. atrox* venom; flavoridin (Tf-fl, P18619, Musial et al., 1990) and flavostatin (Tf-st, P80949, Muruyama et al., 1997) from *Protobothrops flavoviridis* (formerly known as *Trimeresurus flavoviridis*) venom; viridin (Cvv-vr, P31987, Scarborough et al., 1993) from *Crotalus v. viridis* venom; trigramin beta (Tg-trgB, P17495, Dennis

et al., 1990) from *Trimeresurus gramineus* venom; disintegrin EC3B (Ec-EC3B, P81631, Marcinkiewicz et al., 1999) and echistatin (Ec-st, P17347, Gan et al., 1988) from *Echis carinatus* venom. Most of the above amino acid sequences were also subjected to the Jameson-Wolfe antigenic index algorithm (Lasergene software, DNASTAR, Madison, USA) to determine whether the most antigenic domains predicted for each sequence bore any relationship to the intensity of reactivity of the anti-JD9 antibody to whole venom from which the toxin sequence was derived.

3. Results and discussion

The phylogenetic limits to the cross-reactivity of the JD9 antibody was examined by probing an immunoblot of venoms from crotaline snakes of the American (*B. atrox*, *C. atrox*, *C. viridis helleri*, *A. contortrix*) and Asian continents (*P. flavoviridis*) and viperine snakes from Africa (*B. arietans*, *E. ocellatus*, *E. pyramidum leakeyi*, *C. c. cerastes*) and Asia (*D. russelii*) that cause similar haemorrhagic and coagulopathic pathology as *B. jararaca* venom (Fig. 1). Venoms from elapid snakes (*N. naja*, *N. nigricollis*) that typically do not cause haemorrhagic pathology were included to assess the specificity of the antibody.

The reactivity of the JD9 antibody to *B. jararaca* venom (Fig. 1) was restricted to 50–60 kDa component(s), as observed previously (Harrison et al., 2000). Jararhagin has a molecular weight of 52 kDa (Paine et al., 1992; Kamiguti et al., 1991). The JD9-specific antibody reacted strongly with 50–60 kDa proteins in most of the American pit vipers with close phylogenetic links to *B. jararaca* (*B. atrox*, *C. atrox* and *C. v. helleri*). We previously demonstrated that the broad band of *B. jararaca* venom proteins reactive with the JD9 antisera could be resolved, by two dimensional gel immunoblotting of venom, into 3–5 immunologically cross-reactive proteins of distinct isoelectric points. This result therefore suggests that venoms of *B. atrox*, *C. atrox* and *C. v. helleri* contain several distinct proteins expressing conserved antigenic epitopes that further extend the potential efficacy of toxin-specific antibodies.

The reactivity of the JD9 antibody to lower molecular weight components in venoms of *B. atrox* and *C. atrox* species (Fig. 1) probably reflects autolysis or degradation of the nascent 50–60 kDa zinc metalloproteinases into non-enzymatic, functional disintegrins that migrate to approximately 25–30 kDa (e.g. catrocollastatin C which is a derivative of catrocollastatin, a metalloprotease from venom of *C. atrox*; Shimokawa et al., 1997). The lack of reactivity of the JD9 antibody to the jararhagin C molecule (Usami et al., 1994) in *B. jararaca* venom is probably

because the venom sample used here had not been autolysed or degraded.

The JD9 antibody reacted with a decreased intensity to the 50–60 kDa proteins in venoms of the more phylogenetically distant vipers of Asia (*P. flavoviridis* and *D. russelii*) and Africa (*E. ocellatus*, *E. p. leakeyi*, *C. c. cerastes*) and not at all to *B. arietans* or elapid venoms (Fig. 1). The only American viper against which there was no detectable reaction was that of *A. contortrix*. Since viper-induced haemorrhage is primarily exerted by metalloproteinases (Bjarnason and Fox, 1994), this result suggested that the tertiary configurations of metalloproteinases in the venoms of the Asian and African vipers (and *A. contortrix*) were sufficiently distinct to make them immunologically silent to the JD9-specific antibody. To examine this possibility, the genetic databases were searched for zinc metalloproteinases and disintegrin molecules isolated from each snake species, or from the most closely-related species available, and the amino acid sequences aligned to that of JD9 (Fig. 2). Only sequence data corresponding to the JD9 disintegrin/cysteine-rich (DC) domain of jararhagin, initiated with the leucine (L) residue at position 342 in the mature jararhagin protein (Paine et al., 1992), were examined.

