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Detection of venom–antivenom (VAV) immunocomplexes *in vitro* as a measure of antivenom efficacy☆

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ABSTRACT

The measurement of free venom with enzyme immunoassay in serum of patients with snake envenoming is used to confirm snake identification and to determine if sufficient antivenom has been given. Recent studies with Russell's viper (RV; *Daboia russelii*) envenoming have detected free venom post-antivenom despite recovery of coagulopathy. This raises the question as to whether this assay also measures venom–antivenom (VAV) complexes. In this study we developed an assay to measure VAV complexes and investigate the binding of venom and antivenom *in vitro*. The assay consisted of rabbit anti-snake venom IgG attached to a microplate which binds the venom component of VAV and anti-horse IgG antibodies conjugated to horseradish peroxidase to detect the antivenom portion of VAV. A known amount of venom or toxin was incubated with increasing antivenom concentrations and VAV was detected as absorbance at 450 nm and plotted against AV concentration. *Pseudonaja textilis* (brown snake), *Notechis scutatus* (tiger snake), *Oxyuranus scutellatus* (taipan), *Tropidechis carinatus* (rough-scaled snake), *Pseudechis porphyriacus* (red-bellied black snake) and *D. russelii* mixtures with appropriate antivenoms were assayed. Measured VAV initially increased with increasing antivenom concentration until it reached a maximum after which the VAV concentration decreased with further increasing antivenom concentrations. The VAV curves for two Australian snake venom–antivenom mixtures, *Hoplocephalus stephensii* and *Ancanthophis antarcticus*, had broad VAV peaks with two maxima. Two fractions isolated from *N. scutatus* venom and Russell's viper factor X activator toxin produced similar VAV curves to their whole venoms. The antivenom concentration for which the maximum VAV occurred was linearly related to the venom concentration, and this slope or ratio was consistent with that used to define the neutralisation units for Australian antivenoms. The maximal VAV point appears to represent the antivenom concentration where every venom molecule (toxin) is attached to at least one antivenom molecule (antibody) on average and may be a useful measure of antivenom efficacy. *In vivo* this would mean that for a defined antivenom concentration, venom components will be eliminated and are trapped in the central compartment.

1. Introduction

Snake envenoming is a neglected global health issue, and causes large numbers of deaths in the rural tropics, particularly South Asia and Africa (Kasturiratne et al., 2008). Antivenom is the main treatment for snake envenoming but there continues to be shortages of antivenom

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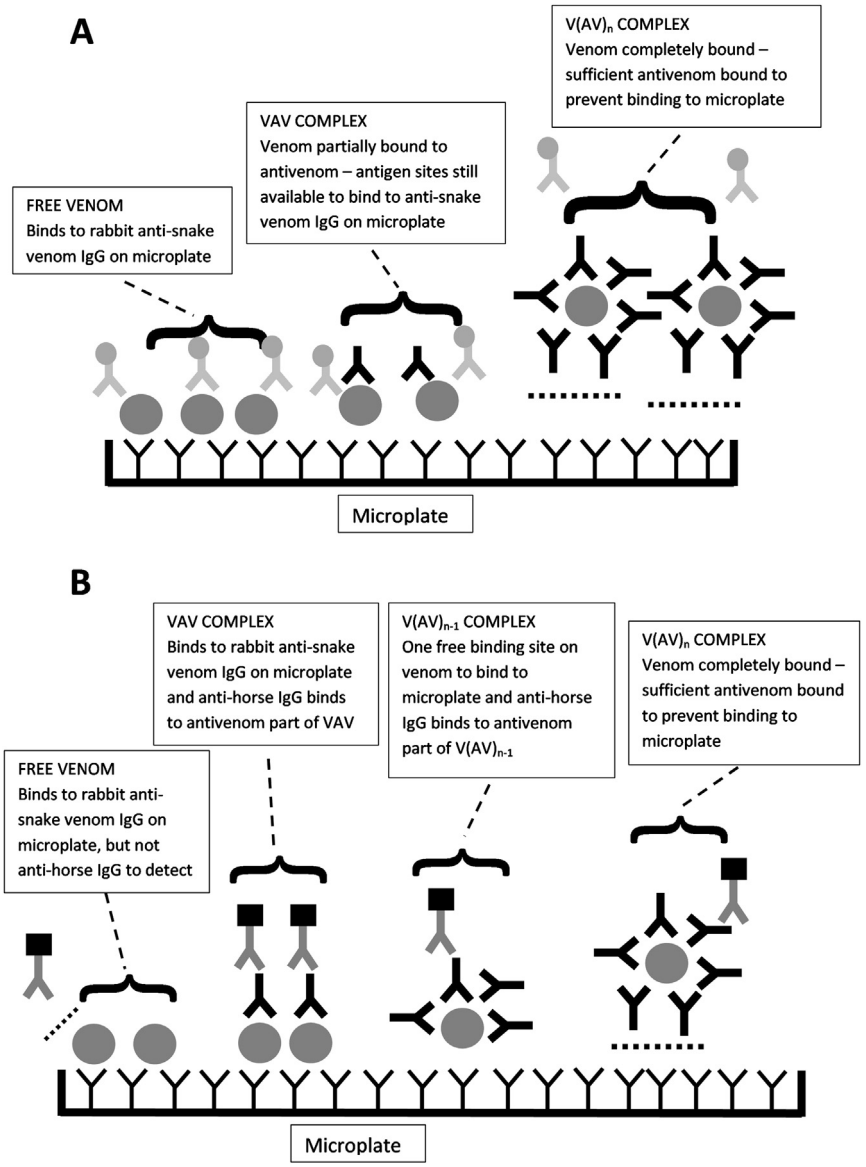

