Investigation of the coagulant effects of Sri Lankan snake venoms and the efficacy of antivenoms

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Bachelor of Medicine and Bachelor of Surgery (MBBS), Master of Philosophy (MPhil)

Thesis submitted in fulfilment of the requirements for obtaining the degree of

DOCTOR OF PHILOSOPHY

in Clinical Pharmacology

School of Medicine and Public Health

University of Newcastle, Australia

February 2016

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Maduwage Kalana Prasad

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Maduwage Kalana Prasad

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I hereby certify that this thesis is in the form of a series of published papers of which I am a joint author. I have included as part of the thesis a written statement from each co-author, endorsed by the Faculty Assistant Dean (Research Training), attesting to my contribution to the joint publications.

Maduwage Kalana Prasad

DEDICATION

I dedicate this thesis to

My beloved parents

Without whom, this could never occurred.

I also dedicate this thesis to

Rohan Pethiyagoda and his family

For their invaluable support throughout my higher studies

and constantly being there for me at time of needs.

ACKNOWLEDGEMENTS

Firstly, I must thank my loving wife Amali, for her dedication to keeping my family life well balanced with my daughter Kalani and my son Inuk, and her kind toleration of my academic life.

Secondly, I thank Aus-Aid Scholarship program, Department of Foreign Affairs and Trade, Government of Australia for supporting my PhD studies at the University of Newcastle, Australia and their leadership development program.

Geoff, I must thank you as my principal supervisor. Thank you so much, not only for taking me on as your student, but for your guidance and inspiration on envenoming research. I have learnt so many things from you, not only science but also leadership qualities and tips of having a balanced life between academics and the family. The atmosphere you created makes for an easy work environment for me to test some of my free ideas on research. Finally, thank you for hosting me in your place during my last three months of the studies with your lovely wife Analee and all your children.

Wayne, as my co-supervisor, I thank you for all your support and guidance over the past four years specially when I was working at your Monash lab.

Margaret, you made my experiments meaningful. I consider you as my unofficial supervisor, who closely supervised my experiments to the finest level and built up my laboratory skills. I leant a lot from you not only about experiments, but also some great attitudes a scientist should develop.

During my whole research career, you were with me from my childhood, Anjana, I thank you for your support and encouragements throughout my studies. Dear sir, Rohan Pethiyagoda, Janaki Pethiyagoda, Ruan and Sarith, I appreciate your enormous support towards my family during our whole stay in Australia constantly being there for us at times of need.

Renai Kearney, as the senior research manager of Clinical Toxicology Research Group, I appreciate your invaluable help throughout my studies. I thank all of the staff of the Clinical Toxicology Research Group, University of Newcastle, including Nicole Ryan, Joyce Cooper, Shane Jenkins, Jennifer Robinson, Leonie Calver, Anna Saiao and Candice Roux for their valuable support during my studies.

As my scholarship liaison officers at the University of Newcastle, Lynne Williams and Anna Carlstrom, I appreciate your support during my studies.

I acknowledge Professor Nick Buckley, Professor Andrew Dawson, Professor Indika Gawarammana, Dilani Pinnaduwa, Fahim Mohamad, Seyed Shahmy, Fathima Shihana for their support with organizing data and samples from Sri Lankan hospitals. I thank the Vice Chancellor, University of Peradeniya, Dean Faculty of Medicine, Heads of the Department of Biochemistry (Professor R. Sivakanesan, Dr P.H.P. Fernando and Professor Shirani Ranasinghe) for all their support. Sanath Valaratne and Amal Wijesekara I appreciate your help on several ways during my study period. I thank Professor S. A. M Kularatne for his help in various ways during the candidature.

I acknowledge the Director General and the staff, Department of Wildlife, Sri Lanka for granting permission to conduct these studies.

I express my heartiest acknowledgement to Mr Dharma Sri Kandamby, former curator, National Maritime Museum, Galle for inspiring me about science of snakes.

Finally, I acknowledge the assistance of the medical and nursing staff at Chilaw hospital, Central West Province and Base Hospital Polonnaruwa Sri Lanka for identifying patients for studies and patients who consented for these studies.

LIST OF PUBLICATIONS INCLUDED AS PART OF THIS THESIS

1. 2013. **Kalana Maduwage**, Fiona E. Scorgie, Seyed Shahmy, Fahim Mohamed, Chandana Abeysinghe, Harendra Karunathilake, Lisa F. Lincz, Christeine A. Gnanathasan, Geoffrey K. Isbister. Factor deficiencies in Hump-nosed pit viper (*Hypnale hypnale*) envenoming. *Clinical Toxicology* 51: 527-31.

2. 2015. Isbister GK, **Maduwage K**, Scorgie FE, Shahmy S, Mohamed F, Abeysinghe C, et al. Venom Concentrations and Clotting Factor Levels in a Prospective Cohort of Russell's Viper Bites with Coagulopathy. *PLoS Neglected Tropical Diseases* 9(8): e0003968. doi:10.1371/journal.pntd.0003968.

3. 2014. **Maduwage K**, O'Leary MA, Scorgie FE, Shahmy S, Mohamed F, et al. Detection of Venom after Antivenom Is Not Associated with Persistent Coagulopathy in a Prospective Cohort of Russell's Viper (*Daboia russelii*) Envenomings. *PLoS Neglected Tropical Diseases* 8(12): e3304. doi:10.1371/journal.pntd.0003304.

4. 2014. **Maduwage K**, O'Leary M, Isbister GK. Diagnosis of snake envenomation using a simple Phospholipase A_2 assay. *Nature Scientific Reports* 4 (4827): DOI:10.1038/srep04827 (2014).

5. 2015. **Maduwage KP**, Scrogie FE, Lincz LF, O'Leary MA, Isbister GK. Procoagulant snake venoms have differential effects in animal plasmas: Implications for antivenom testing in animal models, *Thrombosis Research*, 137; 174–177.

6. 2016. **Maduwage K,** Silva A, O'Leary M, Hodgson WC, Isbister GK. Efficacy of Indian polyvalent snake antivenoms against Sri Lankan snake venoms: lethality studies or clinically focussed *in vitro* studies. *Nature Scientific*. *Reports*. **6**, 26778; doi: 10.1038/srep26778 (2016).

7. 2014. **Maduwage K**, Buckley NA, de Silva HJ, Lalloo DG, Isbister G. Snake antivenom for snake venom induced consumption coagulopathy (Protocol). *Cochrane Database of Systematic Reviews* 2014, Issue 12. Art. No.: CD011428. DOI: 10.1002/14651858.CD011428.

8. 2015. **Maduwage K**, Buckley NA, de Silva HJ, Lalloo DG, Isbister GK. Snake antivenom for snake venom induced consumption coagulopathy (Review). *Cochrane Database of Systematic Reviews*, Issue 6. Art. No.: CD011428. DOI: 10.1002/14651858.CD011428.pub2.

9. 2014**. Maduwage K**, Isbister GK. Current Treatment for Venom-Induced Consumption Coagulopathy Resulting from Snakebite. *PLoS Neglected Tropical Diseases*. 8 (10): e3220. doi:10.1371/journal.pntd.0003220.

LIST OF OTHER PUBLICATIONS DURING CANDIDATURE

(Not included as a part of the thesis)

1. 2015. Isbister GK, **Maduwage K**, Saiao A, Buckley NA, Jayamanne SF, Seyed S, et al. Population Pharmacokinetics of an Indian F(ab')2 Snake Antivenom in Patients with Russell's Viper (*Daboia russelii*) Bites. *PLoS Neglected Tropical Diseases* 9(7): e0003873. doi:10.1371/journal.pntd. 0003873.

2. 2015. O'Leary MA, **Maduwage K**, Isbister GK. Detection of venom after antivenom administration is largely due to bound venom. *Toxicon*. 93: 112-115.

3. 2014. Isbister GK, **Maduwage K**, Page CB. Antivenom cross neutralisation in a suspected Asian pit viper envenoming causing severe coagulopathy. *Toxicon* 90: 286-90.

4. 2013. Isbister GK, **K. Maduwage**, S. Shahmy, F. Mohamed, C. Abeysinghe, H. Karunathilake, CA Ariaratnam, NA Buckley. Diagnostic 20-min whole blood clotting test in Russell's viper envenoming delays antivenom administration. *Quarterly Journal of Medicine* 106(10):925-32.

5. 2013. O'Leary M, **Maduwage K**, Isbister, GK. Use of immunoturbidimetry to detect venom-antivenom binding using snake venoms. *Journal of Pharmacology and Toxicological Methods* 67: 171-181.

6. 2013. **Maduwage K**, Isbister GK, Silva A, Bowatta S, Mendis S, Gawarammana I. Epidemiology and clinical effects of Hump-nosed pit viper (Genus: *Hypnale*) envenoming in Sri Lanka *Toxicon* 61: 11-15.

7. 2016. **Maduwage K**, O'Leary M, Silva A, Isbister GK. Detection of Snake Venom in Post-Antivenom Samples by Dissociation Treatment Followed by Enzyme Immunoassay *Toxins 8*, 130; doi:10.3390.

CONFERENCE PRESENTATIONS DURING CANDIDATURE

1. 2015. Snake antivenom for snake venom induced consumption coagulopathy. *Cochrane Database of Systematic Reviews*. Asia Pacific Association of Medical Toxicology (APAMT), Perth, Australia. (Poster presentation). Conference proceedings, page 56.

2. 2015. Effect of pro-coagulant snake venoms on different animal plasma. Asia Pacific Association of Medical Toxicology (APAMT), Perth, Australia. (Platform presentation). Conference proceedings, page 25.

3. 2015. Snake antivenom for snake venom induced consumption coagulopathy. *Cochrane Database of Systematic Reviews* Australian Society for Medical Research (ASMR) conference, Sydney, Australia (Poster presentation). Conference proceedings, page 40.

4. 2015. Effect of pro-coagulant snake venoms on different animal plasma. Australian Society for Medical Research (ASMR) conference, Hunter Medical Research Institute, Newcastle (March) Australia (Poster presentation). Conference proceedings, page 25.

5. 2015. Efficacy of two Indian polyvalent snake antivenoms against coagulopathy and neurotoxicity of Sri Lankan snake venoms. Australian Society for Medical Research (ASMR) conference, HMRI, Newcastle (March) Australia (Platform presentation). Conference proceedings, page 24.

6. 2014 Diagnosis of snake envenoming using simple phospholipase A_2 assay. North American Centre for Clinical Toxicology (NACCT) conference, New Orleans, USA (Platform presentation, (**Informa Health Care Award for 2015 for best Platform presentation**). *Clinical Toxicology*, 52, page 686.

7. 2014 Diagnosis of snake envenoming using simple phospholipase A_2 assay. The Australian Society for Medical Research New South Wales conference, Sydney Australia. (Poster presentation, (**ASMR Award for Best overall scientific presentation**). Conference proceedings, page 49.

8. 2014. Measurement of venom and clotting functions of Russell's viper envenoming and response to antivenom treatment. European Association of Poisons Centres and Clinical Toxicology (EAPCCT), Brussels, Belgium (Platform presentation). *Clinical Toxicology* (Supplement) 52; 347.

9. 2014 Diagnosis of snake envenoming using simple phospholipase A_2 assay. Conference of Toxicology and Poisoning network of Australasia (TAPNA), Newcastle, Australia (Platform presentation). Conference proceedings, page 15.

10. 2014 Diagnosis of snake envenoming using simple phospholipase A_2 assay. The Australian Society for Medical Research Satellite Scientific Meeting, Hunter Medical Research Institute, Newcastle, Australia. (Poster presentation, **John Morris Scientific Award for the Best overall poster presentation**). Conference proceedings, page 34.

11. 2013. Venom recurrence in Russell's viper (*Daboia russelii*) envenoming in Sri Lanka. 12th Asia Pacific Association of Medical Toxicology Conference (APAMT), Dubai (Platform presentation). Conference proceedings, page 94.

12. 2013. Assessment of efficacy and effectiveness of snake antivenoms in Asia. 12th Asia Pacific Association of Medical Toxicology Conference (APAMT), Dubai, (**Keynote presentation**). Conference proceedings, page 43.

13. 2013. Assessing the efficacy of antivenoms for Sri Lankan venomous snakes. Conference of Toxicology and Poisoning network of Australasia (TAPNA), Newcastle, Australia. (**Keynote presentation**). Conference proceedings, page 14.

14. 2012. Clotting studies and factor deficiencies of Hump-nosed pit viper (*Hypnale hypnale*) envenoming in Sri Lanka, 11th Asia Pacific Association of Medical Toxicology Conference, Hong-Kong (Platform presentation). Hong Kong Journal of emergency Medicine. 19 (6); 427.

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SYNOPSIS

Coagulopathy is the commonest systemic effect of snake envenoming. Despite this there is limited information on the severity and time course of venom-induced consumption coagulopathy (VICC) and the effect of antivenom. Evidence of the efficacy and effectiveness of antivenom is vital to continue antivenom treatment for envenoming. There is increasing evidence that early administration of antivenom is essential, but there is a lack of diagnostic tests of envenoming that can be used to decide on antivenom administration.

The broad aim of this project was to investigate the procoagulant effects of Sri Lankan snake venoms, and the efficacy and effectiveness of antivenoms against these effects. In addition, the study aimed to explore novel methods of testing for envenoming and for the presence of venom in blood.

Snake envenoming cases in Sri Lanka were used with the collection of serial clinical and laboratory data, and blood samples from patients admitted to hospitals in Sri Lanka. Coagulopathy from hump-nosed pit viper and Russell's viper envenoming was investigated by analyzing citrated samples from envenomed patients. Identification of the snake species was by venom specific sandwich enzyme immunoassay (EIA). Antivenom efficacy was assessed in a series of *in-vitro* and *in-vivo* animal studies. Antivenom effectiveness was assessed by undertaking two systematic reviews: Cochrane review of placebo randomized controlled trials and a systematic review of prospective and other controlled trials of antivenom for VICC.

The results provide a much better description of VICC using clotting times and factor levels in both hump-nosed pit viper and Russell's viper envenoming, showing prolonged clotting times and different factor deficiencies. Phospholipase A_2 enzyme levels were investigated as a diagnostic test for snake envenoming and will be key to improving outcomes in snake bite cases as it will allow early identification of envenomed patients so antivenom can be given when it is most effective. The efficacy of two Indian antivenoms was assessed, which showed one to be more efficacious but more importantly explored the difference between lethality studies and clinically focused *in vitro* studies. Two systematic reviews and antivenom for VICC revealed a lack of placebo controlled randomized trials, but that some comparative clinical trials and observational studies provide information on the effectiveness of antivenom.

OVERVIEW

Aims and Objectives

The broad aims of this project were to investigate the coagulant effects of venoms from Sri Lankan snakes, and to investigate diagnostic tests for snake envenoming, as well as the efficacy and effectiveness of antivenoms. To fulfil these aims, the proposed PhD study program consisted of three main study themes.

1. A description of the coagulopathy in hump-nosed pit viper and Russell's viper envenoming including clotting times and clotting factor concentrations.
2. Development of diagnostic tests for envenoming.
3. Investigation of the efficacy and effectiveness of snake antivenoms

Methodology

Ethical approval for demographic data, clinical data and the human blood sample collection from Sri Lankan snake envenomed patients was sought and approval granted by the Ethical Review Committee, Faculty of Medicine, University of Peradeniya, Sri Lanka (approval number: 2011/EC/46 and 2008/EC/26) and and Animal Ethical Review Committee (2012/008), Monash University, Australia. Ethical approval was granted by the University of Newcastle, Australia, (approval number: H2010-1060) for the work done at the University of Newcastle. Informed written consent was obtained from all patients who provided blood samples and clinical information. All subjects had the right to withdraw their participation at any time during the study if they wished.

The majority of the data used for the thesis was from snake envenoming cases in Sri Lanka. This involved the collection of serial clinical and laboratory data, and blood samples from patients admitted to hospitals in Sri Lanka with snake bites. The investigation of coagulopathy in Sri Lankan snake envenoming (Hump-nosed pit viper and Russell's viper) was undertaken by analyzing citrated samples from envenomed patients, including measuring clotting times and clotting factor levels. Development of a diagnostic test for snake envenoming used the same serial blood collection, but used serum samples in which venom concentrations and phospholipase A_2 enzyme levels were measured. Identification of the snake species involved in cases was by a venom specific sandwich enzyme immunoassay (EIA), which has been used widely in the

past to confirm snake identity. Antibodies to Sri Lankan snake venom were prepared especially for this project.

A major issue with evaluating the benefits of antivenom is separating the assessment of the 'efficacy' and 'effectiveness' of antivenoms. We defined the 'efficacy' of antivenom as the ability to bind and neutralise venom-mediated effects under ideal conditions (*in vitro* studies and animal studies of binding and neutralisation) and the 'effectiveness' of antivenom as its ability to reverse or prevent envenoming in human patients and ultimately improve patient outcomes (Isbister, 2010a). Antivenom efficacy was assessed in a series of *in vitro* and *in vivo* animal studies: measurement of the *in vitro* ability of antivenom to bind venom; measurement of antivenom neutralization of *in vitro* coagulopathy and neurotoxicity; and the measurement of the lethal dose 50 (LD_{50}) and effective dose 50 (ED_{50}) in mice. Antivenom effectiveness for the treatment of coagulopathy was assessed by undertaking two systematic reviews: 1) a Cochrane review of placebo randomized controlled trials of antivenom for venom-induced consumption coagulopathy (VICC); and 2) a systematic review of prospective and other controlled trials of antivenom for VICC.

To fulfil these aims, the PhD program consisted of nine chapters covering three sections of study as follows:

1. Coagulopathy in snake envenoming:
    a. Investigate the coagulopathy of hump-nosed pit viper envenoming in Sri Lanka
    b. Investigate the coagulopathy of Sri Lankan Russell's viper envenoming
    c. Explore the correlation between recurrence of venom and the coagulopathy in Russell's viper envenoming

2. Diagnostic tests of snake envenoming:
    a. Investigate the use of snake venom phospholipase A_2 enzyme levels as a diagnostic test of snake envenoming.

3. Efficacy and effectiveness of snake antivenom
    a. Assess the efficacy of Indian snake antivenoms in binding Sri Lankan snake venoms in neutralising coagulopathy and neurotoxicity they cause and preventing lethality.
    b. Explore the susceptibility of various animal plasmas to different procoagulant snake venoms.

c. Cochrane systematic review of snake antivenom for venom-induced consumption coagulopathy.
d. Systematic review of other study designs for antivenom in snake venom induced consumption coagulopathy.

Outcomes

The results of this thesis will provide a much better description of venom-induced consumption coagulopathy using clotting times and factor levels in both hump-nosed pit viper and Russell's viper envenoming. This will provide the basis for evaluating the effectiveness of treatments in VICC, including the use of antivenom and factor replacement. Development of phospholipase A_2 enzyme levels as a diagnostic test for snake envenoming is a key to improving outcomes in snake bite because it will allow the early identification of envenomed patients so that antivenom can be given when it is most effective. Assessment of the efficacy and the effectiveness of antivenoms will provide evidence for the use of antivenom in snake bite and therefore improve clinical outcomes. Understanding the difference between efficacy and effectiveness will result in antivenom being used where it is going to produce the most benefit. In this thesis the efficacy of two Indian antivenoms, VINS and BHARAT, was assessed against four Sri Lankan snake venoms. An important issue that arose during these studies was the role of animals in assessing efficacy for human envenoming, and in particular against coagulopathy. It was found that different animal plasmas had different susceptibility to procoagulant snake venoms, and that only rabbit plasma had a similar susceptibility to human plasma. A systematic review of both placebo randomized controlled trials, comparative clinical trials and observational studies was used to assess the effectiveness of antivenom for VICC.

Link to publications

1. 2013. **Maduwage K**, Scorgie FE, Shahmy S, Mohamed F, Abeysinghe C, Karunathilake H, Lincz LF, Gnanathasan CA, Isbister GK. **Factor deficiencies in Hump-nosed pit viper (*Hypnale hypnale*) envenoming**. *Clinical Toxicology* 51: 527-31.

Although hump-nosed pit viper bites are the commonest cause of snake bite in Sri Lanka, there is limited information on the coagulopathy that occurs. In this paper we measured serial clotting time tests and clotting factor levels of proven hump-nosed pit

viper envenomings in Sri Lanka to understand the effect of venom on the clotting cascade. The study describes the pattern and severity of factor deficiencies and clotting times to better characterise the severity of hump-nosed viper coagulopathy and the potential pathophysiology.

2. 2015. Isbister GK, **Maduwage K**, Scorgie FE, Shahmy S, Mohamed F, Abeysinghe C, Karunathilake H, O'Leary MA, Gnanathasan CA, Lincz LF. **Venom Concentrations and Clotting Factor Levels in a Prospective Cohort of Russell's Viper Bites with Coagulopathy**. *PLoS Neglected Tropical Diseases* 9(8): e0003968. doi:10.1371/journal.pntd.0003968.

Few studies have been published on the severity and time course of coagulopathy in Russell's viper envenoming, despite procoagulant toxins from Russell's viper venom being well characterised as factor V and factor X activators. The recovery of clotting factor levels and clotting times following antivenom administration was poorly characterised with most studies using whole blood clotting times. This paper reports the initial severity and range of abnormal clotting times and factor levels (fibrinogen, factor V, factor VIII and factor X) and then the recovery of after antivenom treatment. In addition, the study found initially high levels of factor VII, VIII and IX which was unusual and thought to be a result of the toxin interfering with the assays *in vitro*.

3. 2014. **Maduwage K**, O'Leary MA, Scorgie FE, Shahmy S, Mohamed F, Abeysinghe C, Karunathilake H, Lincz LF, Gnanathasan CA, Isbister GK. **Detection of Venom after Antivenom Is Not Associated with Persistent Coagulopathy in a Prospective Cohort of Russell's Viper (*Daboia russelii*) Envenomings**. *PLoS Neglected Tropical Diseases* 8(12): e3304. doi:10.1371/journal.pntd.0003304.

Venom recurrence (i.e. detection of venom following undetectable venom immediately post-antivenom) has been reported after envenoming by a number of different vipers and is believed to be associated with recurrence of envenoming due to insufficient antivenom. It has been reported a number of times with Russell's viper envenoming and its significance remains unclear. This study demonstrates that the detection of venom recurrence is not associated with the recurrence of coagulopathy but does occur in patients with initially higher venom concentrations. The explanation for "venom recurrence" appears to be a result of the venom assay measuring bound venom which is shown in an associated publication (2015. O'Leary MA, **Maduwage K**, Isbister GK. Detection of venom after antivenom administration is largely due to bound venom. *Toxicon*. 93: 112-115.)

4. 2014. **Maduwage K**, O'Leary M, Isbister GK. **Diagnosis of snake envenomation using a simple Phospholipase A2 assay.** *Nature Scientific Reports* 4 (4827): DOI:10.1038/srep04827 (2014).

Currently there is no single test that can be used to diagnose envenoming in snake bite patients. The decision to give antivenom has to be based on a constellation of clinical features and laboratory tests, which in some cases may be falsely negative or falsely positive (e.g. whole blood clotting time for coagulopathy - 2013 Isbister GK, **K. Maduwage**, S. Shahmy, F. Mohamed, C. Abeysinghe, H. Karunathilake, CA Ariaratnam, NA Buckley. Diagnostic 20-min whole blood clotting test in Russell's viper envenoming delays antivenom administration. *Quarterly Journal of Medicine*), or develop too late (signs of paralysis). The ability to give antivenom treatment before the development of clinical effects is vital to prevent irreversible effects. This paper is a proof of concept study that shows the potential use of phospholipase A_2 (PLA_2) enzyme levels to diagnose snake envenoming in a range of Sri Lankan snakes and one Australian snake. There was a highly significant correlation between PLA_2 levels and venom concentrations.

5. 2015. **Maduwage KP**, Scorgie FE, Lincz LF, O'Leary MA, Isbister GK. **Procoagulant snake venoms have differential effects in animal plasmas: Implications for antivenom testing in animal models**, *Thrombosis Research*, 137; 174–177.

Although animal models are routinely used to assess the efficacy of snake antivenom, there is little data to support the relevance of animal studies to human envenoming. There is no published information on the susceptibility of different animal plasmas to the procoagulant toxins in snake venoms. This paper describes the wide variation in the susceptibility of seven different animal plasmas to four different procoagulant snake venoms. It demonstrates the significant potential limitation of using animal models to assess the efficacy of antivenom for venom-induced consumption coagulopathy.

6. 2016. **Maduwage K,** Silva A, O'Leary M, Hodgson WC, Isbister GK. **Efficacy of Indian polyvalent snake antivenoms against Sri Lankan snake venoms: lethality studies or clinically focussed *in vitro* studies**. *Nature Scientific. Reports*. **6**, 26778; doi: 10.1038/srep26778 (2016).

Despite Indian antivenom being used for decades to treat snake bite in Sri Lanka, there are no published studies on the assessment of these antivenoms for Sri Lankan snake venoms. In addition, the World Health Organisation currently recommends ED_{50} studies as the 'gold standard' for testing antivenom efficacy. However, such testing ignores the fact that venoms may affect animals and humans in different ways. This paper assesses the efficacy of two Indian antivenoms against Sri Lankan snake venoms and demonstrates that one is superior to the other based on *in vitro* binding, coagulation and neurotoxicity studies. It also demonstrates that ED_{50} studies were inferior in comparing the efficacy of the two antivenoms and future studies should use binding and clinically relevant *in vitro* studies.

7. 2014. Maduwage K, Buckley NA, de Silva HJ, Lalloo DG, Isbister G. **Snake antivenom for snake venom induced consumption coagulopathy (Protocol).** *Cochrane Database of Systematic Reviews*, Issue 12. Art. No.: CD011428. DOI: 10.1002/14651858.CD011428.

8. 2015. **Maduwage K**, Buckley NA, de Silva HJ, Lalloo DG, Isbister GK. **Snake antivenom for snake venom induced consumption coagulopathy (Review).** *Cochrane Database of Systematic Reviews* 2015, Issue 6. Art. No.: CD011428. DOI: 10.1002/14651858.CD011428.pub2.

A Cochrane systematic review was undertaken to investigate the evidence for antivenom effectiveness in VICC. The study found an absence of published placebo randomized controlled trials of antivenom for VICC.

9. 2014**. Maduwage K**, Isbister GK. **Current Treatment for Venom-Induced Consumption Coagulopathy Resulting from Snakebite**. *PLoS Neglected Tropical Diseases*. 8 (10): e3220. doi:10.1371/journal.pntd.0003220.

This systematic review further investigated the effectiveness of antivenom for VICC but included a broader range of studies and study designs including randomised comparative studies, non-randomised studies comparing antivenom to no treatment

and prospective observational studies. The review found few studies that compare antivenom to no antivenom treatment, and that antivenom appears to be effective for VICC in some snakes (Australian elapids), but not for others (*Echis* spp. – carpet vipers).

LIST OF ABBREVIATIONS

aPTT	Activated partial Thromboplastin Time
ED50	Effective dose 50
EIA	Enzyme immunoassay
FFP	Fresh Frozen Plasma
INR	International Normalized Ratio
LD50	Lethal dose 50
PLA_2	Phospholipase A_2
PT	Prothrombin time
MCD	Minimum Clotting Dose
MDD	Minimum Defibrinogenating Dose
MHD	Minimum Hemorrhagic Dose
MMD	Minimum Myotoxic Dose
MND	Minimum Necrotic Dose
SHR	Systemic Hypersensitivity Reactions
TCT	Thrombin Clotting Time
TLEs	Thrombin like enzymes
VICC	Venom induced consumption coagulopathy
WBCT20	20 Minutes Whole Blood Clotting Test
WHO	World Health Organisation

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Table 12. Clinical effects of hump-nosed pit vipers from two different studies. (Ariaratnam et al., 2008), Maduwage et al., 2013).

Table 13. Summary of clinical effects of Sri Lankan snake envenoming based (Kularatne 2003, 2009; Ariartnam et al, 2008; Kularatne et al, 2008; Kularatne et al., 2011; Maduwage et al., 2013 and Gnanathasan et al., 2012).

Table 14. Different preclinical antivenom assessment tests (Anonymous, 2010, Gutierrez et al., 1985, Theakston and Reid, 1983, Laing et al., 1992, Jene et al., 1989, Gutierrez et al., 1992, Chettya et al., 2004).

Chapter One

Literature review

1. LITERATURE REVIEW

Search strategy for the literature review

MEDLINE (1946 to November 2015), EMABSE (1947 to November 2015) and Google scholar (up to first 500 records) were searched using keywords specific to each section.

Section 1:1 and 1:2: “snakebite”, “snake envenoming”, “snake envenomation”, “epidemiology”, “venomous snakes”, “medically important snakes”, “snake venom”, “venom gland”, “venom enzymes”, “toxin”, “coagulopathy”, “neurotoxicity”, “nephrotoxicity”, “local envenoming”, “consumption coagulopathy”.

Section 1.3: “snakebite”, “snake envenoming”, “snake envenomation”, “epidemiology”, “venomous snakes”, “medically important snakes”, “snakebite treatments”, “snake envenoming treatments”, “snake antivenom”, “snake antivenin”, “blood products”, “fresh frozen plasma”, “supportive treatments for envenoming” “heparin”.

Section 1.4 and 1.5: “Snakebite”, “Sri Lanka, snake envenoming”, “snake envenomation”, “epidemiology”, “Russell’s viper”, “Cobra”, “Saw scaled viper”, “Hump-nosed pit viper”, “Ceylon krait”, “venomous snakes”, “Indian snake antivenom”.

Section 1.6: “venom recurrence”, “recurrence of envenoming”, “late effect of envenoming”, “enzyme immunoassay”.

Section 1.7: “snake envenoming”, “snake envenomation”, “diagnosis of envenoming”, “indication of envenoming”, “phospholipase A_2”, “detection of venom”, “enzyme immunoassay”.

Section 1.8 and 1.9: “antivenom”, “antivenin”, “antivenom serum”, “efficacy”, “effectiveness”, “lethality”.

1.1 Snake envenoming

1.1.1. Epidemiology of snake envenoming – a global perspective

Venomous snakebite is considered to be the single most important cause of human injury from any kind of venomous or poisonous animal worldwide (White, 2004). Envenoming and deaths resulting from snakebite are a particularly important public health problem in the tropical world with the highest burden in rural areas of South Asia, Southeast Asia and sub Saharan Africa

(Table 1.1) (Chippaux, 1998, Kasturiratne et al., 2008). People living in these regions face high mortality and morbidity from snake envenoming due to limited heath care access, poor preventive strategies and ineffective antivenom treatments. The true mortality and morbidity from snake envenoming remains unclear and estimates have been hindered because many snakebite patients are not hospitalized or only seek traditional treatments (Fox et al., 2006, Snow et al., 1994). This leads to an underestimation of morbidity and mortality because of the exclusive use of hospital data for snake envenoming (Chippaux, 1998, Kasturiratne et al., 2008, Fox et al., 2006, Swaroop and Grab, 1954). Published estimates of the global burden of snakebite suggest a range from a minimum of 421,000 envenoming cases and 20,000 deaths, up to as many as 1,841,000 envenoming cases and 94,000 deaths (Table 1) (Kasturiratne et al., 2008). The burden of snake envenoming is huge in India. A recent survey has reported that approximately 45,900 snakebite induced deaths occur in India each year (Mohapathra et al., 2011). In addition, the number of people left with permanent disabilities as a result of envenoming is likely to be higher than the number of fatalities (WHO, 2007). Snake envenoming was classified as a neglected tropical diseases by the World Health Organization, due to the lack of national and international attention despite its burden (WHO, 2009). Agricultural workers in the tropical countries are frequently affected by snakes and therefore snake envenoming is considered an occupational disease (Warrell, 2010).

Table 1. Estimates global snake envenoming and deaths taken from Kasturiratne et al., (2008).

Global burden regions	**Estimates of envenoming**	**Estimates of deaths**
Asia Pacific, high income	237,379–1,184,550	15,385–57,636
Oceania	1,460–5,895	229–520
Europe, Eastern	3,961–9, 902	48–128
Latin America, Andean	81,427–137,123	647–3,459
North America, high income	2,683–3,858	5–7
North Africa/Middle East	3,017–80,191	43–78
Sub-Saharan Africa, Central	18,176–47,820	3,529–32,117
Sub-Saharan Africa, East	42,834–74,823	
Sub-Saharan Africa, Southern	1,613–2,296	
Sub-Saharan Africa, West	27,999–294,700	
Total	**420,549–1,841,158**	**19,886–93,945**

1.1.2 Medically important snakes in the world

Of about 3000 snake species worldwide, approximately 600 (20%) are venomous and all of these possess specialised venom producing glands and teeth (fangs) for effective delivery of the venom to the prey or predator (White, 2004). All venomous snakes belong to four snake families - Elapidae, Viperidae, Atractaspididae and Colubridae (Table 2) (White, 2004). These venomous snake families are distributed all over the world except Antarctica, Ireland, New Zealand and the Pacific coast of America.

