Coagulant effects of black snake (*Pseudechis* spp.) venoms and *in vitro* efficacy of commercial antivenom

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ABSTRACT

The coagulant effects of Australasian black snakes (*Pseudechis* spp.) are poorly understood and differ to the procoagulant venoms of most dangerous snakes in Australia. This study aimed to investigate *in vitro* coagulant effects of *Pseudechis* venoms and the efficacy of commercial black snake antivenom (BSAV), tiger snake antivenom (TSAV) and specific rabbit anti-snake IgG to neutralise these effects. Using a turbidimetric assay, all six *Pseudechis* venoms had anticoagulant activity, as well as phospholipase A₂ (PLA₂) activity. Inhibition of PLA₂ activity removed anticoagulant effects of the venoms. *Pseudechis porphyriacus* was unique and had procoagulant activity independent of PLA₂ activity. Both BSAV and TSAV completely inhibited the coagulant and PLA₂ activity of all *Pseudechis* venoms. PLA₂ activity was also inhibited completely by p-Bromophenacyl bromide (pBPPB) and partially by specific anti-*N. scutatus* IgG antibodies. Anti-*N. scutatus* IgG also completely inhibited anticoagulant activity of *Pseudechis* venom. All *Pseudechis* venoms showed immunological cross reactivity with specific anti-snake IgG antibodies to *P. porphyriacus*, *Pseudechis australis* and *Notechis scutatus*. *Pseudechis* venoms have *in vitro* anticoagulant activity that appears to be attributable to PLA₂ activity. Both antivenoms inhibited anticoagulant and PLA₂ activity at concentrations below those occurring in patients treated with one vial of antivenom. There was cross-neutralisation of *Pseudechis* venoms and *N. scutatus* antibodies that might be attributable to immunological similarities between the venoms.

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1. Introduction

The *Pseudechis* genus (black snakes) is one of five groups of clinically important venomous snakes in Australia (White, 1998). *Pseudechis* spp. are widespread across Australia. The two most common and clinically significant black snakes, *Pseudechis porphyriacus* (Red-bellied black snake) and *Pseudechis australis* (Mulga snake) are found

along the East Coast of Australia and throughout mainland Australia respectively (Cogger, 2000). Other members of the genus include *Pseudechis butleri* (Butler's snake), *Pseudechis colletti* (Collett's snake), *Pseudechis guttatus* (Spotted or blue-bellied black snake) and *Pseudechis papuanus* (Papuan black snake) which are less widespread in Australasia and cause far fewer bites (Isbister et al., 2006; Jansen et al., 2007).

The majority of medically important venomous Australian snakes cause a venom induced consumptive coagulopathy (VICC) that results in clotting factor deficiencies and the risk of bleeding (Isbister, 2006; Isbister et al., 2010a, b). This is not the case with *Pseudechis* spp.

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which cause a reversible anticoagulant coagulopathy (Campbell, 1984; Isbister et al., 2006; Churchman et al., 2010). However, despite extensive investigation of procoagulant toxins in Australasian elapids (Isbister, 2009) there has been limited study of the anticoagulant toxins (Madaras et al., 1983). Anticoagulant toxins have been identified in other snake venoms and many of these have been identified as phospholipase A₂ (PLA₂) enzymes (Kini, 2006). PLA₂ enzymes are present in many animals including humans and serve many biological functions. Snake venoms PLA₂ have a toxic activity (e.g. myotoxic, anticoagulant, neurotoxic) in addition to their enzymatic activity and these functions may or may not be independent (Lomonte et al., 2003; Kini, 2006). PLA₂ toxins including myotoxins, platelet inhibitors and anticoagulant toxins have been identified in some *Pseudechis* spp. but a comprehensive study of anticoagulant activity of *Pseudechis* venoms and whether this is associated with PLA₂ activity has not been undertaken (Kamiguti et al., 1994; Laing et al., 1995; Ramasamy et al., 2004; Kini, 2006).