Fig. 1. Schematic diagrams of the "free" venom assay (A) and the VAV assay (B) showing how the venom and venom-antivenom complexes bind in each of the respective assays. Venom (filled grey circles), snake antivenom horse $F(ab')_2$ (thick black "Y"), anti-snake venom rabbit IgG bound to microplate (thin black "Y"), conjugated (detecting) anti-snake venom rabbit IgG (thick grey "Y" with circle attached; A) and conjugated (detecting) anti-horse IgG (thick grey "Y" with black box; B) showing the binding for four different scenarios: 1) free venom in the absence of antivenom binds to the "free" venom assay (A) but not the VAV assay (B); 2) a mixture of antivenom and venom with low concentration antivenom (1:1 ratio) binding in both assays (A and B); 3) a mixture of antivenom and venom with free antibody binding sites on the venom molecules so binds to the VAV assay microplate (B only); 4) a mixture of antivenom and venom with high concentration antivenom which won't bind to the microplates (A and B).

worldwide and there are concerns about the efficacy and safety of many of those currently available (Lalloo 2002; Isbister, 2010). The antivenom dose required to treat a patient for most antivenoms is not clearly defined and is often based on *in vitro* and *in vivo* studies done by the manufacturer and cumulative experience of clinicians regularly treating cases. There has been a trend to increasing doses of antivenom in many countries because of concerns about the efficacy of various antivenoms and patients not rapidly responding to treatment (Isbister, 2010). However, many of the effects of envenoming are irreversible and patient recovery depends on recovery or repair of the damaged tissues or organs.

The measurement of venom concentrations in human serum has been available for decades and has been used to determine if sufficient amounts of antivenom have been administered (Theakston, 1983). It has been assumed that if no free venom is detectable then sufficient antivenom has been administered (Theakston, 1983; O'Leary 2006). If venom is still measurable after antivenom has been

administered it is thought that this represents free venom and insufficient antivenom has been given. We have previously made use of the same technique *in vitro* to show that the addition of increasing concentrations of antivenom to venom gives an exponential decrease in measurable free venom (Isbister et al., 2007; Isbister et al., 2011). The concentration of antivenom at which venom is no longer detectable can then be converted to a dose required for neutralisation.

This approach appears to work well with Australian antivenoms where there is likely to be an excess of antivenom compared to venom, because the commercial antivenom is highly concentrated (O'Leary and Isbister, 2009) and the venom concentration in patients is low due to the small amount of venom delivered by elapids (Kulawickrama et al., 2010; Allen et al., 2012; Isbister et al., 2012). Therefore, venom is rarely detectable after administration of even one vial of antivenom in Australian elapid envenoming (Allen et al., 2012; Isbister et al., 2012). In contrast to this, in many non-Australian snakes, and in particular vipers, the venom concentrations are much higher (10–100 fold) and they are not reduced to zero following the administration of antivenom in a proportion of cases (Phillips et al., 1988; Ho et al., 1990). However, the persistence of venom, or in some cases recurrence of detectable venom, does not always appear to be associated with persistence of envenoming, such as coagulopathy. One explanation for this is that the venom being detected as "free" venom is in fact bound venom or venom–antivenom (VAV) complexes where the ratio of antivenom to venom is low (1–1) so that the VAV complex can still bind to the enzyme immunoassay (EIA) microplate (Fig. 1A).

The suggestion that the assay for free venom can also detect bound venom (or VAV) as well as free venom means that it is important to be able to detect bound venom or VAV complexes. Such an assay would require an antibody to bind to the venom component of the VAV complex and an antibody to the antivenom (i.e. anti-horse antibodies for an equine antivenom). Fig. 1B shows such an assay where antibodies to the venom are attached to the microplate and conjugated anti-horse antibodies are used as the detecting antibody.

