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Detection of venom after antivenom administration is largely due to bound venom

Margaret A. O'Leary [a], Kalana Maduwage [b], Geoffrey K. Isbister [a, *]

[a] School of Medicine and Public Health, University of Newcastle, NSW, Australia
[b] Faculty of Medicine, University of Peradeniya, Peradeniya, Sri Lanka

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ABSTRACT

Detection of recurrent venom post-antivenom in snake envenoming is commonly reported and thought to be due to insufficient antivenom. However, relatively few reports of recurrence have venom measurement, and in most cases patients clinically improve, despite venom detected post-antivenom. We hypothesized that persistent or recurrent venom detection post-antivenom is due to detecting bound venom. Multiple (>4) serum samples were available from 255 Russell's viper (*Daboia russelii*) envenomed patients. Enzyme-linked immunosorbent assay was used to measure venom, antivenom and venom–antivenom (VAV) complexes. In 79/255 (31%) there was persistent/recurrent venom detected despite antivenom being present. In these post-antivenom samples, VAV was also detected at the same time as venom was detected. Anti-horse (aH) antiserum was bound to UltraLink (UL) resin and added to *in vitro* venom–antivenom mixtures, and 15 pre- and post-antivenom samples from patients. There was significantly less free venom detected in *in vitro* venom–antivenom mixtures to which ULaH had been added compared to those without ULaH added. In 9 post-antivenom patient samples the addition of ULaH reduced venom detected by a median of 80% (69%–88%) compared to only 20% in four pre-antivenom samples. This suggests that the detection of persistent/recurrent venom post-antivenom is due to bound and not free venom.

1. Introduction

Snake envenoming is a major public health issue, particularly in resource poor countries, and is now recognised by the World Health Organisation as a neglected tropical disease (Kasturiratne et al., 2008). Antivenom is the main treatment for snake envenoming but there are ongoing issues with availability, effectiveness and dosing. A number of methods are used to assess the efficacy and effectiveness of antivenom, including venom specific enzyme-linked immunosorbent assay (ELISA). The absence of free venom detected by ELISA after antivenom is usually interpreted to indicate that all the free venom is bound and the antivenom has done what it is supposed to do (Theakston, 1983; Theakston et al., 1992).

The recurrence or persistence of venom post-antivenom and the recurrence of envenoming syndromes post-antivenom continue to be major issues in the treatment of snake envenoming. In its most pure form the phenomena of recurrence is the reappearance of venom in the circulation which is associated with a recrudescence of envenoming symptoms, signs and laboratory changes (Boyer et al., 2013). Most authors suggest that this is a result of insufficient antivenom being administered and as further venom is absorbed from the bite site, the antivenom is overwhelmed and the patient is re-envenomed (Ho et al., 1986; Theakston et al., 1992). For this to be true, there would be simultaneous detection of recurrent venom and clinical and/or laboratory evidence of envenoming. However, the vast majority of reports are of recurrent venom detection (antigenaemia) without recurrent envenoming (Ariaratnam et al., 1999; Ho et al., 1986; Phillips et al., 1988; Theakston, 1997). This is demonstrated in the study by Otero et al. where they found recurrence of venom in serum samples in eight of 52 patients, 6–72 h post-antivenom, but no evidence of recurrent coagulopathy. They also had the opposite with two patients who had recurrent coagulopathy at 24 and 48 h, but no detectable venom in their circulation (Otero et al., 2006). There are a few reports where there is both definite recurrence of venom in serum associated with re-envenoming. This has been most recently

* Corresponding author. Department of Clinical Toxicology and Pharmacology, Calvary Mater Newcastle, Edith St, Waratah, NSW 2298, Australia.
E-mail address: geoff.isbister@gmail.com (G.K. Isbister).

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reported with crotaline envenoming in North America (Boyer et al., 2013).

It appears that in cases where venom is detected post-antivenom this is not always associated with re-envenoming and it may be that the venom specific ELISA is detecting more than just free venom (O'leary and Isbister, 2014). A recent study that developed an ELISA to measure venom–antivenom (VAV) complexes demonstrated *in vitro* that for low concentrations of antivenom compared to venom (low antivenom to venom ratio), the free venom assay appeared to detect the VAV complexes as well as free venom (O'leary and Isbister, 2014). This may be an explanation for cases where venom recurrence or persistence is detected in the absence of clinical re-envenoming.

The aim of this investigation was to separately measure bound and free venom post-antivenom *in vitro* and in human serum samples using a previously developed ELISA for bound venom and developing a method to remove bound venom to allow measurement of free venom alone.

