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The molecular basis of cross-reactivity in the Australian Snake Venom Detection Kit (SVDK) ☆

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Abstract

The Snake Venom Detection Kit (SVDK) is of major medical importance in Australia, yet it has never been rigorously characterised in terms of its sensitivity and specificity, especially when it comes to reports of false-negative and false-positive results. This study investigates reactions and cross-reactions of five venoms the SVDK is directed against and a number of purified toxins. Snakes showing the closest evolutionary relationships demonstrated the lowest level of cross-reactivity between groups. This was, instead, far more evident between snakes that are extraordinarily evolutionary separated. These snakes: *Pseudechis australis*, *Acanthophis antarcticus* and *Notechis scutatus*, in fact displayed more false-positive results. Examination of individual toxin groups showed that phospholipase A_2s (PLA_2s) tends to react strongly and display considerable cross-reactivity across groups while the three-finger toxins (3FTx) reacted poorly in all but the *Acanthophis* well. The hook effect was evident for all venoms, particularly *Oxyuranus scutellatus*. The results of this study show considerable variation in toxin detection, with implications in further development of venom detection, both in Australia and other countries.

Keywords: Antivenom; Antibody; ELISA; Venom; Protein

1. Introduction

Snakes have long been a topic of both fascination and medical importance to the community. In Australia, it is uniquely difficult to differentiate between snakes of medical importance due to their similar appearance, colouration and behaviour

☆ *Ethical statement*: This work is not under consideration with any other journal.

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(Cogger, 2000) and clinical effect (Table 1). However, despite these similarities, the snakes of Australia have highly diverse venoms (Fry, 1999).

Venom diversity is a result of a phenomenon known as punctuated evolution, whereby a key adaptation or event leads to an immense expansion of a species (Gould and Eldredge, 1977). In Australia, this took place when the Asian elapids invaded Australia, finding it inhabited only by very primitive pythons with very limited habitats. This left room for both snakes and the composition of their venoms to explode outward, leading to radical shifts in the constitution of the venoms between groups. The major molecular diversity occurs in three main medically important toxin types: three finger toxins (3FTx), phospholipase A_2s (PLA_2s) and prothrombin-activating enzymes (PTA).

Due to the physical similarities between many of the snakes that belong to different immunotypes, a diagnostic method of determining the most appropriate antivenom for treatment in a clinical setting was required. A number of different techniques had been investigated (Muelling et al., 1957; Trethewie and Rawlinson, 1967; Boche and Russell, 1968; Coulter et al., 1974, 1978; Sutherland et al., 1975) before Theakston et al. (1977) adapted the micro-ELISA (enzyme-linked immunosorbent assay) for use in snake venom detection. In its current format, the Snake Venom Detection Kit (SVDK) (CSL Australia, Parkville, Australia) utilises a rapid, freeze-dried, sandwich enzyme immunoassay in which there are eight wells. These include a blank, and a positive and negative control, with the remaining five wells containing pairs of antibodies, each raised against a different snake immunotype and matching one of the five available monovalent antivenoms. One of these antibodies is known as the primary antibody and is attached to the plastic surface of the well, the other is modified by the attachment of peroxidase enzyme and is left free within the well, and is referred to as the conjugate. Upon addition of substrate, the development of a blue colour indicates the presence of a particular venom immunotype and therefore the most appropriate antivenom for treatment. However, in some cases more than one positive well is observed, due to natural cross-reactions between venom (SVDK Technical Information Booklet, 2005) (Table 2).

A degree of cross-reactivity in the SVDK is actually advantageous as it allows the kit to detect venoms that it is not directly targeted against but from related species. However, this can be a problem if the levels of cross-reactivity become high between different groups of venoms, as it can lead to

Table 1
Bioactivities of Australian elapid venoms

Snake group	Presynaptic neurotoxicity	Postsynaptic neurotoxicity	Procoagulant	Anticoagulant	Myotoxic
Acanthophis		X		X	
Notechis	X	X	X		X
Oxyuranus	X	X	X		X
Pseudechis		X		X	X
Pseudonaja	X	X	X		
Sea Snake		X			X

Table 2
Monovalent antivenoms and the snake type neutralised

Type of antivenom	Raised against		Venoms neutralised
	Scientific name	Common name	
Tiger	*Notechis scutatus*	Mainland tiger snake	All species of *Austrelaps*, *Hoplocephalus*, *Notechis*, and *Tropidechis* as well as all *Pseudechis guttatus and Pseudechis porphyriacus*
Brown	*Pseudonaja textilis*	Eastern brown snake	All *Pseudonaja* species
Black	*Pseudechis australis*	Mulga snake	*Pseudechis* species, *Micropechis ikaheka* (New Guinea small-eyed snake)
Death adder	*Acanthophis antarcticus*	Common death adder	All *Acanthophis* species, *Echiopsis curta* (Bardick snake)
Taipan	*Oxyuranus scutellatus*	Coastal Taipan	All *Oxyuranus* species

confusion as to the appropriate choice of antivenom with potentially fatal outcomes (Sutherland and Leonard, 1995). While this situation has been anecdotally described (Winkel et al., 2001; Williams and White, 1990), the molecular basis behind the cross-reactivity has not been investigated.

A second potential limitation of the SVDK is the existence of a phenomenon known as the hook effect (Rodbard, 1988). This is where, at higher concentrations, the binding antibodies of the kit are saturated to the point where a sandwich cannot be formed and the labelled conjugate is, therefore, removed in the wash step. This leads to little, if any, colour observed and as such a negative result may be falsely concluded. While it is anecdotally described (SVDK Technical Information Booklet, 2005), the hook effect has not yet been rigorously characterized.

The aims of this study were to test

- the five primary crude venoms (those that represent the five monovalent antivenoms) to investigate the potential occurrence of false results and
- the differential behaviour of purified representatives of the three major toxin types.