The aim of this study was to develop an assay to measure the venom–antivenom (VAV) complex which will complement the free venom assay. We investigate the binding of venom and antivenom *in vitro* with the assay, which could potentially be used determine if antivenom has bound to venom *in vivo*.

2. Materials and methods

2.1. Materials

All Australian snake venoms were purchased from Venom Supplies, South Australia, including common tiger snake (*Notechis scutatus*), common death adder (*Acanthophis antarcticus*), coastal taipan (*Oxyuranus scutellatus*), rough-scaled snake (*Tropidechis carinatus*), red-bellied black snake (*Pseudechis porphyriacus*), Stephen's banded snake (*Hoplocephalus stephensii*) and the Eastern brown snake (*Pseudonaja textilis*) venoms. Russell's viper (*Daboia russelii*) venom was a gift from Colombo University, Sri Lanka. Saw-scaled viper (*Echis carinatus*) venom was purchased from Sigma. Carpet viper (*Echis ocellatus*) venom was donated by Robert Harrison (Liverpool School of Tropical Medicine). Russell's viper venom factor X activator toxin (RVVFX) was purchased from Haematologic Technologies Inc.

Rabbit anti-snake antibodies were purchased from the West Australian Institute of Medical Research. Hen anti-snake IgY antibodies to *P. textilis* venom were a gift from Frank Madaras (Venom Science Pty Ltd, South Australia). Australian commercial antivenoms were produced by CSL Ltd, including brown snake (BSAV; 1000 U), tiger snake (TSAV; 3000 U), black snake (BlSAV; 18,000 U), taipan (TAV; 12,000 U) and death adder (DAAV; 6000 U). One unit (1 U) of antivenom activity is defined to be the amount required to bind/neutralise 10 μg of venom from the snake species against which the antivenom is raised. Indian polyvalent antivenom was obtained from VINS Bioproducts (Batch No. 1054 Manufactured 09/2008 Expiry 08/2012). Indian polyvalent antivenom is raised against four snake venoms – *D. russelii*, *Notechis naja*, *E. carinatus* and *Bungarus caeruleus*. All commercial antivenoms are of equine origin.

Rabbit anti-horse IgG conjugated with horseradish peroxidise, goat anti-rabbit IgG conjugated with horseradish peroxidise, bovine serum albumin (BSA) and tetramethylbenzidine (TMB) were all purchased from Sigma. All other chemicals used were of analytical grade.

Carbonate buffer is 50 mM, pH 9.5. Blocking solution is 0.5% BSA in phosphate buffered saline (PBS) at pH 7.4. Washing solution is 0.02% Tween 20 in PBS. High binding microplates from Greiner (#655061) were used. Plates were read on a BioTek ELx808 plate reader at 450 nm. All procedures were carried out at room temperature.

2.2. Venom–antivenom complex (VAV) measurement

A known concentration of venom in blocking solution was added to serial dilutions of antivenom in PBS (450 μl), such that the final venom concentration in the mixture was 500, 250, 100, 50 or 0 ng/ml. The mixture was allowed to stand for one hour then applied in triplicate to a microplate as below. Control solutions containing antivenom only were included to allow for subtraction of background absorbance.

Plates were coated with anti-snake venom IgG (100 μl, 1 μg/ml in carbonate buffer) for 1 h at room temperature then at 4 °C overnight. They were then washed once, and blocking solution (300 μl) was applied for 1 h. Plates were washed again and the incubated mixture of venom and antivenom (100 μl) was added. After a further hour, the plates were washed three times and a solution of labelled anti-horse IgG (100 μl, 1 μg/ml in blocking solution) was applied. After a further hour the plates were washed 3 times, and TMB (100 μl) was applied, followed by H_2SO_4 (50 μl, 1 M) to stop the reaction.

For *P. textilis* venom the plate was coated with anti-*P. textilis* IgY (1.5 μg/ml in carbonate buffer). The incubated venom/antivenom mixture comprised *P. textilis* venom (100 ng/ml) and rabbit anti-*P. textilis* IgG (0–100 μg/ml). Detection was with HRP-labelled anti-rabbit IgG at a

dilution of 1:800 of the supplied solution, followed by treatment with TMB as above.

Isolated fractions of *N. scutatus* venom (100 μl, 8 μg/ml in PBS) were mixed with serial dilutions of TSAV antivenom in PBS and VAV was detected using the same method for venoms with labelled anti-horse IgG.