The current clinical knowledge regarding envenoming by black snakes comes mainly from case reports and anecdotes. Effects of envenoming include systemic symptoms, myotoxicity, anticoagulant coagulopathy, and localised effects at the bite site which rarely result in necrosis (Vines, 1978; Campbell, 1984; Laloo et al., 1994; Pearn et al., 2000; Currie, 2004; Isbister et al., 2006; Jansen et al., 2007; Churchman et al., 2010). Myotoxicity appears to be the most important clinical effect and may result in acute renal failure in some cases. The presence and importance of an anticoagulant coagulopathy is more contentious with mixed reports of its presence. From a recent large series of *Pseudechis porphyriacus* envenoming anticoagulant coagulopathy appears to be common, occurring in 61% of patients, and was characterised by a raised activated partial thromboplastin time (aPTT). This anticoagulant coagulopathy was rapidly reversed with administration of one vial of antivenom (Churchman et al., 2010). It is therefore important to characterise the coagulant effects of *Pseudechis* venoms, both anticoagulant and procoagulant, including the efficacy of antivenoms in neutralising these coagulant effects.

This study aimed to investigate the anticoagulant and procoagulant effects of *Pseudechis* venoms for a range of venom concentrations including those found in patients with envenoming. In addition it investigated the effects of black snake antivenom (BSAV) and tiger snake antivenom (TSAV) on the anticoagulant effects.

2. Materials and methods

2.1. Venom and antivenom preparation

P. porphyriacus, *P. australis*, *Notechis scutatus* (Tiger snake) and *Oxyuranus scutellatus* (Taipan) venoms were obtained from Venom Supplies. *P. butleri*, *P. colletti*, *P. guttatus* and *P. papuanus* venoms were gifted by Monash Venom Group. Venom solutions were made to 1.0 mg/ml in 50% glycerol and stored at –20 °C until required.

CSL tiger snake antivenom (TSAV; batch 10402, expiry October 2008) and black snake antivenom (BSAV; batch

07301; expiry July 2007) were obtained from John Hunter Hospital pharmacy. CSL antivenoms are horse derived F(ab')₂ immunoglobulins raised against each of the five clinically important terrestrial snake groups in Australia – *Pseudonaja* (Brown snake), *Notechis* (Tiger snake), *Oxyuranus* (Taipan), *Pseudechis* (Black snake) and *Acanthophis* (Death Adder). One unit (1U) of antivenom is defined as the amount that binds or neutralises 0.01 mg of venom from the respective snake species. Expired CSL antivenoms have been shown to remain active past their stated expiry dates and are suitable for use in *in vitro* experiments (O'Leary et al., 2009).

2.2. Chemicals and other reagents

Fresh frozen plasma was purchased from the Australian Red Cross and aliquots of 10 ml were thawed at 37 °C. Calcium (50 µL 0.4 M CaCl₂) was added immediately prior to the assay. Tris Buffered Saline (TBS) is tris(hydroxymethyl)methylamine 25 mM, NaCl (137 mM), KCl (3.4 mM) adjusted to pH 7.4 with HCl. Innovin® (recombinant tissue factor) was obtained from Dade. Solutions (76 mg/ml) were prepared according to the manufacturer's instructions, then diluted to 10% in TBS.

Anti-*P. australis* venom IgY was purchased from GenWay Biotech Inc (San Diego USA). Rabbit anti-*N. scutatus* venom IgG and rabbit anti-*P. porphyriacus* venom IgG were purchased from the Western Australian Institute of Medical Research and their preparation has been previously described in detail (Isbister et al., 2010a, b). For ELISA experiments, Greiner high-binding 96 well plates were used (#655061). Carbonate buffer used for coating is 50 mM, pH 9.5. Antibodies were biotinylated using the Pierce EZ-Link Sulfo-NHS-LC-Biotin reagent. Washing solution is 0.02% Tween 20 in phosphate buffered saline (PBS). Blocking solution is 0.5% Bovine Serum Albumin in PBS. Streptavidin-horseradish peroxidase (HRP) was purchased from Millipore/Chemicon and tetramethylbenzidine (TMB) from Sigma. p-Bromophenacyl bromide (pBPB) was obtained from Sigma. All other chemicals used were of analytical grade.

