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Diagnosis of snake envenomation using a simple phospholipase A₂ assaySUBJECT AREAS:
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BIOMARKER RESEARCHKalana Maduwage^{1,2}, Margaret A. O'Leary³ & Geoffrey K. Isbister^{1,3}Received
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requests for materials
should be addressed to
G.K.I. (geoff.isbister@
gmail.com)

¹School of Medicine and Public Health, University of Newcastle, NSW 2308, Australia, ²Department of Biochemistry, Faculty of Medicine, University of Peradeniya, Sri Lanka, ³Department of Clinical Toxicology and Pharmacology, Calvary Mater Newcastle, Waratah, NSW 2298, Australia.

Diagnosis of snake envenomation is challenging but critical for deciding on antivenom use. Phospholipase A₂ enzymes occur commonly in snake venoms and we hypothesized that phospholipase activity detected in human blood post-bite may be indicative of envenomation. Using a simple assay, potentially a bedside test, we detected high phospholipase activity in sera of patients with viper and elapid envenomation compared to minimal activity in non-envenomed patients.

Snake envenomation is considered a major medical and public health problem in the rural tropical world¹. Each year there are 1 to 2 million cases of snake envenomation and potentially up to 100,000 deaths worldwide. Despite the burden of illness, the treatment of snake envenomation remains problematic. There is limited availability of antivenom in some countries, unacceptably high reaction rates to antivenom, and difficulties in diagnosing envenomation to allow early antivenom treatment in patients with definite envenomation²⁻⁴. A key issue for improving antivenom treatment in snake envenomation is to have a rapid and accurate test to determine if patients are envenomed and require antivenom⁵. Unfortunately the majority of clinical features and laboratory investigations indicative of envenomation only occur in established envenomation, where antivenom may be of limited or no benefit. It is therefore necessary to develop an early diagnostic test for envenomation that can be done at the bedside and is cheap and available in resource poor settings. Such tests have been developed for poisoning in the resource poor setting^{6,7}.

Phospholipase A₂ (PLA₂) is a common toxin/component in snake venoms including in almost all elapid and viperid venoms⁸⁻¹⁰. Numerous studies have tested PLA₂ activity in snake venoms and it is arguably a standard test when investigating venom activity. However, no studies have tested for PLA₂ activity in the blood of patients with snake envenomation. Although PLA₂ activity is unlikely to represent clinically important toxicity, it indicates that there is venom present in blood, and that there is likely to be systemic envenomation. We aimed to test the hypothesis that envenomed patients would have measurable PLA₂ activity in their blood compared to minimal activity in non-envenomed patients as the basis for a bedside test to diagnose snake envenomation.

Results

There were pre-antivenom samples for 32 patients with Russell's viper (*Daboia russelii*) envenomation, 35 with hump-nosed pit viper (*Hypnale hypnale*) envenomation, 3 with Indian cobra (*Naja naja*) envenomation, 2 with Indian krait (*Bungarus caeruleus*) envenomation, 5 with red-bellied black snake (*Pseudechis porphyriacus*) envenomation and 31 non-envenomed patients. PLA₂ activity was detected in pre-antivenom sera in all patients with snake envenomation. The median PLA₂ activity was 55.7 μmol/ml/min (95% percentiles: 18.0 to 226.2) for Russell's viper envenomation, 13.6 μmol/ml/min (95% percentiles: 9.7 to 24.5) for hump-nosed viper envenomation, 14.8 μmol/ml/min (11.3 to 200.2) for cobra, 17.2 μmol/ml/min (15.8 to 18.7) for krait, and 98 μmol/ml/min (43 to 281) for black snake which were significantly different to non-envenomed patients (Median: 6.0 μmol/ml/min; 95% percentiles 2.3 to 8.4) [Fig. 1].