Snakes in family Elapidae include nearly 300 venomous snake species distributed across Africa, Asia, America and Australia including sea snakes in tropical waters. Cobras, mambas, kraits, coral snakes, sea snakes and Australian snakes are the major groups of snakes belonging to family Elapidae. They are front-fanged snakes with limited fang mobility and most have groove-like fangs to transfer the venom during the bite. Because of the ability to control venom release, elapid bites can cause “dry bites” without any envenoming (Jackson, 2007). Compared to the toxicity of viperid venoms, elapid snake venoms are on the whole more potent although they are likely to deliver smaller amounts of venom because of their smaller fangs (White, 2004).

Snakes in the family Viperidae include most of the other medically important snakes in the world. The vipers are split into two major subfamilies: true vipers (Viperinae, lack of heat sensitive pits) and pit vipers (Crotalinae, possess heat sensitive pits in their head) (Bakken et al., 2012). Heat sensitive pits help pit vipers to locate their warm blooded prey in the dark using infrared detection. All vipers have mobile, rotatable, long, canalised fangs with large venom glands which facilitate venom delivery during a bite. Vipers are distributed across Asia, the Middle East, Europe, America and Africa.

Family Colubridae is the largest snake family in the world. However, only a minority of Colubrid snakes have venom and venom glands. Their fangs are located at rear of the mouth pointing towards the interior of the mouth hence the venom delivery is less effective during a bite (Jackson, 2007). Few Colubrid snakes produce highly potent

venom and they only rarely cause severe human envenoming (Pommier and de Haro, 2007).

Snakes belonging to family Atractaspididae only occur in Africa and the Middle East. These fossorial snakes have front fangs and these fangs come out of the side of their mouth to allow a sideway strike. A few snakes in this family produce highly toxic venom containing sarafotoxins (Kloog et al., 1988).

Table 2. Some major medically important snake species of the world according to White, 2004.

Family	Scientific name	Common name	Distribution
Elapidae	*Notechis* sp.	Tiger snakes	AU
Elapidae	*Pseudonaja* sp.	Brown snakes	Oc
Elapidae	*Pseudechis* sp.	Black snakes	AU
Elapidae	*Tropidechis* sp.	Rough scaled snakes	AU
Elapidae	*Hoplocephalus* sp.	Broad headed snakes	AU
Elapidae	*Acanthophis* sp.	Death adders	Oc
Elapidae	*Oxyuranus* sp.	Taipan	Oc
Elapidae	*Naja haje*	Egyptian cobra	ME
Elapidae	*Naja oxiana*	West Asian cobra	AS
Elapidae	*Naja atra*	Chinese cobra	AS
Elapidae	*Naja naja*	Indian cobra	IN, SE
Elapidae	*Naja nigrocollis*	Black necked splitting cobra	CF
Elapidae	*Ophiophagus hannah*	King cobra	IN
Elapidae	*Bungarus* sp.	Kraits	AS
Elapidae	*Dendroaspis* sp.	Mambas	SF, CF
Elapidae	*Micrurus* sp.	American coral snakes	NA
Elapidae	*Leptomicrurus* sp.	South American coral snakes	SA
Elapidae	*Aspidelaps* sp.	African coral snakes	CF

Elapidae	*Calliophis* sp.	Asian coral snakes	AS
Elapidae	*Maticora* sp.	Long glanded coral snakes	AS
Elapidae	*Enhydrina schistosa*	Beaked sea snake	IPO
Elapidae	*Hydropis* sp.	Sea snakes	IPO
Viperidae	*Daboia russelii*	Russell's viper	IN, SE
Viperidae	*Echis* sp.	Saw scaled viper	IN, AS, CF, ME, NF
Viperidae	*Bitis gabonica*	Gaboon viper	CF, SF
Viperidae	*Crotalus* sp.	North American rattle snakes	NA
Viperidae	*Agkistrodon* sp.	American pit vipers	NA
Viperidae	*Bothrops*	Lancehead vipers	SA
Viperidae	*Lachesis* sp.	Bushmasters	SA
Viperidae	*Bothriechis* sp.	Central and South American pit vipers	SA
Viperidae	*Atropoides* sp.	Jumping pit vipers	NA
Viperidae	*Calloselasma rhodostoma*	Malayan pit viper	AS, SE
Viperidae	*Trimeresurus* sp.	Green pit vipers	AS
Viperidae	*Deinagkistrodon acutus*	Hundred Pace snake	AS
Viperidae	*Hypnale* sp.	Hump-nosed pit vipers	IN
Viperidae	*Cerastes* sp.	Horned vipers	NF
Viperidae	*Causus* sp.	Night adders	CF
Viperidae	*Vipera* sp.	European adder	EU
Colubridae	*Dispholidus typus*	Bloomslang	CF, SF
Colubridae	*Malpolon monospessulanus*	Montpellier snake	AS
Colubridae	*Rhabdophis subminiatus*	Red-necked keelback	AS

AS, Asia; AU, Australia; Oc, Oceania; CF, Central Africa; EU, Europe; IN, Indian subcontinent, including Sri Lanka; IPO, Indo-Pacific Oceans; ME, Middle East; NA,

North America; NF, North Africa; SA, South America; SE, Southeast Asia; SF, Southern Africa.

2. Snake venom

Snake venom is a one of the most complex natural secretions (Harvey, 1991, Ménez, 2003). In some species venom contains more than one hundred different components. The majority are proteins and peptides with enzymatic activity and/or ligand binding activity and make up about 90-95% of the dry weight of the venom (Russell, 2001, Mackessy, 2010). Some of these proteins are responsible for the clinical effects in human envenoming. In addition, snake venoms contain inorganic cations such as sodium, potassium, calcium, zinc, iron and magnesium (Russell, 2001). These cations help to activate some enzymatic proteins in snake venom to produce their biological action (Russell, 2001). Some snake venoms contain carbohydrates, lipids, amines and free amino acids (Russell, 1980). The composition of snake venom greatly varies due to several factors including the individual snake, sex, geographical distribution, external temperature, season, dietary habits and age (Menezes et al., 2006, Daltry et al., 1996, Glenn et al., 1983). Biologically active components of snake venoms can be divided broadly into two main groups - enzymes and non-enzymatic polypeptides (Meier and Stocker, 1994).

3. Venom apparatus

The venom apparatus of snakes are modified salivary glands which produce venom and enable the effective delivery of venom during a bite. The venom apparatus consists of a pair of venom producing glands and modified teeth, called fangs, to inject venom into the prey or the predator (Meier and Stocker, 1994). The arrangement of the venom apparatus is different for Viperids, Elapids and Colubrid snakes (Meier and Stocker, 1994, Jackson, 2007).

Viperids have mobile long fangs located at the front of the upper jaw. When the mouth is closed, the fangs fold back horizontally and can rotate on a horizontal axis (solenoglyphous dentition) transversely when striking (Meier and Stocker, 1994, Weinstein et al., 2010). The fangs of Elapid snakes are located at the anterior end of the upper jaw and they are permanently fixed to the maxillary bone (proteroglyphous dentition) (Weinstein et al., 2010). Venomous Colubrid snakes possess one or more enlarged, posteriorly located grooved fangs in their upper jaw (poisthoglyphous dentition). Colubrid venom glands are small and produce less venom compared to Viperids and Elapids. The venom apparatus is posteriorly located and is a less efficient venom injecting system than the venom apparatus of Viperids and Elapids (Meier and Stocker, 1994). Voluntary contraction of skeletal muscles around the venom gland causes rapid movement of venom to the fangs through the duct system so that it can be injected into the victim during the bite.

4. Snake venom enzymes and toxins

High molecular weight proteins in snake venom will generally have enzymatic activity. Snake venom enzymes act on a number of tissue/organ targets including the coagulation cascade, neuromuscular junction, red cell membrane, skeletal muscle cells, extracellular space and vascular endothelium (Warrell, 2010). Some snake venom enzymes require a metal ion such as calcium or zinc for their catalytic activity (Russell, 2001). Common snake venom enzymes and their biological activities are indicated in Table 3. Enzymatic activity may not necessarily be associated with the toxic activity of all enzymatic toxins. For example, phospholipase toxins cause a range of toxic effects and not all of these require their enzymatic activity (Doley et al., 2010).

Table 3. Common snake venom enzymes and their biological activities.

Enzyme	**Origin**	**Toxicities**

Phospholipase A_2	All species	Neurotoxicity, myotoxicity, cardiotoxicity, haemolysis, anticoagulation, hypotension, local tissue necrosis
Acetylcholinesterase	Elapids, Viperids	Neurotoxicity
Snake venom metalloproteinase	Crotalinae	Haemorrhagic
Thrombin like snake venom serine proteinase	Crotalinae	Fibrinogenolytic
L-amino acid oxidases	Crotalinae, Elapidae	Haemorrhagic, anticoagulant, platelet aggregation
Hyaluronidase	Crotalidae, Elapidae	Local tissue necrosis, haemorrhagic, procoagulant

Based on (Doley et al., 2010, Ahmed et al., 2010, Fox and Serrano, 2010, Phillips et al., 2010, Tan and Fung, 2010, Kemparaju et al., 2010).

Snake venom toxins with low molecular weight polypeptides usually have no enzymatic action. These toxins have evolved to target a wide variety of receptors and ion channels in biological systems with high specificity and affinity (Hegde et al., 2010). Snake venom toxins can cause numerous effects including cardiotoxic, neurotoxic, haemorrhagic and tissue necrotizing effects (Hegde et al., 2010). Their action on specific receptors or ion channels causes derangements in physiology. Hundreds of different toxins belong to a small number of toxin families and their biological activities are outlined in Table 4.

Table 4. Snake venom toxins and their toxicities.

Toxin	**Target**	**Toxicity**
α-neurotoxins	α-7 nicotinic acetylcholine receptors	Neurotoxic
κ-bungarotoxins	Neuronal nicotinic receptors	Neurotoxic
Muscarinic toxins	Muscarinic acetylcholine receptors	Neurotoxic

Fasciculins	Inhibits acetylcholinesterase	Neurotoxic
Calciseptine	L-type Ca^{++2} channels	Cardiotoxic
Haemaxtins	Tissue factor-factor VIIa complex	Anticoagulant
β-cardiotoxin	B-adrenergic receptors	Cardiotoxic
Dendroaspin	Platelet glycoproteins	Inhibit platelet aggregation

Based on (Hegde et al., 2010).

2. Clinical effects of envenoming

A wide range of local and systemic effects have been reported following snake envenoming. Varying degrees of local tissue damage are seen in most venomous snake bites. Coagulopathy, neurotoxicity, myotoxicity and nephrotoxicity are the most common important systemic effects seen in snake envenoming. A combination of several of these clinical effects can be seen in a single snakebite due to the action of different toxins and enzymes in the snake venom (Ariaratnam et al., 2008a, Kularatne, 2003). I will use Sri Lankan examples to illustrate the global consequences and management of snake envenoming in this chapter.

1. Local envenoming

Elapid and viperid envenoming can cause extensive local effects which can result in permanent disability (White, 2004, Gutierrez et al., 2009, Kularatne et al., 2009). Local envenoming effects include pain, swelling, blister formation, bleeding, necrosis and gangrene at the bite site which can be extensive in some cases (Gutierrez et al., 2009, Kularatne et al., 2009). Local necrosis can be complicated by secondary bacterial infections and septicaemia, and extensive local swelling can lead to compartment syndrome (WHO, 1999). Treatments for local tissue injury can result in disfiguration and permanent disability with therapeutic amputations of limb and skin grafting (Kularatne et al., 2009).

Local tissue damage is a major issue following bites by the Indian cobra (*Naja naja*), Central American Crotalids (*Bothrops* sp.) and African cobras (Gutierrez et al., 2009, Kularatne et al., 2009). Snake venom cytotoxins, metalloproteinases, myotoxic phospholipases A_2 and hyaluronidase directly act on local tissues at the bite site and initiate local tissue destruction (Gutierrez et al., 2009). Release of inflammatory mediators also plays an important role in the pathophysiology of local envenoming. Extracellular matrix digestive activity of hyaloronidases enzymes facilitates venom spread and further tissue destruction (Gutierrez et al., 2009).

2. Coagulopathy

Coagulopathy is the most important systemic clinical syndrome caused by snake envenoming in the world (Isbister, 2010b). Viperid snakes commonly cause coagulopathy and certain elapids, including Australian elapids, also cause coagulopathy. The severity of the coagulopathy varies from clinically undetectable to severe with the risk of major haemorrhage (Isbister et al., 2010). Snake venom components that act on the coagulation system are classified according to the part of the coagulation pathway that they affect and include factor V activators, factor X activators, prothrombin activators, thrombin-like enzymes (TLEs), anticoagulant toxins, protein C activators, thrombin inhibitors, fibrinolytic enzymes and plasminogen activators (Table 5) (Lu et al., 2005, Isbister, 2009).

Table 5. Snake venom enzymes that act on the coagulation system.

Enzyme class	**Classification or characteristics**	**Examples**	**In vitro effects**

Prothrombin activators	a. Metalloproteinase, no cofactor required b. Metalloproteinase, Ca^{+2} dependent c. Serine protease, factor Xa-Va like d. Serine protease, factor Xa-like	a. ecarin (*Echis carinatus*) b. carinactivase (*Echis carinatus*) c. pseutarin (*Pseudonaja textilis*) d. notecarin (*Notechis scutatus*)	Produce soluble clot in plasma, or thrombin generation in measuring endogenous thrombin potential
Factor X activators	Metalloproteinases like RVV-X like in Viperid / Crotalid venom Serine proteases in Elapid venom	Russell's viper venom Factor X activators *Bothrops atrox* *Naja atra*	Activates factor X and also IX and protein C
Factor V activators	Thrombin–like serine protease or other protease	Russell's viper factor V activators Thrombocytin (*Bothrops atrox*)	Activates factor V, XIII, VIII, platelet activation
Factor VII activators	Serine protease	Oscutarin (*Oxyuranus scutellatus*)	Activates prothrombin
Thrombin-like enzymes	a. α-fibrinogenase metalloproteinase b. β- fibrinogenase serine protease c. αβ- fibrinogenase serine protease	Atrolase (*Crotalus atrox*) β-finrinogenase (*Vipera lebetina*)	a. Fibrinolytic activity b. b. no Fibrinolytic activity
Plasminogen activators	Serine protease	*Trimeresurus stejnegeri*	Act on plasminogen, similar to fibrinogenolytic action

Based on (Isbister, 2009).

Various terms have been used to describe the procoagulant coagulopathy following snake envenoming including, disseminated intravascular coagulation, defibrination syndrome and procoagulant coagulopathy (Isbister et al., 2010). More recently, venom-induced consumption coagulopathy (VICC) has been introduced because it provides the best description of the coagulopathy (Isbister, 2009). VICC is characterized by low or undetectable levels of fibrinogen following snake envenoming (Isbister, 2009). Different procoagulant toxins in snake venoms (thrombin-like enzymes, prothrombin activators and other coagulation factor activators) activate the clotting pathway at different points resulting in consumption of clotting factors, especially fibrinogen (Isbister et al., 2010). The resulting coagulopathy is due to factor deficiencies and results in an increased risk of bleeding.

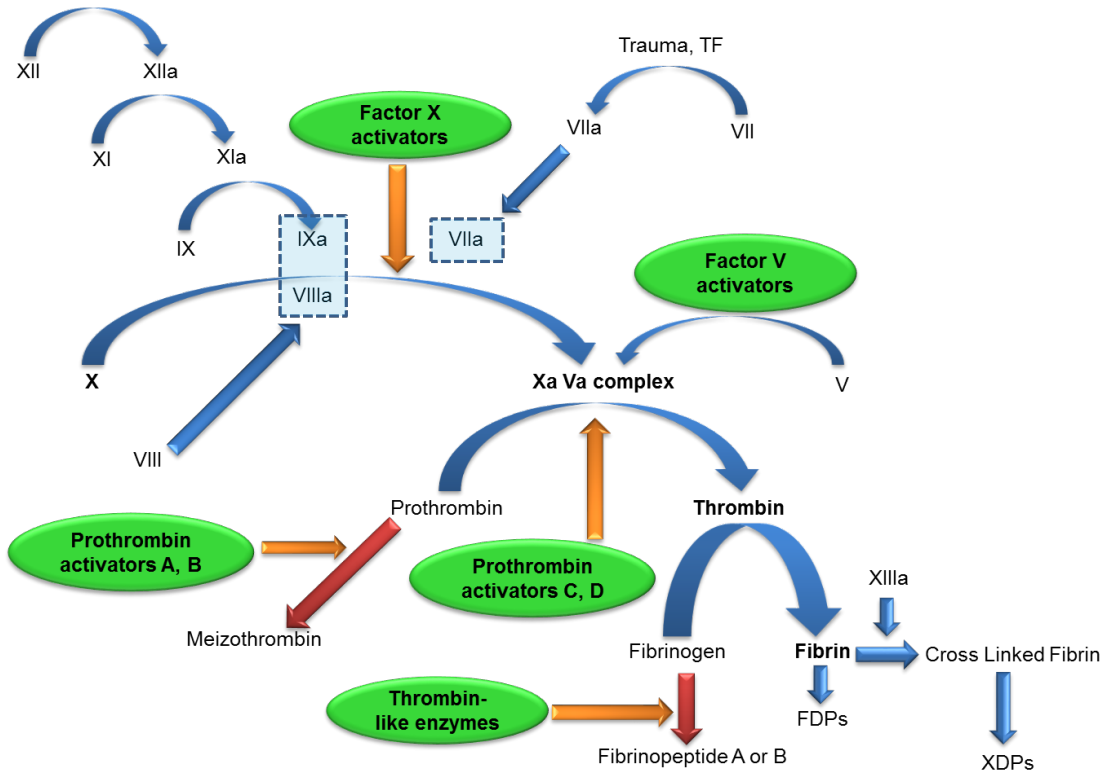

Figure: 1. Clotting pathway showing the major clotting factors and their role in the activation of the pathway and clot formation. The four major groups of snake toxins that activate the clotting pathway are in green and the intermediate or incomplete products they form are indicated in red arrows. There are four major types of prothrombin

activators, which either convert thrombin to form the catalytically active meizothrombin (Group A and B) or to thrombin (Group C and D).

Diagnosis and monitoring of VICC is done with various laboratory clotting times and factor assays (Isbister, 2009). Internationally, the most commonly used test is the 20 minute whole blood clotting test (WBCT20), which is used to detect the coagulopathy following snake envenoming in many parts of the world (Kularatne et al., 2009, Kularatne et al., 2011, Ariaratnam et al., 2008a, Maduwage et al., 2011, Maduwage et al., 2013, Warrell et al., 1977, Sano-Martins et al., 1994). However, the reliability of the WBCT20 as a diagnostic test has come into question for coagulopathy associated with viper envenoming, and there is no standardization of this test. In other parts of the world more common clotting tests such as the prothrombin time (PT; international normalised ratio [INR]), activated partial thromboplastin time (aPTT) and thrombin clotting time (TCT) are used (Isbister, 2010b).

Many patients with VICC may lack clinical features other than bleeding from the bite site or cannula site. However, some patients can develop spontaneous bleeding from gums, gastrointestinal tract (clinically present as melaena or haematemesis) and haematuria (Dart, 1997), but this is usually in snakes with venoms that also contain haemorrhagins. More severe bleeding that may be associated with trauma or pre-existing disease is intracranial bleeding. Bleeding into the pituitary gland has also been reported following viper envenoming and produces subtle or delayed clinical signs compatible with Sheehan's syndrome (Antonypillai et al., 2011, Jeevagan et al., 2012).

3. Neurotoxicity

Neurotoxicity is a common and serious systemic effect of envenoming, especially from elapid and some viperid snakes (Del Brutto and Del Brutto, 2012). Snake neurotoxins do not cross the blood brain barrier because they are large peptides or proteins, and almost always act at the neuromuscular junction to cause a descending paralysis. Paralysis is due to blockade of neuromuscular transmission either at the presynaptic or post synaptic level (Lewis, 2004). Clinically neurotoxicity starts with mild ptosis,

external opthalmoplegia and paralysis of muscles in the face and bulbar region. It then spreads further to respiratory muscles and the peripheries in severe envenoming (Isbister, 2006). The severity of neurotoxicity depends on the species of snake, injected dose of venom and the time between antivenom administration and the bite (Hodgson and Wickramaratna, 2002). Severe neurotoxic envenoming can be fatal due to respiratory paralysis unless there is early administration of effective antivenom or good supportive care. Management of respiratory paralysis includes prolonged ventilation in addition to antivenom treatment (Barber et al., 2013).

Neurotoxins that act on post synaptic nicotinic acetylcholine receptors and presynaptic membranes are called α-neurotoxins and β-neurotoxins, respectively (Barber et al., 2013, de Silva et al., 2011).

4. Nephrotoxicity

Acute kidney injury is not an uncommon complication of snake envenoming and has been increasingly reported in bites from a range of snakes especially following viper envenoming (Kularatne et al., 2009, Ariaratnam et al., 2008a, Herath et al., 2012). It appears to occur either secondary to venom-induced hypotension causing renal ischaemia, myotoxicity, VICC or rarely due to the direct effect of venom. Acute kidney injury manifests as decreased urine output, haematuria, elevated creatinine and urea, and may sometimes result in chronic kidney injury (Maduwage et al., 2011, Herath et al., 2012). Early administration of antivenom and renal replacement therapy may help to prevent acute kidney injury.

5. Other systemic effects

For many snake bites, systemic envenoming may cause predominantly non-specific systemic symptoms such as nausea, vomiting, abdominal pain, headache and, less commonly, diarrhoea, syncope and convulsions (White, 2004, Kularatne, 2003). Persistent vomiting, intractable headache, collapse or convulsions and abdominal pain are more likely to indicate envenoming than anxiety reactions (White, 2004).

3. Treatment of snake envenoming

3.1. Antivenom treatment for snake envenoming

Antivenom is the mainstay of treatment for snake envenoming. Antivenoms consist of polyclonal antibodies to the toxins in snake venoms (Warrell, 1992). They may be whole immunoglobulins (IgG) or fractionated immunoglobulins (Gutierrez et al., 2003). These antibodies are produced in animals, such as horse, sheep, goats and rabbits, that are injected with small amounts of snake venom (Theakston and Warrell, 1991, Heard et al., 1999). The polyclonal nature of antivenoms means that they are able to neutralize multiple venom components (Theakston and Warrell, 1991). Monovalent antivenoms are raised against one species of snakes, while polyvalent snake antivenoms are produced by immunizing with more than one species of snake venom (Theakston and Warrell, 1991).

There are three major types of snake antivenoms - whole IgG, $F(ab')_2$ and Fab (Gutierrez et al., 2003). Whole IgG antivenoms contain intact immunoglobulin G molecules (150kDa) purified by ammonium sulphate or caprylic acid precipitation (Gutierrez et al., 2003, Rojas et al., 1994). $F(ab')_2$ antivenoms are produced by pepsin digestion of whole IgG and are about 100kDa (Gronski et al., 1991). Fab antivenoms are prepared by papain digestion of whole immunoglobulins and are about 50kDa (Gutierrez et al., 2003). These three different types of antivenoms have different pharmacokinetic properties which will influence whether they are able to reach the site of toxin action and their duration of action (presence in the central compartment) (Gutierrez et al., 2003). The three different types of antivenoms also have different rates of adverse effects.

Early administration of antivenom is important so that antivenom can bind with venom components before they distribute to their target sites and cause irreversible toxicity (e.g. pre-synaptic neurotoxicity). Antivenom should be administered intravenously

because local or intramuscular administration will mean that antivenom will not enter the systemic circulation rapidly (Isbister et al., 2008).

Administration of foreign proteins (most commonly horse), which are the basis of antivenoms, can cause hypersensitivity reactions (Leon et al., 2008, Ryan et al., 2015, de Silva et al., 2011) and other reactions such as pyrogenic reactions. Early systemic hypersensitivity reactions (SHR) include skin only SHR, anaphylaxis and severe anaphylaxis based on the features of the reaction (Brown, 2004). Delayed reactions can occur and are referred to as serum sickness. Administration of premedications before antivenom therapy is controversial (Nuchprayoon and Garner, 1999), and a recent randomised controlled trial has shown that for an antivenom with a high reaction rate, premedication with adrenaline reduced reactions (de Silva et al., 2011).

3.2. Supportive treatments and administration of blood products

Initial assessment and resuscitation is critical in the treatment of any snake envenoming (Isbister, 2006). Analgesics, antibiotics and surgery may be required for the treatment of local pain, secondary infections and necrosis that can occur with local envenoming. Intubation and ventilation may be required in neurotoxic envenoming, including intubation for airway protection (bulbar palsy) and mechanical ventilation for respiratory paralysis. A summary of supportive treatment for snake envenoming is given in Table 6.

Table 6. Supportive treatments for snake envenoming.

Supportive treatment	Benefit	Example
Analgesics	Local pain	Any snake bite
Elevation of limb and local swelling management	Prevent the progression of swelling and compartment syndrome	Russell's viper envenoming
Antibiotics	Prevent secondary infections	Cobra bite (Kularatne et al., 2009)

Surgical excision and amputations	Prevent spread of local tissue necrosis	Cobra bite (Kularatne et al., 2009)
Intubation and ventilation	Respiratory paralysis	Krait and cobra venom neurotoxicity (Kularatne, 2009)
Administration of colloid or crystalloids	Correct hypovolemic shock	Any snake envenoming cased to hypovolemic shock (WHO, 1999)
Renal replacement therapy, dialysis	Acute kidney injury, hyperkalaemia	Russell's viper envenoming (Kularatne, 2003, WHO, 1999)
Administration of clotting factors and platelets	Coagulopathy platelet dysfunctions	Russell's viper envenoming, coagulopathy of Australian elapids (WHO, 1999, Isbister et al., 2009)
Adrenalin	As a premedication for prevention of antivenom reaction	(de Silva et al., 2011, Premawardhena et al., 1999)

Based on (Kularatne, 2003, Kularatne, 2009, Kularatne et al., 2009, Ariaratnam et al., 2008a, de Silva et al., 2011, Isbister et al., 2009, Premawardhena et al., 1999, WHO, 1999).

Consumption of clotting factors by various toxins present in snake venom leads to low or undetectable levels of clotting factors which creates a risk of major haemorrhage or even death (Isbister et al., 2010). Once VICC is established and antivenom has been given, it still takes 24 to 48 hours for the full recovery of the clotting factors (Isbister et al., 2010). Therefore, patients with VICC are at an increased risk of developing haemorrhages even after administration of antivenom. Administration of blood products including fresh frozen plasma (FFP) has been suggested for early recovery of VICC (Isbister et al., 2009, Brown et al., 2009). FFP is the most commonly available factor replacement and will replace clotting factors including fibrinogen. However, limited evidence has been published on the effectiveness and the safety of administration of

clotting factors in snake envenoming (Warrell et al., 1977, Isbister et al., 2009, Ferguson et al., 2002). However, reactions to FFP are a recognised complication (Pandey and Vyas, 2012). Furthermore, supplementation of more clotting factors for coagulopathy might worsen the factor consumption by un-neutralized procoagulant toxins in the circulation (Jelinek et al., 2005). Two observational studies suggest that factor replacement leads to earlier recovery of VICC (Warrell et al., 1977, Isbister et al., 2009, Brown et al., 2009, Porath et al., 1992). A recent randomised controlled trial of FFP in VICC, due to Australian elapid envenoming, has shown that FFP speeds the recovery of the coagulopathy (Brown et al., 2009). However, if given too soon after the bite (within 6 hours) it appears to worsen the consumption coagulopathy.

4. Snake envenoming in Sri Lanka

Snakebite is a major medical and public health problem in Sri Lanka. The wide distribution of venomous snakes and the frequent snake human contact during agricultural work is responsible for the high incidence of snake envenoming in the country.

4.1. Epidemiology of snake envenoming in Sri Lanka

Detailed information on snake envenoming is not available for Sri Lanka, although most estimates suggest that the incidence is as high as 0.2% (39,000/20,000,000 population per year, with a case fatality of 0.6% in the year 2007 (Anonymous, 2007). However, the national figure for snakebite is likely to be higher because some victims still do not seek hospital treatment and delayed deaths from envenoming are less likely to be notified as snakebites (Jeyarajah, 1985). Although 100 species of snakes are reported from the island, much of the morbidity and about 95% of the mortality associated with snakebite is due to the highly venomous Russell's viper (*D. russelii*), common cobra (*Naja naja*) and Indian krait (*Bungarus cearuleus*) (de Silva and Ranasinghe, 1983). Saw scaled viper (*Echis carinatus*) is a leading cause of snake envenoming along the coastal plains in the north and eastern provinces of Sri Lanka. Hump-nosed pit vipers

(Genus *Hypnale*) are responsible for the largest number of venomous snakebites on the island due to wide distribution in anthropogenic habitats (Maduwage et al., 2013), but do not cause as severe envenoming. In addition to the above medically important snakes, a large number of mildly venomous snakes from the family Colubridae occur and bites from these occur throughout the island. However, they do not cause significant local or systemic envenoming.

The pattern of snakebite differs from one climate zone to another due to the distribution of venomous snake species (Kasturiratne et al., 2005). Russell's viper, Indian krait and cobra bites frequently occur in the dry zone agricultural areas during the harvesting seasons (Kasturiratne et al., 2005). The number of Russell's viper bites changes with the time of the year because of the rainy season and the two main harvesting seasons of year, when there is close contact between humans and snakes in the paddy fields (Kularatne, 2003). By contrast, Indian krait bites often occur during the night time when people sleep on the floor in wattle-and-daub houses (Kularatne, 2009) and are less affected by the time of year. Government hospital based estimates of snakebites in in Sri Lanka in 2000, data taken from (Kasturiratne et al., 2005) are provided in Table 7.

Table 7. Estimated land snakebite burden in Sri Lanka in year 2000.

Snake	**Zone 1** **Bites (%)**	**Zone 2** **Bites (%)**	**Zone 3** **Bites (%)**	**Zone 4** **Bites (%)**	**Total** **Bites (%)**
Hump-nosed pit vipers	250 (83)	8,600 (65)	4,800 (49)	600 (4)	14,200 (35-45)
Russell's viper	0	3,200 (24)	2,900 (30)	6,600 (47)	12,700 (30-40)
Indian krait	0	0	1,000 (10)	4,200 (30)	5,200 (10-20)

Cobra	0	700 (5)	500 (5)	600 (4)	1,800 (1-10)
Saw scaled viper	0	0	0	600 (4)	600 (1-2)
Mildly venomous snake	50 (17)	700 (5)	500 (5)	1,300 (9)	2,600 (1-10)
Total	**300 (1)**	**13,200 (36)**	**9,700 (26)**	**13,900 (37)**	**37,100**

Zone 1, wet zone, elevation >900m above sea level; Zone 2, wet zone, elevation <900m above sea level; Zone 3, intermediate zone; Zone 4, dry zone based on (Kasturiratne et al., 2005).

4.2. Clinical effects of snake envenoming in Sri Lanka

Clinical effects of Sri Lankan snake envenoming include local envenoming, coagulopathy, neurotoxicity, myotoxicity and nephrotoxicity (Kularatne, 2003, Kularatne et al., 2009, Kularatne, 2009, Kularatne and Ratnatunga, 1999, Maduwage et al., 2011, Maduwage et al., 2013). Local effects from pain, swelling, blister formation and local tissue necrosis have been reported following Indian cobra, Russell's viper, saw scaled viper and hump-nosed pit viper envenoming (Kularatne, 2003, Kularatne et al., 2009, Kularatne et al., 2011, Ariaratnam et al., 2008a). Abdominal pain is a common non-specific systemic effect of Indian krait envenoming (Kularatne, 2009, Ariaratnam et al., 2008b). Coagulopathy including spontaneous systemic bleeding has been reported in Russell's viper and Saw scaled viper envenoming, and less commonly in hump-nosed pit viper envenoming (Kularatne, 2003, Kularatne et al., 2011, Ariaratnam et al., 2008a). Myotoxicity is only reported in Russell's viper bites and nephrotoxicity has been reported following Russell's viper, Saw scaled viper and Hump-nosed pit viper envenoming (Ariaratnam et al., 2008a, Kularatne et al., 2011, Ramachandran et al., 1994). Indian cobra and Indian krait envenoming are the most important causes of neurotoxicity which leads to life threatening neurotoxicity. (Kularatne et al., 2009,

Kularatne, 2009). Russell's viper is the commonest cause of neurotoxicity following envenoming in Sri Lanka, but usually only causes mild to moderate neurotoxicity (Kularatne, 2003, Sitprija, 2006, Phillips et al., 1988). In Sri Lanka, acute kidney injury from snakebite appears to be associated with VICC (White, 2004, Sitprija, 2006).

Clinical manifestations of Russell's viper, Indian cobra, Indian krait, saw scaled viper and hump-nosed pit viper envenoming are given in Tables 8, 9, 10, 11 and 12 respectively. A summary of the clinical manifestations of Sri Lankan snake envenoming are provided in Table 13.

4.2.1. Russell's viper

Table 8. Percentages of different clinical effects of Russell's viper envenoming in Sri Lanka.