2.3. High performance liquid chromatography (HPLC)

HPLC was carried out using a Phenomenex Jupiter column, 5u C18 300Å 250 × 4.6 mm, with mobile phase 15% MeCN (containing 0.1% trifluoroacetic acid) increasing to 53.5% at $t = 60$ min, at a flow rate of 0.5 ml/min. Ultraviolet detection was used at a wavelength of 215 nm.

Fractions were collected of the most clearly-resolved peaks and were subject to MALDI MS analysis on a Bruker Ultraflextreme instrument, followed by trypsin digestion and analysis by MALDI ToF/ToF using MS-peptide mass fingerprint and MS/MS amino acid sequence database search with MASCOT protein sequencing software.

2.4. Analysis

VAV absorbance versus antivenom concentration data was fitted to different curves to obtain the best fit for the data, including the difference of two ligand-binding curves, with *B*max the maximum binding and *K*d the dissociation constant:

$$Y = \frac{B\max_1{}^{*}X}{K\mathrm{d}_1 + X} - \frac{B\max_2{}^{*}X}{K\mathrm{d}_2 + X}$$

and the difference of two exponential curves:

$$Y = y_{\max 1}{}^{*}\left(1 - \mathrm{e}^{-K_1 X}\right) - y_{\max 2}{}^{*}\left(1 - \mathrm{e}^{-K_2 X}\right)$$

These models/curves were used empirically to find the point of maximum absorbance by interpolation and the parameters were not given any biological interpretation. Data were analysed by non-linear regression using Prism 5.03 to fit the curves to the most appropriate model. The best fitting curve was then used to determine the antivenom concentration where the VAV curve was a maximum for each of the venom concentrations. In some cases the data could not be fitted because there was no clear maximum and in these cases the line was drawn directly between the experimental points.

Antivenom concentrations for peak VAV were plotted against the venom concentration and these data were analysed with linear regression to estimate the slope with 95% confidence intervals (95%CI). All analysis and plotting was done in Prism 5.03 for Windows [GraphPad Software, San Diego California USA, www.graphpad.com].

3. Results

3.1. VAV assay of whole venoms

The amount of VAV measured as an increase in absorbance on the VAV assay initially increased with increasing concentrations of mixed equine antivenom until it reached a maximum after which the VAV concentration decreased with further increasing equine antivenom concentrations. This is shown in Fig. 2 for mixtures of five different Australian snake venoms at four different venom concentrations, with increasing mixed antivenom concentrations. For three of the snake venoms the data fitted best to the difference of two exponentials (Fig. 2A, B and C). In two cases the VAV curves did not have a well-defined maximum which is shown for *H. stephensii* venom with TSAV and *A. antarcticus* venom with DAAV, which had broad VAV peaks with two maxima (Fig. 2D and E). *T. carinatus* with TSAV and *P. porphyriacus* with both BlSAV and TSAV had distinct maxima in the VAV curve (data not shown).

E. carinatus and *E. ocellatus* venoms (250 ng/ml) were incubated with Indian polyvalent antivenom and applied to a plate coated with anti-*E. ocellatus* antibodies and *D. russelii* venom (250 ng/ml) was incubated with Indian polyvalent antivenom and applied to a plate coated with anti-*D. russelii* antibodies. Detection was with labelled anti-horse antibodies. Fig. 4 shows a clear VAV peak for *D. russelii* venom but not for *E. carinatus* venom.

The antivenom concentration where there was a peak in absorbance due to VAV increased with increasing venom concentration and was determined using the fitted curves. Fig. 5 shows the linear relationship between the antivenom concentrations for the VAV peak versus venom concentration over the venom range of 50 ng/ml to 500 ng/ml. The slope of the lines can then be interpreted as the ratio of antivenom to venom where there is a peak in absorbance from venom–antivenom complexes. This varied between 0.04 and 0.15 mU/ng for all Australian commercial venoms (Table 1, Fig. 5A) and was 0.09 U/μg (95%CI: 0.07–0.12 U/μg) for *P. textilis* venom, 0.04 U/μg (95%CI: 0.035–0.05 U/μg) for *N. scutatus* venom and 0.08 U/μg (95%CI: 0.06–0.10 U/μg) for *O. scutellatus* venom. For *D. russelii* venom the slope of the line was 180 ng AV/ng (Fig. 5B).

3.2. VAV assay of venom components

To examine the behaviour of individual venom components, we collected four well-defined fractions from the HPLC of *N. scutatus* venom (Fig. 6). Each fraction comprised 6–8% of the total area of the HPLC trace. The fractions were characterised by mass spectrometry and matched to previous structures as: Fraction I – notexin (13,544 Da), fraction II (16,742 Da), fraction III (14,002 Da) and fraction IV – notecarin (46,678 Da). Fraction II could not be matched to a previously isolated structure. Fraction III matched to a phospholipase A2 toxin in *Acanthophis* sp. (acanmyotoxin-3 [fragment]). The prothrombin activator Notecarin has previously been isolated in this manner, and shown to consist of two peaks corresponding to two isoforms (Rao et al., 2003).