PLA₂ activity was compared to venom specific enzyme immunoassays (EIA) for three of the snake venoms. There was good correlation between venom specific EIA and PLA₂ activity in the sera of patients with Russell's viper envenomation ($r = 0.61$; $p = 0.0002$), hump-nosed pit viper envenomation ($r = 0.49$; $p = 0.003$) and in multiple samples from the 5 black snake envenomations ($r = 0.95$; $p < 0.0001$) [Fig. 2].

Venom specific EIA rapidly decreases after antivenom administration and the absence of detectable free venom indicates that sufficient antivenom has been administered¹¹. A similar phenomenon occurred with PLA₂ activity which decreased after antivenom administration and increased with apparent venom recurrence in Russell's viper

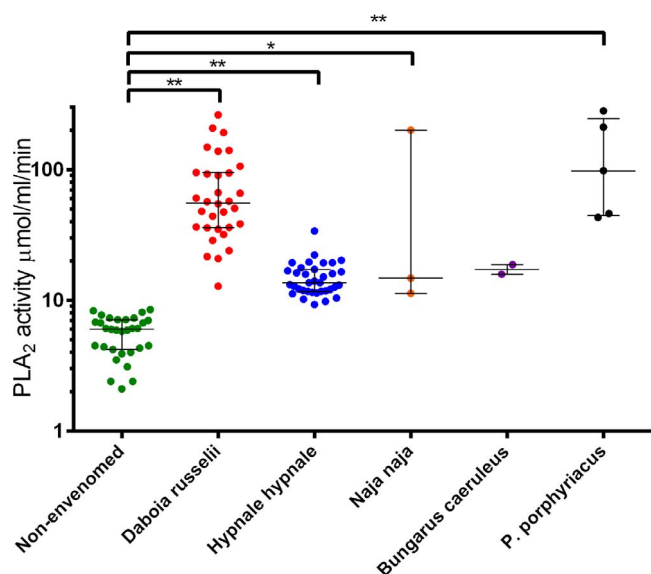


Figure 1 | Scatterplot of the phospholipase A_2 activity ($\mu\text{mol/ml/min}$) for non-venomated patients (green circles) versus patients with Russell's viper envenomation (*D. russelii*; red circles), hump-nosed viper envenomation (*H. hypnale*; blue circles), Indian cobra envenomation (*N. naja*; orange circles), Indian krait (*B. caeruleus*; purple circles) and red-bellied black snake (*P. porphyriacus*; black circles). The 6 groups had significantly different phospholipase A_2 activities ($p < 0.0001$; Kruskal-Wallis) and each snake envenomation group's phospholipase A_2 activity was significantly different to non-venomated samples by Dunn's multiple comparisons [shown with brackets; * = $p < 0.05$, ** = $p < 0.0001$].

envenomation and red-bellied black snake envenomation (Fig 3.). PLA_2 appears to mirror the free venom concentrations and is another potential way to assess the efficacy of antivenom *in vivo*. Interestingly this differs to a previous study where antibodies to the phospholipase did not prevent hydrolysis of a different small chromogenic substrate¹².

Discussion

Currently no single laboratory investigation or bedside test exists to identify patients with systemic envenomation and the diagnosis involves a combination of clinical features and investigations^{3,11}. This usually requires a level of medical knowledge to make the diagnosis and is difficult in resource poor settings. The 20 minute whole blood clotting test has been used as a simple bedside test to identify coagulopathy worldwide^{13,14}. However, it has recently been shown to have a poor sensitivity in the clinical setting⁵ and is of no value for snakes that do not cause a coagulopathy such as kraits and cobras. The PLA_2 assay was positive in both coagulopathic snakes (Russell's viper and Hump-nosed viper) and neurotoxic snakes (kraits and cobras) in our sample of bites by Australian and Asian snakes. A simple bedside version of the PLA_2 assay would therefore be a useful test in resource poor areas with limited access to medical care.

PLA_2 activity in human sera will not determine the snake species responsible for envenomation, but will simply identify that the patient has systemic envenomation. The majority of snake antivenoms that are available are polyvalent so a test that simply indicates that the patient has snake envenomation is sufficient so that polyvalent antivenom can be administered.