The near identical alignment of jararhagin C to JD9 supports the view that this disintegrin/cysteine-rich molecule is derived from jararhagin (Usami et al., 1994). The equally close sequence similarity of the *B. jararaca* venom component, bothropasin, to JD9 indicates the multimeric nature of these metalloproteinases in viper venoms and accounts for the broad band (50–60 kDa) of antigens reactive with the JD9 sera in *B. jararaca* and related viper venoms. The nearly identical amino acid sequence alignment of catrocollastatin to the JD9 sequence mirrored the equally intense immunological reactivity of the JD9 antisera with *B. jararaca* and *C. atrox* venom components.

The amino acid sequence similarity of the DC domains of the African and Asian vipers and *A. contortrix* and disintegrin molecules from American, Asian and African vipers to JD9 was less close and appeared to be restricted to short stretches of sequence containing a high proportion of cysteine residues. To determine whether these conserved domains could account for the relatively modest immunological cross-reactivity of the JD9-specific antisera with the African and Asian vipers (Fig. 1), selected sequences from Fig. 2 were compared using the Jameson-Wolfe antigenic index algorithm (Fig. 3). The latter predicts the antigenic potential of a peptide based upon the hydrophilicity, probability of surface location and charge of adjacent residues (DNASTAR, Madison, USA). The amino terminus of the peptide plots of the botroxostatin and viridin disintegrins were aligned to the 18th residue of the metalloproteinases peptide plot as specified by the clustal alignment shown in Fig. 2.

Unsurprisingly, the antigenic profiles of JD9 and the DC domain of catrocollastatin were nearly identical. The disintegrins, botroxostatin (Ba-st) and viridin (Cvv-vr) and

the DC domains of ecarin (Epl-ec) and ECH1 (Epl-E1) were revealed to have very similar antigenic profiles that matched the antigenic profile of JD9 at residues approximately clustered around positions cysteine, C24-aspartic acid, D36 (line (i)), leucine, L42-glutamine, Q47 (line (ii)) and phenylalanine, F59-glycine, G63 (line (iii)). Similarly, the antigenic domain around positions F133-lysine, K138 (line (iv)) of JD9 appeared to be conserved in Epl-E1, Ac-d and Nn-cob. Without wishing to overstate the biological importance of these analyses of predicted amino acid sequences, they do illustrate the correlation between sequence similarity and the degree of immunological cross-reactivity of toxin-specific antibodies to analogous venom components from snakes of distinct species and genera. However, the association between the predicted conserved antigenic domains and immunoblot cross-reactivity is not infallible. Thus, while the venoms of *A. contortrix* and *N. naja* were not reactive with the JD9 antisera, metalloproteinases from these species shared antigenic domains (defined by line (i) and line (v) (residues L193-asparagine, N206)) with analogous molecules in venoms that were reactive with JD9 antisera.

Having demonstrated the phylogenetic association, and limits, of the polyspecific reactivity of toxin-specific antibody, it was then important to assess whether such antibodies exhibited comprehensive intraspecific reactivity. The protein composition of snake venoms extracted from geographically isolated individuals of a single species often exhibits considerable intraspecific variation (Chippaux et al., 1991; Fox et al., 2002). Snakes of the *B. atrox* complex are the most widely distributed and medically important *Bothrops* species in Central and South America. Species limits within the complex remain poorly understood (Wüster et al., 1997; Wüster et al., 1999). In Brazil, effective treatment of envenoming by any *Bothrops* species, including *B. atrox*, (Theakston and Warrell, 1991) is achieved by administration of polyspecific *Bothrops* antivenoms (e.g. Sôro Antibotrópico, Instituto Butantan, São Paulo and FUNED antivenom, Belo Horizonte, Brazil; Camey et al., 2002) prepared, in the case of Sôro Antibotrópico, by immunising horses with increasing doses of venoms from *B. jararaca* (50% composition), *B. jararacussu* (12.5%), *B. moojeni* (12.5%), *B. neuwiedi* (12.5%), *B. alternatus* (12.5%), (Sano Martins, Instituto Butantan, personal communication). The absence of *B. atrox* venoms in the preparation of this antivenom suggests that its efficacy against this dangerous species complex results from extensive cross-species conservation of antigenic epitopes.