Clinical effect	Jayarajah, 1984	Phillips et al., 1988	Ariaratnam et al., 2001	Kularatne, 2003
Local envenoming				92
Pain	86	95	97	
Swelling		73	97	
Necrosis	4	4	0	
Incoagulable blood	25	59	*	77
Spontaneous systemic bleeding		36		7
Renal involvement	86	27	26	14
Myotoxicity (muscle pain and tenderness)	77	32	19	NR
Neurotoxicity	86	37	88	78
Ptosis		82	65	64
Opthalmoplegia				

* Incoagulable blood is the inclusion criteria for this study; NR, not reported.

Based on published clinical studies from (Jayarajah, 1984; Phillips et al., 1988; Ariaratnam et al., 2011 and Kularatne, 2003).

4.2.2. Indian cobra

Table 9. Clinical manifestations of cobra bites.

Clinical effect	Number of cases (%)
Dry bite	5 (20)
Local effects	20 (80)
mild (swelling only)	7 (35)
moderate (mild necrosis)	5 (25)
severe (extensive necrosis)	8 (40)
desloughing /skin grafting	5
fasciotomy, decompression	2
Necrosis leading to death	2
Neurotoxicity	9 (36)
mild (ptosis, etc)	5 (56)
severe (ventilation needed)	4 (44)
Transient coagulopathy	
spontaneous bleeding	0
positive WBCT20	2 (12)

Based on Kularatne et al, (2009).

1.4.2.3. Indian krait

Table 10. Clinical effects of Indian krait envenoming.

Clinical effect	Kularatne, 2002 Number of cases (%)	Ariaratnam et al., 2008 Number of cases (%)
Local effects	63 (30)	8 (9) mild
Abdominal pain	143 (68)	80 (91)
Alteration of level of consciousness	150 (71)	NR
Respiratory paralysis	101 (48)	56 (64)
Ptosis (neurotoxicity)	147 (70)	84 (95)
Hypokalaemia	149 (71)	NR
Metabolic acidosis	105 (50)	NR
Anterograde memory loss	84 (40)	NR
Delayed neuropathy	38 (22)	NR

NR, Not reported

Based on Kularatne, (2002) and Ariaratnam et al., (2008). (de Silva et al., 2002, Ariaratnam et al., 2008b).

4. Saw scaled viper

Table 11. Clinical manifestations of confirmed saw scaled viper bites.

Clinical effect	Kularatne et al., 2011 Number of cases (%)	Gnanathasan et al., 2012 Number of cases (%)
local effects	19 (73)	46 (96)
coagulopathy (positive WBCT20)	24 (92)	34 (71)
spontaneous bleeding	3 (12)	3 (6)

mild acute renal dysfunction	1 (4)	NR

NR, not reported

Based on Kularatne et al., (2011) and Gnanathasan et al., (2012).

1.4.2.5 Hump-nosed pit vipers

Table 12. Clinical effects of hump-nosed pit vipers from two different studies.

Clinical effect	**Cases (%), Ariaratnam et al., 2008**	**Cases (%), Maduwage et al., 2013**
Local effects	276 (91)	114 (100)
Local necrosis	48 (16)	16 (14)
Systemic envenoming, total	117 (39)	5 (4)
Coagulopathy, positive WBCT20	117 (39)	4 (3)
Spontaneous systemic bleeding	55 (18)	0
Acute renal failure	30 (10)	1 (1)

Ariaratnam et al., (2008), Maduwage et al., (2013).

The reason for the differing incidences of coagulopathy in snake envenoming could be due to the problem with the reliability of WBCT20. The different percentage of spontaneous systemic bleeding and acute renal failure between two studies is possibly due to the different toxicities of *Hypnale hypnale* that inhabit various regions of the island.

Table 13. Summary of clinical effects of Sri Lankan snake envenoming.

Clinical effect	Russell's viper	Indian krait	Cobra	Hump-nosed pit vipers	Saw scaled viper
Local effect	+	uncommon	+	+	+
Coagulopathy	+	–	uncommon	+	+
Neurotoxicity	+	+	+	–	–
Myotoxicity	+	–	–	–	–
Renal injury	+	–	–	+	+
Cardiotoxicity	+	–	–	–	–

Based (Kularatne, 2003, Kularatne et al., 2009, Kularatne et al., 2011, Kularatne, 2009, Ariaratnam et al., 2008a, Ariaratnam et al., 2008b, Maduwage et al., 2013, Gnanathasan et al., 2012).

5. Antivenom treatment for Sri Lankan snake envenoming

The availability of antivenom in government hospitals and increased public awareness of the effective treatment for snakebite has led to increasingly early presentations to hospital for medical treatment for snake envenoming in Sri Lanka (Kularatne, 2000). Currently Indian polyvalent snake antivenoms are used for all cases of Sri Lankan snake envenoming. Three different manufacturers (Haffkine Institute, Mumbai; VINS Bioproducts Limited, Andhra Pradesh; BHARAT Serum and Vaccines Limited, Ambernath) produce a polyvalent snake antivenom raised against Indian krait (*B. caeruleus*), Indian cobra (*N. naja*), saw scaled viper (*E. carinatus*) and Russell's viper (*D. russelii*). All three antivenoms have been manufactured by immunizing horses with these four snake species that also inhabit India. Antivenoms manufactured by Haffkine institute and VINS Bioproducts are $F(ab')_2$ and no information about the type of immunoglobulin has been included in BHARAT Serum and Vaccines Limited. Currently

the Sri Lankan Health Ministry is only importing antivenoms manufactured by VINS Bioproducts and BHARAT Serum and Vaccines Limited.

The product information on VINS and BHARAT antivenoms indicates that 1 ml of reconstituted antivenom neutralizes 0.6 mg of Indian Cobra (*N. naja*) venom, 0.45 mg of Indian krait (*B. caeruleus*) venom, 0.6 mg of Russell's viper (*D. russelii*) venom and 0.45 mg of saw scaled viper (*E. carinatus*) venom. However, the effectiveness of all three Indian antivenoms in Sri Lankan snake envenoming has not been tested in *in-vitro* investigations. Furthermore, all these antivenoms have a high rate of adverse reactions, including life threatening anaphylaxis. None of these Indian antivenoms have antibodies against the venom of the hump-nosed pit viper.

An ovine Fab monospecific antivenom, Polonga-Tab@ was produced against Russell's viper venom in Sri Lanka. The clinical trial found that the Haffkine antivenom was more effective than the monovalent antivenom, Polonga Tab (Ariaratnam et al., 2001). This newly produced antivenom had fewer adverse reactions than the Haffkine antivenom. However, the requirement for a higher initial dose for effective neutralization, the rapid clearance from the circulation and higher cost of treatment, led to the discontinuation of the antivenom (Ariaratnam et al., 2001).

The Indian Haffkine antivenom that was previously used for snake envenoming in Sri Lanka has been reported to be less effective in some studies (Kularatne et al., 2009, Phillips et al., 1988, Theakston et al., 1990). However, almost all these reports have been concerned only about recovery from neurotoxicity, which any antivenom is unlikely to reverse. None of the studies tested the efficacy of Indian antivenoms against the recovery of coagulopathy and other effects. It was also found that Burmese snake antivenom did not neutralize the haemolytic activity of Sri Lankan Russell's viper venom and the composition of the venoms was different for each local snake (Woodhams et al., 1990).

There have been concerns about differences between the venoms of snake species in Sri Lanka and India, and that this may be the reason for antivenom apparently not being effective. However, based on the published evidence, the mortality from snakebite in Sri Lanka is only 0.6% following Indian polyvalent antivenom treatment (Anonymous, 2007). This would actually support the effectiveness of Indian polyvalent antivenom for Sri Lankan snake envenoming, rather than the converse (Suzuki et al., 2010).

Adverse reactions to the equine proteins in snake antivenoms are common. Both acute (non-immune mediated anaphylaxis or pyrogenic) and delayed (serum sickness type) reactions happen following the administration of the antivenom immunoglobulins (Gutierrez et al., 2006, de Silva et al., 2011, Ryan et al., 2015, Leon et al., 2008). The greatest problem is the acute reactions and in most cases they are mild (urticaria, itchiness, flushing, vomiting, and fever), but in some cases they manifest as severe and life threatening anaphylaxis. The reported rate of adverse reactions to the Indian polyvalent antivenoms used in Sri Lanka varies from 43% to 81% (Premawardhena et al., 1999, Kularatne, 2000, Theakston et al., 1990, Seneviratne et al., 2000, Yoganathan, 1973, Karunaratne and Anandadas, 1973). However, only a small proportion (5% to 10%) of reactions are reported as severe reactions (Premawardhena et al., 1999, Kularatne, 2000, Seneviratne et al., 2000).

6. Recurrence of envenoming

Recurrence of envenoming can be defined as the return of any venom effect after that effect has initially resolved (Dart et al., 2001). Local recurrence has been described as the return of progression of local effect after it initially resolves. Coagulopathy recurrence has been described as the return of thrombocytopenia, hypofibrinogenemia, prolongation of prothrombin time or elevation of fibrinogen degradation products after the initial correction (Boyer et al., 1999). Recurrence of coagulopathy has been documented in North American crotalid snake envenoming (Dart et al., 2001, Boyer et al., 1999, Bogdan et al., 2000). There have been a number of explanations for the recurrence, including ongoing absorption of venom from the bite site, dissociation of

venom from the antivenom, variation of the composition of absorbing venom components from the bite site with time (Dart et al., 2001). The recurrence of coagulopathy has been reported following the administration of polyvalent crotalid Fab antivenom for North American crotalid envenoming as recurrent thrombocytopenia and hypofibrinogenemia (Boyer et al., 1999). The reason for this recurrence of envenoming could be explained by the rapid clearance of Fab antivenom from the circulation and ongoing absorption of venom from the bite site.

Recurrence of pit viper venom antigens, after an initial reduction following antivenom treatment, has been described. This is recognised when venom is again being detected in blood. The phenomenon is more commonly observed following Viperid envenoming (Ho et al., 1990, Ariaratnam et al., 1999, Dart et al., 2001, Boyer et al., 1999, Bogdan et al., 2000, Meyer et al., 1997). Measurement of venom in blood after treatment with monovalent ovine Fab antivenom (Polonga Tab) for Sri Lankan Russell's viper (*D. russelii*) envenoming, found the recurrence of venom antigens after the initial treatment. This was explained as a redistribution of venom to the circulation from the bite site (Ariaratnam et al., 1999). Similar venom recurrence has been observed with monovalent $F(ab')_2$ antivenom treatment for Malayan pit viper (*Calloselasma rhodostoma*) envenoming, monovalent ovine Fab antivenom for *Echis ocellatus* bite in Nigeria and ovine Fab antivenom for crotalid envenoming in the United States (Ho et al., 1990, Meyer et al., 1997). Venom recurrence after the administration of Haffkine polyvalent antivenom for Sri Lankan Russell's viper (*D. russelii*) envenoming found that the recurrence happened twice following repeated antivenom doses (Theakston, 1997).

7. Diagnosis of envenoming for antivenom use

Because of the burden of illness, the treatment of snake envenoming remains a huge challenge (Warrell, 2010). There is limited availability of antivenom in some countries, especially in Africa and Asia, unacceptably high reaction rates to antivenom, and difficulties in diagnosing envenoming to allow early antivenom treatment in patients with definite envenoming (Lalloo et al., 2002, Isbister et al., 2012, Ireland et al., 2010).

A key issue for improving antivenom treatment in snake envenoming is to have a rapid and accurate test to determine if patients are envenomed and require antivenom (Isbister et al., 2013b). Unfortunately the majority of clinical features and laboratory investigations indicative of envenoming only occur in established envenoming, where antivenom may be of limited or no benefit. Currently no single laboratory investigation or bedside test exists to identify patients with systemic envenoming and the diagnosis involves a combination of clinical features and investigations (Isbister et al., 2012, Isbister et al., 2013a). 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 (Warrell et al., 1977, Sano-Martins et al., 1994). However, it has recently been shown to have a poor sensitivity in the clinical setting in Russell's viper envenoming (Isbister et al., 2013b) and is of no value for snakes that do not cause a coagulopathy such as kraits and cobras.

It is therefore desirable to develop an early diagnostic test for envenoming that can be carried out at the bedside and is cheap and available in resource poor settings. Such tests have been developed for poisoning in resource poor settings (Shihana et al., 2010a, Shihana et al., 2010b).

8. Assessment of antivenom efficacy

In vitro and *in vivo* testing as well as clinical assessment is mandatory in the development of new antivenoms or when introducing an existing antivenom to a new geographical region (Anonymous, 2010). The median venom lethality (lethal dose; LD_{50}) and median antivenom neutralizing capability (effective dose; ED_{50}) are the only accepted means of testing venom toxicity and antivenom efficacy worldwide based on WHO recommendations (Anonymous, 2010). However, additional preclinical tests have been strongly recommended for assessing antivenoms including tests of neutralisation of specific venom effects for the appropriate snake species in the geographical region the antivenom will be used (Anonymous, 2010). Such testing includes the ability of

antivenom to neutralize haemorrhagic activity, necrotic activity, procoagulant activity and neurotoxic activity (Anonymous, 2010). Assessment of neutralization of venom procoagulant activity is important for antivenoms used for viper envenoming (Reid and Theakston, 1983). *In vitro* assessment of the dose of antivenom (Minimum Clotting Dose, MCD - Effective dose) required to neutralize the procoagulant activity of venom provides a measure of the efficacy of antivenom on procoagulant toxicity (Gutierrez et al., 1985, Theakston and Reid, 1983). Different preclinical antivenom efficacy assessment tests are listed in Table 14.

Table 14. Different preclinical antivenom assessment tests.

Assessing toxicity	**Defined toxic dose of venom**	**Defined neutralizing dose of antivenom**	**Type of test**
Lethality	Median lethal dose (LD_{50})	Median effective dose (ED_{50})	In-vivo, mice
Haemorrhagic activity	Median haemorrhagic dose (MHD)	MHD-effective dose (ED_{50})	In-vivo, mice
Necrotic activity	Median necrotic dose (MND)	MND-effective dose (ED_{50})	In-vivo, mice
Procoagulant activity	Minimum coagulant dose (MCD)	MCD-effective dose	In-vitro
Defibrinogenation activity	Minimum venom defibrinogenation dose (MDD)	MDD- effective dose	In-vitro
Myotoxic activity	Minimum myotoxic dose (MMD)	MMD-median effective dose (ED_{50})	In-vivo, mice or rat
Neurotoxic activity	Time to inhibit 90% of twitch height (t_{90})	Abolish or delaying t_{90}	In vitro, chick nerve muscle preparation in organ bath system; Mouse hemidiaphragm nerve-muscle preparation.

Based on (Anonymous, 2010, Gutierrez et al., 1985, Gutierrez et al., 1992, Theakston and Reid, 1983, Laing et al., 1992, Jene et al., 1989, Chettya et al., 2004).

In-vitro neutralization of procoagulant and anti-clotting activity has also been assessed by turbidimetric assays (Lane et al., 2011). Snake venom-antivenom binding ability has been tested by determination of free venom concentration in mixtures of venom and antivenom by using enzyme immunoassays (EIA) (O'Leary et al., 2014).

9. Effectiveness of snake antivenom for venom-induced consumption coagulopathy (VICC)

Venom-induced consumption coagulopathy is one of the major clinical manifestations of snake envenoming and may be complicated by fatal haemorrhage (Isbister et al., 2010). Antivenom is the primary treatment for snake envenoming including VICC (Lalloo and Theakston, 2003, Isbister, 2010a). Intravenously administered antivenom in patients with snake envenoming binds to circulating toxins to neutralise or eliminate the toxins. The efficacy of antivenom is defined as the ability to bind and neutralise venom-mediated effects under ideal conditions (*in vitro* studies and animal studies of binding and neutralisation) and effectiveness of antivenom is its ability to reverse or prevent envenoming in human patients (Isbister, 2010a), as well as improving patient outcomes.

Antibodies in the antivenom bind to the toxic components in snake venom. Early administration of antivenom will bind the circulating procoagulant toxins and potentially prevent, delay or lessen the severity of VICC. In the majority of patients who have already developed VICC, antivenom is used to neutralise circulating toxins and allow recovery of VICC. However, the effectiveness and mechanism of action of antivenom in already developed VICC remains unclear (Isbister et al., 2009). Recovery of the coagulopathy depends on re-synthesising clotting factors which is not directly affected by antivenom administration (Isbister et al., 2010).

Even though snake antivenom is the mainstay of the treatment for snake envenoming, there is controversy regarding the effectiveness of antivenom for VICC (Isbister et al., 2010). It is unlikely that antivenom can be administered early enough to prevent VICC because the procoagulant toxins in snake venoms act rapidly (Isbister et al., 2010). The more important question is whether the administration of antivenom will speed the recovery of VICC by inactivating the active toxins to allow re-synthesis of clotting factors (Isbister et al., 2010). Thus, only if further factor consumption is occurring due to significant amounts of circulating pro-coagulant venoms, would antivenom be expected to speed recovery. Therefore, it is important to review the evidence of antivenom treatment for VICC.

Few non-randomised trials including comparison groups without antivenom have shown that antivenom was effective for envenoming by some snakes (e.g. *Echis* species in Africa), but not others (e.g. Australasian elapids) (Isbister et al., 2009, Mion et al., 2013). Almost all the studies on the effectiveness of antivenoms have been conducted as comparative trials and without a placebo arm. These studies compared two or more antivenoms or different doses (Otero et al., 1996, Otero et al., 2006, Abubakar et al., 2010, Otero-Patino et al., 2012, Ariaratnam et al., 2001). Therefore, exploration of the evidence for the effectiveness of antivenom for VICC is vital to understand clinical use.

Chapter Two

Hump-nosed pit viper (*Hypnale hypnale*) envenoming causes mild coagulopathy with incomplete clotting factor consumption

Introduction to Chapter Two: Explanation of contribution of the paper titled "Hump-nosed pit viper (*Hypnale hypnale*) envenoming causes mild coagulopathy with incomplete clotting factor consumption" to the overall thesis

Hump-nosed pit vipers (genus *Hypnale*) are the commonest cause of snakebite in Sri Lanka, being responsible for about 35 to 45% of cases (Kasturiratne et al., 2005). Three species have been recognized in the Genus *Hypnale*: *H. hypnale, H. nepa* and *H. zara* (Maduwage et al., 2009). They are widely distributed throughout the island and their anthropogenic habitat preference explains the reason for the large number of bites (Kasturiratne et al., 2005, Ariaratnam et al., 2008a, Maduwage et al., 2013). Previous studies have suggested that Hump-nosed pit viper envenoming predominantly causes local envenoming, less commonly coagulopathy and rarely acute kidney injury (Ariaratnam et al., 2008a, Maduwage et al., 2013). However, various clinical studies have been reported different incidences of coagulopathy, from 4% to 39% (Maduwage et al., 2013, Ariaratnam et al., 2008a). Excepting one study, coagulopathy in *Hypnale* envenoming has been diagnosed based on a 20 minute whole blood clotting test (WBCT20), which has been shown to be unreliable due to lack of standardization (Isbister et al., 2013b). This may be the reason for the different incidence of coagulopathy. One Sri Lankan study reported that 12 of 56 patients had a positive WBCT20 without evidence of spontaneous bleeding (Premawardena et al., 1998). However, 10 of these patients had increased levels of fibrinogen degradation products and 7 had reduced fibrinogen levels (Premawardena et al., 1998). It is likely that the WBCT20 was only positive for severe coagulopathy and mild to moderate coagulopathy may have been missed. Therefore, the frequency and the nature of coagulopathy following *Hypnale* envenoming remains unclear. No study has reported laboratory clotting times and clotting factor concentrations in *Hypnale* envenoming. In addition, no procoagulant toxin has been isolated from *Hypnale* venom and the mechanism of action of *Hypnale* toxins on the clotting cascade is unknown. This chapter investigates the coagulopathy of *Hypnale* envenoming to further understand coagulopathy in Sri Lankan snake envenoming.

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was accepted into Clinical Toxicology for publication on 28th May 2013. I, Maduwage Kalana Prasad, was the first author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

Clinical and laboratory samples were collected under the supervision of clinicians of the hospitals; C. Abeysinghe, H. Karunathilake, C.A Gnanathasan. Clotting factor assays were carried out by E. F. Scorgie and L. F. Lincz. Clinical and laboratory samples collections were coordinated by S. Shahmy and F. Mohamed. Rabbit polyclonal antibodies were produced by A. Silva. Overall supervision of the study was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter Two.

Clinical Toxicology (2013), **51**, 527–531

ISSN: 1556-3650 print / 1556-9519 online
DOI: 10.3109/15563650.2013.811589

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CRITICAL CARE

Hump-nosed pit viper (*Hypnale hypnale*) envenoming causes mild coagulopathy with incomplete clotting factor consumption

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Context. Limited information exists on the coagulopathy caused by hump-nosed pit viper (*Hypnale hypnale*) envenoming. *Objectives.* This study aimed to characterise the coagulopathy in hump-nosed pit viper bites by measuring laboratory clotting times and factor studies. *Materials and methods*. Cases of hump-nosed pit viper envenoming were included from a prospective cohort study of Sri Lankan snake-bite patients. Patient age, sex, snake identification, time of bite and clinical effects were recorded. Patients did not receive anti-venom because no specific anti-venom to hump-nosed vipers exists. All patients received supportive care and serial 20-min whole blood clotting tests (WBCT20). The prothrombin time (PT), international normalised ratio (INR), activated partial thromboplastin time (aPTT), coagulation factors I, II, V, VII, VIII, IX and X, von Willebrand factor (vWF) antigen and D-Dimer concentrations were measured. The median of highest or lowest test result for each patient was reported with interquartile range (IQR). *Results.* There were 80 hump-nosed pit viper bites, median age was 37 years (IQR: 26–51 years) and 48 were male. The WBCT20 was positive in one patient. The median highest INR was 1.9 (1.5–2.2; Range: 1.3 to > 12) and median highest aPTT was 54 s (46–72 s; Range: 35–170 s). There was low fibrinogen [median: 1.3 g/L;1, –1.8 g/L; Range: < 0.2–2.9], low factor VIII levels [median: 23%; 16–37%] and low factor V levels [median: 43%; 23–74%]. D-Dimer concentrations [median: 3.4 mg/L; 2–7.4 mg/L] were slightly elevated. Factors II, VII and X and vWF antigen concentrations were normal. *Discussion and Conclusions.* Hump-nosed pit viper bites result in a mild coagulopathy which is usually not detected by a WBCT20. It is characterised by mild elevation of INR, low fibrinogen and Factors V and VIII which may be consistent with the venom containing a thrombin-like enzyme.

Keywords Clotting factors; *Hypnale*; Hump-nosed viper; Coagulopathy; Snake envenoming; Venom

Abbreviations aPTT, activated partial thromboplastin time; INR, international normalised ratio; IQR, interquartile range; MCC_5, minimum clotting concentration defined as the venom concentration that caused clotting after 5 min; PT, prothrombin time; SV-TLE, snake venom thrombin-like enzyme; VICC, venom induced consumption coagulopathy; VWF:Ag, von Willebrand factor antigen; WBCT20–20 min whole blood clotting test

Introduction

Snake envenoming is now recognised as a major health issue in the rural tropics and in particular in south and southeastern Asia.[1] Venom-induced consumption coagulopathy (VICC) is one of the most common snake envenoming syndromes and results from pro-coagulant snake toxins activating the clotting pathway and causing deficiencies of clotting factors.[2] There is limited information on the severity of VICC and the specific factor deficiencies that occur with different snake species.[3–6] In many cases a 20-min whole blood clotting test (WBCT20) is the only test used to detect and monitor coagulopathy in snake bite. More comprehensive investigation of VICC is important for an understanding and treatment of snake envenoming.

Hump-nosed pit viper (Genus *Hypnale*) bites are considered to be the commonest cause of snake bite in Sri Lanka.[1] Currently three species are recognised in the genus *Hypnale*: *H. hypnale*, *H. nepa* and *H. zara.*[7] Bites by Merrem's hump-nosed pit viper (*H. hypnale*) are commoner than the other two species[8] due to the wide distribution of this species around Sri Lanka.[7] Previous reports and clinical experience suggest that envenoming by hump-nosed pit vipers mainly

Received 31 January 2013; accepted 28 May 2013.

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causes local effects and less commonly coagulopathy and acute kidney injury.[8,9]

Although coagulopathy is reported to be uncommon, it is the most common systemic effect reported in *Hypnale* envenoming.[9–13] *In vitro* studies of *Hypnale* venom show it has mild pro-coagulant activity consistent with clinical reports.[14] However, different studies report different incidences of coagulopathy, from 4%[8] to 39%.[9] Except for one study,[13] the presence of coagulopathy is based on a positive WBCT20,[9–12] which has been shown to be unreliable because there is no standardisation of the method.[15] This is the likely reason for the reports of different frequencies of coagulopathy. Coagulopathy, fibrinolysis and spontaneous systemic haemorrhage have also been described following *H. hypnale* envenoming in India.[16]

One previous study of *Hypnale* envenoming showed that 12 of 56 patients had a positive WBCT20, but no evidence of spontaneous bleeding.[13] Ten of these patients had an increased level of fibrinogen degradation products, and seven of these had reduced fibrinogen levels. None of the patients had any abnormalities in their bleeding time, platelet count, prothrombin time (PT) or partial thromboplastin time with kaolin. It is likely that the WBCT20 will only be positive for severe coagulopathy, so mild to moderate coagulant effects may have been missed in this study.

The frequency of coagulopathy and the importance of VICC in *Hypnale* species envenoming remains unclear. The aim of this study was to characterise the severity and frequency of coagulopathy in Sri Lankan hump-nosed pit viper (*H. hypnale*) bites by measuring laboratory clotting times and factor studies for definite hump-nosed pit viper bites.

Methods

This was a prospective observational study of patients with definite hump-nosed pit viper (*H. hypnale*) bites presenting to Chilaw hospital in Central West Sri Lanka. It was conducted as part of a large cohort study of snake bites presenting to the one hospital.[17] The study was approved by the Ethical Review Committee, Faculty of Medicine, University of Colombo. All patients gave written and informed consent for the collection of data and blood samples.

Patients

Cases of hump-nosed pit viper (*H. hypnale*) envenoming were included between January 2007 and July 2009. Any patient older than 13 years of age and who presented with a snake bite was identified when they arrived in hospital. Snake bite cases were initially considered for the study if the patient brought in the snake and health care staff identified it as a hump-nosed pit viper. Inclusion criteria were cases of suspected hump-nosed viper bites where Sri Lankan hump-nosed pit viper (*H. hypnale*) venom was detected in the patient's serum with venom-specific enzyme immunoassays (EIA). *H. hypnale* is the only *Hypnale* species that occurs in this Central West region of Sri Lanka. Patients taking an anti-coagulant or platelet inhibitor were excluded.

Data collection

Baseline data, including demographic features (age and sex), bite information (species of snake and time of bite), clinical effects (local effects: local pain, swelling, bruising, blistering and necrosis; systemic effects: clinical evidence of coagulopathy including bleeding, neurotoxicity and myotoxicity) complications and treatment were recorded for all patients. The WBCT20 was performed routinely on admission for all patients and repeat testing every 6 h was done by the treating team until discharge.[4,18] Research blood samples were collected from all patients on admission and then after 6 h, 12 h, and every day until discharge, if they did not receive anti-venom. Blood was collected in citrated tubes for coagulation studies and in serum tubes for venom-specific EIA. All samples were immediately centrifuged, aliquoted and frozen at − 20°C and then transferred to a − 80°C freezer within 2 weeks of collection until the completion of the study.

None of the hump-nosed pit viper bites received anti-venom because there is no species specific anti-venom to hump-nosed pit vipers and none of the Indian polyvalent snake anti-venoms currently used in Sri Lanka include antibodies to hump-nosed viper. All patients received supportive care. All decisions regarding treatment were made by the treating clinicians.

Venom-specific EIA

Frozen patient serum was used to test for the presence of *H. hypnale* venom using an EIA. The EIA uses polyclonal antibodies (IgG) raised in rabbits to *H. hypnale* venom. Detection uses biotinylated antibodies and then streptavidin horseradish peroxidise. The limit of detection of the assay is 0.2 ng/mL and the method has been previously described in detail.[17,19,20]

Clotting studies and clotting factor assays

Frozen patient citrated plasma was subjected to a series of clotting tests at a centralised laboratory and included PT [and international normalised ratio; INR], activated partial thromboplastin time (aPTT), levels of factors I (fibrinogen), II (prothrombin), V, VII, VIII, IX and X, von Willebrand factor (vWF) antigen and D-Dimer as previously described.[21] All assays were performed on either a Behring Coagulation System or Sysmex CA-1500 analyzer (Dade Behring, Marburg Germany) using standard coagulometric or immunoturbidometric methods as provided by the manufacturer.

Individual coagulation factor levels were determined by incubating patient plasma and factor deficient plasma with the relevant activator, and the time for clot formation was measured in seconds. For each clotting factor, the amount of factor available in the system was quantified from a standard or reference curve of known factor concentrations versus clotting time. The quantification of vWF:Ag and D-Dimer was done using immunoturbidometric methods.

Statistical analysis

The average number of samples collected per patient was three. Clotting studies and factor levels were measured on

Table 1. Lowest (or highest) value for the clotting tests or factor level results for the 80 patients during their hospital admission. Data is presented as median and 2.5–97.5 percentiles.

	Hump-nose viper	Normal range
INR[†]	1.9 (1.3–7.1)	0.9–1.3
aPTT (s)[†]	54 (37–133)	25–35
Fibrinogen (g/L)[*]	1.3 (< 0.2–2.3)	2–4
Factor II (%)	79 (49–129)	70–120
Factor V (%)	43 (5–115)	70–120
Factor VIII (%)	23 (0.9–85)	70–150
Factor VII (%)	85 (35–163)	70–120
Factor IX (%)	73 (38–125)	70–120
Factor X (%)	85 (43–155)	70–120
vWF Ag (%)	77 (23–189)	50–160
D-dimer (mg/L)	3.4 (0.2–49)	< 0.5

INR, international normalised ratio; aPTT, activated partial thromboplastin time; vWF Ag, von Willebrand factor antigen.
*The limit of detection for fibrinogen is 0.2 g/L.
[†]An INR of 12 was unrecordable, i.e. > 12 and an aPTT of 180 was unrecordable i.e. > 180 s.

all samples for each patient. The highest (INR, aPTT, vWF anti-venom and D-Dimer) or the lowest (fibrinogen II, V, VII, VIII, IX and X) of each test for all samples done in an individual patient was reported for each patient for that test. Descriptive data is presented as medians with inter-quartile ranges (IQR) and ranges. All analyses and graphics were done in GraphPad Prism version 5.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

There were 80 patients with definite hump-nosed pit viper (*H. hypnale*) bites from an initial 94 patients with suspected hump-nosed pit viper bites based on patient or hospital staff identification. The median age 37 years (IQR: 26–51 years) and 48 were male. Local envenoming occurred in 74 (92%) patients. Local effects were reported as follows: local pain, 72 (90%); local swelling, 11 (14%); local blistering, 4 (5%) and local necrosis, 1 (1%). The WBCT20 was positive in one patient (1%) on admission. No patient developed clinical evidence of systemic coagulopathy (including bleeding).

The median highest INR and median highest aPTT in patients bitten by the hump-nosed pit viper were elevated at 1.9 (IQR: 1.5–2.2; Range: 1.3 to > 12) and 54 s (IQR: 46–72 s; Range: 35–170 s), respectively. These abnormal clotting times were associated with low fibrinogen levels [median, 1.3 g/L; IQR: 1.0–1.8 g/L; Range: < 0.2–2.9], low factor VIII levels [median 23%; IQR: 16–37%] and low factor V levels [median 43%; IQR: 23–74%] (Table 1 and Fig. 1). D-dimer concentrations [median 3.4 mg/L; IQR: 2–7.4) were slightly elevated. Factors II, VII and X and vWF antigen concentrations were within the normal range (Table 1). There was no difference in the coagulopathy between patients with and without local effects.

Discussion

This study has shown that hump-nosed pit viper envenoming caused a mild coagulopathy in all patients which in most cases was not detected by a WBCT20. It is characterised by a low but detectable fibrinogen, and low levels of Factors V and VIII. However, the INR was similar to that of patients on therapeutic anticoagulants and none of the patients developed any clinically detectable signs of coagulopathy.