The PLA_2 assay may also be particularly useful in neurotoxic envenomation to diagnose systemic envenomation early, before irreversible neurotoxicity develops. Most neurotoxicity is due to presy-

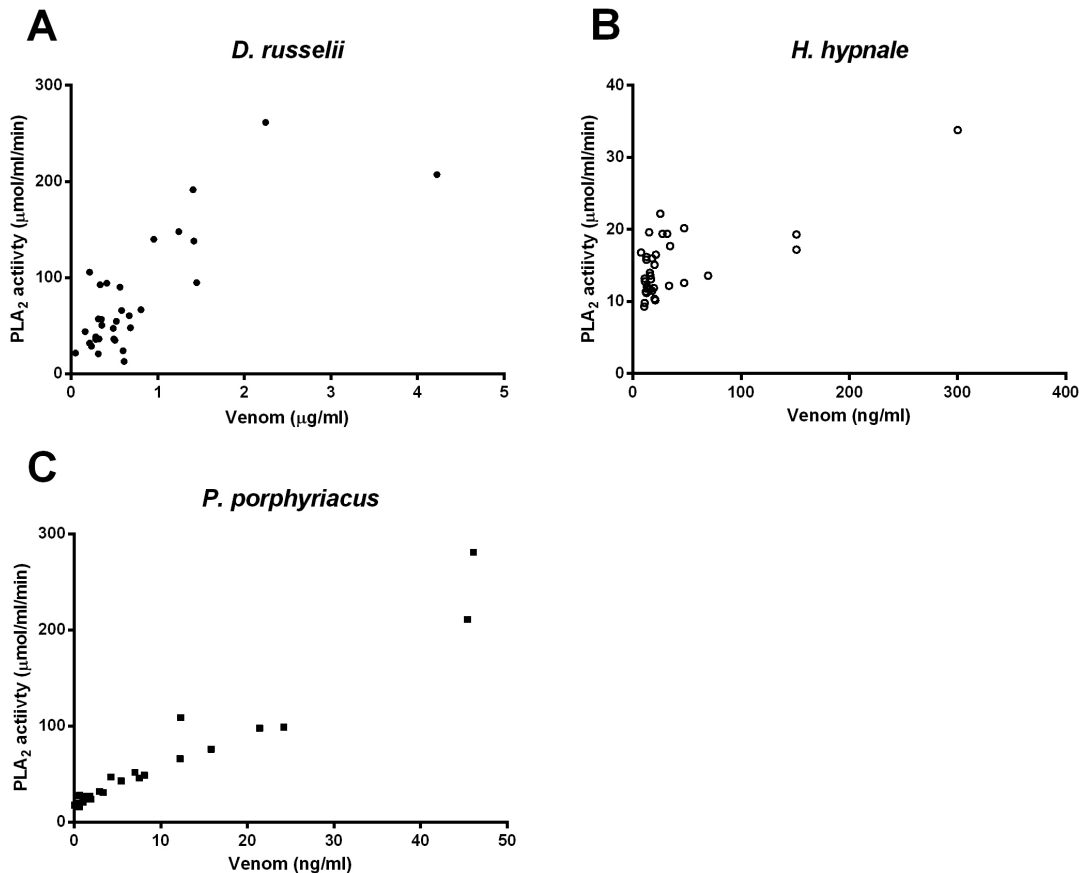


Figure 2 | Plots of phospholipase A_2 activity against venom concentration for samples from patients with envenomation from Russell's viper (*D. russelii*; Panel A), hump-nosed viper (*H. hypnale*; Panel B) and red-bellied black snake (*P. porphyriacus*; Panel C).

