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Use of immunoturbidimetry to detect venom-antivenom binding using snake venoms

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Abstract

Introduction: Immunoturbidimetry studies the phenomenon of immunoprecipitation of antigens and antibodies in solution, where there is the formation of large, polymeric insoluble immunocomplexes that increases the turbidity of the solution. We used immunoturbidimetry to investigate the interaction between commercial snake antivenoms and snake venoms, as well as cross-reactivity between different snake venoms.

Methods: Serial dilutions of commercial snake antivenoms(100µl) in water were placed in the wells of a microtitre plate and 100µl of a venom solution (50µg/ml in water) was added. Absorbance readings were taken at 340nm every minute on a BioTek ELx808 plate reader at 37°C. Limits imposed were a 30 minute cut-off and 0.004 as the lowest significant maximum increase. Reactions with rabbit antibodies were carried out similarly, except that antibody dilutions were in PBS.

Results: Mixing venom and antivenom/antibodies resulted in an immediate increase in turbidity, which either reached a maximum or continued to increase until a 30 minute cut-off. There was a peak in absorbance readings for most Australian snake venoms mixed with the corresponding commercial antivenom, except for *P. textilis* venom and brown snake antivenom. There was cross-reactivity between *Naja naja* venom from Sri Lanka and tiger snake antivenom indicated by turbidity when they were mixed. Mixing rabbit anti-snake antibodies with snake venoms resulted in increasing turbidity, but there was not a peak suggesting the antibodies were not sufficiently concentrated. The absorbance reading at pre-determined concentrations of rabbit antibodies mixed with different venoms was able to quantify the cross-reactivity between venoms. Indian antivenoms

from two manufacturers were tested against four Sri Lankan snake venoms (*D. russelli*, *N. naja*, *E. carinatus* and *B. caeruleus*) and showed limited formation of immunocomplexes with antivenom from one manufacturer.

Discussion: The turbidity test provides an easy and rapid way to compare and characterise interactions between antivenoms and snake venoms.

Introduction

Snake envenoming is now recognised as a global health issue and antivenom is the major treatment.(Kasturiratne, Wickremasinghe et al. 2008) However, there are shortages of antivenom in many parts of the world and there is a need for high quality and effective antivenoms to be developed.(Lalloo, Theakston et al. 2002) An essential part of antivenom development is determining the ability of antivenom to bind to toxins in snake venoms and also the cross-reactivity between particular antivenoms and other snake venoms.(Isbister, O'Leary et al. 2010; Kornhauser, Isbister et al. 2012) This allows effective polyvalent antivenoms to be developed to cover all snakes in a particular geographical region.

The use of immunoprecipitation to determine the extent of reactivity between venoms or venom components (toxins) and antivenoms is well-known.(Williams, White et al. 1994; van der Weyden, Hains et al. 2000). The traditional radial immunodiffusion (RID) process, which is carried out in an agar gel, takes many hours to days to complete. Such long experimental times limit the number of studies that can be done.

Immunoturbidimetry studies the same phenomenon, but in solution rather than on a gel. In solution the formation of large, polymeric insoluble immunocomplexes causes an increase in turbidity. The Ramon flocculation assay is based on this principle and is used as one of the quality check procedures in the manufacture of diphtheria and tetanus vaccines.(Lyng and Bentzon 1987; Preneta-Blanc, Rigsby et al. 2008) For this assay, varying ratios of standardised solutions of toxoid and antitoxin are mixed, and the resulting turbidity of the solution, referred to as flocculation, is observed by naked eye.

The occurrence of flocculation at particular ratios of snake venoms and antivenoms has been long recognised, but there are limited studies on this phenomena.(Schottler 1952) Solano et al used a turbidimeter to monitor the interaction between *Bothrops asper* venom and antivenom to determine how long it took for this interaction to be complete.(Solano, Segura et al. 2010) They noted that turbidity continued to increase over the 30 minute incubation period, but that neutralisation was not dependant on this time.

We found that sufficient turbidity develops to be detectable in the wells of a microtitre plate in a basic filter-based absorbance plate reader. We have used this method of detecting immunoprecipitation in solution to explore the interaction between venoms and commercial antivenoms, or in-house antibodies.