The presence of a mild coagulopathy in all hump-nosed pit viper bites in this study is the likely explanation for the inconsistency seen in previous reports of the coagulopathy. These studies used the WBCT20 to determine the presence of coagulopathy following *Hypnale* envenoming and reported a

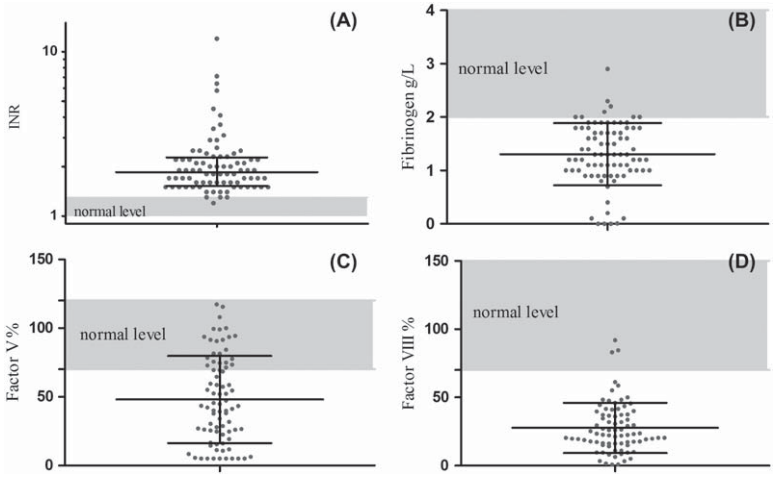

Fig. 1. Scatter plots of peak INR (A), lowest fibrinogen (B), lowest Factor V (C) and lowest Factor VIII (D) with medians and interquartile ranges in 80 patients with hump-nosed pit viper envenoming.

prolonged WBCT20 in 39% of patients in one study,[9] and more recently only 4 of 93 patients (4%).[8] These reports demonstrate the questionable reliability of the WBCT20 for detecting the mild coagulopathy following envenoming by hump-nosed pit vipers. There were six patients with evidence of coagulopathy and who had no reported local effects. This suggests that not all patients with hump-nosed viper envenoming develop significant local effects.

A number of *in vitro* studies have investigated the pro-coagulant effects of *Hypnale* venom. Thrombin-like enzyme activity of *H. hypnale* venom has been reported as 20–22 s/50 µg of venom.[22,23] A more recent study found mild pro-coagulant activity of *H. hypnale* venom with a minimum clotting concentration 5 (MCC_5) value of 4.4 µg/mL.[14] This value is 10- to 50-fold greater (i.e. lower pro-coagulant activity) than 0.08–0.4 µg/mL for Australian elapids, which have highly potent pro-coagulant venoms.[24] These studies would suggest that *Hypnale* venom has a mild thrombin-like effect most likely from the presence of a snake venom thrombin-like enzyme (SV-TLEs) in the venom.

SV-TLEs can act on either the α, β, or both chains of fibrinogen and produce corresponding fibrinopeptides. This leads to consumption of fibrinogen (defibrination) rather than conversion to fibrin and cross-linking to form a clot.[25] Such defibrination is seen commonly with the Malayan pit viper (*Callosellasma rhodostoma*) which is the phylogenetically closest pit viper to *Hypnale* and whose venom contains Ancrod which has fibrinogenolytic Aα activity.[26] It is therefore likely that the hump-nosed pit viper venom contains SV-TLEs which is consistent with the results of our study in which low fibrinogen levels were observed.

The low D-Dimer results in this study are interesting and we assume their slight elevation occurred because very little fibrinogen was converted to fibrin and cross-linked. Thus, the fibrinolysis will not result in cross-linked degradation products (XDPs), and only fibrinogen degradation products (FDPs). The latter are not detected by the newer D-Dimer assays. In contrast, Australian elapids produce VICC that results in very high D-Dimer (100- to 1000-fold elevations) because they contain prothrombin activators that result in the normal conversion of fibrinogen to fibrin with cross-linking, followed by fibrinolysis.[3]

The low concentrations of Factor VIII compared to Factor V in *Hypnale* envenoming are unexpected considering the mildness of the coagulopathy. The Factor VIII concentrations are similar to those seen with VICC following Australasian elapid envenoming, but the latter also causes complete consumption of fibrinogen and Factor V.[3] It is unlikely that this reduction of Factor VIII is just secondary to the consumption of fibrinogen in the case of *Hypnale* envenoming, because Factor V levels are only mildly depressed. Another possibility is that the major pro-coagulant toxin or perhaps another toxin in hump-nosed pit viper venom can activate Factor VIII. Some of the SV-TLEs are known to activate other factors in the clotting cascade,[2] including Factor VIII.[27] Thrombin-like activity in association with Factor VIII activation by *Hypnale* venom could be the possible explanation for the above coagulation results.

One limitation of the study was that timed coagulation tests and clotting factor studies were not available for the full duration of hump-nosed pit viper envenoming. Patients with hump-nosed viper bites were often discharged within 24 h of admission due to the mild clinical effects. Further studies will need to measure clotting tests over a longer period of time to determine when complete recovery of haemostasis occurs. Another limitation of the study was that frozen samples were used for the clotting studies and factor levels. However, this has been done previously in an Australian study.[21] This approach has the advantage of all the studies being done within a short period of time in one laboratory. All samples were centrifuged immediately after collection, aliquoted and then frozen by clinical research assistants who had been trained in sample collection and processing.

Conclusions

This study reports coagulation tests and clotting factor levels following hump-nosed pit viper envenoming showing that it causes mild VICC with mildly decreased levels of fibrinogen and Factor V, and low levels of factor VIII. These findings are consistent with at least SV-TLEs being present in *Hypnale* venom. This study demonstrates the limitations and ongoing problems with using WBCT20 to define coagulopathy in snake-bite patients.

Acknowledgements

We acknowledge the assistance of the medical and nursing staff at Chilaw hospital, Central West Province, Sri Lanka for identifying patients for the study. We acknowledge Andrew Dawson for assistance throughout the study.

Ethical statement

The study was approved by the Ethical Review Committee, Faculty of Medicine, University of Colombo.

Declaration of interest

The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

The study was supported in part by NHMRC Project Grant 631073 and by IRQUE Project Grant C1A2 MBBS SPQEF. Geoff Isbister is funded by an NHMRC Clinical Career Development Award ID 605817. C. Ariaranee Gnanathasan is funded by an IRQUE Project.

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Chapter Three

Venom Concentrations and Clotting Factor Levels in a Prospective Cohort of Russell's Viper Bites with Coagulopathy

Introduction to Chapter Three: Explanation of contribution of the paper titled "Venom Concentrations and Clotting Factor Levels in a Prospective Cohort of Russell's Viper Bites with Coagulopathy" to the overall thesis

Russell's viper (*Daboia russelii*) envenoming is one of the commonest and most medically important snake envenomings in Asia, with bites causing deaths in 2 to 5% of all cases (Kularatne, 2003). Russell's vipers account for 30 to 40% of snake bites in Sri Lanka and the majority of fatal snakebites (Kasturiratne et al., 2005). VICC is the commonest systemic effect of envenoming and can result in major bleeding including intracranial hemorrhages (Kularatne, 2003).

The *in-vitro* procoagulant effects of Russell's viper venom have been well characterized and a number of procoagulant toxins have been isolated (Takeya et al., 1992, Gowda et al., 1994). Russell's viper venom contains factor X and factor V activators which trigger the clotting pathway, resulting in consumption of multiple clotting factors (Isbister, 2009). Although prolonged prothrombin time (PT)/international normalized ratio (INR) and activated partial thromboplastin time (aPTT), decreased fibrinogen, factor V, factor X and elevated D-Dimer concentrations have been previously described (Phillips et al., 1988, Than et al., 1985), there is no information on the dynamics of clotting factor levels and clotting time tests and their response to antivenom. There is also limited information available on the relationship between venom concentration and clotting function. In this chapter I investigated the dynamics of clotting times and clotting factor concentrations in Russell's viper envenoming and explored the effect of antivenom on the time course of VICC.

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was accepted into PLoS Neglected Tropical Diseases for publication on 9th July 2015. I, Maduwage Kalana Prasad, was the second author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

Clinical and laboratory samples were collected under the supervision of clinicians of the hospitals; C. Abeysinghe, H. Karunathilake, C.A Gnanathasan. Clotting factor assays were carried out by E. F. Scorgie and L. F. Lincz. Clinical and laboratory samples collections were coordinated by S. Shahmy and F. Mohamed. Russell's viper venom quantification of patients' samples was carried out by myself under the supervision of Dr Margaret O'Leary. Overall supervision of the study was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter three.

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RESEARCH ARTICLE

Venom Concentrations and Clotting Factor Levels in a Prospective Cohort of Russell's Viper Bites with Coagulopathy

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OPEN ACCESS

Citation: Isbister GK, Maduwage K, Scorgie FE, Shahmy S, Mohamed F, Abeysinghe C, et al. (2015) Venom Concentrations and Clotting Factor Levels in a Prospective Cohort of Russell's Viper Bites with Coagulopathy. PLoS Negl Trop Dis 9(8): e0003968. doi:10.1371/journal.pntd.0003968

Editor: Ana Moura, Instituto Butantan, BRAZIL

Received: February 18, 2015

Accepted: July 9, 2015

Published: August 21, 2015

Data Availability Statement: Data are available from the University of Newcastle NOVA online repository at http://hdl.handle.net/1959.13/1293286.

Funding: The study was supported in part by NHMRC Project Grant 631073 and by IRQUE Project Grant. Geoff Isbister is funded by an NHMRC Senior Research Fellowship ID1061041. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Background

Russell's viper envenoming is a major problem in South Asia and causes venom induced consumption coagulopathy. This study aimed to investigate the kinetics and dynamics of venom and clotting function in Russell's viper envenoming.

Methodology/Principal Findings

In a prospective cohort of 146 patients with Russell's viper envenoming, we measured venom concentrations, international normalised ratio [INR], prothrombin time (PT), activated partial thromboplastin time (aPTT), coagulation factors I, II, V, VII, VIII, IX and X, and von Willebrand factor antigen. The median age was 39y (16–82y) and 111 were male. The median peak INR was 6.8 (interquartile range[IQR]:3.7 to >13), associated with low fibrinogen [median,<0.01g/L;IQR:<0.01–0.9g/L), low factor V levels [median,<5%;IQR:<5–4%], low factor VIII levels [median,40%;IQR:12–79%] and low factor X levels [median,48%; IQR:29–67%]. There were smaller reductions in factors II, IX and VII over time. All factors recovered over 48h post-antivenom. The median INR remained >3 at 6h post-antivenom but had reduced to <2, by 24h. The aPTT had also returned to close to normal (<50sec) at 24h. Factor VII, VIII and IX levels were unusually high pre-antivenom, median peak concentrations of 393%, 307% and 468% respectively. Pre-antivenom venom concentrations and the INR ($r = 0.20$, $p = 0.02$) and aPTT ($r = 0.19$, $p = 0.03$) were correlated (non-parametric Spearman analysis).

Conclusions

Russell's viper coagulopathy results in prolonged aPTT, INR, low fibrinogen, factors V, VIII and X which recover over 48h. Severity of clotting abnormalities was associated with venom concentrations.

Author Summary

Snake envenoming is an important health issue in many parts of the world and coagulopathy is one of the commonest manifestations. Russell's viper envenoming occurs throughout south-east and south Asia and most commonly causes a venom induced consumption coagulopathy which may be complicated by bleeding. There is a limited understanding of the time course of the coagulopathy, including the specific clotting factor deficiencies and their relationship to the time course of venom concentrations. In this study, we measure the venom concentrations and clotting factor concentrations throughout the course of the patient admission. The study confirms that Russell's viper envenoming causes prolonged clotting times and deficiencies of factors V, X, VIII and fibrinogen which recover over 2 days post-antivenom. In addition, the prothrombin time and activated partial thromboplastin times correlated with venom concentrations. Factor VII, VIII and IX levels were very high and may be related to venom activity, although further research is required.

Introduction

Snake envenoming is a major health issue in the Asia-Pacific region with between 250,000 and 1 million cases occurring annually.[1] Russell's viper (*Daboia russelii*) is one of the most medically important snakes in the region,[1, 2] with bites causing death in 2 to 5% of cases, accounting for the majority of fatal snakebites in Sri Lanka[3]. Russell's viper envenoming results in local effects, venom induced consumption coagulopathy (VICC), mild neurotoxicity and renal injury.[3, 4] VICC is the commonest systemic manifestation and in some cases results in mucosal bleedings and less commonly major bleeding including intracranial haemorrhage.[3, 5–7]

The *in vitro* procoagulant effects of Russell's viper venom have been well characterised and a number of procoagulant toxins have been isolated and used in laboratory assays for decades.[8–10] Russell's viper venom contains both factor X and factor V activators which trigger the clotting pathway early on, resulting in consumption of multiple clotting factors.[11–13] Previous studies have shown that VICC resulting from Russell's viper envenoming causes overall haemostatic disturbances which manifest in prolonged prothrombin time (PT)/international normalised ratio (INR) and activated partial thromboplastin time (aPTT), as well as decreased levels of fibrinogen, factor V and factor X and elevated D-Dimer concentrations.[14–17] However, there is limited information on the dynamics of clotting factor levels in Russell's viper envenoming and their response to antivenom treatment.

A number of studies have measured venom concentrations in patients with Russell's viper envenoming, and some have suggested that there is recurrence of venom post-antivenom.[5, 18] However, there is limited information on the dynamic relationship between venom concentrations and clotting factor levels, and whether the detection of venom post-antivenom is associated with further clotting factor consumption.

This study aimed to explore the dynamic changes in venom and clotting factor levels in VICC, including the effect of antivenom treatment following Russell's viper envenoming.

Methods

This was a prospective observational study of serial venom concentrations and clotting factor levels in definite Russell's viper (*D. russelii*) envenomed patients admitted to a single hospital in Sri Lanka. Patients were recruited as part of a large cohort study of snakebites admitted to the Base Hospital Chilaw in Central West Sri Lanka[19]. The study was approved by the Ethical Review Committee, Faculty of Medicine, University of Colombo, Sri Lanka. All patients gave written and informed consent for the collection of clinical data and blood samples.

Patients

All patients (>13 years old) admitted with a suspected or definite snake bite between January 2007 and July 2009 were identified on admission to hospital. From these only definite cases were included if Russell's viper venom was detected with a venom-specific enzyme immunoassay (EIA). All admission samples were tested for Russell's viper venom. VICC was defined as coagulopathy (abnormal PT/INR) with evidence of consumption (low/undetectable fibrinogen or elevated D-Dimer more than 10 times the upper limit of normal) and an INR > 1.5.[20] Severe or complete VICC was defined as an INR > 13 (unrecordable).

Data collection

Baseline data, including demographic data (age and sex), information on the snake bite (snake type, time of bite), clinical effects (local effects: local pain, swelling, bruising, blistering and necrosis; systemic effects: features of coagulopathy including bleeding and neurotoxicity) and antivenom treatment (dose and time of administration) were recorded prospectively for all patients. Research blood samples were collected from all patients on admission and then at regular time intervals during their admission. Blood was collected in citrated tubes for clotting times and coagulation studies and in serum tubes for venom-specific EIA. All samples were immediately centrifuged, aliquoted and frozen initially at -20°C, and then transferred to a -80°C freezer within 2 weeks of collection. All patients received Indian polyvalent snake antivenom manufactured by VINS Bioproducts Limited (batch number: ASV 42C/06, 1030) or BHARAT Serum and Vaccines Limited, India (batch number: 5346KD4, LY 55/05, LY 32/04, A5307035).

Venom-specific enzyme immunoassays (EIA)

A sandwich EIA was used to measure Russell's viper venom in serum samples and has previously been described.[7, 21, 22] In brief, polyclonal IgG antibodies were raised against Russell's viper (*D. russelii*) venom in rabbits[23]. Antibodies were bound to microplates as well as being conjugated to biotin for a sandwich EIA with the detecting agent being streptavidin-horseradish peroxidase. All samples were measured in triplicate, and the averaged absorbance converted to a concentration by comparison with a standard curve based on serial dilutions of venom using a sigmoidal curve. The assay does not cross-react with *Hypnale* venom, the only other medically important snake in Sri Lanka that cause coagulopathy (excepting *Echis carinatus*—saw-scaled viper—in the north). [22]

Clotting studies and clotting factor assays

Frozen citrated plasma samples were used for all clotting times and clotting factor studies including prothrombin time (PT)/international normalised ratio [INR] and activated partial thromboplastin time (aPTT). Levels of factors I (fibrinogen), II (prothrombin), V, VII, VIII, IX and X, von Willebrand factor antigen (VWF:Ag) and D-Dimer were all measured. All assays

were done using either standard coagulometric or immunoturbidimetric methods as provided by the manufacturer and were performed on a Behring Coagulation System (BCS) or Sysmex CA-1500 analyzer (Dade Behring, Marburg, Germany) [24]. Individual clotting factor levels were determined by mixing patient plasma with plasma deficient in the factor being measured and the time for clot formation measured in seconds. The amount of factor present in the sample was quantified by comparing with a standard or reference curve produced using serial dilutions of plasma deficient in the factor mixed with normal plasma, against the clotting time. The quantification of von Willebrand factor antigen (VWF:Ag) and D-Dimer was done using immunoturbidometric methods.

Data analysis

The average number of samples collected from the patients was four (range 1 to 10). To describe the peak effect of the venom on the clotting pathway, the maximal (longest) clotting time or minimum (lowest) factor level was determined for each patient. Factor levels and clotting times were then reported as medians and interquartile ranges of the maximum (PT/INR, aPTT, D-Dimer) or minimum (Fibrinogen, Factors II, V, VIII, IX, X) for each level over the time course of the admission.

For visual analysis of concentration time data, median factor concentrations were plotted versus time to provide empirical estimates of the average/median changes over time in the coagulation studies and the factor levels. Time zero was defined as the time of antivenom administration. This was done by binning the data based on the time post-snake antivenom and then calculating the median factor level/clotting time for each bin and the median time for each bin (ie. for the specified time period). The median factor level/clotting time was then plotted versus the median time. For factors VII, VIII and IX, the median and interquartile range of the levels of these factors was taken from the bin where the peak occurred because of the unusually high pre-antivenom levels of these three factors. Separate plots were also made for complete VICC where the INR was unrecordable (INR $>$ 13) and partial VICC where the INR was abnormal but still recordable (1.5 $<$ INR $<$ 13).

To investigate whether there was an association between the venom load (i.e. amount of venom delivered by the snake) and the severity of VICC, correlations between pre-venom concentrations and the PT/INR, aPTT and all clotting factor levels were tested with non-parametric Spearman correlation analysis. In addition clotting tests and factor studies versus pre-antivenom venom concentrations were plotted (with the line of best fit and 95% confidence intervals).

All analyses and graphics were done in GraphPad Prism version 6.01 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

There were 146 patients with Russell's viper bites. The median age was 39 years (Range: 16 to 82 years) and 111 (76%) were male. All patients had VICC (low fibrinogen and elevated PT) and 70 (48%) had neurotoxicity. Local effects were reported in 134 (91%) patients and systemic bleeding developed in 14 (10%) patients. The median pre-antivenom venom concentration was 201ng/ml (IQR: 74 to 435ng/ml; Range: 1 to 1521ng/ml) which dropped to a median concentration of 2ng/ml (IQR: 0 to 9ng/ml) after the administration of antivenom.

The median peak INR in the patients was elevated at 6.8 (IQR: 3.7 to $>$13) as was the median peak aPTT of $>$180sec (IQR: 91.3 to $>$ 180sec). The abnormal clotting times were associated with a low fibrinogen [median, $<$0.01g/L; IQR: $<$0.01 to 0.9g/L], low factor V levels [median $<$5%; IQR: $<$5 to 4%], low factor VIII levels [median; 24%; IQR: 10 to 41%] and mildly decreased factor X levels [median 48%; IQR: 29 to 67%] over the course of the patient

Table 1. The median, interquartile range (IQR) and range of the minimum (Factors I, II, V, VII, VIII, IX, X) or maximum (PT/INR, aPTT, D-Dimer) factor concentrations/clotting times measured for the 146 patients during their hospital admission.

Factor Concentration or Clotting times	Normal Range	Median	Interquartile range	Range
Prothrombin time (PT) sec	9–14	69	36–180	12–180
INR	0.9–1.3	6.8	3.7 –>13	1.3 –>13
aPTT (s)	25–35	> 180	91.3 –> 180	29 –> 180
Fibrinogen (g/L)	2–4	< 0.01	<0.01–0.9	<0.01–3
Factor II (%)	70–120	60	49–74	10–120
Factor V (%)	70–120	< 5	<5–4	<5–61
Factor VII (%)	70–120	63	43–123	15–1203
Factor VIII (%)	70–120	24	10–41	1–335
Factor IX (%)	70–120	88	66–109	2–860
Factor X (%)	70–120	48	29–67	<0.01–152
VWF:Ag (%)	50–160	176	100–245	39–523
D-dimer (mg/L)	< 0.5	134	20–450	1–905

doi:10.1371/journal.pntd.0003968.t001

admissions. The median of the highest or median of the lowest factor concentrations/clotting times are given in Table 1. There were smaller reductions in factors II, VII and IX over the course of the patient admission. The INR, fibrinogen, factors V and X recovered over 48 hours post-antivenom (Figs 1 and 2). The median INR remained greater than 3 at 6 hours post-antivenom, but had reduced to less than 2, by 24 hours (Fig 1). The aPTT had also returned to close to normal (< 50sec) at 24 hours (Fig 1). There were smaller reductions in most factor levels in partial versus complete VICC (S1 Fig).

Factors VII, VIII and IX levels were very high prior to antivenom and then dropped dramatically into the normal range or to low levels (FVIII) post-antivenom (Fig 2). The median peak factor VII levels were 393% (IQR: 85 to 698%), factor VIII levels were 307% (IQR: 160 to 400%) and factor IX levels were 468% (IQR: 331 to 704%) respectively (Fig 2).

There was a statistical association between pre-antivenom venom concentrations and the INR ($r = 0.20$, $p = 0.02$), aPTT ($r = 0.19$, $p = 0.03$) and factor IX ($r = -0.36$, $p<0.001$), and there were trends for factor V ($r = -0.17$, $p = 0.058$), factor X ($r = -0.17$, $p = 0.05$) and VWF:Ag ($r = -0.18$, $p = 0.053$) (S2 Fig). There were no statistical associations for pre-antivenom venom concentrations and fibrinogen, factors II, VII, VIII and D-Dimer (S2 Fig).

Discussion

The study shows that VICC resulting from Russell's viper envenoming is characterised by an elevated INR and aPTT associated with low fibrinogen, factor V, VIII and X levels. There was an association between the pre-antivenom venom concentrations and the severity of the coagulopathy, mainly with the INR and aPTT. The coagulopathy resolved over a period of 48 hours after the administration of antivenom consistent with VICC from other snakes.[24, 25] An unusual finding was the very high levels (above the normal range) of factor VII, VIII and IX prior to antivenom treatment which then returned to normal ranges soon after antivenom treatment. It is unclear the exact reason for these high factor levels but may be related to venom activity in the sample *in vitro*.

Russell's viper venom contains factor V and factor X activators which convert these factors to their activated forms (i.e. Va and Xa), explaining the low factor V and factor X levels in human envenoming (Fig 3).[11, 12, 26, 27] Activation of factor V and X results in the formation of the prothrombinase complex (XaVa) which activates the whole clotting cascade by converting prothrombin to thrombin. This then leads to the consumption of fibrinogen, factor

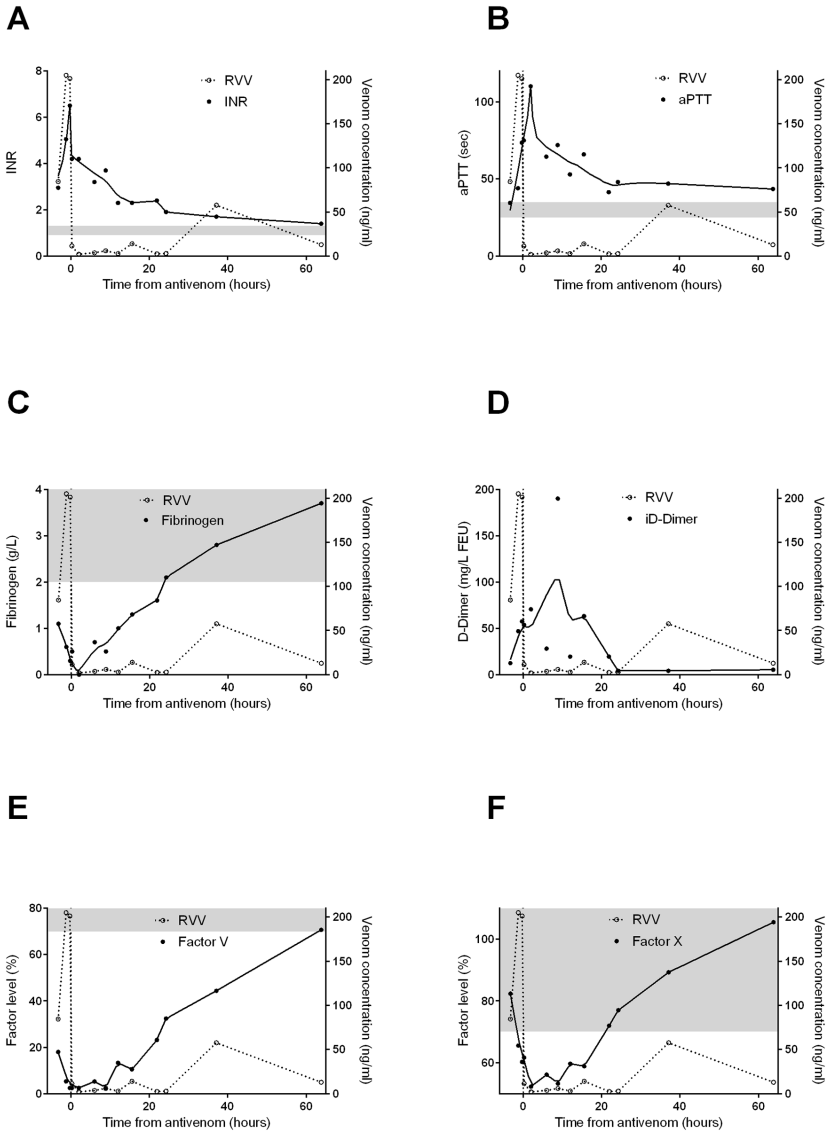

Fig 1. Plots of median venom concentrations (open circles [○] and dashed lines; all panels), clotting times and factor concentrations (filled circles [●]) versus time post-antivenom for 146 patients with Russell's viper envenoming including INR [A], aPTT (sec) [B], fibrinogen (g/L) [C], iD-dimer (mg/L FEU) [D], factor V (%) [E] and factor X (%) [F]. Black lines represent the interpolated median factor concentration time curves and the shaded area is the normal range for each test. INR—international normalised ratio; aPTT—activated partial thromboplastin time; RVV—Russell's viper venom.

doi:10.1371/journal.pntd.0003968.g001

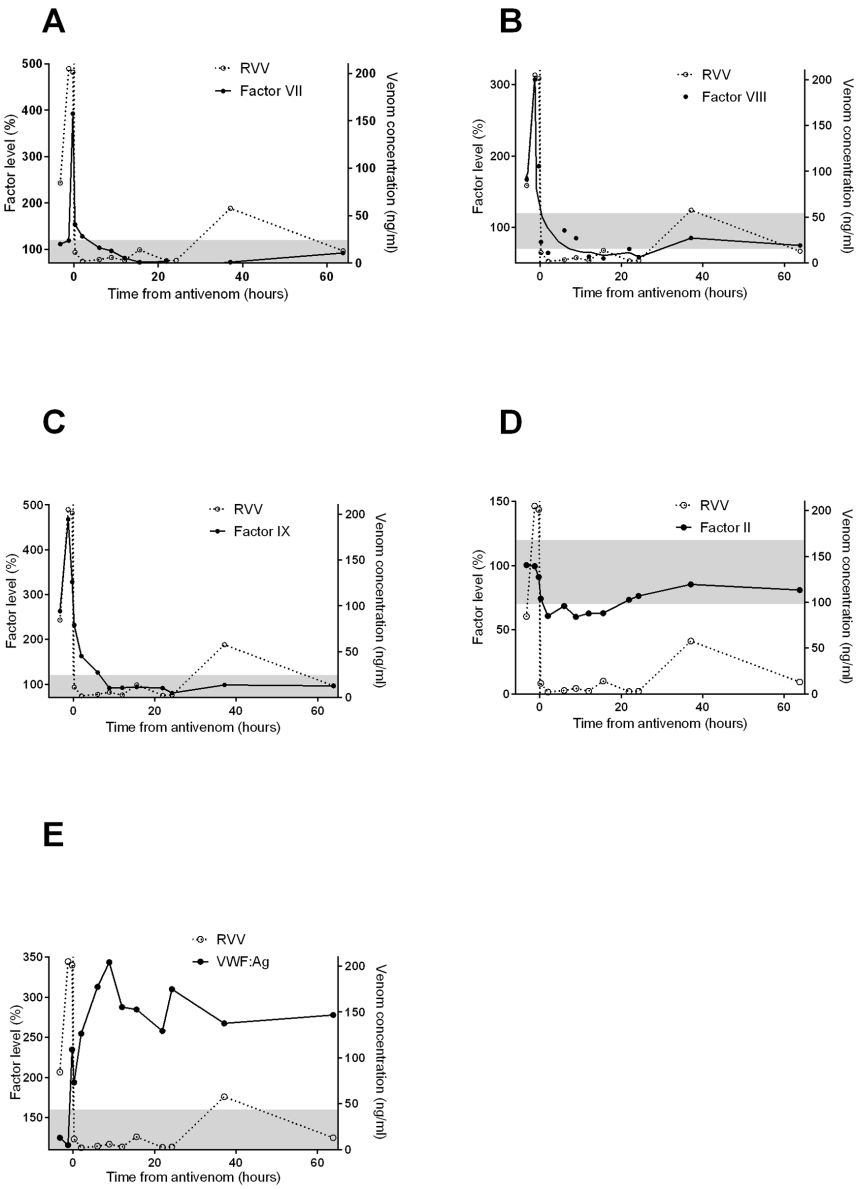

Fig 2. Plots of median venom concentrations (open circles [○] and dashed lines; all panels), clotting times and factor concentrations (filled circles [●] and lines) versus time post-antivenom for 146 patients with Russell's viper envenoming including factor VII (A; thick lines), factor VIII (B), factor IX (C), factor II (D) and v-WF antigen (E). The black lines represent the interpolated median factor concentration time curves and the shaded area is the normal range for each test. RVV—Russell's viper venom; VWF:Ag—von Willebrand factor antigen.

doi:10.1371/journal.pntd.0003968.g002

VIII and further consumption of factor V. The multiple factor deficiencies result in the prolonged INR and aPTT, and activation of the clotting cascade leads to increased fibrinolysis and therefore elevated D-Dimer levels. A similar pattern of factor deficiencies has been described in previous studies of patients envenomed by Russell's vipers.[14–17] Four studies showed the initial drop in fibrinogen to low levels followed by a recovery over 24 to 48 hours. [14–17] Two studies also reported serial measurements of factor V and factor X, with initial low levels that recovered over 24 hours consistent with our study.[15, 16]

The recovery of the coagulopathy occurred over a period of 48 hours, although the rate of recovery differed for each of fibrinogen, factor V and factor VIII. The median INR was still greater than 3 at 6 hours, suggesting that 6 hours if too early to determine the effect of antivenom (Fig 1). The median INR was 2.3 at 12 hours and then less than 2 at 24 hours suggesting that patients had only a mild coagulopathy at this time (Fig 1).

The presence of very higher factor VII, VIII and IX levels (392%, 313% and 463% respectively; Fig 2) prior to antivenom is an unexpected finding, although earlier studies have reported high values in a small number of patients.[15, 16] A possible explanation for this finding is that the presence of Russell's viper venom (RVV) factor X activator toxin in the sample results in falsely high factor levels. The *in vitro* activity of the toxin would appear similar to the activity of factor VII, VIII and IX. For example, in the case of factor VII, the presence of active RVV in the sample will result in a shorter clotting time because the RVV factor X activator has the same activity as factor VII (i.e. both VII:TF and RVV factor X activator convert factor X to Xa). Interpolation of this shorter clotting time on the standard curve for factor VII results in a factor VII level greater than 100% (S3 Fig). A similar phenomena occurs with both factor VIII and factor IX because the VIIIa:IXa complex also activates factor X to Xa (intrinsic pathway). Previous studies that have measured factor VII, VIII and IX levels have also found normal to high values, but because they have only measured factors at one time point, they are difficult to interpret.[14, 16] The time course of these three factors, with high levels only occurring in the pre-antivenom samples, and then normal factor VII levels or low factor VIII and IX levels post-antivenom, also suggests that these high levels prior to antivenom are due to the assay and not VICC (Fig 2).

There was only moderate reduction in factor II, and moderate reductions in factors VII and IX after the initial high pre-antivenom values of the latter two. This is most likely because none of these factors are directly activated by the venom (as are factors V and X), or are factors that are completely consumed when the clotting pathway is completely activated (factor VIII). There is a large excess of factor II (prothrombin), so even if all the fibrinogen is converted to fibrin (consumed), prothrombin levels will only partially decrease. Factor VII and IX are also not activated and consumed in VICC. VWF:Ag levels were mildly elevated which is most likely an indirect consequence of the clotting pathway being activated.