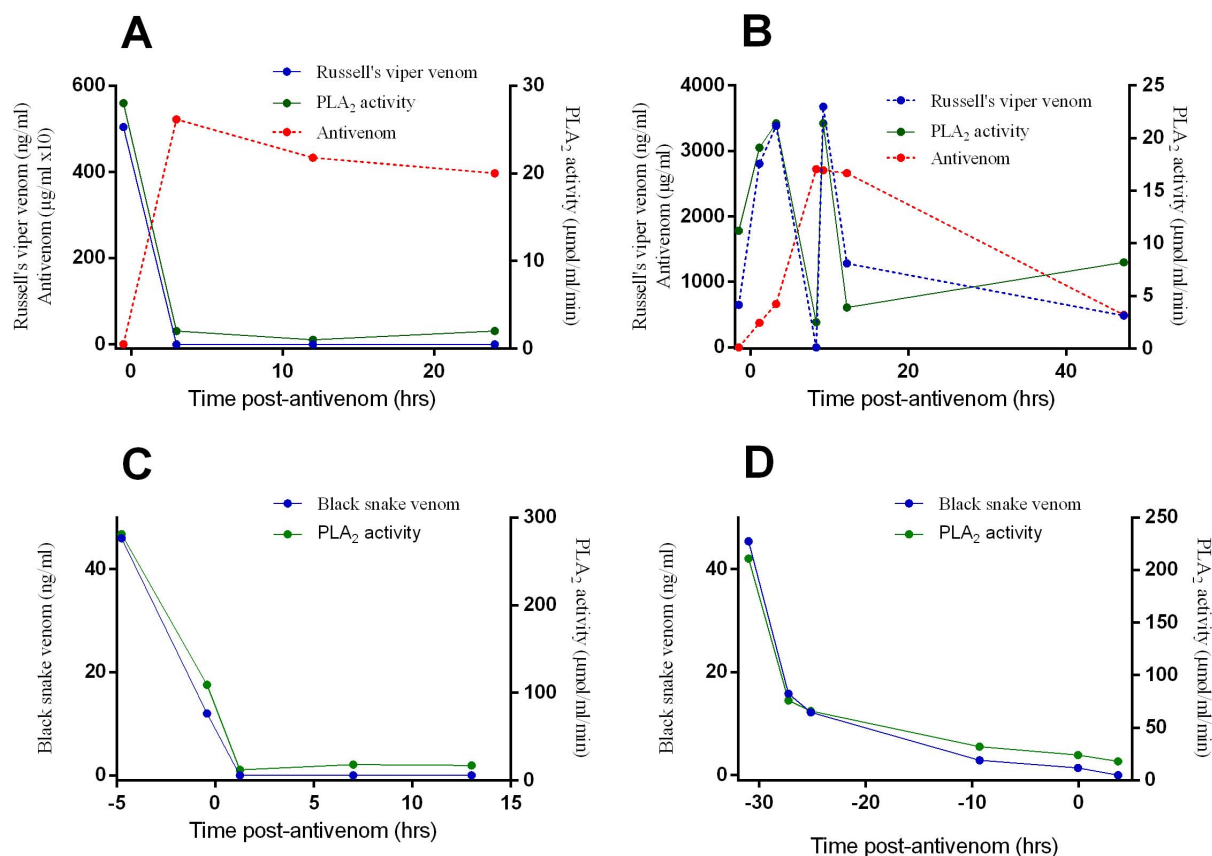


Figure 3 | Plots of free venom concentration (ng/ml; blue circles and lines), phospholipase A_2 activity ($\mu\text{mol/ml/min}$; green circles and lines) and antivenom concentrations ($\mu\text{g/ml}$; red circles and dashed lines) versus time in a patient with Russell's viper envenomation where venom was undetectable post-antivenom (Panel A), a patient with Russell's viper envenomation where there was venom recurrence post-antivenom (Panel B), a patient with red-bellied black snake envenomation given early antivenom (Panel C) and a patient with red-bellied black snake envenomation given late antivenom (Panel D).

naptic neurotoxins (e.g. β -bungarotoxin in krait; *Bungarus* spp.), which cause irreversible nerve injury that can only be prevented and not reversed by antivenom. Once paralysis develops antivenom is ineffective. Venom is injected at the bite site and moves rapidly to the circulation. However, the onset of clinical neurotoxicity occurs over hours because it takes further time for the neurotoxins to distribute to the neuromuscular junctions and bind to the presynaptic membrane. It is therefore likely that PLA_2 activity can be measured in patient serum within 30 to 60 minutes of the bite as venom enters the circulation. This would then allow patients with systemic envenomation to be identified rapidly and given antivenom prior to the development of neurotoxicity.