2. Materials and methods

2.1. Venom collection

The following venoms were purchased as a freeze-dried product from Venom Supplies Pty. Ltd., Tanunda, South Australia: *Acanthophis antarcticus* from South Australia; *Notechis scutatus* from Victoria; *Oxyuranus scutellatus* from Queensland; *Pseudechis australis* from Eyre Peninsula; and *Pseudonaja textilis* from South Australia.

2.2. Preparative reverse phase-high-performance liquid chromatography (RP-HPLC)

RP-HPLC was used to purify individual toxins in order to determine their behaviour relative to crude venom. Preparative RP-HPLC was performed using an AKTABasic HPLC (Amersham Biosciences, USA) equipped with a multi-wavelength tuneable UV detector and a Frac 950 fraction collector. Samples (up to 5 mL) were loaded onto a Brownlee Aquapore 300 Octadecyl silica (C18) semi-preparative RP-HPLC cartridge column (Perkin-Elmer) with 20 μm nominal particle size, 300 Å pore size, 10 cm × 10 mm i.d. (internal diameter) at a 2.5 mL/min flow rate.

A linear gradient (0–70% organic modifier, 1%/min) from buffer A (0.1% trifluoroacetic acid (TFA) (v/v) in water) to buffer B (70% acetonitrile (v/v), 0.09% TFA (v/v) in water) was used to elute bound material. Fractions of 3 mL volume were collected.

2.3. Mass spectrometry

Mass spectrometry was used to determine the molecular composition and purity of RP-HPLC peaks. Mass spectrometry was performed using the Agilent 1100 series LC/MSD trap (Agilent Technologies, USA). Samples of interest were introduced into the Agilent 1100 with a syringe at a rate of 2 μL/min.

2.4. Sequencing

Amino acid sequencing was used to confirm the identity of pure fractions. Isolated fractions were N-terminally sequenced using Edman degradation chemistry on an Applied Biosystems 477A Protein Sequencer.

2.5. SDS-PAGE

SDS-PAGE was used to confirm the molecular weight of large, hydrophobic fractions putatively containing prothrombin activators that do not resolve well by mass spectrometry. Toxin was loaded onto a 12% SDS-PAGE, in reducing conditions. Prior to loading, samples were incubated in 2.3% sample buffer, containing 0.1% mercaptoethanol, for 5 min at 100 °C.

2.6. Activated partial thromboplastin time (APTT)

As they were N-terminally blocked, the identity of prothrombin-activating toxins was rather confirmed by APTT assays. APTT assay was performed using the STA- APTT assay kit, in combination with the fully automated coagulation analyser STA, both manufactured by Diagnostica STAGO, France. The snake venom and toxin (100 μL) were each added to 2 mL of adult pooled plasma samples (individual plasma samples obtained from healthy adults). The APTT assay was initiated within 1 min following the addition of venom, toxin, bovine Factor Xa or saline to the plasma. Results are

expressed in seconds and represent the time required for clot formation.

2.7. Snake venom detection kit method

Tenfold dilutions of dried venom or purified toxin were made in the Yellow Sample Diluent (YSD) of the SVDKS (provided by CSL Australia) to give final concentrations ranging from 100 pg/mL to 10 mg/mL. A volume of 100 μL of each sample in YSD was added to each of the wells of a SVDK test strip. Strips were mixed by gentle agitation and incubated for 10 min at room temperature. Strips were then washed with eight cycles of pyrogen-free water with an automated washer (WellWash 4; Denley Instruments, Sussex, England). A volume of 50 μL of SVDK chromogen (tetramethylbenzidine) and 50 μL of SVDK peroxide were added to each well. Strips were again mixed and incubated at room temperature for 10 min after which 50 μL of 0.5 M H_2SO_4 stop solution was added to all wells and mixed by gentle agitation. Optical densities were read within 5 min at 450 nm with a reference wavelength of 560 nm (Wallac1420 Victor Multilabel counter, Perkin-Elmer, USA). The antibodies used in the SVDK are made in rabbits by hypersensitisation with venoms shown to be representative of the five defined venom immunotypes that match the five available monovalent antivenoms. The intention is to create antibodies that detect venom immunotypes rather than species-specific venoms. The rabbit antisera are purified using fast protein liquid chromatography and are not further purified using affinity chromatography as this is likely to result in species-specific antibodies that are too specific for the clinical purpose. Further information on the SVDK formulation is unavailable due to commercial confidentiality.

2.8. SVDK result interpretation

For a test to be valid it must have a negative control well optical density (OD) of less than 0.125 and a positive control well OD of greater than 1. Test wells with OD greater than 0.15 are considered positive by the manufacturer based on in-house testing.

2.9. Statistics

All statistics were performed using Prism 4.0 (GraphPad Software, USA). Data are presented as mean ± standard error of the mean (SEM), with n indicating the number of experiments. Curves were analysed by use of a two-way analysis of variance (ANOVA). In all cases a P value of <0.05 was taken as significant (Fig. 1).

3. Results

3.1. Purification of toxins

RP-HPLC of *A. antarcticus* and *N. scutatus* venom each yielded 19 peaks. *P. australis* venom provided nine peaks. Masses corresponding to 3FTx (~6–8 kDa) and PLA_2 (~12–14 kDa) toxins were searched for amongst these peaks using electrospray mass spectrometry, with only fractions containing a single component chosen for SVDK testing (e.g. Fig. 2 showing *Acanthophis* toxins).

3.2. Determination of toxin identity

Amino-terminal sequencing of the toxins was achieved by Edman degradation, resulting in the identification of the first 15 residues (Fig. 3). Protein identity was determined through BLAST searching for sequence homology (http://au.expasy.org/tools/blast). Two toxins could not be identified in this manner: a 23 kDa toxin from *A. antarcticus* and a putative prothrombin activator from *N. scutatus*. The *A. antarcticus* 23 kDa toxin was N-terminally blocked so its identity could not, at this time, be confirmed but based on molecular weight and retention time is most likely to be a CRISP toxin. The *N. scutatus* PTA is both a large glycoprotein and therefore unable to be accurately sized by mass spectrometry and was also N-terminally blocked. Its identity was confirmed by initially running on a 12% SDS gel for separation based on size, as these toxins are well characterised as being approximately 50 kDa (e.g. Tans et al., 1985; Swiss-Prot-P81428, P83370), and then testing in plasma (Table 3) for the well-documented prothrombin activation activity.