2.3. Clotting studies

Clotting studies were performed using a turbidimetric assay described elsewhere (O'Leary and Isbister, 2010). In brief, procoagulant activities were tested by adding 1:1 serial dilutions of *Pseudechis* venom (starting at 1 µg) in 100 µL TBS to wells of a 96-well microtitre plate. Clotting was triggered by adding 100 µL of recalcified fresh frozen plasma, incubated at 37 °C. Wells were mixed for 5 s and optical density at 340 nm at 37 °C was recorded immediately and at 30 s intervals in a BioTek ELx808 plate reader. Clotting time was calculated based on previous definitions as the lag time before a 0.02 increase in optical density (OD) at 340 nm from the mean of the first two values (O'Leary and Isbister, 2010). Procoagulant activity of *Pseudechis* venoms was determined as a shortening of the clotting time compared to no venom addition.

Anticoagulant activity was measured in a similar way but recombinant tissue factor (Innovin; including synthetic

Fig. 1. Procoagulant effects of *Pseudechis* venoms. Clotting times for increasing concentrations of venom in the absence of another triggering agent showing the absence of procoagulant activity for *P. australis* venom (A) and the procoagulant activity of *P. porphyriacus* venom (B). Other *Pseudechis* venoms behaved as for *P. australis* venom, showing no procoagulant activity (data not shown).

phospholipids and calcium) was pre-mixed with *Pseudechis* venoms and used to trigger clot formation, which occurred in less than 30 s with Innovin alone. When venom was added an increase in the clotting time was then used as a measure of anticoagulant activity. This was done by adding 1:1 dilutions of *Pseudechis* venoms (starting at 50 ng or 5 ng) and 380 µg of Innovin in 100 µl TBS to wells of 96-well microtitre plate. Anticoagulant activity was defined as the venom concentration that caused a doubling of the Innovin induced clotting time from 22 to 44 s. Procoagulant studies for *P. porphyriacus* and all anticoagulant clotting studies were done in triplicate and the mean value and standard error of the mean used.

For antivenom neutralisation studies 25 ng of *Pseudechis* venom, 380 µg Innovin and serial doubling dilutions of BISAV (starting at 45mU) or TSAV (starting at 75mU) in 150 µl TBS were added to wells of a 96-well microtitre plate. Venom or antivenom concentrations were recorded as ng/ml of plasma or mU/ml of plasma respectively. The minimum inhibitory concentration of each antivenom was defined as the concentration required to completely inhibit anticoagulant activity (normalise clotting time back to less than 30 s).

Neutralisation studies with monovalent rabbit anti-snake IgG were carried out as above, beginning the 1:1 serial dilutions of either anti-*P. porphyriacus* or anti-*N. scutatus* IgG at 40 µg/well, and with *P. australis* or *P. porphyriacus* venoms at 5 ng/ml.

2.4. Enzyme immunoassays

A sandwich enzyme immunoassay was used to quantify known concentrations of each *Pseudechis* venom using antibodies to either *P. porphyriacus*, *P. australis* or *N. scutatus* venoms to detect the venoms to measure cross-reactivity. For example, a range of concentrations of *P. australis* venom was put on a plate coated with anti-*P. porphyriacus* antibodies and then detected with anti-*P. porphyriacus* antibodies. Three 96-well microtitre plates were coated with 100 µl/well of 3.75 µg/ml rabbit anti-*P. porphyriacus* IgG, 2.1 µg/ml rabbit anti-*N. scutatus* IgG or

975 ng/ml anti-*P. australis* IgY in carbonate buffer for 1 h at room temperature and kept at 4 °C overnight. Plates were washed once and 300 µl of blocking solution was added to each well. Plates were washed again and 100 µl venom dilutions in triplicate were added as follows and left for 1 h at room temperature: 1:1 dilutions in blocking solution of standards (*P. porphyriacus*, *N. scutatus* and *P. australis* venoms for each of the three plates respectively) from 2.5 ng/ml to 0.039 ng/ml; and 1.25, 0.63 and 0.31 ng/ml of all other venoms. Plates were washed three times and 100 µl biotinylated anti-*P. porphyriacus* rabbit IgG, anti-*N. scutatus* rabbit IgG or anti-*P. australis* IgY were added to the respective plates at an optimum titrated concentration. Plates were left for 1 h at room temperature, washed four times and 100 µl/well of streptavidin-HRP (1.5 µg/ml) was added. Plates were washed four times and 100 µl/well of TMB was added. Plates were left for up to 10 min for a colour change to appear before adding 50 µl 1 M H₂SO₄. Plates were incubated for 1 h at room temperature before each wash stage and were read 20 min after acid addition at 450 nm in a BioTek ELx808 plate reader.