Methods and Materials

Australian snake venoms including common tiger snake (*Notechis scutatus*), black tiger snake (*N. ater*), rough-scaled snake (*Tropidechis carinatus*), mulga snake (*Pseudechis australis*), red-bellied black snake (*P. porphyriacus*), common death adder (*Ancanthophis antarcticus*), coastal taipan (*Oxyuranus scutellatus*), Stephen's banded snake (*Hoplocephalus stephensii*), and the Eastern brown snake (*Pseudonaja textilis*) venoms were obtained from Venom Supplies, South Australia. Venoms from *Daboia russelli* (Russell's viper) and *Naja naja* (Indian cobra) venom were obtained from Colombo University, Sri Lanka. *Echis carinatus* (saw-scaled viper) and *Bungarus caeruleus* (Indian krait) venoms were purchased from Sigma. *E. ocellatus* (carpet viper) venom was donated by Robert Harrison (Liverpool School of Tropical Medicine). Stock solutions of venoms of 1 or 2 mg/ml in 50% glycerol are stored at -20°C. **Russell's viper venom factor X activating factor (RVVX) was obtained from Haematologic Technologies Inc, VT USA.**

Rabbit anti-snake antibodies were obtained from the Western Australian Institute of Medical Research as approximately 1mg/ml in phosphate buffered saline (PBS). IgY from hens immunised with *P. textilis* venom was a gift from Frank Madaras (Venom Science Pty Ltd, South Australia), and is 3 mg/ml in PBS. The CSL antivenoms used were: Brown snake (BSAV; batch #0559-10801), Tiger snake (TSAV; batch #10702), Black snake (BSAV; batch #0543-07301), Taipan (TAV; batch #0548-05601), Death adder (DAAV; batch #0557-07701). Solutions of 20 U/ml in water were prepared. The Indian polyvalent antivenoms used were: VINS(batches #1054-2000, #01AS11114-2012, #01024/10-11-2010) and BHARAT (batch #A511006-2011). Indian polyvalent

antivenoms are raised against four snake venoms – *D. russelli*, *N. naja*, *E. carinatus* and *B. caeruleus*. All commercial antivenoms are IgG of equine origin. Solutions of 40 mg/ml in water were prepared.

Serial dilutions of snake antivenom (100µl) in water were placed in the wells of a microtitre plate. The plate was placed in a BioTek ELx808 plate reader at 37°C, and 100µl of a venom solution (50µg/ml in water) was added. The final venom concentration was 25µg/ml. Readings were taken at 340nm every minute, including a 3 second shake step. Preliminary experiments found that after mixing venom and antivenom there was an immediate increase in the reading which either reached a plateau or continued to increase. Limits imposed were a 30 minute cut-off for the experiment and a value of 0.004 as the lowest significant maximum increase.

Reactions with rabbit antibodies were carried out similarly, except that the antibody dilutions were in PBS instead of water.

Results

1. *Snake venoms and Australian antivenoms*

Experiments were undertaken in water because turbidity did not develop in PBS for Australian snake venoms. Figure 1 shows the increase and peak in turbidity for the major Australian snake venoms mixed with the corresponding antivenom for increasing concentrations of antivenom. The antivenom concentration for the maximum turbidity was estimated by interpolation (Table 1) and varied for each venom. *P. textilis* venom and BSAV only had a small increase and no distinct peak. The combination of *P. textilis* venom and BSAV was repeated for a range of venom concentrations, but this did not result in the appearance of a well-defined maximum either.

The cross reactivity of *N. naja* venom (Cobra from Sri Lanka) with TSAV was tested by comparing the combinations of *N. scutatus* venom/TSAV and *N. naja* venom/TSAV for increasing concentrations of TSAV (Figure 2). There was a peak at a TSAV concentration of approximately 2500 mU/ml which was significantly lower than the peak for *N. scutatus* venom.

2. *Snake venoms and rabbit monovalent antibodies*

Figure 3A shows increasing turbidity with increasing concentrations of antibodies against six Australian snake venoms and rabbit anti-*N. ater* antibodies and that a peak does not occur. It appears that the preparations of these rabbit antibodies are not sufficiently concentrated to reach a peak of maximum turbidity with venom. Figure 3B depicts

absorbance reading versus time for venom with increasing concentrations of antibody, showing the rapid formation of the immunocomplex.