3.3. SVDK analysis

The results observed with the primary venoms in the SVDK varied considerably, with *P. textilis* showing no cross-reactivity (Fig. 4a) and *O. scutellatus* (Fig. 4b) showing negligible amounts. The whole venoms that showed the most considerable interactions between each other were *P. australis* (Fig. 5a) and *N. scutatus* (Fig. 6a), with a lesser, but

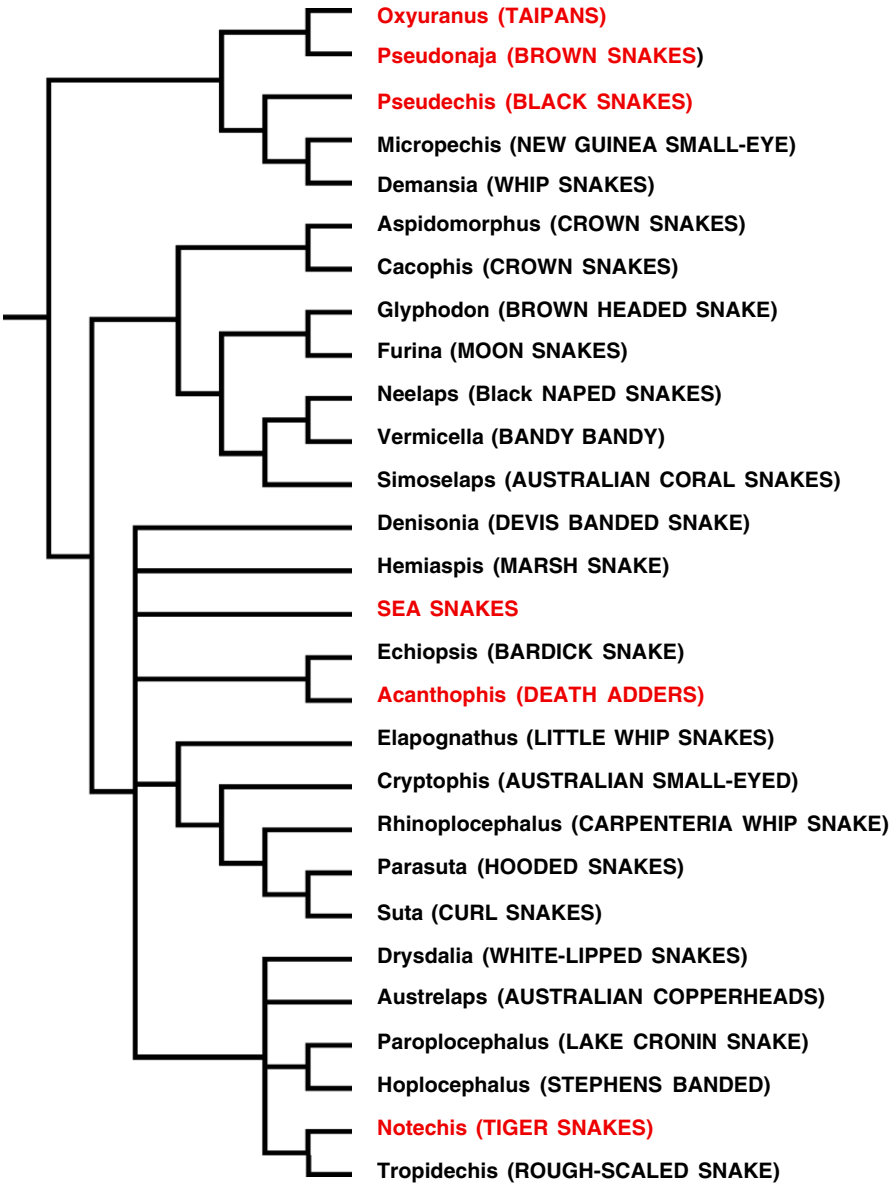

Fig. 1. Taxonomical relationships of Australian elapid snakes. Genera highlighted in red are those for which monovalent antivenoms are made.

still substantial degree also observed with *A. antarcticus* (Fig. 7a).

The SVDK was shown to be very sensitive to all five of these venoms. It was able to detect as little as 1 ng/mL of the venoms of each of *N. scutatus* and *P. textilis* and just 10 ng/mL of *O. scutellatus* and *P. australis*. The least sensitive was *A. antarcticus* venom, which was not detected until 100 ng/mL was added. In terms of the hook effect, *N. scutatus* was detectable over the widest range of concentrations (a 10-million-fold increase in concentration), followed by *P. textilis*, *P. australis* and *A. antarcticus*, with *O. scutellatus* working over the narrowest range, which is still a 10,000-fold span.

The individual toxin types reacted differently for different venoms. The *P. australis* PLA_2 reacted strongly in the *Pseudechis* well, mimicking the crude reaction ($P = 0.1163$), and it also displayed pronounced cross-reactivity with both the *Notechis* and *Acanthophis* wells (Fig. 5b) while the *P. australis* 3FTx showed no reaction in any well at any of the concentrations tested (Fig. 5c).