The percent cross-reactivity was calculated as the ratio of the measured concentration for the *Pseudechis* venom to the measured concentration for the venom corresponding to the antibodies on the plate (i.e. *P. porphyriacus*, *P. australis* or *N. scutatus*).

2.5. PLA₂ assays

PLA₂ activities of *Pseudechis* venoms were measured using the PLA₂ assay kit (Cayman # 765001). Venoms were diluted to 100 ng/ml in assay buffer. Venom (1 ng) +/- 4.5mU BISAV, 0.75mU TSAV or 2 µg anti-*N. scutatus* IgG in 10 µl of assay buffer were added to separate wells of a 96-well microtitre plate. Bee venom PLA₂ (5–10 ng) or no venom in 10 µl of assay buffer was added to separate wells as a positive control and background activity measurement respectively. CSL antivenom mixtures and anti-NsV were incubated with venoms at room temperature for 15 or 30 min respectively. DTNB (10 µl) and assay buffer (5 µl) were added to each well. Plates and diheptanoyl thio-PC

Fig. 2. Anticoagulant effects of *Pseudechis* venoms. Clotting times for increasing concentrations of venom in the presence of Innovin for *P. porphyriacus*, *P. australis*, *P. butleri*, *P. colletti*, *P. guttatus* and *P. papuanus*.

(substrate) were incubated separately at 37 °C before being mixed for 2 s. Samples were read immediately and at 30 s intervals for 10 min at 405 nm in a BioTek ELx808 plate reader. PLA₂ activity is equivalent to the change in concentration of product which in turn is proportional to change in OD at 405 nm. PLA₂ activity was therefore expressed as nmol of product/ml/min/ng of venom.

2.6. Treatment of venoms with pBPB

Para-bromophenacyl bromide (pBPB) is a phospholipase inhibitor, which acts by blocking a histidine residue at the active site (Volwerk et al., 1974). pBPB was dissolved in

ethanol and a portion added to water to give a concentration of 50 µg/ml in 0.1% ethanol. 100 µl of this (5 µg) was added to venom (25 µg) in PBS (400 µl) and the solutions allowed to stand for 2 h at room temperature. Control solutions were prepared at the same time without pBPB, or without venom. These solutions were tested for PLA₂ and anticoagulant activity.

In an alternative procedure, a solution of pBPB in ethanol was prepared (5 mg/ml) and 20 µl of this was added to solutions of control *P. porphyriacus* venom (200 µg) in TBS (2 ml). After 20 h at room temperature, excess pBPB was removed by dialysis (90 min in 200 ml TBS). Control venom solutions were prepared by the same

Fig. 3. Plots of Innovin triggered clotting times for all six *Pseudechis* venoms (250 ng/ml) versus increasing antivenom concentration when either BISAV (A) or TSAV (B) is premixed with the venom. The clotting time is returned to normal (<30 s) at higher antivenom concentrations.

procedure, without the addition of pBPB. These solutions were compared for coagulant and anticoagulant activity over a range of concentrations and PLA₂ activity.

2.7. Data analysis

Standard curves for ELISAs were calculated by non-linear regression using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Other venoms were compared to the standard to give a percentage equivalent concentration and therefore percentage immunological cross-reactivity.

Table 1

Minimum inhibitory concentration (MIC) of BISAV and TSAV and equivalent percentage of one vial of antivenom required to achieve this concentration in a human patient. The proportions (%) of one vial are based on 18000U of BISAV and 3000U of TSAV being administered intravenously into a plasma volume of 3000 ml giving 6U/ml and 1U/ml respectively.