Fraction II and Notecarin bound poorly to the rabbit anti-*N. scutatus* antibodies used to coat the plate so VAV measurement with these fractions was not possible. Notexin and fraction III produced VAV curves similar to whole venom, but with maxima displaced to higher or lower TSAV concentrations compared to whole venom (Fig. 7).

RVVFX, the FX-activating component from RVV, was mixed with Indian polyvalent antivenom and assayed for VAV. A set of VAV curves was obtained at RVVFX = 50, 100,

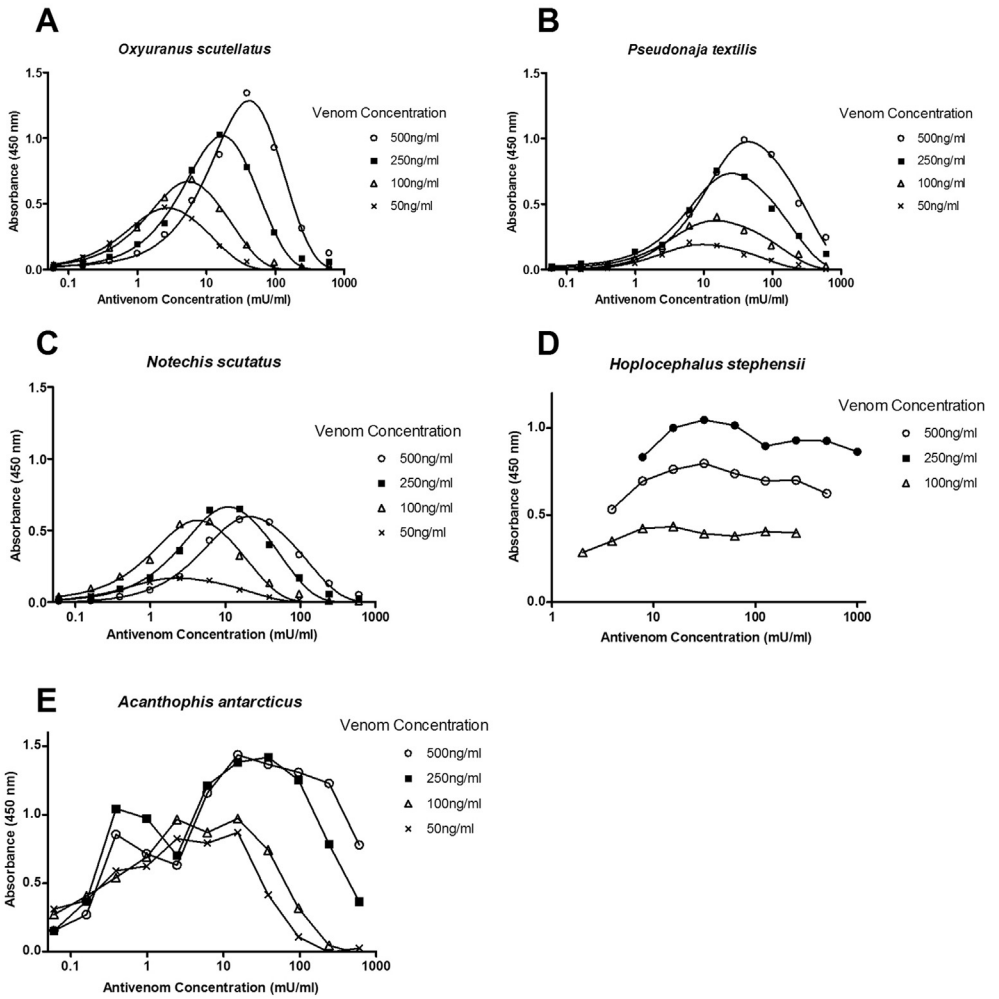

Fig. 2. Logarithmic-linear plots of venom–antivenom (VAV) measurements versus antivenom concentration for mixtures of venom and antivenom, at three or four concentrations of venom (ng/ml) – *O. scutellatus* (A), *P. textilis* (B), *N. scutatus* (C), *H. stephensii* (D) and *A. antarcticus* (E). The plotted points are the average of three absorbances. For panels A–C the line is a predicted curve based on the subtraction of two exponential curves (see text). For panels D and E a curve could not be fitted and the lines connect experimental points.