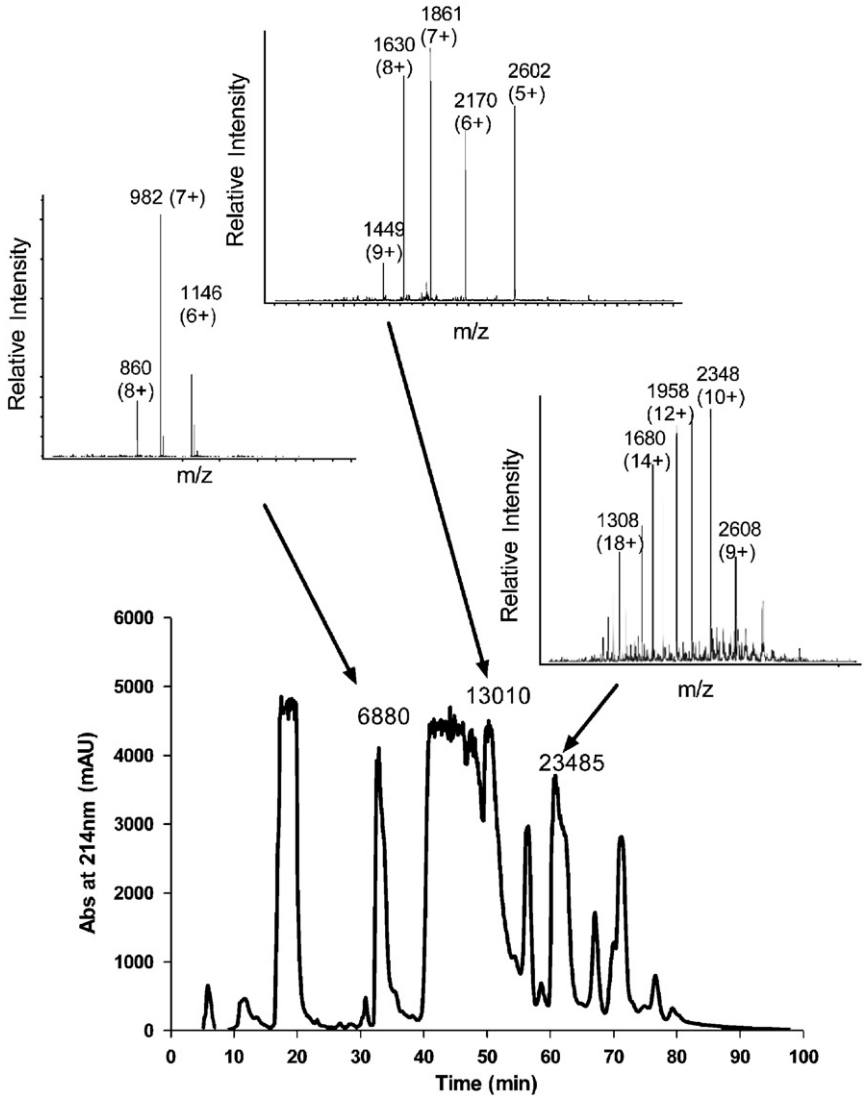

Fig. 2. HPLC trace of *Acanthophis antarcticus* venom; mass spectrometry results are shown above fractions used in this study.

A. antarcticus had the strongest response to its PLA_2 (Fig. 7b), with a robust signal also present with the *A. antarcticus* 3FTx (Fig. 7c). Both of these toxins showed only negligible levels of cross-reactivity. The 23 kDa toxin also reacted strongly as an *Acanthophis*, showing some cross-reactivity in the form of an *Pseudechis* positive at higher concentrations (Fig. 7d).

The *N. scutatus* PLA_2 showed almost equal levels of *Notechis* and *Acanthophis* positives, with a lesser degree of *Pseudechis* cross-reaction (Fig. 6b), while the 3FTx reacted in the SVDK with a strong positive in the *Acanthophis* well and only weak *Notechis* and *Pseudechis* reactions (Fig. 6c) The *N. seutatus* PTA reacted in the *Notechis* well with similar strength to its PLA_2, also showing a strong *Pseudechis* positive and weak *Acanthophis* positive. A summary of these results is presented in Table 4.

All purified toxin curves (other than that of the *P. australis* PLA_2) gave P values of <0.05 and so can be thought to be different from each of their respective crude venom curves.

4. Discussion

The SVDK has been in use in its current format since 1988 (Cox, 1988). Despite this, no comprehensive studies have been undertaken relating to the

a

1. MQCCNQQSSQ PKTTTTCPGG
2. MTCCNQQSSQ PKTTTICAGG
3. MQCCNQQSSQ PKTTT
4. M?CCNQQSSQ PKTTT
5. MTCCNQQSSQ PKTTT

b

1. NLYQFGGMIQ CANKGARSWL
2. NLVQFSYLIQ CANHGRRPTR
3. NLIQFGNMIQ CANKGSRPSL
4. NLIQFRKMIQ QANKG
5. NLTQFGGMIQ CANKG
6. NLHQFHNMIQ CANNR

Fig. 3. N-terminal sequence comparison of isolated toxins with previously reported sequences (Swiss-Prot accession numbers are shown in parentheses). (a) (1) *Acanthophis antarcticus* (P01434); (2) *Pseudechis australis* (P25497); (3) *Acanthophis antarcticus* 6880 Da component; (4) *Notechis scutatus* 6690 Da component; (5) *Pseudechis australis* 6758 Da component. (b) (1) *Acanthophis antarcticus* (P81237); (2) *Notechis scutatus* (P00609); (3) *Pseudechis australis* (P04056); (4) *Acanthophis antarcticus* 13010 Da component; (5) *Notechis scutatus* 13212 Da component; (6) *Pseudechis australis* 13920 Da component.