Venom	BISAV		TSAV	
	MIC (mU/ml) ^a	Equivalent % of 1 vial	MIC (mU/ml) ^a	Equivalent % of 1 vial
<i>P. porphyriacus</i>	113	2	94	10
<i>P. australis</i>	56	1	47	5
<i>P. butleri</i>	113	2	94	10
<i>P. colletti</i>	113	2	94	10
<i>P. guttatus</i>	113	2	47	5
<i>P. papuanus</i>	113	2	94	10

^a These antivenom concentrations correspond to concentrations observed in envenomed patients.

3. Results

3.1. Effects of black snake venoms on coagulation

P. porphyriacus had procoagulant activity for venom concentrations greater than 100 ng/ml. All other *Pseudechis* venoms showed no procoagulant activity even for venom concentrations of up to 10 µg/ml (Fig. 1). All *Pseudechis* venoms had anticoagulant activity which was concentration dependent and had a sigmoidal response with a saturation effect at higher concentrations for all *Pseudechis* venoms except *P. porphyriacus* venom (Fig. 2). The anticoagulant activities of *P. porphyriacus*, *P. australis*, *P. butleri*, *P. colletti*, *P. guttatus* and *P. papuanus* were 62.5, 12.5, 3.13, 6.25, 25 and 6.25 ng/mL of plasma, respectively. *P. porphyriacus* venom had a non-linear increase in clotting time until a peak anticoagulant effect at about 125 ng/ml of plasma. Above 125 ng/ml the clotting time decreased consistent with procoagulant activity in a concentration dependent manner.

3.2. Effect of antivenom and rabbit IgG on venom activity

Both BISAV and TSAV were able to completely inhibit the anticoagulant activity of 250 ng/ml of all black snake venoms (Fig. 3). The minimum inhibitory concentration of the two antivenoms for each venom was less than observed antivenom concentrations in patients and equivalent to less than 2% of a vial of BISAV and less than 10% of a vial of TSAV (Table 1). TSAV was able to neutralise the procoagulant effects of *P. porphyriacus* at a lower concentration of 2000mU/ml compared to BISAV which required greater than 8000mU/ml. Rabbit anti-*P. porphyriacus* IgG and rabbit anti-*N. scutatus* IgG inhibited the anticoagulant activity of *P. porphyriacus* venom in a dose dependent manner at similar concentrations of IgG. Rabbit anti-*N. scutatus* IgG also inhibited the anticoagulant activity of *P. australis* venom.

3.3. Immunological cross-reactivity

All *Pseudechis* venoms cross-reacted with antibodies raised against *P. porphyriacus*, *P. australis* and *N. scutatus* to varying degrees (Table 2). *P. australis* and *P. porphyriacus* are the least cross-reactive venoms within the *Pseudechis* genera with other venoms showing intermediate cross reactivity. In comparison to *P. australis* venoms, *P. colletti*, *P. guttatus* and *P. papuanus* have very high immunological cross reactivity where as *P. butleri* and *P. porphyriacus* have decreasing cross reactivity to *P. australis* antibodies. All the *Pseudechis* venoms were between 9% and 20% cross reactive with antibodies raised against *N. scutatus*.

3.4. Phospholipase activity

All *Pseudechis* venoms had similar PLA₂ activity (nmol of product/ml/min/ng) with values of 9.3, *P. australis* 18, *P. butleri*, 9.5, *P. colletti*, 11.7, *P. guttatus*, 13.4, *P. papuanus* and 15.5, *P. porphyriacus*. When venoms were preincubated with antivenoms, both BISAV and TSAV reduced PLA₂ activity of the venoms to below 4% of that of venom alone (Fig. 4). However, anti-*N. scutatus* venom IgG only partially

Table 2

Immunological cross reactivity of all six *Pseudechis* venoms, *N. scutatus* and *O. scutellatus* venoms with antibodies to *P. porphyriacus*, *P. australis* and *N. scutatus*.