To assess the *B. atrox* venom-neutralising potential of the JD9 antibody, *B. atrox* complex venoms extracted from several individual specimens in distinct geographic areas in South and Central America were fractionated by SDS-PAGE and stained with Coomassie blue or blotted onto incubated with the Sôro Antibotrópico antivenom (1:10, 000) or JD9 antibody (1:2000) (Fig. 4). It was evident that

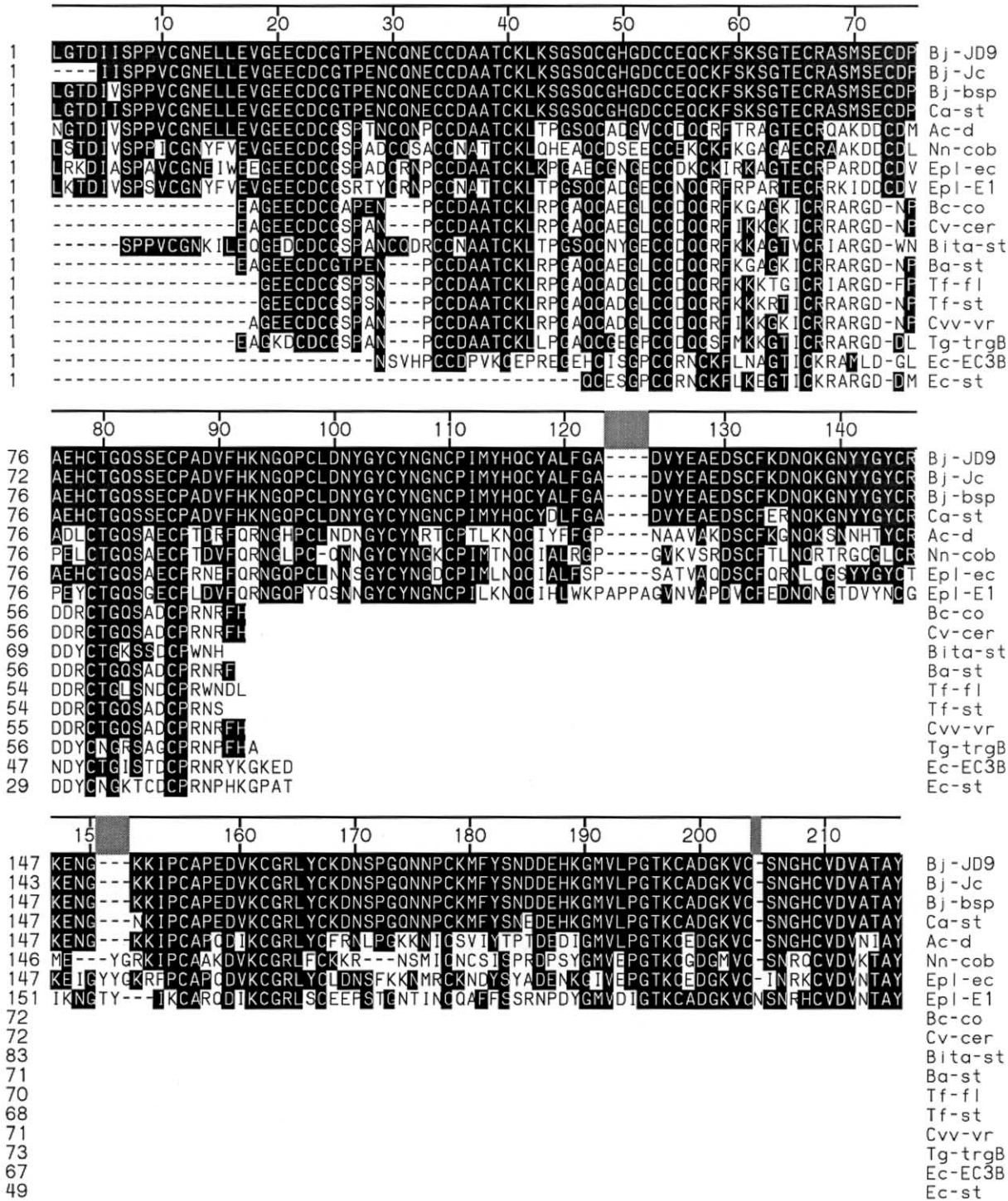


Fig. 2. Amino acid sequence identity between JD9 and analogous venom molecules from snakes of distinct species and genera. The following sequences were aligned to the JD9 sequence (Bj-JD9): jararhagin C (Bj-Jc) and bothrospasin (Bj-bsp) from *B. jararaca* venom; catrocollastatin (Ca-st) from *C. atrox* venom; a metalloproteinase (Ac-d) from *A. contortrix laticinctus* venom; cobrin (Nn-cob) from *N. naja* venom; ecarin (Epl-ec) and ECH1 (Epl-E1) from *E. p. leakeyi* venom; cotiarin (Bc-co) from *B. cotiara* venom; cerebrin (Cv-cer) from *C. v. cerberus* venom; bitistatin (Bit-a-st) from *B. arietans* venom; batroxostatin (Ba-st) from *B. atrox* venom; flavoridin (Tf-fl) and flavostatin (Tf-st) from *P. flavoviridis* venom; viridin (Cvv-vr) from *C. v. viridis* venom; trigramin beta (Tg-trgB) from *T. gramineus* venom; disintegrin EC3B (Ec-EC3B) and echistatin (Ec-st) from *E. carinatus* venom. Residues shaded in black are identical to the JD9 amino acid sequence.