250 and 500 ng/ml, showing a concentration of AV at VAV_{max} as 8, 18, 36 and 66 μg/ml (Fig. 3). The slope of this line, 135 ng AV/ng RVVX, compares to the value of 180 ng AV/ng, found for the whole *D. russelii* venom (Fig. 4B).

4. Discussion

The VAV assay will only detect bound antivenom because the microplate is coated with an anti-snake venom antibody which binds the venom, and detection is with labelled anti-horse antibodies which bind equine antivenom (Fig. 1B). *In vitro* this provides a measure of antivenom-venom binding for increasing concentrations of antivenom. The curve increases with increasing binding of antivenom (antibodies) to free venom until a point where increasing amounts of antivenom (antibodies) prevent the venom-antivenom complex binding to the microplate, because there are no longer any free antibody binding sites (epitopes) on the venom molecules (Fig. 1B). The concentration of antivenom at which the VAV peak occurs is the concentration at which every venom component, on average, must be attached to at least one antivenom molecule. This gives us a new measure of antivenom efficacy. In addition, it provides an assay to measure bound venom *in vivo* and to determine if venom detected post-antivenom using the free venom assay is bound.

At low concentrations of antivenom, the antivenom binds to the venom molecules in a one to one ratio to form VAV complexes. The VAV complex still has free binding sites on the venom molecule which allows further antivenom to bind with increasing concentrations to form $V(AV)_2$, $V(AV)_3$, … $V(AV)_n$ where n is the maximum number of antibody binding sites on a venom molecule. However, at least one binding site must remain free and exposed for the venom-antivenom complex to bind to the anti-snake venom antibodies on the microplate. In other words, $V(AV)_n$ cannot bind to the microplate (Fig. 1B). This is the reason that initially as the antivenom concentration

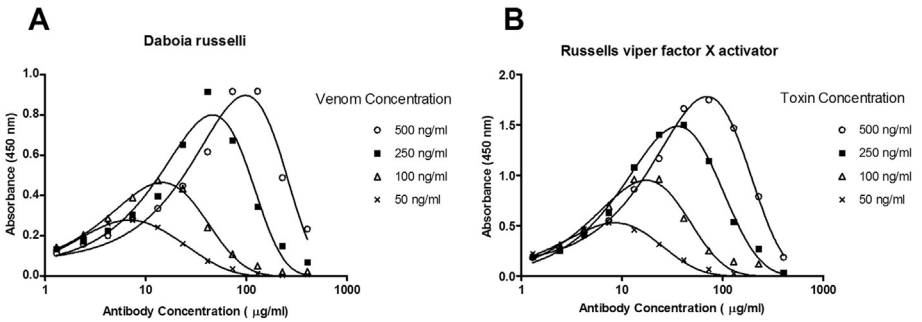

Fig. 3. Logarithmic-linear plots of venom–antivenom (VAV) measurements versus antivenom concentration for mixtures of *D. russelii* venom (A) or *D. russelii* Factor X activator [RVVFX] (B) with serial dilutions of Indian polyvalent antivenom (μg/ml), at four concentrations of venom or toxin. The plotted points are the average of three absorbances. The line is a predicted curve based on the subtraction of two exponential curves.

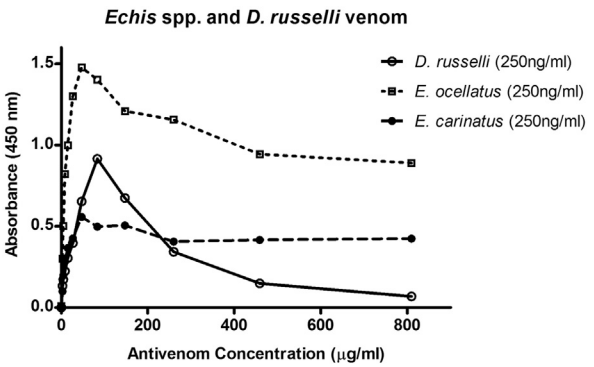

Fig. 4. Linear plots of venom–antivenom (VAV) measurements versus antivenom concentration for mixtures of three venoms (*D. russelii*, *E. carinatus* and *E. ocellatus*) with serial dilutions of Indian polyvalent antivenom (μg/ml). The plotted points are the average of three absorbances. A curve could not be fitted and the lines connect points.

Table 1
Ratio of the concentration of antivenom to the concentration of venom at the maximum VAV absorbance for Australian commercial antivenoms (CSL Ltd.) and Australian elapid snakes.