Table 3
Activated partial thromboplastin time results

	Time (s)	Observation
Plasma	49	No clot
Plasma + *Notechis scutatus* venom	<10	Clot
Plasma + 50 kDa *Notechis scutatus* toxin	<10	Clot
Plasma + FXa	21.6	Clot
Plasma + saline	53.9	No clot

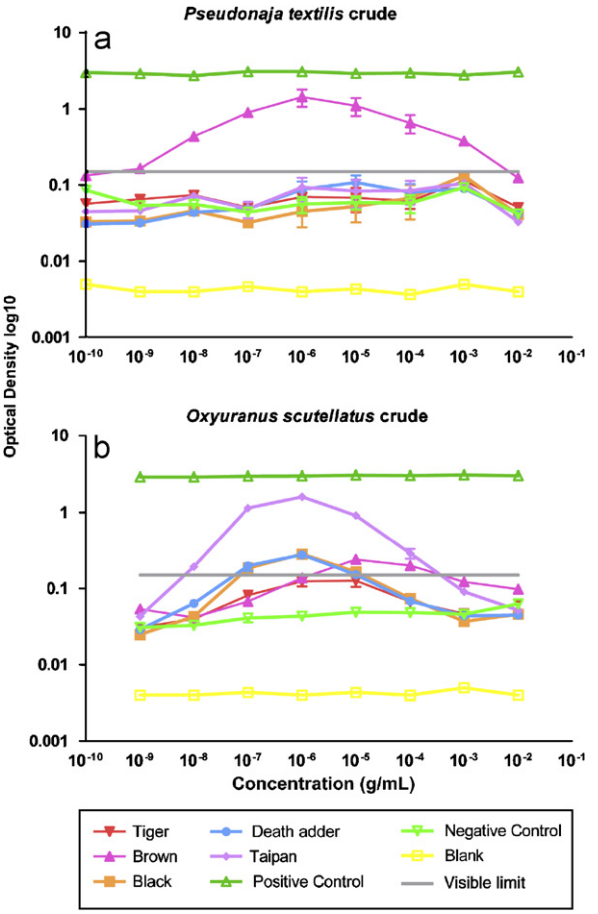

Fig. 4. SVDK results: (a) crude *Pseudonaja textilis* venom, $n = 3$ for 10^{-7}–10^{-4} g/mL; (b) crude *O. scutellatus* venom, $n = 3$ for 10^{-7}–10^{-4} g/mL. Error bars are ±SEM (those not seen are within symbol dimensions).

reaction of venoms within the kit, especially at higher concentrations or the behaviour of individual toxin types. This is true regardless of evidence that there are problems associated with the SVDK, which may lead to clinical confusion, including anecdotal reports of both false-negative (Bruce Wentworth, CSL Australia, personal communication) and false-positive results (Winkel et al., 2001; Williams White, 1990).

Despite the evolutionary closeness of *P. textilis* to *O. scutellatus* and *P. australis* (Fig. 3), only negligible levels of cross-reactivity were observed between these groups. However, the results of this study imply that *O. scutellatus* venom is the most susceptible of the primary venoms to the hook effect, with levels of just 1 mg/mL being undetectable by the SVDK. This is a level that is theoretically very possible to be observed clinically. Measurements of the amount of *O. scutellatus* venom remaining on the skin (the preferred sampling site for SVDK testing) averaged 0.9 mg in mice and was proven to be as high as 4.6 mg (Morrison et al., 1982). One human post-mortem study, using radioimmunoassay methods, has also shown that skin and subcutaneous tissues from an *O. scutellatus* victim contained 2 mg of venom per gram of tissue (Sutherland et al., 1980).

This hook effect is also of some concern with the three venoms, *P. australis* (Fig. 5a), *N. scutatus* (Fig. 6a) and *A. antarcticus* (Fig. 7a). These three venoms, while being extraordinarily evolutionarily distinct (Fig. 1), also show the most extensive cross-reactivity. This decrease in sensitivity occurs at a different rate for each well of the SVDK and so at certain concentrations, it can become impossible to

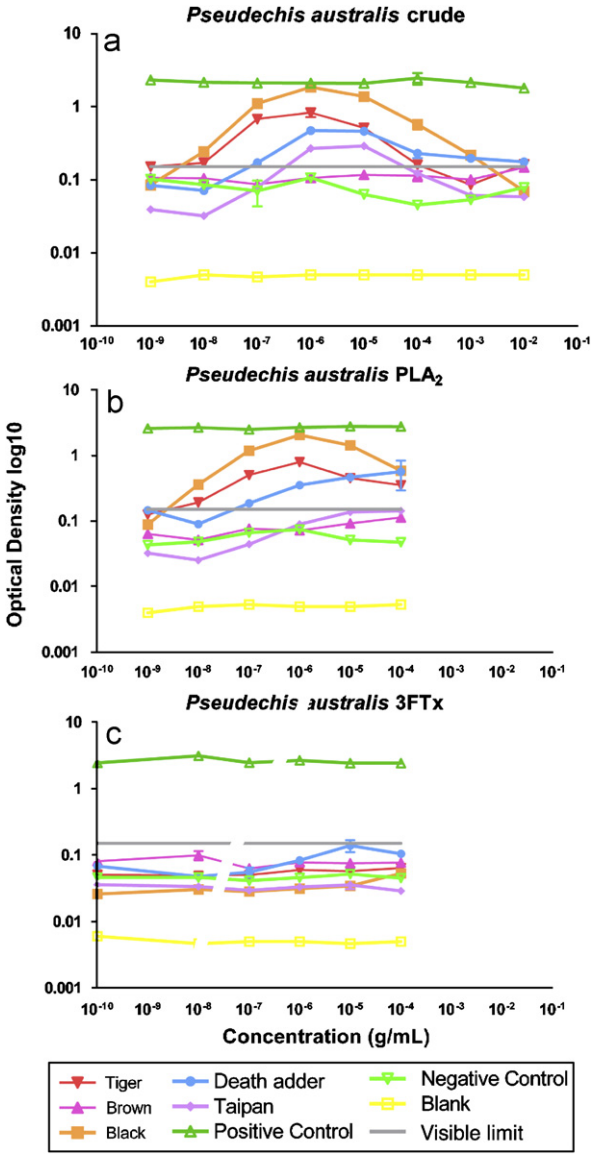

Fig. 5. *Pseudechis australis* SVDK results: (a) crude venom, $n = 3$ for 10^{-7}–10^{-4} g/mL; (b) phospholipase A2, $n = 3$ for 10^{-7}–10^{-4} g/mL; (c) three-finger toxin, $n = 3$ for 10^{-8}–10^{-5} g/mL. Error bars are ±SEM (those not seen are within symbol dimensions).

distinguish between the primary venom and the cross-reaction itself. This can be observed at a concentration of 100 μg/mL of *N. scutatus* venom, where there is an equal intensity of colour in each of the *Notechis*, *Pseudechis* and *Acanthophis* wells.