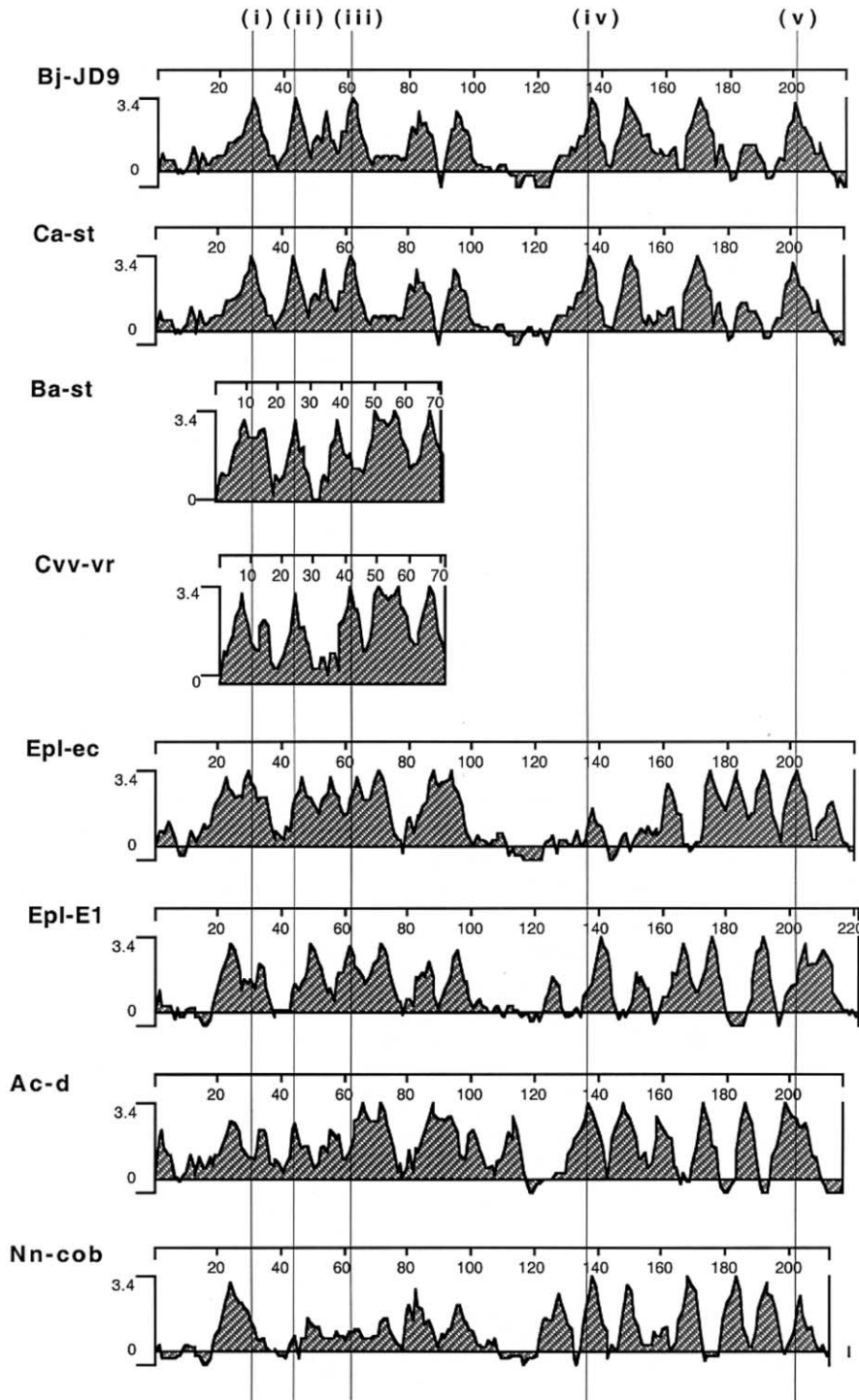


Fig. 3. Alignment of the predicted antigenic domains of the JD9 sequence and analogous venom molecules from snakes of distinct species and genera. The predicted antigenic domains (Jameson-Wolfe algorithm (DNASTAR)) of the peptide sequence of JD9 (Bj-JD9) from *B. jararaca*; catrocollastatin (Ca-st) from *C. atrox*; batroxostatin (Ba-st) from *B. atrox*; viridin (Cvv-vr) from *C. v. viridis*; ecarin (Epl-ec) and ECH1 (Epl-E1) from *E. p. leakeyi*, a metalloproteinase (Ac-d) from *A. contortrix laticinctus* and cobrin (Nn-cob) from *N. naja* were vertically aligned according to their sequence identity to JD9 (Fig. 2). The numerical amino acid sequences are shown above each peptide. The vertical lines (i–v) indicate conserved domains in venom molecules from snakes of distinct species or genera as discussed in the text.