There was a rebound in venom concentrations approximately 40 hours after antivenom (Figs 1 and 2) which we have previously shown to not be associated with recurrent coagulopathy.[28] A recent study has shown that this re-appearance of venom is due to bound venom being detected by the venom specific EIA.[29] This is entirely consistent with the results in this study. There was recovery of all clotting factors and clotting times despite this rebound in measured venom This further supports that venom-specific EIA is measuring bound venom post-antivenom.

The study found a correlation between venom concentrations and the severity of the coagulopathy measured by the INR and aPTT. An earlier study by Than et al found a similar association between venom concentrations and the severity of the coagulopathy.[15] There was an association between venom concentrations and factor V and Factor X, consistent with the Factor V activator and Factor X activator toxins in the venom.

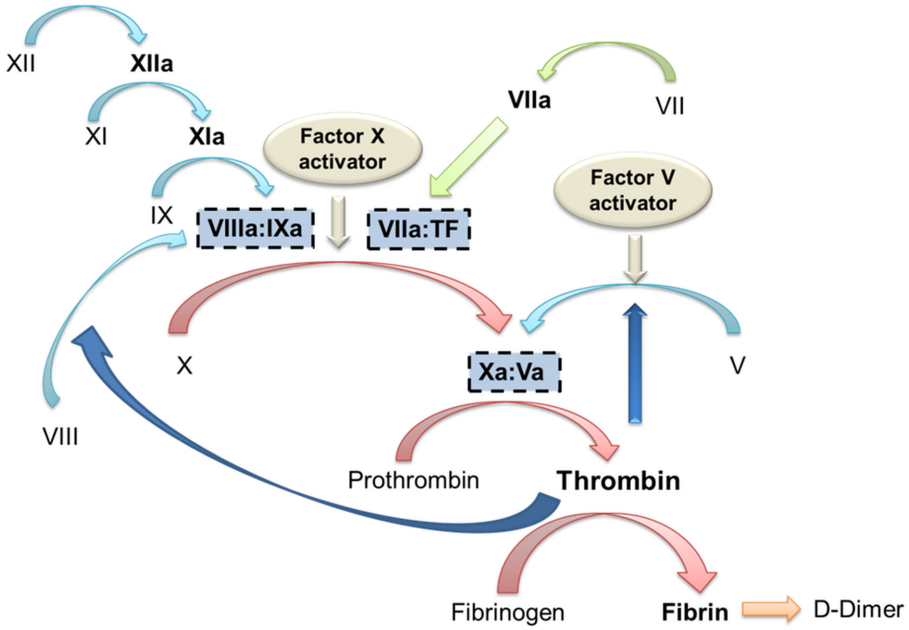

Fig 3. Diagrammatic representation of the clotting pathway and the points where Russell's viper factor X and factor V activators cause activation of the clotting pathway.

doi:10.1371/journal.pntd.0003968.g003

There are a number of limitations of the study. Coagulation studies are best done on samples collected fresh from the patient. This was not possible in this study because of the resource limitations in the hospital where the patients were treated. However, all samples were immediately centrifuged, aliquoted and frozen to preserve the functional activity of the clotting factors. The results were in keeping with another study of VICC in Australian elapids which used a similar approach successfully.[24]Platelet counts, platelet function testing and tissue factor assays were not done in this study. These may play a role in haemostatic disturbances, particularly platelets, and should be investigated in future studies.

Another limitation was that the decision to give antivenom was made by the treating clinician and not the investigators. However, the majority of these patients were recruited to a randomised controlled trial comparing two different infusion rates of antivenom. This meant that all patients had a standardised antivenom administration.

Supporting Information

S1 Fig. Plots of median venom concentrations (red circles and lines; all panels), clotting times and factor concentrations (blue circles and lines) versus time post-antivenom for 51 patients with complete VICC (closed circles [●] and lines; column 1) and 94 with partial VICC (open circles [○] and dashed lines; column 2), including INR [A], fibrinogen (g/L)

[B], iD-dimer (mg/L FEU) [C], factor V (%) [D] and factor X (%) [E]. The shaded area is the normal range for each test. INR—international normalised ratio; RVV—Russell's viper venom.
(TIF)

S2 Fig. Plots of clotting tests and factor levels versus pre-antivenom (AV) venom concentrations showing the linear regression with 95% confidence intervals for international normalised ratio (INR; A), activated partial thromboplastin time (aPTT; B), factor V (C), factor X (D), factor VIII (E), factor IX (F), v-WF antigen (G) and fibrinogen (H) a. VWF: Ag—von Willebrand factor antigen.
(TIF)

S3 Fig. Diagram of the steps in the factor VII assay and the standard curve that is used to convert clotting times measured in the assay to factor VII concentrations. The standard curve is made by diluting normal plasma with factor VII deficient plasma and measuring a range of clotting times for decreasing dilutions of factor VII concentrations. In a normal sample where there is normal factor VII concentrations the measured clotting time will be approximately 12 seconds which corresponds to 100% factor VII concentration. Factor deficient patient samples will have a longer clotting time and therefore lower factor concentrations. However, when factor X activator from Russell's viper venom (RVV) is present in the sample this will result in a shorter clotting time because RVV factor X activator has the same action as factor VII. In the diagram the clotting time is 2 seconds which is interpolated as 400% factor VII concentration. In this way the factor assay is a surrogate measure for the toxin activity.
(TIF)

Acknowledgments

We acknowledge the assistance of the medical and nursing staff at Chilaw hospital for identifying patients for the study. We acknowledge Andrew Dawson for assistance throughout the study.

Author Contributions

Conceived and designed the experiments: GKI LFL CAG. Performed the experiments: FES KM MAO. Analyzed the data: KM LFL GKI. Contributed reagents/materials/analysis tools: LFL. Wrote the paper: FES KM MAO LFL CAG CA HK FM SS. Patient recruitment: FM SS CA HK.

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Chapter Four

Detection of Venom after Antivenom Is Not Associated with Persistent Coagulopathy in a Prospective Cohort of Russell's Viper (*Daboia russelii*) Envenomings

Introduction to Chapter Four: Explanation of contribution of the paper titled "Detection of Venom after Antivenom Is Not Associated with Persistent Coagulopathy in a prospective Cohort of Russell's Viper (*Daboia russelii*) Envenomings" to the overall thesis

Venom recurrence is defined as the detection of venom in post-antivenom samples after the initial decrease to zero or low concentrations. Venom recurrence has been described following bites by many vipers worldwide, including Russell's viper envenoming. The main question has been whether the detection of venom in post antivenom samples is associated with further envenoming, and in the case of coagulopathy, worsening VICC. Therefore, we aimed to investigate the recurrence of venom detection following Russell's viper envenoming and whether this is associated with a recurrence of envenoming (coagulopathy) in this chapter.

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was accepted into PLoS Neglected Tropical Diseases for publication on 30th September 2014. I, Maduwage Kalana Prasad, was the first author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

Clinical and laboratory samples were collected under the supervision of clinicians of the hospitals; C. Abeysinghe, H. Karunathilake, C.A Gnanathasan. Clotting factor assays were carried out by E. F. Scorgie and L. F. Lincz. Clinical and laboratory samples collections were coordinated by S. Shahmy and F. Mohamed. Russell's viper venom quantification of patients' samples was carried out by myself under the supervision of Dr Margaret O'Leary. Overall supervision of the study was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter Four.

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Detection of Venom after Antivenom Is Not Associated with Persistent Coagulopathy in a Prospective Cohort of Russell's Viper (*Daboia russelii*) Envenomings

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Abstract

Background: Venom recurrence or persistence in the circulation after antivenom treatment has been documented many times in viper envenoming. However, it has not been associated with clinical recurrence for many snakes, including Russell's viper (*Daboia* spp.). We compare the recovery of coagulopathy to the recurrence or persistence of venom in patients with Russell's viper envenoming.

Methodology/Principal Findings: The study included patients with Russell's viper (*D. russelii*) envenoming presenting over a 30 month period who had Russell's viper venom detected by enzyme immunoassay. Demographics, information on the snake bite, and clinical effects were collected for all patients. All patients had serum collected for venom specific enzyme immunoassay and citrate plasma to measure fibrinogen levels and prothrombin time (international normalised ratio; INR). Patients with venom recurrence/persistence were compared to those with no detectable recurrence of venom. There were 55 patients with confirmed Russell's viper envenoming and coagulopathy with low fibrinogen concentrations: 31 with venom recurrence/persistence, and 24 with no venom detected post-antivenom. Fibrinogen concentrations increased and INR decreased after antivenom in both the recurrence and non-recurrence patients. Clinical features, laboratory parameters, antivenom dose and length of hospital were similar for both groups. Pre-antivenom venom concentrations were higher in patients with venom recurrence/persistence with a median venom concentration of 385 ng/mL (16–1521 ng/mL) compared to 128 ng/mL (14–1492 ng/mL; $p = 0.008$).

Conclusion: Recurrence of Russell's viper venom was not associated with a recurrence of coagulopathy and length of hospital stay. Further work is required to determine if the detection of venom recurrence is due to the venom specific enzyme immunoassay detecting both venom-antivenom complexes as well as free venom.

Citation: Maduwage K, O'Leary MA, Scorgie FE, Shahmy S, Mohamed F, et al. (2014) Detection of Venom after Antivenom Is Not Associated with Persistent Coagulopathy in a Prospective Cohort of Russell's Viper (*Daboia russelii*) Envenomings. PLoS Negl Trop Dis 8(12): e3304. doi:10.1371/journal.pntd.0003304

Editor: Robert A. Harrison, Liverpool School of Tropical Medicine, United Kingdom

Received July 21, 2014; **Accepted** September 30, 2014; **Published** December 18, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. The data are available at http://hdl.handle.net/1959.13/1051772.

Funding: The study was supported in part by NHMRC Project Grant 631073. GKI is funded by an NHMRC Senior Research Fellowship ID1061041. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Snake envenoming is a major public health issue in many resource poor countries in the rural tropics [1]. Understanding the underlying pathophysiology of snake envenoming and the effect of antivenom is essential to improving health outcomes. An important part of investigating snake envenoming is the detection and measurement of venom in human sera, which confirms the type of snake (diagnosis) as well as assessing the efficacy of antivenom. As such, venom concentrations are measured before and after antivenom and the absence of venom in blood post-antivenom has been interpreted to mean that sufficient antivenom has been administered and envenoming will resolve. This has been used as an important end-point in recent studies in Australia showing that one vial of antivenom is sufficient for the treatment of all Australian elapids [2]. The persistence or recurrence of venom after antivenom administration has been interpreted as insufficient antivenom being administered and there being ongoing envenoming.

Author Summary

Snakebite is a major public health problem and understanding the effectiveness of antivenom is essential to improving health outcomes. The measurement of venom in blood has been used to assess the effectiveness of antivenom. The absence of venom post-antivenom indicating that sufficient antivenom has been given, and the persistence or recurrence of venom indicating that insufficient antivenom has been given. There are numerous reports of venom recurrence with viper bites, including Russell's viper bites. However, it remains unclear if venom recurrence is always an indicator of inadequate antivenom and recurrence of clinical envenoming. In this study, we compare patients with and without the persistence or recurrence of venom who develop coagulopathy after Russell's viper bites. There was no difference in the recovery of the coagulopathy between the two groups of patients demonstrating that for Russell's viper envenoming, venom recurrence or persistence was not associated with the recurrence or persistence of clinical effects such as coagulopathy. Patients with detectable venom after antivenom did have higher pre-antivenom venom concentrations. Further investigation is required to interpret venom concentrations post-antivenom.

The phenomenon of persistent or recurrent venom in patients following antivenom administration has been reported many times for a number of snakes, including Russell's viper (*Daboia russelii* and *D. siamensis*) [3–8], Malayan Pit viper (*Calloselasma rhodostoma*) [9,10], Carpet viper (*Echis ocellatus*) [11], (*Echis pyramidum*) [12], Western Diamond back rattle snake (*Crotalus atrox*) [13–15], and Lancehead vipers (*Bothrops* species) [16]. Most studies have been conducted in Russell's viper because of the importance of this snake in South Asia, and have reported recurrence rates ranging from 7 to 95% [3–8,17]. To date, the recurrence of venom detected by EIA in sera post-antivenom has been interpreted as a failure of the initial antivenom dose to be effective or sufficient. Most experts usually suggest that there is ongoing absorption of venom from the site of the bite to the systemic circulation due to the large dose of venom injected by vipers [4–6]. Although it is often assumed and stated that there is ongoing clinical envenoming or recurrent clinical envenoming associated with the detection of venom post-antivenom, this has never been conclusively proven. In the case of Russell's viper envenoming it is not clear whether there is a recurrence of coagulopathy with the recurrence of venom in the circulation.

Russell's viper venom contains factor X and factor V activators which result in the activation of the clotting pathway manifesting as venom induced consumption coagulopathy (VICC) [18,19]. VICC in Russell's viper envenoming is characterised by a prolonged prothrombin time (PT) or international normalised ratio (INR), decreased levels of fibrinogen, decreased levels of factor V, decreased levels of factor X, and elevated d-Dimer concentrations [6,20–22]. Once antivenom is administered there is a resolution of VICC with normalising of the clotting function times and replenishing of the clotting factors including a gradual increase in fibrinogen levels.

We hypothesized that if sufficient antivenom had been administered, then there would be an improvement in clotting function despite persistent or recurrent venom being detected using EIA. The aim of this study was to compare the recovery of VICC in patients with and without venom recurrence/persistence. The recovery of VICC was assessed primarily by the recovery of fibrinogen levels over time.

Methods

This was a prospective observational cohort study of definite Russell's viper (*Daboia russelii*) bites that compared patients with and without recurrence or persistence of venom post-antivenom. It was conducted as part of a large cohort study of patients with snakebites presenting to the Base Hospital Chilaw in Central West Sri Lanka [23]. The study was approved by the Ethical Review Committee, Faculty of Medicine, University of Colombo and Faculty of Medicine, University of Peradeniya, Sri Lanka. All patients gave written and informed consent for the collection of clinical data and blood samples.

Patients

Patients over 13 years of age who presented with Russell's viper envenoming and coagulopathy from January 2007 to July 2009 were included in the study. Cases were only included if Russell's viper venom was detected in the patients' serum with the Russell's viper venom-specific EIA. We included only patients who had citrate and serum samples collected before antivenom administration, and who had at least three samples collected up to 24 hours after antivenom. The median number of samples collected from the patients was 5 (Range: 3 to 10).

Data collection

The following data were collected from patients prospectively: age and sex, time of the snakebite, clinical effects (local effects, coagulopathy, systemic bleeding [haematemesis, bleeding gums or haematuria], neurotoxicity [ptosis, ophthalmoplegia] and non-specific systemic symptoms), antivenom treatment (timing and dose) and hospital length of stay. Additional blood samples were collected from all patients on admission and then for at least 24 hours after antivenom treatment. Blood was collected in citrated tubes for coagulation studies and in serum tubes for venom-specific EIA. All samples were immediately centrifuged, aliquotted and frozen at $-20^{\circ}C$ and then transferred to a $-80^{\circ}C$ freezer within 2 weeks of collection until the completion of the study. All patients received Indian polyvalent snake antivenom manufactured by VINS Bioproducts Limited (batch number: ASV 42C/06, 1030) or BHARAT (batch number: 5346KD4, LY 55/05, LY 32/04, A5307035) Serum and Vaccines Limited, India. Both are equine $F(ab')_2$ antivenoms.

Venom specific enzyme immunoassays (EIA)

Russell's viper venom concentrations were measured in serum samples by sandwich EIA which has previously been described [23–26]. In brief, polyclonal IgG antibodies were raised against Russell's viper (*D. russelii*) venom in rabbits as previously described [27]. These were bound to the microplates as well as being conjugated to biotin for a sandwich EIA with the detecting agent streptavidin-horseradish peroxidase. All samples were measured in triplicate, and the averaged absorbance converted to a concentration by comparison with a standard curve based on serial dilutions of venom using a sigmoidal curve.

Fibrinogen and prothrombin time (PT) assays

Prothrombin times (PT), international normalised ratio (INR) and fibrinogen concentrations were measured in platelet free citrated plasma samples. All assays were performed using standard coagulometric methods on the Behring Coagulation System (Siemens, Marburg, Germany) or Sysmex CA-1500 coagulation

Table 1. Comparison of patients with Russell's viper envenoming and venom recurrence/persistence and non-recurrence of venom, including clinical features of envenoming, coagulopathic parameters, treatment and outcomes.

	Non-recurrence	Venom recurrence
Number of patients	24	31
Male:female	17:7	26:5
Median age (years) (range)	38 (19 to 70)	35 (16 to 82)
Local envenoming	16	20
Systemic bleeding	1	5
Neurotoxicity (ptosis)	9	25
Type of antivenom, VINS: BHARAT	5: 17	11: 18
Median lowest fibrinogen g/L	0.8 (0.01–1.3)	0.5 (0.01–1.5)
Median highest INR	4.8 (2.6–13)	6.4 (2–13)
Median pre antivenom RV venom concentration ng/ml (range)	128 (14–1492)	385 (16–1521)
Median dose of antivenom (vials)	10	10
Number of patients received repeat antivenom doses	2	14
Median length of hospital stay (days)	2 (1 to 5)	3 (1 to 9)

INR, International normalised ratio.
doi:10.1371/journal.pntd.0003304.t001

analyser (Siemens, Marburg, Germany), respectively. Briefly, the PT was determined by mixing patient plasma and Innovin reagent (Dade Behring Inc, USA) and the time taken for fibrin clot formation was measured. The INR was calculated from the PT according to the recommended formula. The fibrinogen concentration was determined by mixing a 1:10 dilution of patient plasma in Owrens Veronal Buffer, in a 2:1 ratio with Dade Thrombin Reagent (Siemens Healthcare Diagnostic Inc, USA) and measuring the time to fibrin clot formation. The fibrinogen concentration was then determined from the time to clot formation according to a standard curve of serially diluted standard human plasma in g/L.

Data analysis

Patients with venom detected after the administration of antivenom, whether after an initial decrease in venom concentrations (recurrence) or no initial decrease in venom concentration (persistence), were defined as patients with venom recurrence/persistence. These patients were then compared to patients where venom was never detected after the administration of antivenom. Time to the recovery of fibrinogen concentration and INR, pre-antivenom venom concentrations, clinical effects (coagulopathy [INR>1.5], neurotoxicity [ptosis], systemic bleeding and local envenoming), number of antivenom doses administered and length of hospital stay, were compared between the venom recurrence or persistent group and the group of non-recurrence patients.

Continuous variables (venom concentrations, time to fibrinogen and INR recovery, and length of hospital stay) are reported as median values with interquartile ranges (IQR) and ranges. Continuous variables were compared with the Mann-Whitney test (non-parametric). All analyses and graphics were done in GraphPad Prism version 6 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

Results

Russell's viper envenoming was confirmed in 173 patients by EIA, but only 55 patients were included in the analysis due to adequate numbers of blood samples. In 24 patients, venom was not detected in serum after the administration of antivenom (Table 1; Fig. 1A). In 31 patients there was recurrence or persistence of Russell's viper venom after antivenom administration (Table 1; Fig. 1B).

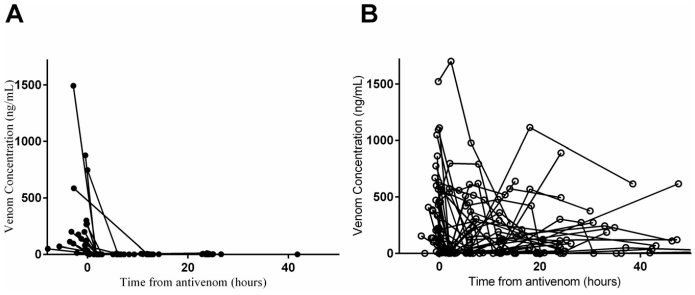

Fig. 1. Concentrations of Russell's viper venom (ng/ml) versus time in the patients without recurrence (Panel A) compared to patients with persistence/recurrence (Panel B).
doi:10.1371/journal.pntd.0003304.g001

All 55 patients developed VICC with an abnormal INR and low or undetectable fibrinogen in the pre-antivenom or admission blood sample. Clinical features, coagulopathic parameters, pre-antivenom venom concentrations, antivenom dose and length of hospital stay are shown in Table 1. The severity of the coagulopathy as determined by the median highest INR and median lowest fibrinogen, was similar for patients with recurrence or persistence of venom versus patients without recurrence (Table 1). Pre-antivenom venom concentrations were significantly higher in patients with venom recurrence or persistence with a median venom concentration of 385 ng/mL (16 to 1521 ng/mL) compared to patients without recurrence/persistence with a median venom concentration of 128 ng/mL (14 to 1492 ng/mL; $p = 0.008$; Fig. 2).

The median time to recovery of the fibrinogen concentration to 1 g/L was 11.5 hours (0.3 to 34.9 h) in patients with venom recurrence/persistence compared to 12.3 hours (1.8 to 55.3 h) in patients without venom recurrence which was not significantly different ($p = 0.75$; Figs. 3A and 3B). Similarly, the median time to correct the INR to less than 2 was 11.8 hours (0.3 to 32.9) for patients with venom recurrence or persistence compared to 12.3 hours (5.8 to 55.3 h) in patients without recurrence which was not statistically significant ($p = 0.21$;Figs. 4A and 4B).

Patients in both groups were treated with either VINS or BHARAT Indian polyvalent antivenoms and a median of 10 vials of antivenom was given in both groups. However, 14 patients in the recurrence group had multiple doses of antivenom and only two patients in the non-recurrence group had repeated doses of antivenom. Administration of the second dose of antivenom in these patients was based on a positive whole blood clotting test 20 minutes (WBCT 20). However, in ten out of 14 patients in the recurrence group who had a second dose of antivenom, the coagulopathy had already resolved with a normal INR and fibrinogen, not consistent with the positive WBCT 20 done at the time. The remaining two patients in the recurrence group, and two patients in the non-recurrence group who had additional antivenom, were in the recovery phase of the coagulopathy (S1 Figure). The median length of hospital stay was 3 and 2 days in recurrence and non-recurrence groups, respectively, which was not statistically significant (Table 1).

Discussion

This study shows that the measurement of venom post-antivenom, either seen as the persistence of venom or the recurrence of venom, was not associated with ongoing coagulopathy in Russell's viper bites. The time to recovery of fibrinogen, the time to recovery of INR, and length of hospital stay were similar in both the venom recurrence/persistence group and the non-recurrence group. However, pre-antivenom venom concentrations were higher in recurrence/persistence group compared to the non-recurrence group.

Recurrence of coagulopathy associated with the Russell's viper venom recurrence has been described previously in single case reports [4,5,10]. All of these studies have used the WBCT 20 or methods measuring clot quality to provide evidence for recurrence of coagulopathy. In addition, the so-called venom recurrence was recognised many months after the patient was treated and discharged, when the venom assays were done. Ariaratnam et al. selectively describe a single case from a study of 35 patients where the WBCT 20 became abnormal after antivenom administration, thus prompting a second dose of antivenom. This was later found to coincide with the measurement of venom recurrence. However, venom recurrence occurred in the majority of the patients in their study and in 11 patients given only a single dose of 2 g antivenom (PolongaTab), they state that all patients had recurrence and persistence of venom for a mean time of 72 hours, despite all having immediate reversal of systemic envenoming including coagulopathy [4]. A different interpretation of the data is that Ariaratnam et al show that recurrent venom antigenaemia is not associated with coagulopathy in the majority of their cases, similar to our study, and in one case only there was an abnormal WBCT 20 at the time of the recurrence. This single abnormal WBCT 20 was potentially an error and the investigators did not confirm coagulopathy with other clotting studies. Lack of sensitivity of WBCT 20 for the detection of Russell's viper coagulopathy has

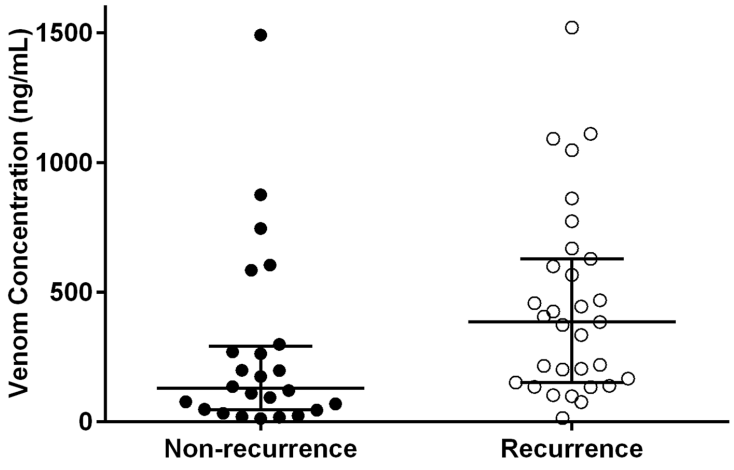

Fig. 2. Scatterplot of the pre-antivenom Russell's viper venom concentrations (ng/ml) in the patients without recurrence (filled circles) compared to patients with persistence/recurrence (open circles).
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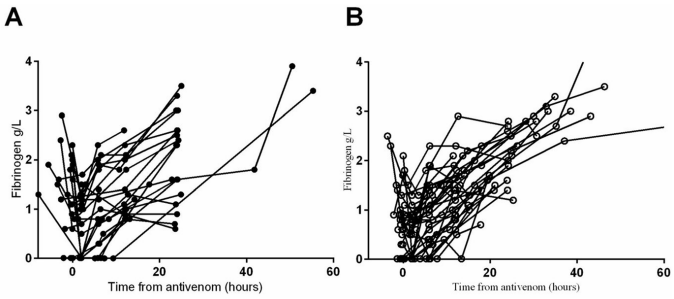

Fig. 3. Fibrinogen levels (g/L) versus time in Russell's viper envenoming comparing patients without recurrence (Panel A) to patients with persistence/recurrence (Panel B).
doi:10.1371/journal.pntd.0003304.g003

been recently demonstrated [28], and is the probable explanation for the recurrence of coagulopathy in previous reports [4,10] as well as the 10 abnormal WBCT 20 that occurred in our study.

The case reported by Theakston is a single case in which the reported venom concentrations were very low (<15 ng/mL) compared to ours and other studies measuring Russell's viper venom [3,4,6–8,29]. Ho et al report a number of cases but there is no correlation between the recurrence of venom and the recurrence of an abnormal WBCT 20 (Table 3; Ho et al.), except in one patient. This again suggests that recurrent coagulopathy did not occur with the measurement of venom recurrence [10]. Recurrence of coagulopathy in Russell's viper envenoming has not been described in any of the studies that used formal laboratory coagulation studies to assess the coagulopathy including fibrinogen concentration or clotting factor assays [6,21,30,31].

Venom recurrence has been documented with other vipers [10–13,15,16]. In a number of cases this is similar to previous studies of Russell's viper where there is little evidence to support recurrence of clinical envenoming. However, for some snakes there appears to be recurrence of clinical and/or laboratory envenoming at the same time as there is venom recurrence, but in these cases the recurrence appears to occur days after the bite, unlike the 12 to 24 hours in our study [15]. This is demonstrated clearly in a phase II clinical trial by Boyer et al of American vipers where the venom recurrence occurs approximately one week after the administration of antivenom in patients given Fab antibodies compared to no recurrence in those given $F(ab')_2$ antibodies [15].

The recurrence reported in American viper envenoming appears to be different to that seen with reports from Asian and African vipers. It occurs much later and has only been recognised with the change from an $F(ab')_2$ antivenom to an Fab antivenom. The recurrence is thought to be due to the rapid clearance of Fab and ongoing persistence of venom. Persistent coagulopathy has been reported in North American Crotalid envenoming for many years [32–37] and is thought to be due to slow absorption of venom. Therefore, the mismatch of Fab antivenom pharmacokinetics with rapid elimination and the slow venom absorption are the likely reason for the recurrence of coagulopathy. The recent study by Boyer et al confirms this [15].

It has always been assumed that venom specific EIA only detects free venom. It reasonably follows that the presence of free venom detected after antivenom administration means that insufficient antivenom has been given. A recent study has shown that in fact the traditional venom specific EIA (as originally developed by Theakston [38]) can detect bound venom or venom-antivenom (VAV) complexes under certain conditions [39]. If there are high concentrations of antivenom such that venom molecules are surrounded by antivenom and there is excess free antivenom, no venom is detected by the traditional venom EIA. However, at lower concentrations of antivenom (lower ratio of antivenom to venom), where every venom component is not completely surrounded by antivenom but attached to at least one antivenom molecule or antibody on average, the traditional venom EIA will detect bound venom or VAV complexes (see figure 1 O'Leary and

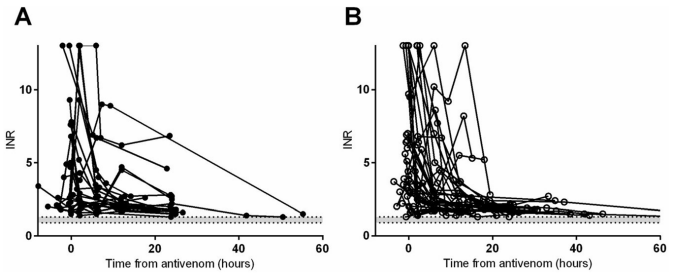

Fig. 4. International normalised ratio (INR) versus time in Russell's viper envenoming comparing patients without recurrence (Panel A) to patients with persistence/recurrence (Panel B).
doi:10.1371/journal.pntd.0003304.g004

Isbister 2014 [39]). It was therefore proposed that the detection of venom in sera post-antivenom may not mean that insufficient antivenom has been administered, because the assay is detecting bound venom. This is not surprising since it is well known that the assay for digoxin will detect digoxin bound to digoxin antibodies after the administration of digoxin antibodies [40,41]. This VAV assay was developed with $F(ab')_2$ antivenom [39].

We found that patients with persistence or recurrence of venom had significantly higher pre-antivenom venom concentrations (Table 1). This is consistent with the hypothesis that the venom assay detects VAV complexes, because patients with a high venom load will have a lower ratio of antivenom to venom molecules and therefore, VAV complexes that can be detected (Fig. 1B). In contrast, patients with lower concentrations of Russell's viper venom will have the venom molecules completely surrounded by the same dose of antivenom, so VAV complexes cannot be detected and there is no apparent venom recurrence with EIA (Fig. 1A). Clearly, if there is insufficient antivenom there will be persistence of free venom in addition to VAV complexes. Further assays need to be developed to help distinguish free venom from bound venom to confirm that there is no free venom present in samples collected post-antivenom in our study.

An unusual finding in the study was that repeat dosing of antivenom was more common in the group with venom recurrence or persistence. This could be interpreted to mean that patients with recurrence were given further antivenom because of suspected recurrent or ongoing envenoming or coagulopathy. This is partly correct because these patients still had an abnormal WBCT 20 which was the likely reason for a second dose of antivenom being given. However, all of these patients had a normal INR or an improving INR showing that the coagulopathy was in fact resolving, and that the WBCT 20 was incorrect.

Another difference between two groups was that patients with persistence or recurrence were more likely to have neurotoxicity and systemic bleeding. This is again consistent with this group having higher venom concentrations and it therefore making sense that they had more severe envenoming. Both systemic bleeding and neurotoxicity (mostly ptosis) do not rapidly reverse with antivenom, so their persistence after antivenom does not indicate recurrent envenoming. In fact, this also further explains why repeat doses of antivenom were given in this group because of the common misconception that neurotoxicity will immediately resolve with antivenom, and if it hasn't, the patient needs more antivenom.

Our study shows that the recurrence or persistent of venom post-antivenom does not necessarily mean that insufficient or inefficacious antivenom has been given. For the doses of antivenom given in these patients with Russell's viper envenoming, sufficient antivenom had been given and the coagulopathy was recovering. Further work is required to clarify the best measures of antivenom efficacy in vivo.

Supporting Information

S1 Figure Fibrinogen levels (g/L) versus time in the two patients in the recurrence group and two patients in the non-recurrence group who had additional antivenom and were in the recovery phase of the coagulopathy.
(JPG)

S1 Checklist STROBE checklist.
(DOCX)

Acknowledgments

We acknowledge the assistance of the medical and nursing staff at Chilaw Hospital, Central West Province, Sri Lanka. We acknowledge Andrew Dawson and Indika Gawarammana for assistance throughout the study.

Author Contributions

Conceived and designed the experiments: KM MAO GKI LFL. Performed the experiments: KM MAO FES SS FM CA HK CAG. Analyzed the data: KM GKI. Contributed reagents/materials/analysis tools: FES LFL MAO. Wrote the paper: KM MAO FES FM GKI.