Cross-reactivity between different venoms and rabbit monovalent antibodies was compared at a pre-determined concentration of antibodies. Table 2 provides the absorbance found for a range of snake venoms using the antibodies. Figure 3C shows the cross-reactivity between four Asian snake venoms (*D. russelli*, *E. carinatus*, *B. caeruleus* and *N. naja*) and antibodies to *D. russelli* venom. Figure 3D shows the cross-reactivity between *E. ocellatus* and *E. carinatus* venoms and antibodies to *E. ocellatus*.

3. Sri Lanka snake venoms and Indian Polyvalent Antivenom

Four batches of antivenom from the two manufacturers were tested against four Sri Lankan venoms (*D. russelli*, *N. naja*, *E. carinatus* and *B. caeruleus*) (Figure 4). The figures show that the two newer batches of VINS antivenoms react equally well with all three venoms, while the older VINS antivenom batch reacts only weakly with *E. carinatus* and *B. caeruleus* venom. BHARAT antivenom forms a substantial immunoprecipitate only with the *N. naja* venom. **Reaction with a single venom component was also detected. Mixtures of RVVX with VINS antivenom showed a turbidity increase over a similar range of antivenom concentrations as for the whole venom.**

Discussion

This study has shown that turbidity formation can be used to detect the interactions between snake venoms and antivenoms (or antibodies). In the case of Australian snake venoms and antivenoms it was possible to determine the point of maximum interaction between venoms and antivenoms for a number of the snake venoms. This was not possible for all snake venoms, but the procedure provides a useful method of testing for cross-reactivity between different snake venoms and antivenoms or antibodies, and supports previous studies of cross-neutralisation between antivenoms raised against one snake and venom of a different snake, in some cases a snake from a different genus and geographical location.

Snake venoms contain numerous different proteins, each of which may have none, one or several epitopes, all with different affinities towards a polyclonal antivenom. The formation of insoluble immune complexes between venom and antivenom will not necessarily include every component of the venom. Nevertheless, a distinct maximum in turbidity develops at a certain ratio of venom to antivenom (V:AV) which must signify that the majority of venom components are behaving in a similar fashion. This is most likely the reason that venom specific enzyme immunoassays are able to quantify snake venom concentrations despite using polyclonal antibodies to trap and detect multiple toxin components. (Kulawickrama, O'Leary et al. 2010) As the concentration of antivenom increases, the composition of the solution changes from $(V)_2AV$ to $(VAV)_n$ where n is large and the species $(VAV)_n$ is the insoluble immune complex. As the concentration of antivenom increases further, the predominant species becomes the smaller and more soluble $V(AV)_m$, where $m \geq 2$.

In general, the turbidity curves are skewed towards lower antivenom concentrations, which would be a result of antibodies with the greatest affinity binding more rapidly. This is notably not the case with *B. caeruleus* venom, to which antibodies are slower to bind, and which requires three times as much antivenom to reach maximum turbidity as do *D. russelli* or *Echis* venom.

In the case of CSL Ltd antivenoms, turbidity curves provided a measure of the amount of antivenom required for most of the Australian snake venoms, comparable to the zone of equivalence obtained by radial immunodiffusion. For *O. scutellatus* and *P. australis* venoms at a concentration of 25 µg/ml, the greatest turbidity develops at an antivenom concentration of 2500mU/ml – i.e. an AV:V ratio of 100 mU/µg or 1U antivenom per 10µg of venom. This corresponds to the CSL definition of 1U of antivenom being the amount required to neutralise 10µg of venom.(White 2001) In this turbidity assay, antivenom concentrations less than this will be in the region of venom excess, and antivenom concentration greater will be in the region of antivenom excess; both these regions produce less precipitation of immunocomplexes. The assay therefore provides a semi-quantitative approach to assessing antivenoms from at least one commercial manufacturer.