These results/observations were not anticipated. It seemed probable that the snakes most closely related would be those to show the most interaction between groups. The cause of this cross-reactivity is not, therefore, strictly associated with the taxonomic relationships of the snake groups to one another. As this may be due to a retained biochemical similarity between their venoms, individual toxin types were therefore isolated to test their specific reactions in the SVDK. Venoms chosen were based on relative chemical composition: dominated by 3FTx (*Acanthophis*), dominated by PLA_2 (*Pseudechis*) or not dominated by either (*Notechis*).

The domination of *P. australis* venom by PLA_2s is reflected in the fact that this toxin is so well recognised in the *Pseudechis* well of the SVDK (Fig. 5b). This toxin would most probably also account for the cross-reactivity seen with the crude venom. The PLA_2 from *A. antarcticus* also reacted very strongly in the kit (Fig. 7b), possibly accounting for most of the crude venom's reaction. This is interesting as PLA_2s make up only a very small fraction of the whole venom and have only recently been isolated (van der Weyden et al., 1997; Chow et al., 1998; Sim, 1998). *Notechis* PLA_2 reacted in the *Notechis* well only at higher concentrations (Fig. 6b), giving a high level of *Acanthophis* and a lower level of *Pseudechis* cross-reactivity. This is slightly unusual given the comparative abundances of PLA_2 in these venoms and that they are responsible for a significant percentage of the *Notechis* clinical effects, causing both presynaptic neurotoxicity and myotoxicity (Sutherland and Tibballs, 2001).

The *P. australis* 3FTx, on the other hand, seems immunogenically unreactive, providing no positive result at any of the concentrations tested (Fig. 5c). This could be due to the fact that this toxin is found in such small amounts in the *P. australis* venom that the antibodies raised against it are only present in insignificant amounts compared with those against the PLA_2. However, the lack of cross-reactivity with the *Acanthophis* well is surprising considering the tremendous diversity of 3FTx present (Fry et al., 2002). In contrast, *Acanthophis* venoms are instead overwhelmingly made up of 3FTx (Fry, 1999) and, as such, that is the toxin that would have been expected to show the strongest signal and demonstrate the greatest cross-reactivity. This was not, however, the case. While the *A. antarcticus* 3FTx was detected by the SVDK, it was only over a relatively narrow range and virtually no cross-reactivity was observed (Fig. 7c). These toxins may simply not produce a strong immune response compared with the other toxins, and as such there are relatively few detecting antibodies present in the