Venom	Mean % cross reactivity		
	<i>P. porphyriacus</i>	<i>P. australis</i>	<i>N. scutatus</i>
<i>P. australis</i>	18	100	20
<i>P. butleri</i>	21	46	13
<i>P. colletti</i>	56	95	18
<i>P. guttatus</i>	51	100	9
<i>P. papuanus</i>	33	100	14
<i>P. porphyriacus</i>	100	16	17
<i>N. scutatus</i>	9	0	98
<i>O. scutellatus</i>	0	0	7

neutralised PLA₂ activity with between 23 and 38% remaining compared to venom alone (Fig. 4). PLA₂ activity of *N. scutatus* venom was completely neutralised by anti-*N. scutatus* venom IgG.

3.5. Effect of pBPB treatment on phospholipase and coagulant activity

pBPB treatment completely inhibited PLA₂ activity of all *Pseudechis* venoms and also neutralised their anticoagulant activity (Fig. 5; data not shown). However, the procoagulant activity of pBPB-treated *P. porphyriacus* venom was unaffected by pBPB treatment (Fig. 5).

4. Discussion

This study demonstrates that all *Pseudechis* venoms have *in vitro* anticoagulant effects which could be

neutralised with antivenom concentrations easily achieved by the administration of less than one vial of BISAV or TSAV. In addition, the coagulant effects of *P. porphyriacus* were unique and showed both anticoagulant activity at lower concentrations typically seen in human envenoming and procoagulant activity for higher concentrations (Churchman et al., 2010). This explains some of the contradictory findings from previous *in vitro* studies (Chester and Crawford, 1982; Marshall and Herrmann, 1983; Tan and Ponnudurai, 1990) and clinical series of *P. porphyriacus* envenoming (Pearn et al., 2000). It was previously believed that *P. porphyriacus* venom only had procoagulant effects and caused only a mild silent coagulopathy (Chester and Crawford, 1982; Marshall and Herrmann, 1983; Tan and Ponnudurai, 1990; Pearn et al., 2000). The current study showed that procoagulant effects occur at venom concentrations in excess of those measured in human envenoming cases and this explains the absence of VICC seen clinically (Churchman et al., 2010). The fall in anticoagulant activity after the peak effect seen in Fig. 2 is explained by the procoagulant activity of the venom overcoming the anticoagulant effects at these venom concentrations. Further work is required to clarify this interaction. This dual procoagulant-anticoagulant effect has been demonstrated for *Calloselasma rhodostoma* and *Agkistrodon contortrix* venoms before but no explanation for its existence has been offered (Dambisya et al., 1994).

An anticoagulant coagulopathy has now been reported in envenoming by *P. porphyriacus* (Churchman et al., 2010), *P. australis* (Campbell, 1984; Currie, 2004), *P. colletti* (Isbister et al., 2006) and *P. papuanus* (Lalloo et al., 1994), but was not evident in *P. guttatus* (Jansen et al., 2007) envenoming. However, the small number of cases does not rule out the

Fig. 4. The effect of BISAV (4.5mU), TSAV (0.75mU), rabbit anti-*N. scutatus* venom IgG antibodies (aNsV IgG; 2 µg) and p-BPB on the phospholipase activity of all six *Pseudechis* venoms. Results were not obtained for p-BPB treated *N. scutatus* venom, p-BPB treated bee venom PLA₂ and *N. scutatus* venom premixed with antivenom.

Fig. 5. The effect of pBPB treatment on the procoagulant (A) and anticoagulant (B) activity of *P. porphyriacus* venom.

possibility of a coagulopathy in the latter and there is potential for problems with standard laboratory assays detecting the anticoagulant coagulopathy which is likely the reason that it was not identified in *P. porphyriacus* envenoming earlier (Churchman et al., 2010).