Although a number of the Australian snake venoms had an absorbance peak at an antivenom concentration where the AV:V ratio is close to the CSL definition of neutralisation, this was not the case for all snakes (Figure 1). One exception was *A. antarcticus* venom with DAAV where two peaks occur. This might be the result of the presence of two populations of antibodies with very different affinities. Radial

immunodiffusion of this venom with CSL DAAV also produced more than one precipitin line.(van der Weyden, Hains et al. 2000)

The other exception was *P. textilis* venom interacting with BSAV. The lack of development of substantial turbidity between this venom and antivenom is anomalous, especially since it occurs with in-house IgY antibody raised against *P. textilis* venom. This indicates that a sufficient proportion of the venom components do possess the requisite minimum of two antibody binding sites necessary for (VAV)_n formation. We have found that BSAV binds to *P.textilis* venom and prevents its clotting activity.(Isbister, Woods et al. 2010)

The size of the peaks/curves (area under the curve), is likely to be a function of both the number of epitopes in the venom component mixture, and the proportion and affinity of the antibodies directed against these. For neutralisation, the attachment of just one antivenom molecule should be sufficient as attested by the efficacy of monoclonal antibodies.

In the experiments with monovalent rabbit antibodies, the increasing absorbance readings with antibody concentration can be taken as an indicator of cross-reactivity with other venoms. Table 2 and Figure 3A show there is cross-reactivity between *T. carinatus* and *Notechis* spp. antibodies, consistent with the difficulty of distinguishing envenoming by these genera by enzyme immunoassay,(Gan, O'Leary et al. 2009) whereas *Hoplocephalus* antibodies show no interaction with *T. carinatus* and *Notechis* spp. venom consistent with studies of this venom.(Isbister, White et al. 2011)

In addition, use of the procedure with the in-house antibodies raised to snake venoms, provides a useful screening test for the viability of antibodies, whether in a newly-obtained preparation, or in a solution that has been stored for some time. The test could replace the lengthier enzyme immunoassay in these applications, and could serve as an additional parameter for batch characterisation, as it does with some vaccines.(Lyng and Bentzon 1987; Preneta-Blanc, Rigsby et al. 2008)

The interaction between Sri Lankan Cobra (*N. naja*) and Australian TSAV (Figure 2), supports previous studies demonstrating cross-reactivity between *N. haje* (Egyptian cobra) venom and TSAV, using enzyme immunoassay and in vitro neurotoxicity studies.(Kornhauser, Isbister et al. 2012)

A limitation of this approach is that the lack of formation of insoluble immunocomplexes does not preclude the possibility of neutralisation of specific components of the venom. For example, we have found that anti-*N. scutatus* antibodies can reduce the phospholipase activity of *P. porphyriacus* venom,(Lane, O'Leary et al. 2011) but there was not sufficient interaction between *P. porphyriacus* venom and anti-*Notechis* antibodies for turbidity to develop (Figure 3A, Table 2). Similarly, the oldest batch of VINS antivenom was just as effective as the newer batches in preventing clotting by *E. carinatus* venom (data not shown), but there was no formation of insoluble immunocomplexes with this antivenom. It has been suggested that reactions between antibodies and antigens occur almost immediately, and that subsequent polymerisation of the initial, soluble complex to large insoluble species occurs on a time scale of minutes to hours.(Gorgani, Easterbrook-Smith et al. 1996) Thus, while the development of turbidity

indicates that reaction has occurred, the lack of development does not suggest that neutralisation has not occurred or will not occur.

We have showed that turbidity develops for a single toxin, RVVX, which is consistent with the antivenoms neutralising the procoagulant effect of Russell's viper venom. However, this method is best used to screen antivenoms against venoms, and not for testing against individual toxins which may not have been isolated for many snake venoms. The ability of an antivenom to neutralise individual toxins or better, specific activities of venoms (procoagulant, neurotoxic etc.), should be undertaken with separate assays that measure functional activities of toxins.

Conclusion

The use of this turbidity test to compare and characterise antivenoms gives a rapid and easy preliminary investigation of the properties of batches of antivenom or antibodies, complementing information obtained from binding studies and functional tests. With the proviso that lack of turbidity formation does not imply lack of neutralisation, the formation of turbidity is a positive indication of antibody binding.

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Figures

Figure 1: Increase in absorbance resulting 30 minutes after the addition of seven snake venoms to their corresponding CSL Ltd antivenoms. Tiger snake antivenom was used for *T. carinatus* and *H. stephensii*. Experiments were repeated in triplicate for *A. antarcticus*, *P. australis* and *O. scutellatus*. All venoms were at a concentration of 25µg/ml.