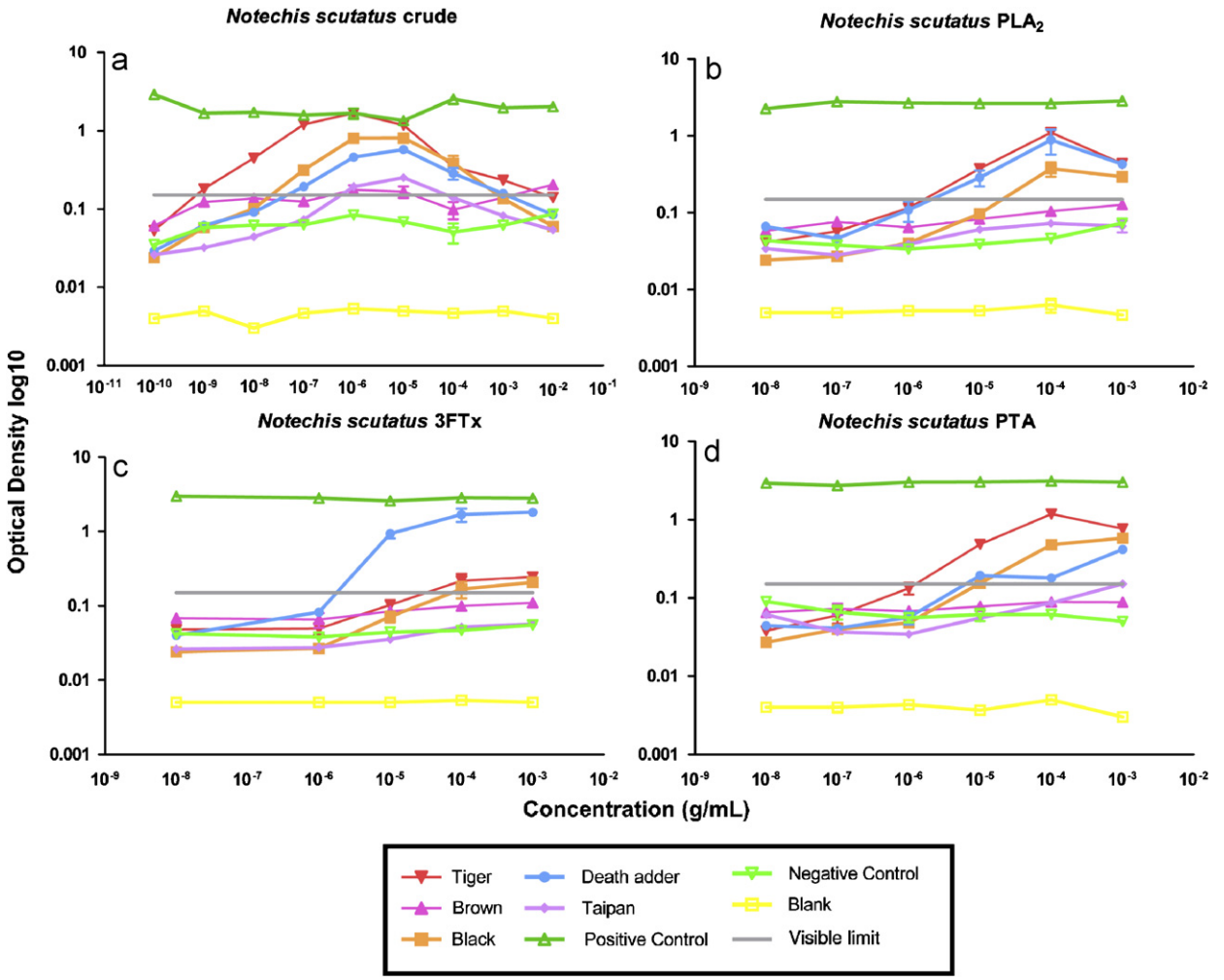

Fig. 6. *Notechis scutatus* SVDK results: (a) crude venom, $n = 3$ for 10^{-7}–10^{-4} g/mL; (b) phospholipase A_2, $n = 3$ for 10^{-6}–10^{-3} g/mL; (c) three-finger toxin, $n = 3$ for 10^{-6}–10^{-3} g/mL; (d) prothrombin activator, $n = 3$ for 10^{-7}–10^{-4} g/mL. Error bars are ±SEM (those not seen are within symbol dimensions).

wells of the other immunotypes. Of further interest is the reaction of the *N. scutatus* 3FTx (Fig. 6c), which is actually detected as *Acanthophis*. This supports the theory that this particular toxin type has its immune response dwarfed by the other components present and is only able to be detected by the *Acanthophis* well due to the fact that these 3FTx make up an overwhelming majority of *Acanthophis* venom.

A third dominant and medically important *Notechis* toxin was chosen for testing: a PTA. This provided a reasonable *Notechis*-positive reaction (Fig. 6d), however again only at higher concentrations, and this did not explain the pattern of reactivity of the crude venom (Fig. 6a). It also gave a moderate reaction in the *Pseudechis* well, accounting for some of the cross-reactivity observed with it. Perhaps the most remarkable reaction seen with this toxin though is the slight positive seen in the *Acanthophis* well. This is interesting as a PTA has never been characterised from an *Acanthophis* venom and this reaction gives a fair indication that there is in fact one present. This is further supported by our find of a toxin whose size is consistent with PTAs of other venoms (e.g. Tans et al., 1985; Swiss-Prot-P81428, P83370).

A 23 kDa toxin was also isolated from *A. antarcticus* venom (Fig. 7d). Its retention time and size are consistent with those of a CRISP toxin (cysteine-rich secretory protein), which are ubiquitous among snake venoms (Fry et al., 2003). CRISP toxins are also known to sometimes be N-terminally