The anticoagulant effects of all *Pseudechis* venoms at 250 ng/ml were completely neutralised by both BISAV and TSAV used at concentrations equivalent to far less than those likely to occur in patients administered one vial of antivenom (Table 2). Both antivenoms also neutralised PLA₂ activity of all six *Pseudechis* venoms and have been previously shown to prevent but not reverse myotoxic effects of *Pseudechis* venoms (Ramasamy et al., 2004). Because myotoxicity and the anticoagulant coagulopathy are the major effects of *Pseudechis* envenoming it appears that both antivenoms are effective at neutralising the clinically relevant toxicity. Furthermore *Pseudechis* venom concentrations of 250 ng/ml rarely occur in human envenoming with median concentrations for *P. porphyriacus* and *P. papuanus* envenoming of 19 ng/ml (Churchman et al., 2010) and 39 ng/ml (Lalloo et al., 1994) respectively. Therefore one vial of antivenom is likely to be sufficient in human envenoming. This is supported by a recent study of

P. porphyriacus envenoming where one vial of TSAV bound all venom *in vivo* (Churchman et al., 2010).

PLA₂ activity has been previously demonstrated in *Pseudechis* venoms (Ramasamy et al., 2004). The elimination of both anticoagulant and PLA₂ activity of *Pseudechis* venoms by pBPB suggests that the anticoagulant toxin is a PLA₂ toxin which is consistent with other anticoagulant toxins (Kini, 2006). It is also likely that all *Pseudechis* venoms contain similar anticoagulant PLA₂ toxins as PLA₂ activity was inhibited by both CSL antivenoms but further work is required to isolate the individual toxins and demonstrate this. The procoagulant activity in *P. porphyriacus* venom is likely to be due to a previously identified toxin that is similar but smaller than the prothrombin activators in other Australasian elapids (St Pierre et al., 2005). The procoagulant effect was only neutralised by antivenom and not pBPB treatment which is consistent with it being a separate toxin that is not a PLA₂. Further work is required to confirm that the procoagulant activity is not due to a PLA₂ toxin.

Neutralisation of *Pseudechis* venoms by TSAV could be due to the current production methods of the commercial antivenom which mean that monovalent antivenoms are actually polyvalent so TSAV would contain antibodies raised against *Pseudechis* venoms (O'Leary and Isbister, 2009). However, the current study also demonstrated immunological and functional cross-reactivity between antibodies raised against *N. scutatus* and *Pseudechis* venoms. Rabbit anti-*N. scutatus* IgG was able to partially inhibit PLA₂ activity of all *Pseudechis* venoms and completely inhibit anticoagulant activity of *P. australis* and *P. porphyriacus* venoms. This demonstrates a similarity between PLA₂ enzymes with different toxicological properties from more distantly related elapid species since *N. scutatus* does not cause an anticoagulant coagulopathy. Immunological and functional cross-reactivity between components of venoms from different species and respective monovalent antibodies has been previously demonstrated for *Pseudonaja textilis*, *O. scutellatus* and *N. scutatus* venoms and may become important for future antivenom design (O'Leary et al., 2007; Isbister et al., 2010). This study provides further support for the development of toxin specific antivenom rather than species specific antivenom.

The effect of antivenom on the procoagulant effect of *P. porphyriacus* was unusual in that larger concentrations of BISAV were required to neutralise these effects than TSAV compared to similar concentrations for anticoagulant effects (Table 1). This may be because BISAV is raised against *P. australis* venom which does not contain a procoagulant toxin where as TSAV is raised against *N. scutatus* which contains the prothrombin activator Notecarin which has similar homology to the procoagulant toxin identified in *P. porphyriacus* (St Pierre et al. 2005).

Pseudechis venoms all show anticoagulant activity at concentrations similar to those seen in human envenoming cases. This effect is important in human envenoming after *Pseudechis* bites since anticoagulant coagulopathy occurs early in envenoming and appears to be rapidly reversed by antivenom (White, 2001; Isbister et al., 2006; Churchman et al., 2010). It therefore could be used as both an early indicator of envenoming and a clinical indicator of

antivenom response. We have previously shown this for *P. porphyriacus* venom in patients but further work is required to demonstrate this for the other *Pseudechis* species (Churchman et al., 2010). Further to this, the anti-coagulant toxin appears to be a PLA₂ enzyme that is inhibited by both BISAV and TSAV as well as anti-*N. scutatus* IgG, therefore demonstrating cross-reactivity between antibodies raised against venoms of unrelated snake species (Wagstaff et al., 2006; Wagstaff et al., 2009; Isbister et al., 2010).

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Ethical statement

No ethics approval was required.

Conflict of interest

None.

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