Figure 2: Increase in absorbance 30 minutes after the addition of *N. scutatus* venom (25 $\mu\text{g/ml}$) and *N. naja* venom (25 $\mu\text{g/ml}$) to tiger snake antivenom (TSAV).

Figure 3A: Increase in absorbance 30 minutes after the addition of six different venoms to rabbit anti-*N. ater* IgG. All venoms were at 25 $\mu\text{g/ml}$.

Figure 3B: Development of turbidity over time, resulting from addition of *N. scutatus* venom to increasing concentrations ($\mu\text{g/ml}$) of rabbit anti-*T.carinatus* antibodies.

Concentration of venom is $25\ \mu\text{g/ml}$

Figure 3C: Increase in absorbance 30 minutes after the addition of *D. russelli* venom (closed circle, thick line), *E. carinatus* venom (open circle, dashed line) and *N. naja* venom (open square, dashed line) with increasing concentrations of antibodies against *D. russelli*. *B. caeruleus* venom gave no increase. All venoms at 25 μ g/ml.

Figure 3D: Increase in absorbance 30 minutes after the addition of *E. ocellatus* venom (25 μ g/ml; closed circle, thick line) and *E. carinatus* venom (25 μ g/ml; open square, dashed line) to increasing concentrations of antibodies against *E. ocellatus*.

Figure 4: Increase in absorbance 30 minutes after the addition of *D. russelli* venom, *N. naja* venom, *E. carinatus* venom and *B. caeruleus* to increasing concentrations of four different batches of Indian Polyvalent antivenom. All venoms were at a concentration of 25 µg/ml.

Table 1: Formation of insoluble immunocomplexes between snake venoms and antivenoms. The venom concentration was 25µg/ml.

Venom	Antivenom	Antivenom concentration (mU/ml) for maximum absorbance
<i>N. scutatus</i>	TSAV	2500
<i>N. ater</i>	TSAV	3750
<i>T. carinatus</i>	TSAV	5350
<i>H. stephensi</i>	TSAV	670
<i>P. porphyriacus</i>	TSAV	no peak
<i>P. porphyriacus.</i>	BISAV	no peak
<i>P. australis</i>	BISAV	2500
<i>O. scutellatus</i>	TAV	2500
<i>P. textilis</i>	BSAV	no peak
<i>A. antarcticus</i>	DAAV	2500 and 625
<i>N. naja</i>	TSAV	2500

TSAV – tiger snake antivenom; BISAV – black snake antivenom; TAV – taipan antivenom; BSAV – brown snake antivenom’ DAAV – death adder antivenom

Table 2: Formation of insoluble immunocomplexes between snake venoms and rabbit monovalent antibodies. All venom concentrations were 25µg/ml

Antibody	Antibody concentration (µg/ml)	Venom	Δ Abs at 340nm
anti- <i>N.scutatus</i>	1000	<i>N. ater</i>	0.2
		<i>N. scutatus</i>	0.062
		<i>P. porphyriacus</i>	0.03
		<i>T. carinatus</i>	0.07
anti- <i>N.ater</i>	1000	<i>H. stephensi</i>	0.02
		<i>N. ater</i>	0.24
		<i>N. scutatus</i>	0.18
		<i>T. carinatus</i>	0.04
		<i>P.aust.</i>	0.05
		<i>P. porphyriacus</i>	0.05
anti- <i>T.carinatus</i>	700	<i>N. scutatus</i>	0.19
		<i>T. carinatus</i>	0.29
anti- <i>H.stephensi</i>	500	<i>H. stephensi</i>	0.11
		<i>N. scutatus</i>	0
		<i>T. carinatus</i>	0
anti- <i>P.porphyriacus</i>	750	<i>N. scutatus</i>	0
		<i>P. porphyriacus</i>	0.23
anti- <i>P.textilis</i> IgY *	1500	<i>P. textilis</i>	0.08
anti- <i>O.scutellatus.</i>	500	<i>O. scutellatus</i>	0.03
		<i>P. textilis</i>	0
anti- <i>E.ocellatus</i>	500	<i>E. carinatus</i>	0
		<i>E. ocellatus</i>	0.11
anti- <i>D.russelli</i>	750	<i>D. russelli</i>	0.04

		<i>E. carinatus</i>	0.01
		<i>N. naja</i>	0.01
		<i>B. caeruleus</i>	0

* IgY, not rabbit IgG