Venom	Antivenom	Slope (U/μg)
P. porphyriacus	TSAV	0.05
P. porphyriacus	BlSAV	0.05
T. carinatus	TSAV	0.17
P. textilis	BSAV	0.09
O. scutellatus	TAV	0.08
N. scutatus	TSAV	0.04

TSAV – tiger snake antivenom; BlSAV – black snake antivenom; BSAV – brown snake antivenom; TAV – taipan antivenom.

increases and the proportion of antivenom in the mixture increases, there is an increasing amount of VAV detected. The maximum or VAV peak occurs when further binding of antivenom results in decreasing free antibody binding sites on venom molecules, resulting in decreasing binding to the microplate. Rather simplistically, the VAV peak is when there is on average of mainly $V(AV)_{n-1}$ in the antivenom/venom mixture and this means that there is at least one antivenom molecule is attached to each venom molecule.

This is a rather simplistic description of what occurs because venom consists of different toxins and each toxin is likely to have a different number of epitopes (antibody binding sites) depending on toxin size and antigenicity. In addition, antivenom is a polyclonal antibody mixture with antibodies to different toxins and different toxin epitopes with varying affinities. However, the stepwise formation of $V(AV)_k$ complexes (where $1 < k < n$) applies to the behaviour of the whole population of venom (toxins) and antivenom molecules, regardless of the fact the venoms contain dozens of different proteins, each with several epitopes, and that the antivenoms are themselves polyclonal. For most antivenom-venom pairs, the range of affinities for the first and second binding sites is not large, otherwise the VAV peak would not be so well-defined in

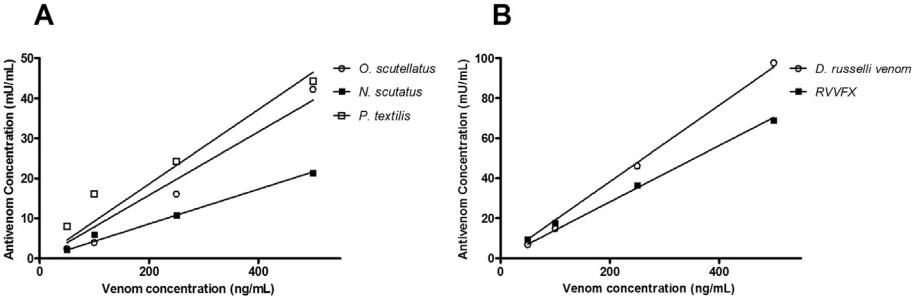

Fig. 5. Plot of the antivenom concentration at which the maximum VAV absorbance occurred versus the concentration of venom for three Australian venoms (*O. scutellatus*, *P. textilis* and *N. scutatus*) [A] and for *D. russelii* venom and its major toxin – RVVFX [B].

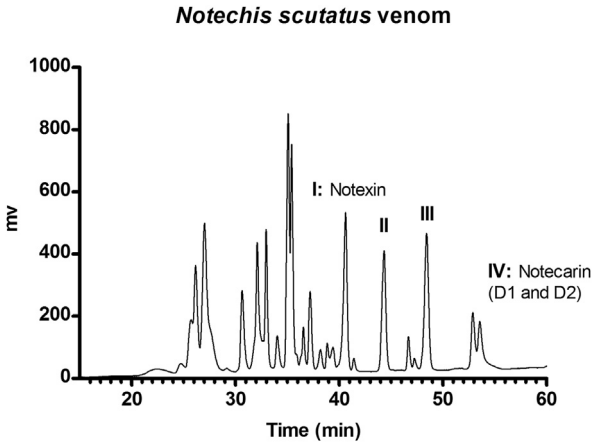

Fig. 6. High performance liquid chromatography trace of *N. scutatus* venom showing the four collected fractions.

the majority of cases. Therefore we consider an averaged set of antivenom-venom pairs for a range of n and k. The observation that the VAV curves of individual venom components are not too dissimilar to those of whole venom supports this view (Figs. 3B and 7).

In some cases, a well-defined VAV curve was not obtained (Fig. 2D, E and 4). For *A. antarcticus* venom and death adder antivenom there appeared to be two maxima within the overall curve (Fig. 2E), suggesting an overlapping of two distinct populations of venom–antivenom complexes in the mixture, possibly due to the presence of epitopes of very different affinity or different toxins. Nevertheless, the curves do return towards zero, showing that the venom can be fully neutralised by the antivenom. *H. stephensii* venom, with tiger snake antivenom gave a broad peak, possibly suggesting a low affinity of this venom for tiger snake antivenom. We have previously shown that *H. stephensii* venom requires more tiger snake antivenom for neutralisation than does *N. scutatus* venom (Isbister et al., 2011), consistent with the fact that *H. stephensii* venom is not used to immunise horses for antivenom production.