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Chapter Five

Diagnosis of snake envenomation using a simple phospholipase A_2 assay

Introduction to Chapter Five: Explanation of contribution of the paper titled "Diagnosis of snake envenomation using a simple phospholipase A_2 assay" to the overall thesis

The second main objective of the thesis was to investigate diagnostic tests of snake envenoming to help decide whether to give antivenom following a definite or suspected snake bite. Antivenom is not indicated in every snakebite due to the large number of dry bites. Administration of antivenom is associated with severe anaphylaxis, especially in Asia with Indian antivenoms. Therefore, the decision to give antivenom is critical in the management of snake envenoming. Currently there is no single test for the diagnosis of systemic envenoming and clinicians usually decide to give antivenom based on the clinical features of envenoming (e.g. bleeding, ptosis or anuria) or abnormal laboratory test (e.g. positive WBCT20). Unfortunately most clinical features of envenoming and laboratory tests become abnormal after it is too late to prevent or reverse the effect of envenoming. Therefore, diagnosis of envenoming before development of clinical manifestation is critical to decide to early antivenom treatment.

Snake venom phospholipase A_2 (PLA_2) is a common toxic enzyme distributed widely in almost all snake venoms. We aimed to detect snake venom PLA_2 in envenomed patients to diagnose envenoming compared to low or undetectable levels in non-envenomed patients. The manuscript titled "Diagnosis of snake envenomation using a simple phospholipase A_2 assay" makes up this chapter.

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was accepted into Nature Scientific Reports for publication on 9th April 2014. I, Maduwage Kalana Prasad, was the first author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

Snake venom quantification of patients' samples and Phospholipase A_2 concentrations were carried out by myself under the supervision of Margaret O'Leary. Overall supervision of the study was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter Five.

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SUBJECT AREAS:
DIAGNOSTIC MARKERS
BIOMARKER RESEARCH

Received
27 February 2014

Accepted
9 April 2014

Published
29 April 2014

Correspondence and requests for materials should be addressed to G.K.I. (geoff.isbister@gmail.com)

Diagnosis of snake envenomation using a simple phospholipase A_2 assay

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Diagnosis of snake envenomation is challenging but critical for deciding on antivenom use. Phospholipase A_2 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 world[1]. 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 envenomation[2–4]. A key issue for improving antivenom treatment in snake envenomation is to have a rapid and accurate test to determine if patients are envenomated and require antivenom[5]. 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 A_2 (PLA_2) is a common toxin/component in snake venoms including in almost all elapid and viperid venoms[8–10]. Numerous studies have tested PLA_2 activity in snake venoms and it is arguably a standard test when investigating venom activity. However, no studies have tested for PLA_2 activity in the blood of patients with snake envenomation. Although PLA_2 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 envenomated patients would have measureable PLA_2 activity in their blood compared to minimal activity in non-envenomated 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-envenomated patients. PLA_2 activity was detected in pre-antivenom sera in all patients with snake envenomation. The median PLA_2 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-envenomated patients (Median: 6.0 μmol/ml/min; 95% percentiles 2.3 to 8.4) [Fig. 1].

PLA_2 activity was compared to venom specific enzyme immunoassays (EIA) for three of the snake venoms. There was good correlation between venom specific EIA and PLA_2 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 administered[11]. A similar phenomenon occurred with PLA_2 activity which decreased after antivenom administration and increased with apparent venom recurrence in Russell's viper

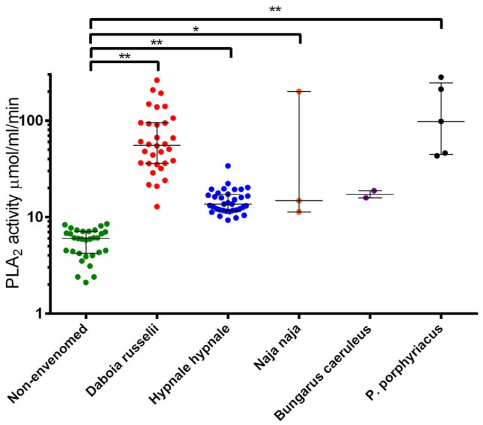

Figure 1 | **Scatterplot of the phospholipase A_2 activity (µmol/ml/min) for non-envenomated patients (green circles) versus patients with Russell's viper envenomation (*D.* russelii; red circles), hump-nosed viper envenomation (*H. hypnale*; blue circles), Indian cobra envenomation (*N. naja*; orange 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-envenomated 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 substrate[12].

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 setting[5] 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-

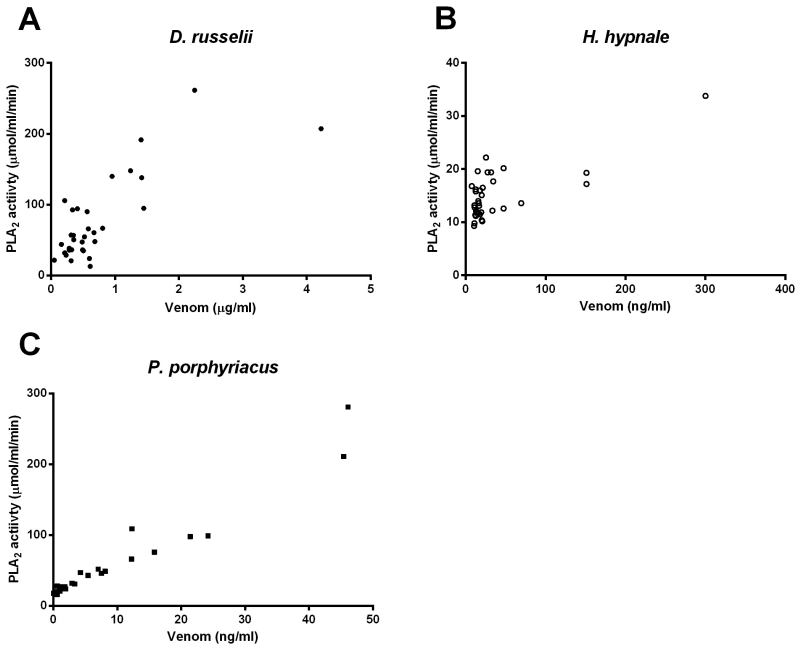

Figure 2 | **Plots of phospholipase A_2 activity against venom concentration for samples from patients with envenomation from Russell's viper (*D. russelii*; Panel A), hump-nosed viper (*H. hypnale*; Panel B) and red-bellied black snake (*P. porphyriacus*; Panel C).**

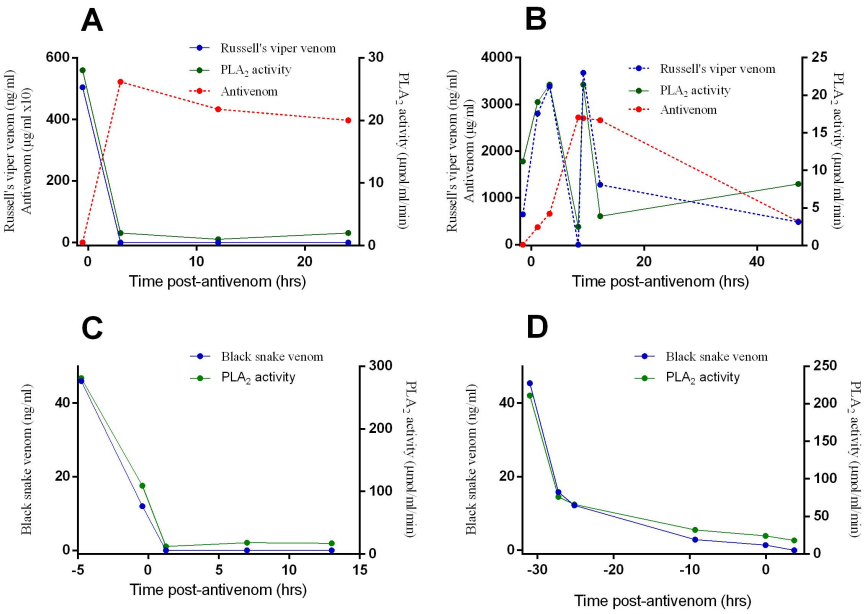

Figure 3 | Plots of free venom concentration (ng/ml; blue circles and lines), phospholipase A_2 activity (µmol/ml/min; green circles and lines) and antivenom concentrations (µg/ml; red circles and dashed lines) versus time in a patient with Russell's viper envenomation where venom was undetectable post-antivenom (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 2011as part of prospective cohort studies and randomised controlled trials[3]. 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 project[19]. 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 sPLA$_2$ assay kit (#765001Cayman 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 PLA_2 activity, including before and after antivenom.

The PLA_2 activities of patient sera from different snake groups and the non-envenomated snake bites were compared with the non-parametric Kruskal-Wallis test and multiple comparisons with Dunn's test. Correlation between PLA_2 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.

How to cite this article: Maduwage, K., O'Leary, M.A. & Isbister, G.K. Diagnosis of snake envenomation using a simple phospholipase A_2 assay. *Sci. Rep.* **4**, 4827; DOI:10.1038/srep04827 (2014).

Chapter Six

Efficacy of Indian polyvalent snake antivenoms against Sri Lankan snake venoms: lethality studies or clinically focussed *in vitro* studies

Introduction to Chapter Six: Efficacy of Indian polyvalent snake antivenoms against Sri Lankan snake venoms: lethality studies or clinically focussed *in vitro* studies to the overall thesis

Sri Lankan snake envenoming has been treated by Indian polyvalent antivenoms for many decades due to unavailability of specific antivenom for Sri Lanka. Many studies have been published on the lack of effectiveness of Indian antivenom for Sri Lankan snake envenoming. Moreover, there is a high rate of severe reactions to the Indian antivenoms reported throughout the Island. Coagulopathy and neurotoxicity are the commonest systemic effects in Sri Lankan snake envenoming. However, there are no studies done to test the efficacy of Indian Polyvalent antivenoms for coagulopathy and neurotoxicity of Sri Lankan snake venoms. Therefore, it is vital to test the efficacy of Indian polyvalent antivenoms for all four major Sri Lankan snake venoms. This is the central chapter of my thesis with a study entitled "Efficacy of two Indian polyvalent snake antivenoms against coagulopathy and neurotoxicity of Sri Lankan snake venoms".

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was published in Scientific Reports (Nature Publishing Group) on 27th May 2016. I, Maduwage Kalana Prasad, was the first author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

Part of the lethality neutralization experiments were carried out by A. Silva. In-vitro efficacy studies were carried out by myself under the supervision of Margaret O'Leary. In-vitro neurotoxicty experiments were carried out by myself under the supervision of Wayne C. Hodgson. Overall supervision of the study was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter Six.

SCIENTIFIC REPORTS

OPEN

Received: 14 January 2016
Accepted: 06 May 2016
Published: 27 May 2016

Efficacy of Indian polyvalent snake antivenoms against Sri Lankan snake venoms: lethality studies or clinically focussed *in vitro* studies

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In vitro **antivenom efficacy studies were compared to rodent lethality studies to test two Indian snake antivenoms (VINS and BHARAT) against four Sri Lankan snakes. *In vitro* efficacy was tested at venom concentrations consistent with human envenoming. Efficacy was compared statistically for one batch from each manufacturer where multiple vials were available. In binding studies EC_{50} for all VINS antivenoms were less than BHARAT for *D. russelii* [553 μg/mL vs. 1371 μg/mL;$p = 0.016$), but were greater for VINS antivenoms compared to BHARAT for *N. naja* [336 μg/mL vs. 70 μg/mL;$p < 0.0001$]. EC_{50} of both antivenoms was only slighty different for *E. carinatus* and *B. caeruleus*. For procoagulant activity neutralisation, the EC_{50} was lower for VINS compared to BHARAT - 60 μg/mL vs. 176 μg/mL ($p < 0.0001$) for Russell's viper and 357 μg/mL vs. 6906 μg/mL ($p < 0.0001$) for Saw-scaled viper. Only VINS antivenom neutralized *in vitro* neurotoxicity of krait venom. Both antivenoms partially neutralized cobra and didn't neutralize Russell's viper neurotoxicity. Lethality studies found no statistically significant difference in ED_{50} values between VINS and BHARAT antivenoms. VINS antivenoms appeared superior to BHARAT at concentrations equivalent to administering 10 vials antivenom, based on binding and neutralisation studies. Lethality studies were inconsistent suggesting rodent death may not measure relevant efficacy outcomes in humans.**

Snake envenoming is recognised as a major global health issue with large numbers of deaths and cases of envenoming occurring each year in tropical and sub-tropical regions of the world[1]. Although antivenom is the accepted treatment for snake envenoming, there is a shortage of antivenoms in Africa and Asia and in some cases this has led to the use of antivenom made in one country being used in a different country, that may not be effective against snake venoms in that geographical region[2–5]. Over the last two decades there have been concerns about the efficacy of some antivenoms and whether sufficient doses are being used[6,7]. In some cases this has led to changes in dosing based on such concerns and in others the development of new antivenoms[8].

A particular problem with developing antivenoms is testing their efficacy and more importantly the potential efficacy in humans, including appropriate dosing. The World Health Organisation (WHO) recommends the use of median lethal dose (LD_{50}) and median effective dose (ED_{50}) for assessing the efficacy of antivenoms[9]. However, there are significant animal ethical considerations because of the numbers of animals that are required for these tests, and there are concerns about extrapolating the death of a rodent to clinical effects in humans.

Although the exact mechanism of venom-induced death is unclear in rodents, death may be due to the effects of post-synaptic neurotoxins or early cardiovascular collapse[10]. In contrast, pre-synaptic neurotoxins are important in human envenoming[11], as well as toxins that cause coagulopathy, myotoxicity and nephrotoxicity. For example, a recent study has demonstrated that rodent plasma is highly resistant to procoagulant toxins that are highly relevant for human envenoming[12]. This means that the use of death of a rodent as a test of efficacy for snake venoms that cause coagulopathy is problematic.

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Vial Type	Dry powder wt. (mg/vial)	Percent protein	Protein content/vial (mg)
VINS2000 (1054)	800	30.7%	246
VINS2008 (1061)	614	26.4%	162
VINS2010* (01011/10–11)	658 (535–815)	30.3% (23–36)	198 (157–238)
VINS2011 (01AS11112)	801	40.9%	328
VINS2012 (01AS11114)	798	62.7%	500
BHARAT2011* (A5311006)	390 (155–510)	25.2% (23–27)	98 (39–125)

Table 1. The dry powder weight of antivenom, the percentage of proteins per mg of antivenom and the amount of protein per vial in VINS and BHARAT antivenoms. *The median value and range is reported for VINS2010 and BHARAT2011 based on testing 10 vials from each batch.

Other *in vitro* and *in vivo* investigations are available to test the efficacy of antivenom to neutralise relevant pathophysiological effects induced by snake venoms and may provide more clinically useful information[13–16]. For snakes that cause coagulopathy in humans it would seem more appropriate to test the efficacy of antivenom to neutralise procoagulant venom activity in human plasma, rather than the efficacy of antivenom in preventing death in a rodent. The fields of venomics and antivenomics are also beginning to improve our understanding of the different composition of venoms and their interaction with antivenoms[17,18]. However, it will be important to ensure that antivenoms are tested in functional studies of clinically relevant venom effects.

Currently all antivenom used in Sri Lanka is supplied from India and there are limited data regarding the efficacy of Indian antivenoms against Sri Lankan snakes[19]. Concerns were raised about the effectiveness of Indian polyvalent antivenoms in Sri Lanka in 2012 and there were sufficient concerns about the efficacy of antivenom for the treatment of Russell's viper (*Daboia russelii*) envenoming that the Government requested independent testing of the antivenoms. Treating doctors had observed an increased number of hospital deaths and reactions to the antivenom.

The aim of this study was to investigate the efficacy of two different Indian polyvalent antivenoms comparing a range of *in vitro* studies to classical lethality studies. In doing so we aimed to test the ability of the antivenoms to neutralise 1) the procoagulant effect of two viper venoms–Russell's viper (*D. russelii*) and the saw-scaled viper (*Echis carinatus*); 2) neurotoxic effect of three snake venoms–common krait (*Bungarus caeruleus*), common cobra (*Naja naja*) and Russell's viper (*D. russelii*); comparing these to LD_{50}/ED_{50} studies.

Results

All *in vitro* experiments were done at venom concentrations measured in cases of human envenoming which was 1.7 μg/ml for *D. russelii*, 0.5 μg/ml for *E. carinatus*, 1.4 μg/ml for *N. naja* and 0.15 μg/ml for *B. caeruleus*. Multiple batches of antivenoms from both VINS Bioproducts Limited and BHARAT Serum and Vaccines Limited were tested and compared. To statistically compare the efficacy of the two antivenoms one batch from each manufacturer was compared where multiple vials were available–VINS (01011/10/11; 2010) and BHARAT (A5311006; 2011Jan).

Protein quantification. The median dry powder weight (mg) of antivenom, the median percentage of protein per mg of antivenom and the median dry powder weight (mg) of protein per antivenom vial were measured (Table 1). All batches of VINS antivenom had a higher protein concentration than the batch of BHARAT antivenom tested (Table 1). The mean protein content of 10 vials of VINS antivenom from the same batch (01011/10–11) was 198 mg (Standard Deviation [SD]: 28 mg), which was significantly higher than the mean protein content of 10 vials of BHARAT antivenom from the same batch (A5311006), 98 mg SD: 29 mg; $p < 0.0001$; unpaired t-test).

Venom-antivenom binding studies. The median effective concentration (EC_{50}) for antivenom binding was the concentration of antivenom that bound 50% of free venom antigens at clinically relevant venom concentrations. The EC_{50} for both antivenoms are given in Table 2 and the venom-antivenom binding curves are shown graphically in Fig. 1 and Supp Figure 1. The EC_{50} values of *D. russelii* venom for all VINS antivenoms were less than for BHARAT antivenoms, and was 553 μg/mL (95% confidence intervals [CI]: 237–1289 μg/mL) for VINS 2010 compared to 1371 μg/mL (95% CI: 956–1965 μg/mL) for BHARAT 2011, which was statistically significantly different ($p = 0.016$). The EC_{50} values for *N. naja* venom of all VINS antivenoms were greater than all BHARAT antivenoms, 336 μg/mL (95% CI: 325–347 μg/mL) for VINS 2010 compared to 70 μg/mL (95% CI: 53–91 μg/mL) for BHARAT 2011 ($p < 0.0001$). The EC_{50} values for *E. carinatus* and *B. caeruleus* venoms of VINS and BHARAT antivenoms did not appear to differ markedly (Table 2, Fig. 1), but was statistically significantly different when comparing the two batches for *B. caeruleus* [97 μg/mL (95% CI: 85–110 μg/mL) for VINS versus 157 μg/mL (95% CI: 121–205 μg/mL) for BHARAT; $p = 0.002$], but not for *E. carinatus* [165 μg/mL (95% CI: 156–172 μg/mL) for VINS versus 187 μg/mL (95% CI: 168–208 μg/mL) for BHARAT; $p = 0.053$] (Supp Figure 1).

Neutralization of procoagulant activity of D. russelii and E. carinatus venoms. VINS antivenoms were able to neutralise the procoagulant activity of both venoms at much lower antivenom concentrations than BHARAT antivenom, based on lower median effective concentrations against procoagulant activity (EC_{50}; Table 3, Fig. 2, Supp Figure 2). The EC_{50} against procoagulant activity of *D. russelii* for VINS was 60 μg/mL (95% CI: 44–81 μg/mL) compared to 176 μg/mL (95% CI: 149–207 μg/mL) for BHARAT, which was significantly different ($p < 0.0001$). Much higher concentrations were required by both antivenoms to neutralise the

Vial Type	Median effective concentration (EC_{50}) μg/mL			
	1.7 μg/mL of *D. russelii* venom	0.5 μg/mL of *E. carinatus* venom	1.4 μg/mL of *N. naja* venom	0.15 μg/mL of *B. caeruleus* venom
VINS2008 (1061)	324 (133–791)	165 (160–170)	253 (231–277)	131 (108–157)
VINS2010 (01011, 01013, 01023, 01024)	553 (237–1289)	165 (156–172)	336 (325–347)	97 (85–110)
VINS2011 (01AS11112)	248 (103–599)	158 (157–159)	281 (238–332)	155 (130–186)
VINS2012 (01AS11114)	262 (100–686)	166 (163–169)	142 (133–152)	42 (31–56)
BHARAT 2011Jan (A5311006)	1371 (956–1965)	187 (168–208)	70 (53–91)	157 (121–205)
BHARAT 2011Apr (A5311013, A5311014)	1051 (837–1319)	167 (165–170)	59 (53–66)	132 (111–158)

Table 2. Median effective concentration (EC_{50}) with 95% confidence intervals for VINS and BHARAT antivenoms binding of 1.7 μg/mL of *D. russelii*, 0.5 μg/mL of *E. carinatus*, 1.4 μg/mL of *N. naja* and 0.15 μg/mL of *B. caeruleus* venom antigens.

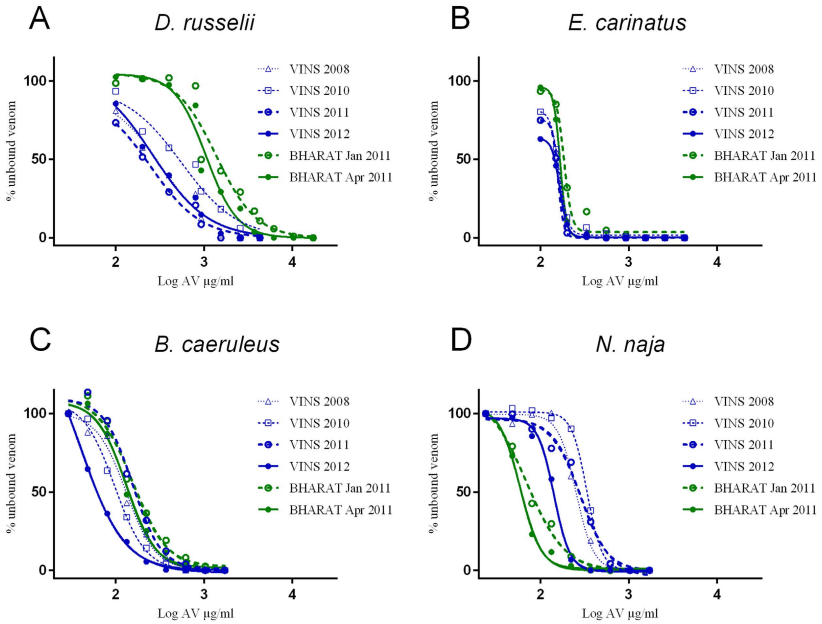

Figure 1. Plots of percent free (unbound) venom versus the logarithm of the antivenom concentration for six batches of antivenom, four VINS and two BHARAT antivenoms showing the binding capacity for (**A**) *D. russelii*, (**B**) *E. carinatus*, (**C**) *N. naja* and (**D**) *B. caeruleus* venoms.

procoagulant effect of *E. carinatus* despite there being lower concentrations of venom present. (Table 3, Fig. 2). The EC_{50} against procoagulant activity of *E. carinatus* for VINS was 357 μg/mL (95% CI: 87–1458 μg/mL) compared to 6906 μg/mL (95% CI: 4859–9817 μg/mL) for BHARAT, which was significantly different ($p < 0.0001$).

Neurotoxicity of B. caeruleus, N. naja and D. russelii venoms. *B. caeruleus* venom (3 μg/ml; N = 3) caused rapid inhibition ($t_{90} = 33.5$ min) of nerve-mediated twitches in the chick biventer preparation, while *N. naja* (N = 4) and *D. russelii* (N = 3) venoms only caused partial inhibition of nerve-mediated twitches at a concentration of 3 μg/ml (Fig. 3A). *E. carinatus* venoms (N = 3) did not cause inhibition of nerve-mediated twitches at any dose. For the increased concentration of 30 μg/ml, *N. naja* ($t_{90} = 29.5$ min) and *D. russelii* ($t_{90} = 44.0$ min) venom did inhibit nerve-mediated twitches (Fig. 3A, Table 4), confirming that these venoms were less neurotoxic than krait venom. *B. caeruleus* and *N. naja* venom significantly inhibited responses to exogenous ACh and CCh (Fig. 4A), while having no significant effect on the response to KCl, indicating the presence of postsynaptic

Vial Type	Median effective concentration (EC_{50}) μg/mL	
	1.7 μg/mL of *D. russelii* venom	0.5 μg/mL of *E. carinatus* venom
VINS2008 (1061)	48 (45–52)	645 (443–939)
VINS2010 (01011, 01013, 01023, 01024)	60 (44–81)	357 (87–1458)
VINS2011 (01AS11112)	42 (37–48)	367 (169–793)
VINS2012 (01AS11114)	49 (40–60)	330 (142–766)
BHARAT 2011Jan (A5311006)	176 (149–207)	6906 (4858–9817)
BHARAT 2011Apr (A5311013, A5311014)	84 (67–105)	859 (410–1800)

Table 3. Median effective concentrations (EC_{50}) of antivenom in neutralising the procoagulant activities of 1.7 μg/mL of *D. russelii* venom and 0.5 μg/mL of *E. carinatus* venom in human plasma for VINS versus BHARAT antivenoms.

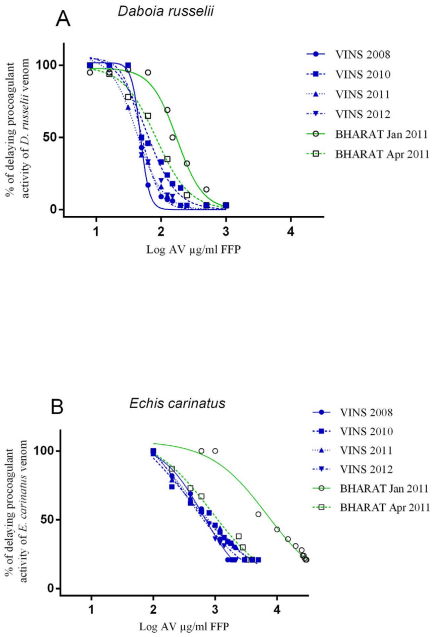

Figure 2. Effect of VINS and BHARAT antivenoms on delaying the procoagulant activities of 1.7 μg/mL of *D. russelii* (**A**), 0.5 μg/mL of *E. carinatus* (**B**) venom on human plasma.

neurotoxins in *B. caeruleus* venom, but not excluding pre-synaptic activity. *D. russelii* venom only partially inhibited the response to exogenous ACh and CCh (Fig. 4).

Neutralization of neurotoxicity of *B. caeruleus*, *N. naja* and *D. russelii* venoms. Addition of the recommended concentration of VINS and BHARAT antivenom did not prevent the inhibition of twitches induced by any of the venoms, so five times the recommended concentration of the antivenoms was used as per previous studies[20]. At this concentration VINS antivenom effectively prevented *B. caeruleus* venom induced twitch inhibition compared to no effect with BHARAT antivenom (Fig. 3B, Table 4). There was partial recovery of the response to exogenous ACh and CCh with VINS and BHARAT antivenoms following *B. caeruleus* venom (Fig. 4B). However, both VINS and BHARAT antivenoms had minimal effect in preventing *N. naja* venom induced twitch inhibition (Fig. 3C, Table 4), and no effect in preventing the partial inhibition of twitches induced by *D. russelii* venom (Fig. 3D, Table 4).

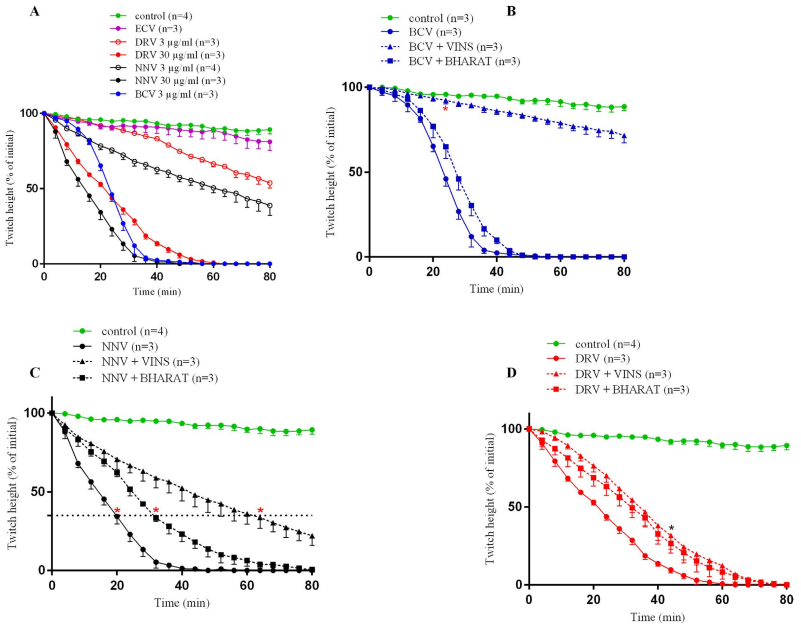

Figure 3. Plots of twitch height (mean ± standard error of the mean [SEM]) versus time - (**A**) The neurotoxic effect of *B. caeruleus, N. naja, D. russellii* and *E. carinatus* venoms alone on indirect twitches of the chick biventer nerve–muscle preparation; and the effect of prior administration (10 min) of VINS and BHARAT antivenoms (five times the recommended dose) to (**B**) *B. caeruleus* (3 µg/ml) venom (*Twitch height of BCV + VINS after 24 min is significantly different from the BCV and BCV + BHARAT and not different from the controls: $p < 0.05$, one way ANOVA followed by Bonferroni's post-hoc test), (**C**) *N. naja* (30 µg/ml) venom (*Time taken for the maximum drop of twitch height in all tissues in NNV + VINS, i.e. by 65% is significantly prolonged compared to similar twitch inhibition in NNV and NNV + BHARAT: $p < 0.05$, Kruskal-Wallis test followed by Dunn's multiple comparison test), and (**D**) *D. russelii* (30 µg/ml) venom on indirect twitches of the chick biventer nerve–muscle preparation (*Twitch height of DRV + VINS and DRV + BHARAT after 44 min is not different from DRV while all above are different from the controls: $p < 0.05$, one way ANOVA followed by Bonferroni's post-hoc test). BCV; *B. caeruleus* venom, NNV; *N. naja* venom, ECV; *E. carinatus* venom and DRV; *D. russelii* venom.

t_{90} (mean [SD])	Venom alone	Venom + VINS antivenom	Venom + BHARAT antivenom
B. caeruleus (3 µg/ml)	33.5 [2.5]	Delayed (see Fig. 3B)	40.0 [2.6]
N. naja (30 µg/ml)	29.5 [5.1]	Delayed (see Fig. 3C)	52.0 [9.3]
D. russelii (30 µg/ml)	44.0 [3.5]	61.5 [11.9]	57.5 [9.5]

Table 4. Inhibition of indirect stimulation of chick biventer cervicis nerve-muscle preparations (time to 90% inhibition [t_{90}]; mean and standard deviation [SD]) by *B. caeruleus, N. naja* and *D. russelii* venoms and the effect of preventing twitch height inhibition by VINS and BHARAT antivenoms. Five times the recommended amount of antivenom was used for each antivenom.

Lethality neutralization studies. Lethality and neutralization studies for the antivenoms were investigated by calculating the median lethal dose (LD_{50}) and the median effective dose (ED_{50}) values in mice. LD_{50} values of *D. russelli* and *B. caeruleus* venoms were less than that of *E. carinatus* and *N. naja* venom in mouse experiments (Table 5). VINS antivenom appeared to be slightly more effective in the neutralization of lethality induced by *D. russelli* and *B. caeruleus* venoms compared to BHARAT antivenom, but this was not statistically significant (Table 5). VINS was almost twice as effective against *N. naja* venom compared to BHARAT, which was almost statistically significant. There was no numerical difference in the ED_{50} values for *E. carinatus* venom

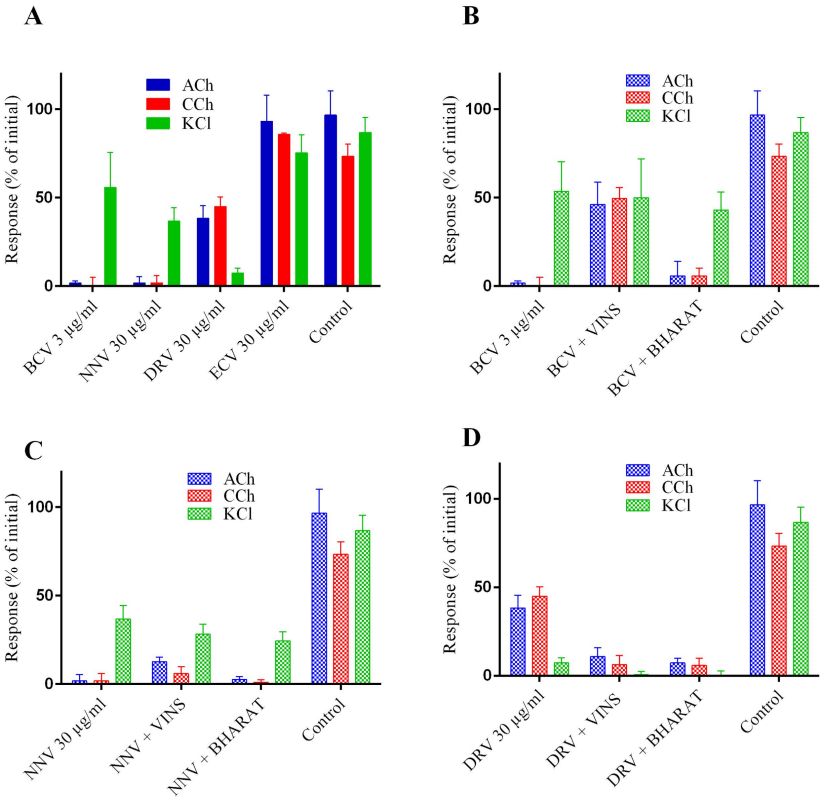

Figure 4. Response to acetylcholine (ACh), carbachol (CCh) and potassium chloride (KCl) for venom alone (**A**) and with five times recommended doses of VINS and BHARAT antivenoms; (**B**) *B. caeruleus* (3 µg/ml) venom, (**C**) *N. naja* (30 µg/ml) venom and (**D**) *D. russelii* (30 µg/ml) venom on indirect twitches of the chick biventer cervicis nerve–muscle preparation. BCV; *B. caeruleus* venom, NNV; *N. naja* venom, ECV; *E. carinatus* venom and DRV; *D. russelii* venom.