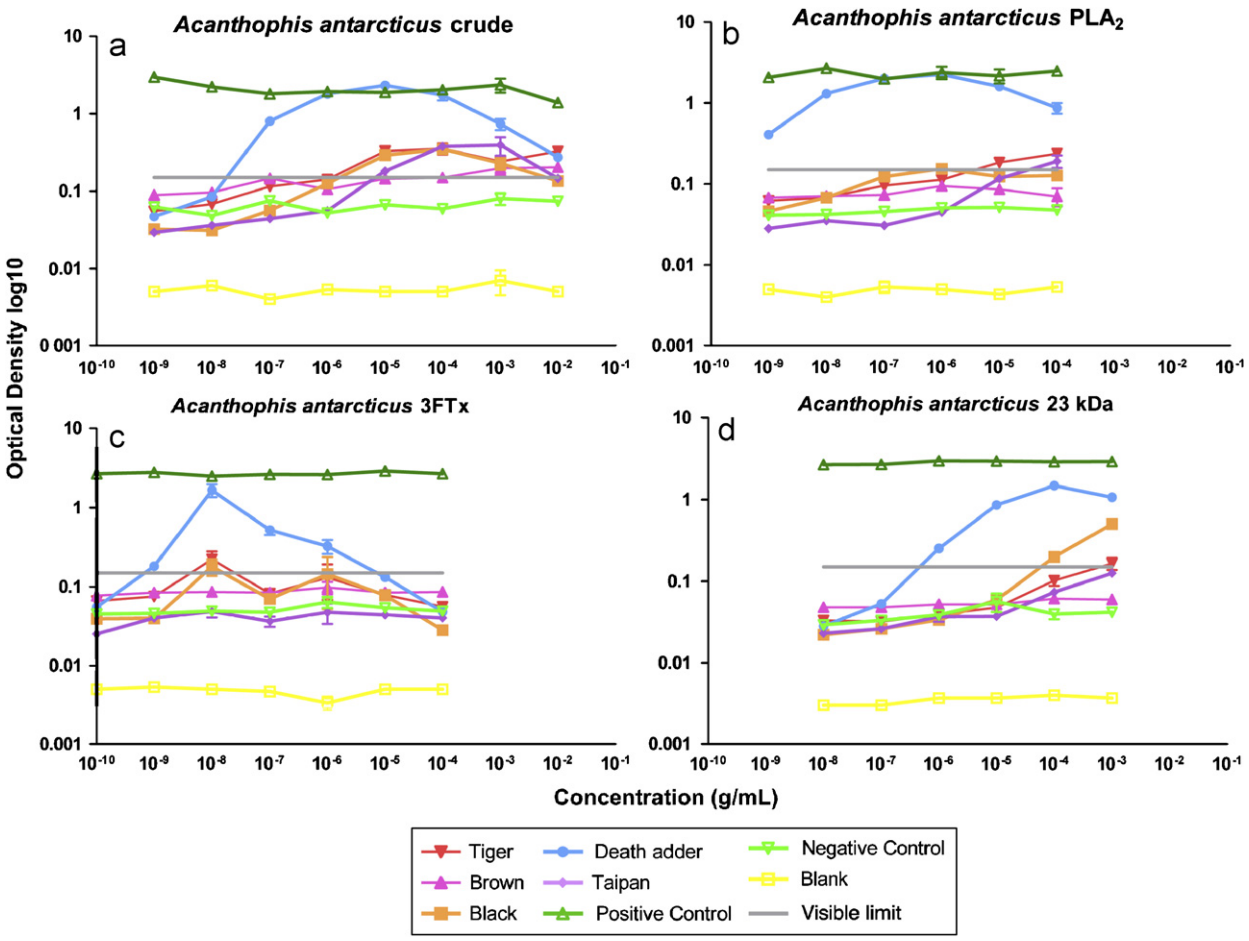

Fig. 7. *Acanthophis antarcticus* SVDK results: (a) crude venom, $n = 3$ for 10^{-6}–10^{-3} g/mL; (b) phospholipase A2, $n = 3$ for 10^{-7}–10^{-4} g/mL, (c) three-finger toxin, $n = 3$ for 10^{-9}–10^{-6} g/mL; (d) 23 kDa toxin, $n = 3$ for 10^{-6}–10^{-3} g/mL. Error bars are ±SEM (those not seen are within symbol dimensions).

blocked (Fry et al., 2002). However, due to this, absolute identity cannot be confirmed by normal Edman degradation sequencing. This toxin required higher concentrations to be detected in the *Acanthophis* well. However, at these higher concentrations it also gave a reasonably strong *Pseudechis* signal, perhaps accounting for the *Pseudechis* cross-reactivity observed with the crude venom as CRISP toxins have been purified and well characterised from *Pseudechis* venoms (Brown et al., 1999).

The hook effect was demonstrated for all toxins tested with the exception of the *Notechis scutatus* PTA, for which the limited amount of toxin purified was not sufficient to observe the loss of sensitivity. The individual toxins were generally detected over a narrower range and with sharper peaks than the crude venoms, as no single toxin was responsible for all of the detecting antibodies in any well, although the *P. australis* PLA_2-detecting antibodies are extremely dominant in the *Pseudechis* well. These results show that developing a kit that detects toxin types rather than whole venom would not be as viable an idea as has been previously thought (Van Dong et al., 2003). This type of a kit would probably still display false positives at higher concentrations (as observed with the *P. australis* PLA_2 and all three of the *N. scutatus* toxins tested), while it may remove the desired cross-reactivity that allows the kit to detect antigenically similar venoms, it is not directly targeted against. This has implications for both Australia and other countries that are now in the process of developing venom detection techniques.

Overall, the SVDK was found to function incredibly well. It demonstrated the ability to detect whole venom over an immense range, on average

Table 4
Summary of SVDK results

Antigen tested	Primary reaction	Range (g/mL)	Peak (μg/mL)	Visible cross-reactions[a]
A. antarcticus crude	Death adder	10^{-7}–>10^{-2}	10	Tiger Black Taipan
A. antarcticus PLA_2	Death adder	<10^{-9}–>10^{-4}	1	Tiger
A. antarcticus 3FTx	Death adder	10^{-9}–10^{-5}	0.01	Tiger Black
A. antarcticus 23kDa toxin	Death adder	10^{-6}–>10^{-3}	100	Black Tiger
N. scutatus crude	Tiger	10^{-9}–10^{-2}	1	Black Death adder Taipan
N. scutatus PLA_2	Tiger	10^{-5}–>10^{-3}	100	Death adder Black
N. scutatus 3FTx	Death adder	10^{-5}–>10^{-3}	? (1 mg/mL)	Tiger Black
N. scutatus PTA	Tiger	10^{-6}–>10^{-3}	100	Black Death adder
O. scutellatus Crude	Taipan	10^{-8}–10^{-4}	1	Black Death adder Brown
P. australis crude	Black	10^{-8}–10^{-3}	1	Tiger Death adder Taipan
P. australis PLA_2	Black	10^{-8}–>10^{-4}	1	Tiger Death adder
P. australis 3FTx	–	–	–	–
P. textiles crude	Brown	10^{-9}–>10^{-3}	1	–

[a]Arranged in descending order of optical density strengths.

some 100,000-fold increase in concentration, with levels of just 1 ng/mL being visibly perceived as positive. It is, however, important to note that while there was little evidence of false positives, given the level of variability seen in the detection of these primary venom's toxin types, they are likely to be much more evident in other venoms that the kit is not directly targeted against. Venoms such as the *Austrelaps superbus* and *Pseudechis colletti* have been, in particular, shown to cross-react strongly in both the *Notechis* and *Pseudechis* wells, with the former requiring treatment with *Notechis* antivenom and the latter with *Pseudechis*. This variability in toxin detection is also very apparent in a clinical case of envenomation by *Suta suta* where a negative reaction was observed in the SVDK. Despite this, treatment with polyvalent antivenom was able to reverse the neurotoxic effects (BGF, personal observations), which were most probably due to abundant 3FTx present in *Suta* venom (Fry et al., 2003), a toxin group demonstrated in this study as being not well detected by the kit.

This study is only the beginning of potential studies into the SVDK. The exact cause of the *Notechis* pattern of reaction has not been elucidated. None of the toxin tested can account for the whole of the crude *Notechis scutatus* curve. It may be that another toxin, which is present in lower amounts but is more immunogenically dominant, is responsible. The complex mixture of antigens in the venom may also lead to a complex mixture of antibodies in the SVDK, and while none of these toxins reacts particularly strongly on their own, when they are all being detected together a synergistic response is observed, providing the crude venom curve. More toxins must be tested before this riddle can be solved, including testing different combinations of pure toxins in order to determine whether they react in a concerted manner.

5. Conclusions

These results show, for the first time, the general robustness of the SVDK for the identification of the

primary venoms. The occurrence of false positives was found to be low and, remarkably, most apparent between greatly divergent venoms. The cross-reactivity observed was therefore due, not to the closeness of groups, but rather to the evolution of their venoms, leading to toxins of similar antigenicity between the different immunotypes.

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