2. Materials and methods

2.1. Materials

Russell's viper (*Daboia russelii*) venom was obtained from Colombo University, Sri Lanka. Common brown snake (*Pseudonaja textilis*) venom was purchased from Venom Supplies. Rabbit anti-*D. russelii* venom antibodies were purchased from the Western Australian Institute of Medical Research and then biotinylated using Pierce EZ-Link Sulfo-NHS-LC-Biotin. Streptavidin conjugated with horseradish peroxidase was purchased from Chemicon/Millipore. Indian polyvalent antivenom (equine $F(ab')_2$ antibodies raised against *D. russelii*, *Echis carinatus*, *Naja naja* and *Bungarus caeruleus*) was purchased from VINS Bioproducts (Batch No. 1054; Manufactured 09/2008; Expiry 08/2012) and BHARAT Serum and Vaccines Limited, India (Batch No. A5311006 Manufactured 01/2011; Expiry 12/2014). Australian brown snake antivenom (BSAV; 1000 U) was produced by CSL Ltd. UltraLink Biosupport resin #53110 was purchased from Pierce. Rabbit anti-horse whole serum fractionated antiserum #H9383, rabbit anti-horse IgG labelled with horseradish peroxidase #H6917 and tetramethylbenzidine (TMB) were bought from Sigma. Protein measurement was carried out using the Bradford reagent (Bio-Rad #500-0205). Carbonate buffer is 0.05 M pH 9.5. Bovine serum albumin (BSA) is bought from Bovogen. Blocking solution was made up as 0.5% BSA in phosphate buffered saline (PBS) and washing solution was 0.02% TWEEN20 in PBS. All procedures are carried out at room temperature unless otherwise indicated. Microplates used for the ELISA were Greiner high binding #655061 and were read in a BioTek Synergy HT plate reader at 450 nm for the ELISA.

2.2. Patient samples

Serial serum samples were available for analysis from patients with Russell's viper envenoming presenting to three hospitals in Sri Lanka from January 2007 to July 2012 (Isbister et al., 2012, 2013b). Patients (>13 years of age) presenting with Russell's viper envenoming were included if venom was detected in the patients' serum with a Russell's viper venom specific ELISA, at least four serum samples at different time points were available for analysis and a pre-antivenom sample was available. Serial samples were also available from patients with brown snake envenoming who were recruited to the Australian snakebite project (Allen et al., 2012). All serum samples were immediately centrifuged after collection, then aliquoted and stored at −80 °C until analysed. Ethics approval was obtained from Colombo University and the University of Peradeniya to cover hospitals in Sri Lanka and approval was obtained from several Human Research and Ethics Committees to cover all Australian hospitals involved (Allen et al., 2012).

2.3. Enzyme-linked immunosorbent assays (ELISA)

2.3.1. Venom specific ELISA

A venom specific ELISA was used to measure Russell's viper and brown snake venom as previously described (Kulawickrama et al., 2010). Microplates were coated with anti-*D. russelii* venom IgG antibodies (100 μL/well of 1 μg/ml in carbonate buffer) for 1 h at room temperature and overnight at 4 °C. The plates were washed and blocking solution (300 μL/well) applied for one hour. The plates were again washed and the serum sample (100 μL) applied for one hour. The plates were washed three times and biotinylated anti-*D. russelii* venom IgG antibodies (100 μL/well of 0.6 μg/ml in blocking solution) applied for one hour. The plates were washed three times and streptavidin horseradish peroxidase (100 μL/well of 0.15 μg/ml in blocking solution) applied for one hour. Finally the plates were washed three times and TMB (100 μL) applied followed by 1 M H_2SO_4 (50 μL) to stop the reaction. All samples were measured in triplicate and averaged. The absorbance was converted to a concentration using a standard curve of serial dilutions of venom on a sigmoidal curve.

For measurement of Russell's viper venom in patient serum, samples were prepared as dilutions of 1:80 and 1:400. For measurement of Russell's viper venom in mixtures with antivenom, the samples were prepared by adding a constant amount of Russell's viper venom to serial dilutions of antivenom in blocking solution. The mixtures were allowed to stand for an hour before diluting and applying to the plate. The venom specific ELISA for brown snake venom was the same but used anti-*P. textilis* venom IgG antibodies.

2.3.2. ELISA for antivenom

The procedure for measuring antivenoms was similar to the venom specific ELISA but the plate was initially coated with either Russell's viper venom (for Indian antivenom) or brown snake venom (for brown snake antivenom). Detection was with horseradish peroxidase labelled anti-horse antibodies (1 μg/ml). Samples were measured at an initial dilution of 1:3000.

2.3.3. ELISA for venom–antivenom (VAV) complexes

The measurement of VAV has been recently described (O'leary and Isbister, 2014). In brief the plates are prepared in the same way as for venom measurement by coating with rabbit anti-venom IgG antibodies. However, the detection is the same as for antivenom measurement using horseradish peroxidase labelled anti-horse antibodies (1 μg/ml). Samples were measured at a dilution of 1:80.

2.4. Preparation of UltraLink resins-anti-horse (ULaH) antibodies

A suspension of the UltraLink resin and anti-horse antiserum (ULaH) was made up by binding anti-horse antiserum (6 mg) to UltraLink resin (77 mg) following the manufacturer's directions. Protein measurement of the supernatant demonstrated that there was greater than 85% binding to the UltraLink resin. The ULaH was then made up to a total volume of 1.7 ml suspension in PBS and stored at 4 °C.

A suspension of the UltraLink resin and anti-*D. russelii* venom IgG antibodies (ULaRVV) was made up by binding anti-*D. russelii* venom IgG (1.6 mg) to UltraLink resin (40 mg) following manufacturer's directions. Protein measurement of supernatant demonstrated 75% binding to the resin to have occurred. The

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