Venom	LD_{50} µg/g body weight of mice (95% confidence intervals)	ED_{50} µg/g body weight of mice (95% confidence intervals) for VINS 2010	ED_{50} µg/g body weight of mice (95% confidence intervals) for BHARAT 2011 (Jan)
D. russelii	0.102 (0.075–0.121)	0.248 (0.175–0.375)	0.412 (0.310–0.505)
E. carinatus	0.664 (0.519–0.806)	1.188 (0.862–1.666)	1.177 (0.690–2.112)
N. naja	0.665 (0.482–0.978)	0.770 (0.484–1.052)	1.375 (0.986–1.632)
B. caeruleus	0.196 (0.148–0.251)	0.250 (0.090–0.520)	0.334 (0.212–0.590)

Table 5. Lethality dose 50 (LD_{50}) and effective dose 50 (ED_{50}) of VINS and BHARAT antivenoms for *D. russelii*, *E. carinatus*, *N. naja* and *B. caeruleus* venom. *ED 50 is calculated for neutralizing doses of five times LD_{50} values of each venom.

(Table 5). The ER_{50} for *D. russelli* venoms was 2.06 for VINS compared to 1.24 for BHARAT; for *E. carinatus* venom was 2.79 for VINS compared to 2.82 for BHARAT; for *B. caeruleus* was 3.92 for VINS compared to 2.93 for BHARAT; and for *N. naja* was 4.32 for VINS compared to 2.42 for BHARAT.

Discussion

This study has shown that VINS antivenom has a higher protein content and overall a greater *in vitro* efficacy against the medically important effects of most snake venoms in Sri Lanka, compared to BHARAT antivenom. VINS antivenom performed better against the clinically relevant effects of three snakes, being more efficacious

against the *in vitro* procoagulant activity of *D. russelii* and *E. carinatus* venoms, and the neurotoxic effects of *B. caeruleus* venom. A concerning finding was that the lethality and ED_{50} studies did not reflect these findings. The ED_{50} for the two antivenoms was not statistically significantly different for *D. russelii*, *B. caeruleus* and *E. carinatus*, in contrast to VINS being more efficacious against clinically relevant effects–coagulopathy and neurotoxicity. The study also found some variation in the protein content and efficacy of the different VINS antivenom batches from 2008 to 2012, with decreased protein content and reduced capacity of antivenoms binding venom antigens, for antivenoms manufactured in 2008 and 2010.

In a previous study of Russell's viper envenoming where antivenom concentrations were measured in 86 patients after administration of 10 vials of antivenom, the median antivenom concentration was 2.2 mg/ml a median of two hours after administration of 10 vials of antivenom[21]. Tables 2–4 show that this concentration is sufficient for complete binding of all four venom antigens, neutralisation of procoagulant effects and neutralisation of neurotoxicity by VINS antivenom. However, this was not true for BHARAT antivenom in which larger concentrations were required to bind the venom antigens and neutralise the procoagulant effects of *E. carinatus* venom. Both antivenoms required higher concentrations to neutralise the procoagulant effect of *E. carinatus* venom suggesting they are not as efficacious against this venom, although this concentration appears to be sufficient for VINS antivenom. The inferior efficacy of BHARAT antivenoms was consistent with the lower protein content in these antivenoms.

An unusual finding was that BHARAT antivenom had a significantly higher venom antigen binding capacity for *N. naja* venom than VINS antivenom. However, BHARAT antivenom was less effective in neutralising the neurotoxic effects of *N. naja* venom and had a higher ED_{50} compared to VINS. This differed to the other venoms and suggests that BHARAT antivenom has a higher titre to cobra venom antigenic components that may not be toxic. One study found that BHARAT antivenom was ineffective against the neurotoxicity of Pakistani cobra (*N. naja*) and Pakistani krait (*B. sindarus*) at similar concentrations[22]. Unfortunately this study did not test other antivenoms and concluded that antivenom in general was ineffective against Pakistani snake neurotoxicity[22]. A more recent study found that higher titres of three different antivenoms (Indian, Thailand and Taiwan) were required to neutralise the neurotoxic effects[23]. Another study found that VINS antivenom bound more avidly to Indian compared to Sri Lankan cobra venom, also suggesting possible geographical variability in the venoms, particularly the neurotoxic activity[24].

A limitation of the study was that only the most recent batches of BHARAT antivenoms were available to be tested and only multiple vials from one batch of each type of antivenom was available. However, the two recent batches of BHARAT were inferior to the recent batches of VINS tested against the important clinical effects of the medically important snakes in Sri Lanka and a direct comparison between batches with multiple vials found VINS to be statistically significantly more efficacious than BHARAT antivenoms. In addition, the variability within these two batches was much greater for BHARAT than for VINS. We have previously shown that expired antivenoms up to 10 years old, formulated as liquids, have lost minimal activity, even after extended periods at room temperature[25]. Antibodies in the solid form would be expected to be as least as stable.

The units of antivenom differed for the lethality studies compared to the *in vitro* studies because the lethality studies are dosed on mouse body weight. This is another reason that lethality studies are problematic because it is difficult to relate μg/g body weight to the amount of venom in human bites. For the binding and *in vitro* studies we used concentrations measured in human snake envenoming cases. The difference in units did not affect the conclusions of the study because the relative efficacy of the two antivenoms differed in lethality studies compared to the *in vitro* studies. In addition, the ER_{50} was also calculated to provide another comparison without units.

Another limitation was that the study did not test the efficacy of the antivenom against other known venom effects, such as myotoxicity or nephrotoxicity[26]. There are no well tested methods of assessing nephrotoxicity[26]. One study reports only minor changes in renal function in an *in vivo* murine model, evidenced by protein and erythrocytes in urine, but not renal histology or measurements of creatinine. Myotoxicity is only a minor clinical problem in envenoming by all of these snakes[27]. One study reported an unusually high early increase in creatine kinase in mice 3 hours post-injection of *D. russelii* venom, which is too early to be due to systemic myotoxicity and not consistent with a previous *in vivo* examination of systemic myotoxicity due to snake venoms in rats[28]. Coagulopathy is the most important clinical effect in Sri Lankan Russell's viper and saw-scaled viper envenoming, so testing the efficacy of antivenom against the procoagulant effect is most appropriate. Other haemotoxic venom effects were also not tested, such as haemorrhagic effects and platelet toxicity. Although haemorrhagic effects are important for *E. carinatus* neither of the other haemotoxic effects are important in *D. russelii* bites.

Neurotoxicity is the most important clinical effect in *B. caeruleus* envenoming[27], so testing antivenom against neurotoxicity was most appropriate. In addition, we tested the efficacy of the antivenoms to bind to venom antigenic components as a general assessment of antivenom efficacy. There was good correlation between binding efficacy and efficacy against medically important clinical effects, except for cobra neurotoxicity where BHARAT antivenom was found to bind more effectively. VINS antivenom had excellent binding efficacy for *D. russelii*, and more recent vials had better binding efficacy for *B. caeruleus* and *E. carinatus*. The binding efficacy was statistically significantly better for VINS for all snakes except *E. carinatus*. VINS antivenoms were also more efficacious in neutralising procoagulant effects for *D. russelii* and *E. carinatus*, and neurotoxic effects for *B. caeruleus*. Neither antivenom was able to neutralise the neurotoxic effects of *D. russelii* venom. Although neurotoxicity occurs in about half of *D. russelii* bites in Sri Lanka it is rarely life-threatening[29]. Neurotoxicity is only reported for Russell's viper bites in Sri Lanka and Southern India[30], so the venoms used in making the Indian antivenoms may not contain these neurotoxins[31]. This supports assessing antivenoms using tests of *in vitro* efficacy against clinically relevant toxicity.

In contrast to efficacy as assessed by *in vitro* binding and neutralisation efficacy, efficacy assessed by traditional ED_{50} testing against lethality in mice, was not statistically significant between the antivenoms for any of the venoms. The relative efficacy of the two antivenoms based on ED_{50} values was not consistent with any of the testing

against important clinical effects in any of the snakes, suggesting that relying on such testing is problematic. The reason for this is that death in animals (e.g, mice in this study) could be due to a range of toxicities including some clinically irrelevant toxic effects important in human envenoming. It is entirely possible that post-synaptic neurotoxins or early cardiovascular collapse are major causes of lethality in rodents[32], but are far less important in humans, in which presynaptic neurotoxins and procoagulant toxins are more prominent. A recent study has found that the procoagulant toxins in snake venoms have different effects on human and animal plasmas, making interpretation of efficacy of antivenom in rodent models problematic[12]. Based on the results of the current studies it would appear to be more appropriate to use clinically relevant *in vitro* studies of antivenoms against venom effects, and great care should be taken when interpreting *in vivo* animal models. However, further work is required on other snakes worldwide to confirm our findings for all antivenoms.

VINS antivenom appears to be the more efficacious compared to BHARAT antivenom. A dose of 10 vials is sufficient to bind all free venom antigens from these four snakes for venom concentrations found in patients with human envenoming. In addition, this dose was also able to neutralise the procoagulant effects *in vitro* of *D. russelii* venom and *E. carinatus* venom, and the neurotoxic effects of *B. caeruleus*. In contrast, the lethality studies did not appear to provide as useful an assessment or comparison of the efficacy of the two antivenoms.

Methods

Materials. Indian polyvalent snake antivenom was obtained from VINS Bioproducts Limited (Hyderabad, Andra Pradesh, India) and from BHARAT Serum and Vaccines Limited (Mumbai Maharashtra, India). Details of the antivenoms tested are given in Supplementary Table 1. All antivenoms were reconstituted according to the manufacturer's instructions. Russell's viper (*D. russelii*), common cobra venom (*N. naja*), Saw-scaled viper (*E. carinatus*) and common krait (*B. caeruleus*) venoms were collected in Sri Lanka. Stock solutions of venom was prepared as 1 mg/mL in 50% Glycerol and stored at −20 °C.

Bradford reagent (Bio-Rad, Catalogue # 500–0205) and Bovine Gamma Globulin (Bio-Rad, Catalogue # 500–0208) were used for protein quantification. Tris-buffered saline (TBS) was used to make up dilutions of antivenom for neutralization of *D. russelii* and *E. carinatus* venom procoagulant activity studies. Fresh frozen plasma was obtained from the Australian Red Cross and aliquots of 10 mL were thawed at 37 °C. Tetramethylbenzidine (TMB) from Sigma, bovine serum albumin (BSA) from Bovogen, Australia and Streptavidin-conjugated horseradish peroxidase (Streptavidin HRP) from Millipore Chemicon were used for the binding studies. Blocking solution is 0.5% BSA in phosphate buffered saline (PBS). Washing solution is 0.02% TWEEN 20 in PBS. Polyclonal monovalent rabbit IgG to Russell's viper venom was obtained by injection of rabbit with *D. russelii* venom followed by purification of the serum on a Protein G-Sepharose column and was carried out at the Western Australian Institute of Medical Research. Polyclonal monovalent rabbit IgG to *E. carinatus*, *N. naja* and *B. caeruleus* venom were obtained by injection of rabbits with the corresponding venoms, followed by purification of the serum on a Protein G-Sepharose column and was carried out at the Faculty of Medicine and Allied sciences, Rajarata University, Sri Lanka. Rabbit IgG antibodies were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce # 21335).

Binding and neutralisation studies of *D. russelii* venom were undertaken at venom concentrations measured in cases of human envenoming and taken as 1.7 μg/ml, the 97th percentile of venom concentrations in a previous study of 257 patients with pre-antivenom venom concentrations ranging from 0.0033 to 14.8 μg/mL[21]. For *E. carinatus* venom the venom concentration used was 0.5 μg/mL based on the maximum concentrations measured in one study of *Echis ocellatus* envenoming[33]. For *N. naja* and *B. caeruleus* venoms the venom concentrations was again taken as the 97th percentile which was 1.4 μg/ml for *N. naja* and 0.15 μg/ml for *B. caeruleus* based on nine envenomed patients from Sri Lanka for each venom. Only one batch of antivenom from each manufacturer were compared statistically -VINS (01011/10–11; 2010) and BHARAT (A5311006; 2011Jan)–because multiple vials were available.

Protein quantification. Quantification of the protein content in each antivenom was undertaken using the Bradford protein assay method[34] Inter- and intra-batch protein quantification was carried out for all 36 vials of antivenom. Bradford reagent (150 μl) was added to a solution of antivenom in PBS (150 μl). After 10 minutes absorbance at 595 nm was measured on a Bio-Tek ELx808 plate reader. Concentrations of proteins were calculated with reference to a standard curve based on bovine gamma-globulin. Samples were measured at three dilutions.

Venom-antivenom binding studies. The following antivenom vials were used for antivenom venom binding studies–VINS 1061; VINS 01011/10–11; VINS 01013/10–11; VINS 01023/10–11; VINS 01024/10–11; VINS 01AS11112; VINS 01AS11114; BHARAT A5311006; BHARAT A5311013; BHARAT A5311014. Solutions of increasing concentrations of antivenom (0 to 4.3 mg/mL for *E. carinatus* venom and 0 to 17.1 mg/mL for *D. russelii, N. naja* and *B. caeruleus* venom) in blocking solution (0.5% Bovine Serum Albumin in PBS) were incubated with venom (*D. russelii* 1.7 μg/mL, *E. carinatus* 0.5 μg/mL, *N. naja* 1.4 μg/mL and *B. caeruleus* 0.15 μg/mL) for one hour at room temperature. Unbound venom antigens were detected using a sandwich enzyme immunoassay (EIA) as previously described[35]. In brief, Greiner Microlon 96-well high-binding plates were coated with the four different monovalent rabbit anti-snake venom IgGs (1 μg/mL) in carbonate buffer (50 mM, pH 9.6), kept at room temperature for 1 h and then at 4 °C overnight. The plates were then washed once with PBS containing 0.02% TWEEN 20 and 300 μL of blocking solution of 0.5% BSA in PBS was added. After 1 h the plates were washed again, and 100 μL of venom–antivenom mixture was applied, after first diluting appropriately in blocking solution applied as (1:400 for *D. russelii,* 1:25 for *E. carinatus,* 1:140 for *N. naja,* 1:15 for *B. caeruleus*) dilutions in blocking solution. The plates were allowed to stand for 1 h and then washed three times. Next, biotinylated anti-snake venom IgG (*D. russelii* 0.5 μg/mL, *E. carinatus* 8 μg/mL, *N. naja* 0.12 μg/mL and *B. caeruleus* 1 μg/mL in blocking solution) was added. After standing for a further hour the plates were washed again. Streptavidin-horseradish

peroxidase (100 μL, 0.1 μg/mL in blocking solution) was added and left for 1 h. The plate was then washed three times and 100 mL of TMB reagent added and colour allowed to develop for 3.5 min. The reaction was stopped by the addition of 50 mL of 1 M H_2SO_4. All samples were measured in triplicate, and the averaged absorbance converted to a concentration of the venom of interest by comparison with a standard curve based on serial dilutions of venom.

Neutralization of procoagulant activity of D. russelii and E. carinatus venom. The same antivenom vials used for *D. russelii* and *E. carinatus* venom binding studies were used for neutralization studies. The ability of antivenom to neutralise the procoagulant activity of *D. russelii* and *E. carinatus* venoms was measured using the turbidimetric method[13]. Solutions of increasing concentrations of antivenom in TBS (0 to 1 mg/mL for *D. russelii* venom and 0 to 30 mg/mL for *E. carinatus* venom) were incubated with 1.7 μg/mL of *D. russelii* venom or 0.5 μg/mL of *E. carinatus* venom for 30 min at 37 °C in a 96 well plate. Fresh frozen plasma (100 μl) containing 40 μl of 0.4 M $CaCl_2$/mL and venom-antivenom solution were added simultaneously to each well using a multichannel pipette. After a 5-second shaking step, the optical density at 340 nm was monitored every 30 s for 20 min. The clotting time was defined as the time until the rapid increase in absorbance, as calculated by Gen5 software (supplied with the Biotek ELx808 plate reader).

***In vitro* neurotoxicity studies.** VINS (01AS11114) and BHARAT (A5311014) antivenom batches were used to investigate the neutralisation of the neurotoxic effects of Russell's viper (*D. russelii*), Common cobra (*N. naja*) and Indian krait (*B. caeruleus*) venoms. Chicks (4 to 10-day-old males) were killed by CO_2 inhalation and exsanguination, and the two biventer cervicis muscles were removed from the back of the neck. Each muscle was attached to a wire tissue holder and placed in a 5 mL organ bath filled with physiological salt solution with the following composition (mM): NaCl, 118.4; $NaHCO_3$, 25; glucose, 11.1; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2 and $CaCl_2$, 2.5. The organ baths were bubbled with carbogen (95% O_2, 5% CO_2) and maintained at a temperature of 33–34 °C under a resting tension of 1 g. Motor nerves were indirectly stimulated every 10 s (0.2 ms duration) at supramaximal voltage using a Grass S88 stimulator. The tissues were equilibrated for 10–15 min after which d-tubocurarine (10 μM) was added, and the subsequent abolition of twitches confirmed the selective stimulation of the motor nerves. The tissues were then washed repeatedly until twitch height was restored. Contractile responses to acetylcholine (ACh; 1 mM for 30 s), carbachol (CCh; 20 μM for 60 s), and KCl (40 mM for 30 s) were measured in the absence of stimulation[36]. At the conclusion of the experiment, responses to ACh, CCh, and KCl were measured again. Each of the four venoms at two concentrations (3 and 30 μg/mL) were initially added to the organ bath without antivenom to determine the neurotoxic potency of each venom. Only *B. caerulus* venom had significant neurotoxicity at concentrations seen in human envenoming (3 μg/mL). Neurotoxicity potency was measured as the t_{90} which is the time required to cause 90% inhibition of the initial twitch height, for a given concentration of venom, expressed as a mean +/− SD. For some venoms, where the twitch height inhibition for a particular venom/treatment did not reach 90%, the time to maximum twitch inhibition observed in all tissues of that treatment/venom group was considered for all the groups for comparison.

Antivenom was added to the bath and incubated for 10 min. *D. russelii* (30 μg/mL), *N. naja* venom (30 μg/mL) or *B. caeruleus* venom (3 μg/mL) was then added and left in contact with the tissue for 90 min. The antivenom concentration used was based on the manufacturer's instructions (1 ml of antivenom neutralises 0.6 mg *N. naja* venom, 0.45 mg *B. caerulus* venom and 0.6 mg of *D. russelii* venom). Experiments were done for both the recommended dose and five times the recommended dose.

Lethality neutralization studies. Lethality neutralization studies on antivenoms were tested by calculating the median lethal dose (LD_{50}) and the median effective dose (ED_{50}) values in mouse experiments. ICR (Institute of Cancer Research) mice, both sexes, weighing 18–20 g were used for all experiments. The following antivenom vials VINS0101110/11 and Bharat A5311006 were used for lethality studies. For this study, 250 mg/ml solutions of the antivenoms were prepared for venom-neutralization studies which was higher than the manufacturer's recommended concentration because the recommended concentration was too dilute and therefore too large a volume for administration to mice

Assessment of the LD_{50} and ED_{50} experiments followed the methods described by the World Health Organisation (WHO) (2010)[9]. After dose ranging studies, varying doses of venom or venom and antivenom mixtures were injected to multiple groups of five mice in both LD_{50} and ED_{50} experiments. In all ED_{50} studies, five times the LD_{50} of the respective venom was mixed with varying amounts of antivenom and incubated at 37 °C for 30 min before injection. All injections were intravenous to the tail veins and were made to a final constant volume of 250 μl by adding normal saline. In both LD_{50} and ED_{50} studies, death/survival rates were recorded for 48 hours. The ED_{50} values were expressed in μg of antivenom per g body weight of mouse (μg/g) to neutralize the challenge dose of venom. The median effective ratio (ER_{50}) was also calculated using the following formula,

$$ER_{50} = nLD_{50}/ED_{50}$$

where n is the number of LD_{50} doses given (5 in this study).

Ethics Approvals. Ethical approval for the chick experiments was obtained from the Monash University Animal Ethics Committee 2012/008. All animal experiments were conducted in the Animal House, Faculty of Medicine and Allied Sciences, Rajarata University of Sri Lanka according to the Council for International Organizations of Medical Sciences (CIOMS) guidelines on animal experimentation[37]. Animal ethics clearance for the study was obtained from the Ethics review committee, Faculty of Medicine and Allied Sciences, Rajarata university of Sri Lanka ERC 2012/038.

Analysis of results. Standard curves for enzyme immunoassays and calculations of EC_{50} were fitted by non-linear regression. For the *in vitro* neurotoxicity data the twitch heights were analysed by one way ANOVA followed by Bonferroni post-hoc tests, and the time to a reduction in twitch heights by Kruskal-Wallis test followed by Dunn's multiple comparison test. Standard error of the mean (SEM), standard deviations (SD) and 95% confidence intervals (95% CI) were all calculated for parametric and non-parametric data respectively. The difference in protein content between vials from one batch of each of the two antivenoms was compared using an unpaired t-test. Log EC_{50} values were compared using the extra sum of the squares F test in Prism when comparing one antivenom from each manufacturer. The 95% confidence intervals of the ED_{50} values were compared to determine if the ED_{50} values were significantly different. Statistical significance was set at $p < 0.05$. All analyses and graphics were done in GraphPad Prism version 6.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com, except for the calculation of the LD_{50} and ED_{50} which was done using the probit method[38] using SPSS statistical software version 20.0.

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Acknowledgements

We acknowledge the assistance of staff from the South Asian Clinical Toxicology Research Collaboration, including Shaluka Jayamana, Indika Gawarammana and other medical staff involved in the study. We thank Kolitha Sellahewa for obtaining the antivenoms. P.K. Gamage, S. Hettiarachchi, N. Pathirana and T. Elpitiya (Rajarata University of Sri Lanka) for the assistance in mouse assays. We thank the Department of Wildlife Conservation, Sri Lanka for granting permission for this study (permit number: WL/3/2/20/14). Special thanks to Rohan Pethiyagoda (Wildlife Heritage Trust of Sri Lanka) for his support.

Author Contributions

K.M., A.S., W.C.H. and G.K.I. designed the study; K.M., A.S. and M.L. undertook the laboratory studies; K.M., A.S. and G.K.I. undertook the analysis; K.M. drafted the manuscript and all authors contributed to the final version; G.K.I. takes responsibility for the manuscript.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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

How to cite this article: Maduwage, K. *et al.* Efficacy of Indian polyvalent snake antivenoms against Sri Lankan snake venoms: lethality studies or clinically focussed *in vitro* studies. *Sci. Rep.* **6**, 26778; doi: 10.1038/srep26778 (2016).

Chapter Seven

Procoagulant snake venoms have differential effects in animal plasmas: implications for antivenom testing in animal models

Introduction to Chapter Seven: Procoagulant snake venoms have differential effects in animal plasmas: implications for antivenom testing in animal models" to the overall thesis

Procoagulant coagulopathy is the commonest systemic effect of any snake envenoming in the world. This is due the activation of clotting cascade by pro-coagulant snake toxins in snake venom. Different pro-coagulant toxins have been identified and they activate a specific reaction in the clotting cascade. Factor V and X activators in Russell's viper venom, prothrombin activators in Australian elapid venom and snake venom thrombin like enzymes in pit viper venoms are major classes of pro-coagulant snake toxins identified.

Animal models have been used to test the efficacy of snake antivenoms used for pro-coagulant snake envenoming. According to the World Health Organization guidelines on testing the efficacy of snake antivenoms, calculation of the lethal dose 50 and effective dose 50 are essential to establish their efficacy for any snake venom. However, little information is available on the susceptibility of different animals to various snake toxins, especially the pro-coagulant toxins. Therefore, the prediction of the efficacy of antivenoms based on animal models might not be accurate for human envenoming. I aimed to explore the susceptibility of various animal plasmas for different pro-coagulant snake toxins. This is vital to understand the usefulness of using animal models to predict the snake venom toxicities during initial anitvenom assessments. Therefore, the study titled "Pro-coagulant snake venoms have differential effects in animal plasmas: implications for antivenom testing in animal models" is justifiable to include under the subheading of efficacy and effectiveness of snake antivenoms.

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was published into Thrombosis research journal on 2nd December 2015. I, Maduwage Kalana Prasad, was the first author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

Part of the coagulation assays were carried out by E. F. Scorgie and L. C. Lincz. In-vitro efficacy studies were carried out by myself under the supervision of Margaret O'Leary. Overall supervision of the study was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter seven.

Thrombosis Research 137 (2016) 174–177

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Procoagulant snake venoms have differential effects in animal plasmas: Implications for antivenom testing in animal models

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ARTICLE INFO

Article history:
Received 11 August 2015
Received in revised form 19 October 2015
Accepted 1 December 2015
Available online 2 December 2015

ABSTRACT

Background: Animal models are used to test toxic effects of snake venoms/toxins and the antivenom required to neutralise them. However, venoms that cause clinically relevant coagulopathy in humans may have differential effects in animals. We aimed to investigate the effect of different procoagulant snake venoms on various animal plasmas.

Methods: Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen and D-dimer levels were measured in seven animal plasmas (human, rabbit, cat, guinea pig, pig, cow and rat). *In vitro* clotting times were then used to calculate the effective concentration (EC_{50}) in each plasma for four snake venoms with different procoagulant toxins: *Pseudonaja textilis*, *Daboia russelli*, *Echis carinatus* and *Calloselasma rhodostoma*.

Results: Compared to human, PT and aPTT were similar for rat, rabbit and pig, but double for cat and cow, while guinea pig had similar aPTT but double PT. Fibrinogen and D-dimer levels were similar for all species. Human and rabbit plasmas had the lowest EC_{50} for *P. textilis* (0.1 and 0.4 μg/ml), *D. russelli* (0.4 and 0.1 μg/ml), *E. carinatus* (0.6 and 0.1 μg/ml) venoms respectively, while cat plasma had the lowest EC_{50} for *C. rhodostoma* (11 μg/ml) venom. Cow, rat, pig and guinea pig plasmas were highly resistant to all four venoms with EC_{50} 10-fold that of human.

Conclusions: Different animal plasmas have varying susceptibility to procoagulant venoms, and excepting rabbits, animal models are not appropriate to test procoagulant activity. *In vitro* assays on human plasma should instead be adopted for this purpose.

1. Introduction

Venom induced consumption coagulopathy (VICC) is a major systemic syndrome that occurs as a consequence of snake envenoming with procoagulant toxins, including envenoming by many Viperid snakes, certain elapids, including Australian elapids and a few Colubrid (rear fang) snakes [1]. VICC is therefore one of the most medically important envenoming syndromes worldwide. VICC results from the activation of the clotting pathway by procoagulant toxins in the venom which then causes consumption of clotting factors, leaving snakebite victims at risk of major haemorrhage [2]. Snake venom toxins which act on the coagulation cascade are classified according to the part of coagulation pathway where they act and include factor V activators, factor X activators, prothrombin activators, and snake venom thrombin-like enzymes (TLEs) or fibrinogenases [2].

Antivenom therapy is regarded as the foremost treatment for snake envenoming. Although it is widely recommended for VICC there is limited evidence to support its effectiveness [3]. The effectiveness of antivenom in humans and the dosing is generally based on animal studies. Animal models have been used to test the efficacy of antivenom against lethality induced by procoagulant snake venoms, including calculation of lethal dose 50 (LD_{50}), effective dose 50 (ED_{50}) and *in vivo* venom defibrinogenating activity [4,5]. These tests are carried out in mice and sometimes rats, and are used to compare the efficacy of antivenoms against lethality and procoagulant coagulopathy. However, there are concerns about the relevance of such lethality models in animals with regard to VICC in humans. It is likely that rodents are highly susceptible to post-synaptic snake neurotoxins [6] so the lethality of a venom in a rat may have nothing to do with the procoagulant effect of venom that is the primary cause of morbidity and mortality in humans.

Another consideration is that there may be a significant difference in the susceptibility of various animals to procoagulant snake venoms, and this would make interpretation of lethality studies of rodents problematic when applying them to VICC in humans. For example if VICC does not occur in mice at venom concentrations that cause VICC in humans, then VICC is unlikely to be the mechanism of death in mice studies. There is little information on the effect of different procoagulant snake venoms on the coagulation pathway in animals, or whether procoagulant

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http://dx.doi.org/10.1016/j.thromres.2015.12.002
0049-3848/

coagulopathy is a factor in their death by envenoming [7,8]. The effect of procoagulant snake venom on animal plasma may be different to that on human plasma, due to variation of clotting factors, factor levels and activation in different animals. Only one previous study has investigated clotting times and factor levels in rat plasma [9], while there exists no published literature for other animal plasmas.

In a preliminary experiment to investigate the dynamic effects of venom on clotting in an *in vivo* rat model, we found that we were unable to produce a coagulopathy in rats with a sub-lethal dose of (the highly procoagulant in humans) *P. textilis* venom (unpublished results). Even with a much higher dose of brown snake venom where cardiovascular collapse and death occurred within 60 min of venom administration, the prothrombin time (PT) remained normal in rat plasma. This suggested that there was a difference in susceptibility of rat plasma to procoagulant snake toxins compared to human plasma.

This study aimed to compare the *in-vitro* procoagulant effect of four snake venoms - *Pseudonaja textilis* (brown snake; prothrombin activator toxin), *Daboia russelli* (Russell's viper; factor X activator toxin), *Echis carinatus* (Carpet viper; prothrombin activator toxin) and *Calloselasma rhodostoma* (Malayan pit viper; thrombin-like enzyme toxin) — on seven different animal plasmas — human, rabbit, cat, guinea pig, pig, cow and rat.

2. Methods

2.1. Materials

P. textilis venom purchased from Venom Supplies Pty Limited, South Australia; *D. russelli* venom was a gift from the Faculty of Medicine, University of Colombo, Sri Lanka; *E. carinatus* venom (batch # V8250) was purchased from Sigma; and *C. rhodostoma* was a gift from Wayne Hodgson, Monash venom group, Melbourne, Australia. Human fresh frozen plasma (batch # 8,449,298; contains citrate) was obtained from the Australian Red Cross. Rabbit and Guinea pig plasmas were collected from the animals using a non-heparinized catheter into citrated tubes which were immediately centrifuged and the plasma separated and stored at −80 °C until used. Citrated rat (batch # P2516), pig (batch # P2891), cow (batch # P4839) and cat plasma (batch # P3276) were purchased from Sigma and reconstituted in water according to the manufacturer's instructions and used the same day for experiments.

2.2. Haemostatic function tests and clotting factor levels

A prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, factor V, factor VIII, factor X and D-dimer levels were measured in each animal plasma. All assays were carried out using either standard coagulometric or immunoturbidimetric methods as provided by the manufacturer and were performed on a Behring Coagulation System (BCS) or Sysmex CA-1500 analyser (Dade Behring, Marburg, Germany) as previously reported [10,11]. Factor V, factor VIII and factor X levels were determined by mixing animal plasma with human plasma deficient in the factor being measured and the time for clot formation measured in seconds. The amount of factor present in the sample was quantified by comparing with a standard or reference curve produced using serial dilutions of human plasma deficient in the factor mixed with normal human plasma, against the clotting time. The quantification of D-dimer was done using immunoturbidometric methods.

2.3. Effect of procoagulant venoms on animal plasma clotting times

Procoagulant effect was assessed by measuring the clotting times after adding each of the four snake venoms to each of the seven animal plasmas. For each experiment serial dilutions of the snake venom were mixed with the animal or human plasma according to the method described by O'Leary and Isbister [12]. Each venom solution (50 μl) in Tris Buffered Saline, pH 7.4 was placed in a well of a 96 well microtitre plate at room temperature or at 37 °C in a BioTek Synergy HT plate reader. The animal or human plasma (50 μl) with 40 μl of 0.4 M $CaCl_2$/ml was simultaneously added to the wells with a multichannel pipette. After 5-s of shaking to mix, the optical density was monitored at 340 nm every 30 s for 20 min. A plot of optical density versus time was produced. The lag time, defined as the time when absorbance becomes 0.02 units greater than the average of the first two absorbance measurements, was taken as the clotting time as this represents the abrupt rise in the optical density as a clot starts forming.