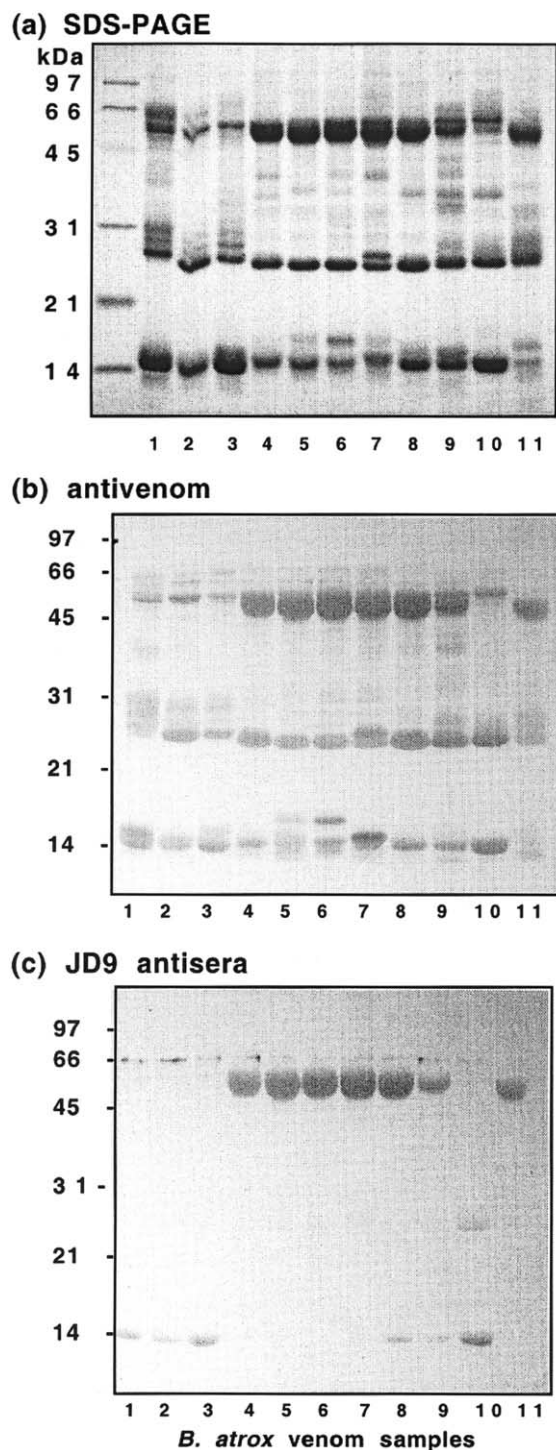


Fig. 4. Immunological reactivity of the JD9 antibody and *Bothrops* polyspecific antivenom with venoms from snakes of the *B. atrox* complex distributed throughout Central and South America. 12.5 μ g samples of pooled venoms extracted from individual *B. atrox* complex snakes from (1) Belize (*B. asper*), (2) Bahia, NE Brazil, (3) Rinópolis, São Paulo state, Brazil, (4) Serra da Mesa, northern Goiás, Central Brazil, (5) Suriname, (6) São Bento,

despite the proteomic variation of the *B. atrox* complex venoms (Fig. 4a) the antivenom reacted with all components in all the venoms (Fig. 4b), emphasising the value of this antivenom. The polyspecific nature of the JD9 antibody was clearly demonstrated from its reactivity with the 50–60 kDa bands of venoms from *B. atrox* complex snakes originating from central Brazil (lane 4), Suriname (lane 5), Maranhão State and the mouth of the Amazon, NE Brazil (lanes 6, 7, respectively), Amazonian Ecuador (lane 8), Venezuela (lane 9) and *B. asper* venom from SW Ecuador (lane 11). However, the non-reactivity of JD9 antisera to molecules of slightly higher molecular weight in venoms from *B. asper* in Belize (lane 1) and *B. atrox* complex species from NE Brazil and N Venezuela (lanes 2, 3 and 10, respectively) showed that the JD9 antiserum was not as wide in its cover as the antivenom. The *B. atrox* complex and *B. asper* snakes containing the 50–60 kDa venom components that were reactive and were not reactive to JD9 could not be grouped by geographic, mitochondrial DNA or snake-age criteria (unpublished). While it would be important to the toxin-specific antivenom hypothesis if the 60 kDa molecules in venoms in lanes 1–3 and 10 were haemorrhagic metalloproteinases, the identity and function of the latter molecules falls beyond the scope of this study. The relatively incomplete reactivity of the JD9 antibody to venom components of the *B. atrox* complex suggests that a toxin-specific approach to antivenom production requires a greater knowledge of the target venoms than is necessary for conventional antivenom development.