In this study we assessed the PLA_2 activity of only a small number of snakes. In addition, we did not compare the increase in PLA_2 in snake envenomation patients to patients with acute pancreatitis, sepsis and rheumatoid arthritis^{15,16}, which may cause false positive results. This may not be a major problem because the test would be used in cohorts of patients bitten by snakes where the pre-test probability for snake envenomation is high. In contrast the test would be of much less value in cohorts of patients with suspected bites or clinical features where false positives would be more problematic. Larger cohorts of patients are required to determine if this is a major problem. There would be huge benefits for a PLA_2 test because it would allow for fast, simple and easy identification of systemic envenomation, so that antivenom can be given. The PLA_2 kit used in this study requires a microplate reader and is designed to run tests as batches making it expensive for testing when the patient presents. Several methods have been used to measure PLA_2 activity in serum samples^{12,17,18}. It would therefore

be feasible to develop a PLA_2 assay that is inexpensive, allows individual tests to be done and can be done at the bedside or in a basic laboratory setting.

Methods

Patients with Russell's viper (*Daboia russelii*), hump-nosed pit viper (*Hypnale hypnale*), cobra (*Naja naja*) and krait (*Bungarus caeruleus*) envenomation were recruited prospectively from snakebites presenting to Chilaw Hospital (Western Province) and Polonnaruwa Hospital (North Central Province) in Sri Lanka between 2007 and 2011 as part of prospective cohort studies and randomised controlled trials³. The studies were approved by the Ethical Review Committee, Faculty of Medicine, University of Colombo. Patients with no evidence of envenomation but who had been bitten by a snake were also recruited. In addition, cases of black snake (*Pseudechis porphyriacus*) were included from the Australian snakebite project¹⁹. The design of the Australian snakebite project has previously been described in detail and approval has been obtained from the Hunter New England Area Health Human Research Ethics Committee and nineteen other Human Research and Ethics Committees covering all institutions involved around Australia^{19,20}. Informed consent was obtained from all patients and the experiments were undertaken in accordance with the National Health and Medical Research Council guidelines.

Clinical data and serial serum and citrate samples were collected for all snakebites. Serum samples were tested with venom specific enzyme immunoassay (EIA) for Russell's viper venom and hump-nosed pit viper venom for Sri Lankan cases, and black snake venom for Australian cases, to confirm envenomation^{3,19}. Clotting studies on citrate samples and clinical data were used to confirm systemic envenoming^{3,21,22}. Non-envenomed cases were defined as patients with a suspected snakebite where no venom was detected in serum samples, clotting studies were normal and the patient remained asymptomatic.

Serum samples were analysed for PLA_2 activity by Cayman s PLA_2 assay kit (#765001 Cayman Chemical Company, USA) according to manufacturer instructions. We compared the PLA_2 activity of Russell's viper, hump-nosed pit viper and black snake envenomed patients with non-envenomed patients. Furthermore, we investigated the correlation between PLA_2 activity and venom specific EIA



concentration in three different snakes. In selected patients we measured serial serum samples for PLA₂ activity, including before and after antivenom.

The PLA₂ activities of patient sera from different snake groups and the non-venomated snake bites were compared with the non-parametric Kruskal-Wallis test and multiple comparisons with Dunn's test. Correlation between PLA₂ activity and venom concentrations was compared with Spearman's test. Data is presented as medians and interquartile ranges or 95% percentiles, and ranges.

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Author Contributions

K.M. conducted the laboratory experiments, analysed the data and drafted the manuscript; M.A.O. assisted with the laboratory experiments; G.K.I. designed the study, assisted in data analysis and edited the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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