Another example of limited neutralisation is shown by the VAV curves produced by *Echis* venoms with Indian

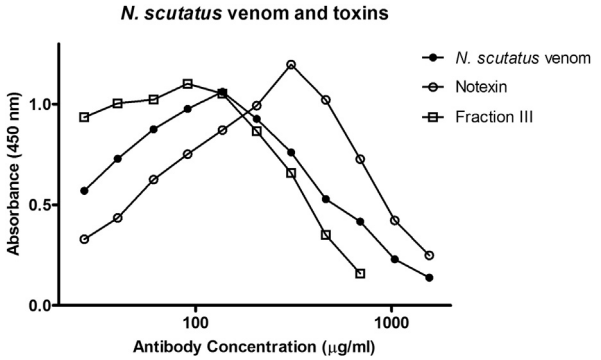

Fig. 7. Logarithmic-linear plots of venom–antivenom (VAV) measurements versus antivenom concentration for mixtures of tiger snake whole venom or fractions of the venom with TSAV. Concentration of whole venom and of components was 1000 ng/ml. The plotted points are the average of three and a curve could not be fitted so lines connect points.

polyvalent antivenom. *E. carinatus* venom is one of the four against which the polyvalent antivenom is raised, but this antivenom is reportedly not suitable for *E. ocellatus* (Warrell, 2008). We applied both venoms, after incubation with Indian polyvalent antivenom, to a plate coated with anti-*E. ocellatus* antibodies. Besides showing cross-reactivity between the *Echis* venoms, in that *E. carinatus* binds to the plate and *E. ocellatus* binds to Indian polyvalent antivenom, the VAV curves show no sharp maxima (Fig. 4). This suggests that after attachment of the first antibody in the polyvalent antivenom, there is little or no further binding. In contrast, the VAV curve of *D. russelii* shows the venom quickly becomes saturated with antivenom and therefore unable to bind to the plate.

Most measurements of circulating immune complexes are for the investigation of autoimmune diseases or serum sickness. Immune complex formation between snake venoms and antivenoms has been investigated previously by Sanny, using size-exclusion HPLC (Sanny, 2011), and by ourselves, using turbidimetry (O'Leary et al., 2013) and enzyme immunoassay (O'Leary et al., 2006). This study supports a stepwise process of VAV formation, and indicates the amount of antivenom required such that each venom component is attached to at least one antivenom molecule.

The data was fitted to the difference of two exponential curves empirically to allow the point of maximum absorbance to be determined by interpolation. This concept is related to the classical precipitin reaction wherein at the ratio of antibody:antigen which gives the maximum amount of cross-linking, immune-complexes become insoluble and thus visible in a gel. The same phenomenon can be observed turbidimetrically in solution which has been shown for venoms and antivenoms (O'Leary, Maduwage et al., 2013). In the measurement of VAV, the maximum signal or VAV peak occurs when there is on average $V(AV)_{n-1}$ in the antivenom/venom mixture, which means that each venom molecule is attached to at least one antivenom molecule (antibody). This can then be used as a marker of efficacy because it means that all venom molecules (toxins) are bound to at least one antibody, so they cannot distribute to their site of action and/or can be eliminated.

This antivenom:venom ratio measured over a range of venom concentrations as a slope appears to be constant (Fig. 5). Table 1 gives results obtained for some Australian snake venoms with the commercial antivenoms. Interestingly, these values of between 0.4 and 1.7 U required for 10 μg of venom, compare to the original definition from manufacturer of antivenom activity as 1 U being sufficient to neutralise 10 μg of venom (Sutherland and Tibballs, 2001). While "neutralise" is not defined, it can be argued that the attachment of at least one antivenom molecule (antibody) to a venom molecule, even if not near the active site of the molecule, is sufficient to prevent it from leaving the circulation and render it susceptible to removal by the reticulo-endothelial system or by circulating phagocytes.

In this study we have only shown the detection of VAV in *in vitro* mixtures of venom and antivenom. A more useful application of this assay would be to measure VAV in

patients' sera after the administration of antivenom, particularly in cases where there remains detectable venom using the free venom assay or in cases where there is venom recurrence. In the former the VAV assay may show that detectable venom is in fact all VAV, so that all of the venom molecules *in vivo* are bound to at least one antivenom molecule. In the case of purported venom recurrence the VAV assay may also show that there is only bound venom present (i.e. VAV), so there is not true venom recurrence. The VAV assay will therefore provide a useful tool for the investigation of free and bound venom in envenomed patients.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Author contributions

MAO designed the assay; MAO undertook the experimental work; MAO and GKI undertook the analysis; MAO and GKI wrote the paper; GKI takes responsibility for the manuscript.

Ethical

No approval was required because neither animals or human material was used in this study.

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