This study illustrates that while the development of toxin-specific antivenom will not be as facile as conventional antivenom, it is not scientifically unrealistic. Recently it was demonstrated experimentally that specific antibody neutralisation of a haemorrhagic metalloproteinase of *B. jararaca* venom reduced the severity of coagulopathy; inhibition of one arm of venom-induced pathology may have unforeseen clinical benefits (Anai et al., 2002). An earlier report from our group described a monoclonal antibody (SV-1/F10) that possessed potent haemorrhage-neutralising activity against *E. ocellatus* venoms from Nigeria and Ghana (Iddon et al., 1988). Other reports of monoclonal antibodies raised against viper venoms (Perez et al., 1984; Pukrittayakamee et al., 1983) and elapid neurotoxins (Boulain et al., 1982; Menez, 1985; Menez et al., 1986) also indicated the venom-neutralising potential of toxin-specific antibodies. We have previously shown that DNA immunisation is a potent means of generating toxin-specific, venom-neutra-

Maranhão state, Brazil, (7) Belém, Amazon delta, Brazil, (8) Morona Santiago and Pastaza provinces in Amazonian Ecuador, (9) Guanare, Portuguesa state, Venezuela (10) Falcón, N. Venezuela and (11) SW Ecuador (*B. asper*) were fractionated by SDS-PAGE and stained with Coomassie blue (a) or transferred to nitrocellulose filters that were incubated with (b) Soro Antibiotrópico antivenom (diluted 1:10,000) or (c) antisera from mice immunised with JD9 DNA (diluted 1:2000).

lising antibody (Harrison et al., 2000) and that DNA immunisation protocols exist to significantly increase antivenom titres (Harrison et al., 2002). The present report describes the exciting possibility that the increasing availability of venom toxin sequence data can in the future be exploited to design DNA constructs to generate antibodies specific to toxin domains that are conserved across phylogenetic boundaries.

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