Table 1
Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen and D-dimer concentrations in different animal plasmas.

Plasma	PT (sec)	INR	aPTT (sec)	Fibrinogen (g/L)	D-dimer (mg/dL)
Human	11	1.3	17	2.6	<0.17
Rabbit	12	1.3	14	2.4	<0.17
Rat	11	1.3	18	2.3	<0.17
Cat	19	1.9	48	3.2	<0.17
Cow	25	2.4	52	2.5	0.21
Pig	11	1.2	16	2.0	0.79
Guinea pig	21	2.1	15	1.6	<0.17

2.4. Data analysis

In previous studies the procoagulant effect of snake venoms has been expressed as a minimum clotting concentration for a clot to occur at 5 min [13]. However, this only allows comparison for a single clotting time of 5 min. In this study we plotted 30 s/[clotting time(s)] against the logarithm of the venom concentration. Taking the inverse of the clotting time converts a normal long clotting time to a small number or basal measure (no procoagulant effect), and the shortest clotting time that can be measured on the plate reader of 30 s, to 1 (maximal procoagulant effect). This provides a normalised measure of the clotting effect, ranging from basal values (no effect) where the clotting time is long (30 s/600 + s) to 1 (maximal effect) where the clotting time is the shortest (30 s/30 s). This produces normalised concentration-clotting curves which can be fitted with a standard sigmoidal curve (Hill slope = 1) to calculate the effective concentration 50 (EC_{50}), which is the concentration that results in a procoagulant effect halfway between the minimal (bottom) or no clotting effect and the maximal (top) clotting effect (Fig. S1). Graph Pad Prism software, version 6 was used to graph the data and estimate the EC_{50}.

3. Results and discussion

Haemostatic function tests (PT and aPTT), fibrinogen concentration and D-dimer levels are presented in Table 1. The PT was similar to human plasma for rat, rabbit and pig plasmas. However, the PT was about double for cat, cow and guinea pig plasma. The aPTT was similar in human, rabbit, rat, pig and guinea pig while it was more than three

Table 2
Effective concentration 50 (EC_{50}) values for seven different animal plasmas with each of four snake venoms — *Pseudonaja textilis*, *Daboia russelli*, *Echis carinatus* and *Calloselasma rhodostoma* venoms.

Plasma	EC_{50} μg of venom/ml of plasma (on clotting times)			
	Pseudonaja textilis	*Daboia russelli*	*Echis carinatus*	*Calloselasma rhodostoma*
Human	0.1	0.3	0.6	28
Rabbit	0.4	0.1	0.1	32
Rat	1.4	9.5	5.6	284
Cat	3.0	No clot	100	11
Cow	13	125	646	121
Pig	1.3	17	14	40
Guinea pig	1.3	1.1	5.8	49

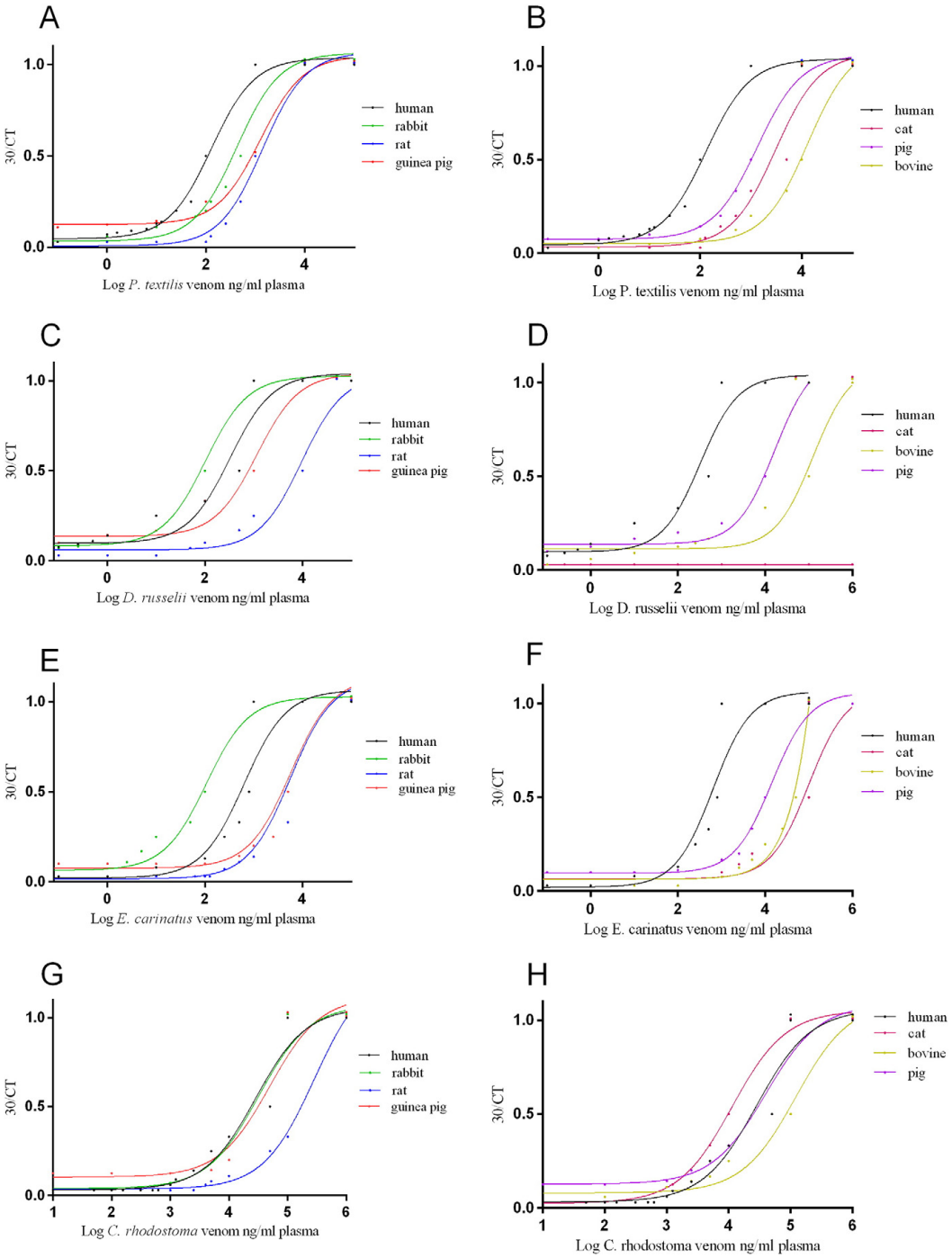

Fig. 1. Plots of 30/clotting time versus log venom concentration demonstrating the procoagulant effect of *Pseudonaja textilis* (A, B), *Daboia russelli* (C, D), *Echis carinatus* (E, F) and *Calloselasma rhodostoma* (G, H) for human, rabbit, rat and guinea pig plasmas (A, C, E, G) and for human, cat, bovine and pig plasmas (B, D, F, H).

times higher in cow and pig plasmas. Fibrinogen concentrations were similar for all species as were the D-dimer levels. However, there were huge variations in the other factor levels (factor V, VIII and X) between species (Table S1).

Problems with estimating haemostatic function (prothrombin time, aPTT and thrombin time) and factor concentrations in rat plasma with reagents used for human assays have been previously described [9]. There is almost no information on normal coagulation and factor levels in other animals, excepting one study of the differences in rabbit and human coagulation factor X and its implications [7,8]. The present study suggests that clotting function and at least fibrinogen and D-dimer levels are reasonably similar across these animal plasmas.

Unfortunately, the results of the other factor levels measured were highly variable (Table S1). This is most likely due to the fact that this assay requires factor deficient plasma (of the factor of interest), and these are only available for human plasma. The assay therefore required mixing the animal plasma with the factor deficient human plasma (e.g. human plasma deficient in factor V for the factor V assay). This is the likely reason for the variable results and has been previously recognised by Garcia-Manzano et al. [9]. To measure other factors in animal plasmas will require obtaining specific animal plasmas that are factor deficient.

The EC_{50} values for the different snake venoms on each animal plasma are shown in Table 2 and Fig. 1. Human and rabbit plasmas had the lowest EC_{50} values for *P. textilis* (0.1 and 0.4 μg/ml), *D. russelli* (0.4 and 0.1 μg/ml), *E. carinatus* (0.6 and 0.1 μg/ml) venoms, while cat plasma had the lowest EC_{50} value for *C. rhodostoma* (11 μg/ml) venom. Cow plasma was resistant to all four procoagulant venoms with high EC_{50} of 13.1 for *P. textilis* venom to 646 μg/ml for *E. carinatus* venom. Rat, pig and guinea pig were also resistant to the procoagulant venoms with EC_{50} values 10-fold that of human and rabbit for all venoms.

Animal models are frequently used in pre-clinical efficacy studies of snake antivenoms. Estimation of LD_{50} and ED_{50} of snake antivenoms for mice or rat models are considered the gold standard for testing the efficacy of antivenoms [5]. Moreover, neutralization of snake venom de-fibrinogenating activity is also tested using *in-vivo* mice models [4]. Results of these animal experiments continue to be used to compare the efficacy of antivenoms, and estimation of initial dose of antivenoms for human clinical trials [14].

Wide variation of the EC_{50} values for different animal plasmas for different procoagulant snake venoms in this study, suggests that different animal plasmas have varying susceptibility to procoagulant toxins. Therefore, predicting the efficacy of antivenoms for coagulopathy in snake envenoming, based on animal models (LD_{50} and ED_{50}), is problematic and unlikely to represent what happens in humans. In a recent study, sheep plasma was used to assess antivenom efficacy against the procoagulant effect of *C. rhodostoma* [15]. Although sheep plasma was not tested in our study, the results suggest that the use of non-human plasma to test antivenom efficacy is also highly problematic. Excepting rabbits, animal models including ones using rodents or non-human plasma, are not appropriate to test procoagulant activity. Assessment of antivenom efficacy and dosing should be done using human plasma [4,13].

The reasons for some animal plasmas being resistant to the procoagulant snake toxins may be due to prey species (e.g. rat, guinea pig) favouring mutations that provide protection against snake venom proteins. Such an adaptation has been shown for opossums that prey on pit-vipers, where they have developed mutations in the gene encoding von-Willebrand's factor which is targeted by the pit-viper venom [16].

The study once again emphasizes the problems with using animal studies to determine the effectiveness and dose of treatments to be used in humans. Different animal plasmas appear to have varying susceptibility to procoagulant snake venoms. Rodent plasmas were much more resistant to the procoagulant venoms and for some snakes 2 to 3 orders of magnitude higher venom concentrations were required to cause clot formation. This was despite similar PT, aPTT, fibrinogen and D-dimer levels for most of the different animal plasmas without the addition of venom, suggesting that the differences in susceptibility to snake venoms is due to the toxins in the venoms. Factor levels could not be accurately measured in any of the animal plasmas because factor deficient animal plasmas were not available.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.thromres.2015.12.002.

Funding

The research was funded in part by an NHMRC Program Grant 1055176. Geoff Isbister is funded by an NHMRC Senior Research Fellowship ID1061041.

Author contributions

GI, MO and KM designed the study, KM, FS and LL did the coagulation assays, KM and MO did the venom procoagulant assays; KM and GI did the analysis, KM and GI wrote the manuscript and all authors contributed, GI takes responsibility for the manuscript.

Conflicts of interest

None of the authors has a conflict of interest.

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Chapter Eight

Snake antivenom for snake venom induced consumption coagulopathy (A Cochrane Review)

Introduction to Chapter Eight: Snake antivenom for snake venom induced consumption coagulopathy (A Cochrane Review)to the overall thesis

Placebo randomized control trial evidence is considered as the best scientific evidence to establish the effectiveness of a drug for a disease or condition. Snake antivenoms are widely used for snake envenoming including venom-induced consumption coagulopathy. However, the level of evidence that is available to use antivenom for VICC is questionable. Cochrane systematic review is the best model to explore the evidence of using antivenom for VICC. Therefore, I aimed to explore the availability of placebo randomized controlled trial evidence for the use of antivenom for VICC. Under the subtitle of efficacy and effectiveness of antivenom the study titled “Snake antivenom for snake venom-induced consumption coagulopathy (A Cochrane Review)” is relevant to my overall thesis.

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was published into *Cochrane Database of Systematic Reviews* (Protocol) on December 2014 and Full Review article was published into *Cochrane Database of Systematic Reviews* on June 2015. I, Maduwage Kalana Prasad, was the first author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

According to requirement of protocol search for the studies, selection of studies was carried out by Professor G. K. Isbister. Suggestions and directions were provided by Nick Buckley, David Lalloo and Janaka De Silva. Overall supervision of the study was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter Eight.

Snake antivenom for snake venom induced consumption coagulopathy (Protocol)

Maduwage K, Buckley NA, de Silva HJ, Lalloo DG, Isbister G

This is a reprint of a Cochrane protocol, prepared and maintained by The Cochrane Collaboration and published in *The Cochrane Library* 2014, Issue 12

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[Intervention Protocol]

Snake antivenom for snake venom induced consumption coagulopathy

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Editorial group: Cochrane Injuries Group.
Publication status and date: New, published in Issue 12, 2014.

Citation: Maduwage K, Buckley NA, de Silva HJ, Lalloo DG, Isbister G. Snake antivenom for snake venom induced consumption coagulopathy. *Cochrane Database of Systematic Reviews* 2014, Issue 12. Art. No.: CD011428. DOI: 10.1002/14651858.CD011428.

ABSTRACT

This is the protocol for a review and there is no abstract. The objectives are as follows:

To assess the effects of antivenom for the recovery from VICC in people with snake envenoming.

BACKGROUND

Description of the condition

Snake envenoming is a major medical problem in the tropical world. The estimated burden of snake bite is approximately 421,000 cases of envenoming with 20,000 fatalities annually, although there may be as many as 1,841,000 envenomings and 94,000 deaths (Kasturiratne 2008).

Venom induced consumption coagulopathy (VICC) is one of the major clinical manifestations of snake envenoming and may be complicated by fatal haemorrhage (Isbister 2010a). VICC has previously been referred to by a number of different terms, including disseminated intravascular coagulation, defibrination syndrome and procoagulant coagulopathy (Isbister 2010b). VICC results from the action of snake procoagulant toxins on human coagulation factors causing consumption of these clotting factors leading to multiple factor deficiencies (Isbister 2009a). There are many examples of procoagulant snake toxins that cause VICC, including prothrombin activators in *Echis carinatus*, *Pseudonaja textilis, Notechis scutatus* venoms (Rosing 2001; Rosing 1992; Joseph 2001), factor X activators in *Dabois russelii, Bothrops atrox, Cerastes cerastes, Bungarus, Ophiophagus* venom (Tans 2001), factor V activators in *Bothrops atrox, Naja naja oxiana, Vipera* venom (Rosing 2001), thrombin-like enzymes in *Agkistrodon contortrix contortrix* venom (Swenson 2005) and plasminogen activators in *Trimeresurus stejnegeri* venom (Sanchez 2006). VICC can result in bleeding if there is trauma or spontaneous haemorrhage in cases where the venom also contains a haemorrhagin (e.g. *E. carinatus*). Major haemorrhage in vital organs, such as intracranial haemorrhage, is the most serious issue and is often fatal.

A number of laboratory clotting times and clotting factor studies are used to diagnose and monitor VICC, including the prothrombin time (PT)/International normalised ratio (INR), the activated partial thromboplastin time (aPTT), and the 20 minute whole blood clotting test (WBCT20). These play a major role in diag-

nosis, assessment and treatment of VICC (Isbister 2010a).

Description of the intervention

Antivenom is the primary treatment for snake envenoming (Isbister 2010c; Lalloo 2003). Antivenoms contain polyclonal antibodies raised against one or more snake venoms. They may contain whole IgG immunoglobulins, but more commonly, pepsin or papain digested fragments of IgG immunoglobulins such as F(ab')2 or Fab. They are made by injecting venom into either horses, sheep or goats, and then collecting blood and separating out the specific antibodies to the snake venom. Intravenous administration of antivenom to patients with snake envenoming binds to circulating snake toxins which aims to neutralise or eliminate the toxins and therefore prevent or reverse the clinical effects of envenoming. Monovalent antivenoms are raised against a single snake species, while polyvalent antivenoms are raised against more than one species.

Immediate hypersensitivity reactions to the foreign proteins (immunoglobulins) in snake antivenoms are the major adverse effect of antivenom treatment, including life threatening anaphylaxis (de Silva 2011; Gawarammana 2004; Lalloo 2003; Isbister 2012; Nuchprayoon, 1999). Manufacturing protocols and methods of snake antivenoms are different in various regions in the world and the standardization of snake antivenom production remains problematic.

How the intervention might work

Antibodies in the antivenom bind to the toxic components in snake venom. Early administration of antivenom will bind the circulating procoagulant snake toxins and potentially prevent, delay or lessen the severity of VICC. In the majority of patients who have already developed VICC, antivenom is used to neutralise circulating toxins and allow recovery of VICC. However, the effectiveness and mechanism of action of antivenom in already developed VICC remains unclear (Isbister 2009b). Recovery of the coagulopathy depends on re-synthesizing clotting factors which is not directly affected by antivenom administration (Isbister 2010a).

Why it is important to do this review

Even though snake antivenom is the mainstay of the treatment for snake envenoming, there is controversy regarding the effectiveness of antivenom for VICC (Isbister 2010a). It is unlikely that antivenom can be administered early enough to prevent VICC because the procoagulant toxins in snake venoms act rapidly (Isbister 2010a). The more important question is whether the administration of antivenom will speed the recovery of VICC by inactivating the active toxins to allow re-synthesis of clotting factors (Isbister 2010a). Recent observational clinical studies on Australian elapid envenomation, indicated that neither early (vs. late) antivenom nor higher doses of antivenom (> 1 vial) were associated with more rapid recovery in VICC (Isbister 2009b; Allen 2009). In contrast, in *Echis* envenomation in Africa, the use of antivenom does appear to speed the recovery of the coagulopathy (Mion 2013). We aim to examine the clinical trial evidence regarding effectiveness of snake antivenom for VICC from all snake species.

OBJECTIVES

To assess the effects of antivenom for the recovery from VICC in people with snake envenoming.

METHODS

Criteria for considering studies for this review

Types of studies

All randomised controlled trials in humans.

Types of participants

People of any age with snake envenoming who have already developed snake venom induced consumption coagulopathy. Diagnosis of venom induced consumption coagulopathy must be based on positive whole blood clotting test 20 (WBCT20) and/or elevated INR more than 2.

Types of interventions

Intravenous administration of snake antivenom regardless of the type of antivenom or the dose. People who were not treated with antivenom will be the comparision group.

Types of outcome measures

Primary outcomes

1. Mortality

Secondary outcomes

1. Major haemorrhages
2. Time to improve clotting studies (e.g. time to INR <2; time to improve WBCT 20)
3. Immediate systemic hypersensitivity reactions
4. Serum sickness

Calculation of information size requirements.

Snakebite mortality is very variable and has contributors other than VICC such as neurotoxicity, myotoxicty and acute renal injury. However, for simplicity we have taken the mortality rate from (Kasturiratne 2008) which estimates an overall case-fatality of around 5%. Using GPower, the estimated sample size required in order to show this rate could be halved would require 2504 people in total.

Search methods for identification of studies

In order to reduce publication and retrieval bias we will not restrict our search by language, date or publication status.

Electronic searches

The Cochrane Injuries Group's Trials Search Co-ordinator will search the following:

1. Cochrane Injuries Group Specialised Register (latest version);
2. The Cochrane Central Register of Controlled Trials (*The Cochrane Library*) (latest issue);
3. Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R) 1946 to present;
4. Embase Classic + Embase (OvidSP) 1947 to present;
5. ISI Web of Science: Science Citation Index Expanded (1970 to present);
6. ISI Web of Science: Conference Proceedings Citation Index-Science (1990 to present);
7. Current Awareness in Clinical Toxicology (latest update);
8. TOXLINE (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE) (latest version);
9. Clinicaltrials.gov (www.clinicaltrials.gov);
10. WHO International Clinical Trials Registry Platform (http://apps.who.int/trialsearch/);
11. Open Grey (http://www.opengrey.eu/)(latest version).

We will adapt the MEDLINE search strategy (Appendix 1) as necessary for each of the other databases: the added study filter is a modified version of the Ovid MEDLINE Cochrane Highly Sensitive Search Strategy for identifying randomised trials and to the Embase search strategy we will add the study design terms as used by the UK Cochrane Centre (Lefebvre 2011).

Searching other resources

We will search the reference lists of all relevant studies and contact experts in the field in order to identify ongoing and completed studies. We will also run a search on Google and Google Scholar restricting the search results from 1947 to present, and will review at least 500 results to find relevant studies.

Data collection and analysis

We will perform a systematic review following the instructions given in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011).

Selection of studies

Two review authors (KM and GI) will independently scan the titles and abstracts of all articles identified by the search strategy. If either or both authors identifies the article as possibly being a report that meets the inclusion criteria, we will obtain the full text of the published article. Both authors will review the full text of each article to determine if the article meets the inclusion criteria. We will resolve disagreement between two authors at this stage by consensus. We will provide details of included and excluded studies in the appropriate tables with the review.

Two authors will independently review each article that meets inclusion criteria, and extract data from the article onto a standard data extraction form. We will then compare these data forms. In the event of disagreement between the authors, we will seek the opinion of a third person (NB). For ambiguous studies and where there are insufficient data, we will attempt to contact the authors of the articles for further clarification and more information. We will grade the studies for quality, using the instructions given in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011).

Data extraction and management

Two authors will extract data on the following items onto a standard form.

- General information about the article (title of the article, source, publication year, years the study was conducted, language of publication, etc)
- Clinical trial characteristics: design, diagnostic ascertainment, standard care provided, randomisation, allocation concealment, interventions, drop-out and lost to follow up rates, definitions of outcomes, and methods of outcome assessment.
- Patients: inclusion and exclusion criteria, sample size, base line characteristics (e.g. age of the patients, past history of bleeding, anticoagulant therapy or coagulation disorders, clinical severity on enrolment etc.)
- Interventions: type of antivenom (polyvalent or monovalent), manufacturer, dose of antivenom (number of vials

or mg), duration of administration, timing of administration of antivenom after the bite.

- Outcomes: mortality, major haemorrhage (according to the definition by ISTH), time to improved clotting function defined as either the time to INR <2 or time until a negative WBCT 20, length of hospital stay, systemic hypersensitivity reactions.

Assessment of risk of bias in included studies

Two review authors (KM and GI) will independently assess the included studies for risk of bias in the following areas. We will assess risk of bias using the suggested domains and guidance provided in the Cochrane Collaboration's tool for assessing risk of bias as detailed in section 8.5 of the Cohcrane Handbook for Systematics review of Interventions (Higgins 2011). We will assess random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attribution bias), selective reporting (reporting bias), and other sources of bias (in particular, funding source). If there is insufficient information we will initially judge domains as "unclear risk" and will attempt to clarify the risk of bias by contacting the study authors. We plan to include all studies irrespective of the risk of bias; however, we plan to perform a sensitivity analysis. If the sensitivity analysis shows substantial differences, we will present alternative estimates that exclude studies with high or unclear risk of bias.

- **Sequence generation of the randomisation process**

Low risk: using random number tables, computer random number generation, coin tossing, stratified or block randomisation, shuffling cards or envelopes, throwing dice, drawing lots or other valid methods
High risk: "Quasi" randomisation, date of birth, day of visit, ID or record results, alternate allocation
Unclear risk: not described or not enough information to make a clear judgement

- **Allocation concealment**

Low risk: allocation concealment is described and would not allow either the investigator or participants to know or influence treatment group assignment at the time of study entry. Acceptable methods include central randomisation (phone, web, pharmacy) or sequentially numbered, opaque sealed envelopes
High risk: the method of allocation is not concealed (e.g. random sequence known to staff in advance, envelopes or packaging with out all safeguards or a non-randomised and predictable sequence
Unclear risk: trial either did not describe the method of allocation concealment or reported an approach that clearly was not adequate

- **Blinding of participants and personnel**

Low risk: blinding, and unlikely that the blinding could have been broken, or no blinding, or incomplete blinding but outcome unlikely to be influenced
High risk: no blinding, incomplete or broken blinding and outcome likely to be influenced
Unclear risk: not described or not enough information to make a clear judgment

- **Blinding of outcome assessment**

Low risk: Blinding of outcome assessors was clearly maintained, or no blinding but measurements unlikely to be influenced
High risk: no blinding, or broken blinding, and measurements likely to be influenced
Unclear risk: not described or not enough information to make a clear judgment

- **Intention-to-treat (ITT) analysis**

Low risk: Specifically reported that ITT analysis undertaken by the authors, or report that makes it unmistakable that ITT was undertaken for the primary analysis
High risk: No report that could be an ITT analysis is presented
Unclear risk: It is uncertain from the report whether the results presented are an ITT analysis

- **Incomplete outcome data**

Low risk: No missing data, reasons for missing data not related to outcomes, missing data balanced across groups and proportion missing or plausible effect size not enough to have a clinically relevant effects
High risk: reasons related to outcome and imbalance in number or reasons, proportions missing or plausible effect size enough to have clinically relevant effect, "as treated" analysis with substantial departure from allocation, inappropriate use of imputation
Unclear risk: not described or not enough information to make a clear judgment

- **Selectiveness of outcome reporting**

Low risk: method is available and all pre-specified outcomes of interest are reported in the pre-specified way, protocol not available but it is clear that all pre-specified and expected outcomes of interest are reported
High risk: outcomes not reported as pre-specified or expected eg, missing, added, subset, unexpected measurement or methods. Outcomes reported incomplete and cannot enter a meta-analysis
Unclear risk: not described or not enough information to make a clear judgment

- **Reporting bias**

Methods for dealing with reporting bias in reviews of diagnostic accuracy studies are relatively underdeveloped. Consequently, we will interpret our results cautiously and with an awareness of the likelihood of reporting bias. We will consider using funnel plots.

- **Other sources of bias**

Low risk: Studies appears to be free of other sources of bias such as imprecision (eg small sample size), diversity (eg, inadequate dose, unusual population)

High risk: baseline imbalance, blocked randomisation in unblinded trials, non-randomised studies, recruitment bias in cluster-randomised trials
Unclear risk: not described or not enough information to make a clear judgment
Based on the above elements, we will classify each study as having a "high", "low" or "unclear" risk of bias.

Measures of treatment effect

We will define measures of treatment effects as follows;
Dichotomous data
We will present dichotomous data outcomes as risk ratios (RR) with 95% confidence intervals (CI) for individual trials.
Continuous data
We will present continuous data outcomes with mean difference (MD) and 95% CI. We will calculate mean difference if possible as these results are easier for clinicians and readers to interpret; we will use standardised mean difference when different scales are used in the trials.
Ordinal data
We will report the types of adverse events and complications.

Unit of analysis issues

Individual participants are the unit of analysis. To answer our primary question (does antivenom improve VICC compared to no antivenom treatment) we will in the first instance simply combine all active intervention groups of the study into a single group and compare their outcomes to the control groups(s) not receiving antivenom. We may also explore comparison of doses or types of antivenom (post-hoc).

Dealing with missing data

We will contact the authors of the original studies if essential data are missing from their trial reports. If we receive no reply after eight weeks, we will extract the available data from the published reports. We will assess the missing data and attrition rates for each of the included studies and report the number of participants who are included in the final analysis as a proportion of all participants in the study.

Assessment of heterogeneity

We will evaluate statistical heterogeneity using the Chi^2 test to assess for heterogeneity between trials, and the I^2 statistics for quantifying heterogeneity across studies (roughly interpreted as follows: 0 to 30%: probably not important; 31 to 60%: may represent moderate heterogeneity; 61 to 75%: may represent substantial heterogeneity; 76 to 100%: very considerable heterogeneity) as outlined in (Deeks 2011). We expect considerable heterogeneity due to considerable variation across trials in setting, snake, intervention and outcomes. We intend to use a random effects model to account for this heterogeneity in any summary estimates of effect. We may also (post hoc) look for plausible explanations of heterogeneity. We will discuss the implications of heterogeneity and how they relate to external validity in the discussion.

Assessment of reporting biases

Systematic difference between reported and unreported findings are referred to as reporting bias. We will include selective outcome reporting assessment as part of the risk of bias table and also under ITT analysis.
We will assess publication biases by using funnel plots when there are at least 10 studies included in the meta-analysis.

Data synthesis

We will analyse the data using Review Manager software (Review Manager). We will express results for dichotomous outcomes as risk ratios (RR) with 95% confidence intervals (CI) and continuous outcomes as mean differences (MD). We will present data in a GRADE Summary of Finding table according to the methods as described in the *the Cochrane Handbook* (Schünemann 2011). The summary of findings table will include mortality, major haemorrhages, time to improved clotting (eg, time to INR <2 or time to normalised WBCT20), immediate systemic hypersensitivity reactions and serum sickness as outcomes.
Dichotomous outcomes such as mortality, number of haemorrhages, number of immediate type hypersensitivity reactions, number of cases of serum sickness will be presented as RR with 95% confidence intervals for individual trials. For dichotomous data meta-analysis we will use a Mantel-Haenszel random-effect model. For continuous outcomes (time to improve clotting studies) that have been recorded as a mean difference or standardised mean difference and SD or 95% confidence interval, we will use an inverse variance, random-effects model for the analysis. If we find two or more studies assessing the same outcomes we will perform meta-analysis. If meta-analysis is not possible we will write a narrative summary of the study findings and follow alternative methods as described in *the Cochrane Handbook* (Deeks 2011).

Subgroup analysis and investigation of heterogeneity

Where possible (if sufficient data and information are available) we will perform subgroup analysis based on the following factors, which are thought to affect outcomes after VICC.
1. Type of snake envenoming (Elapids and Viperids)
2. Type of snake antivenoms
3. Dose of antivenom

Sensitivity analysis

We will restrict sensitivity analyses to include studies with both adequate allocation concealment and blinded outcome assessment.

ACKNOWLEDGEMENTS

We would like to acknowledge the Cochrane Injuries Group editors for their advice and support.

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* *Indicates the major publication for the study*

APPENDICES

Appendix 1. Search strategy

Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R)

1. exp snakes/ or exp boidae/ or exp colubridae/ or exp elapidae/ or exp viperidae/
2. (snake* or viper*).ab,ti.
3. exp snake venoms/ or exp elapid venoms/ or exp viper venoms/
4. snake venoms.mp.
5. ((venom* or bite*) adj3 snake*).ab,ti.
6. (envenomation or venom-induced or antivenom* or antivenin*).ab,ti.
7. (snake adj3 poisonous).ab,ti.
8. exp Antivenins/
9. exp Viper Venoms/
10. exp Viperidae/
11. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9 or 10
12. randomi?ed.ab,ti.
13. randomized controlled trial.pt.
14. controlled clinical trial.pt.
15. placebo.ab.
16. clinical trials as topic.sh.
17. randomly.ab.
18. trial.ti.
19. Comparative Study/
20. 12 or 13 or 14 or 15 or 16 or 17 or 18 or 19
21. (animals not (humans and animals)).sh.

22. 20 not 21
23. 11 and 22

Google and Google Scholar

snake envenoming OR snake envenomation OR coagulopathy OR venom induced OR coagulation abnormalities OR snake antivenom OR snake antivenin AND (randomized controlled trial or randomised controlled trial).

CONTRIBUTIONS OF AUTHORS

All authors contributed to this protocol.

DECLARATIONS OF INTEREST

None known.

SOURCES OF SUPPORT

Internal sources

- Library services, University of Newcastle, NSW, Australia.

Support to find the references for the review.

External sources

- No sources of support supplied

Snake antivenom for snake venom induced consumption coagulopathy (Review)

Maduwage K, Buckley NA, de Silva HJ, Lalloo DG, Isbister GK

This is a reprint of a Cochrane review, prepared and maintained by The Cochrane Collaboration and published in *The Cochrane Library* 2015, Issue 6

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[Intervention Review]

Snake antivenom for snake venom induced consumption coagulopathy

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Editorial group: Cochrane Injuries Group.
Publication status and date: New, published in Issue 6, 2015.
Review content assessed as up-to-date: 30 January 2015.

Citation: Maduwage K, Buckley NA, de Silva HJ, Lalloo DG, Isbister GK. Snake antivenom for snake venom induced consumption coagulopathy. *Cochrane Database of Systematic Reviews* 2015, Issue 6. Art. No.: CD011428. DOI: 10.1002/14651858.CD011428.pub2.

ABSTRACT

Background

Snake venom induced consumption coagulopathy is a major systemic effect of envenoming. Observational studies suggest that antivenom improves outcomes for venom induced consumption coagulopathy in some snakebites and not others. However, the effectiveness of snake antivenom in all cases of venom induced consumption coagulopathy is controversial.

Objectives

To assess the effect of snake antivenom as a treatment for venom induced consumption coagulopathy in people with snake bite.

Search methods

The search was done on 30 January 2015. We searched the Cochrane Injuries Group's Specialised Register, the Cochrane Central Register of Controlled Trials (CENTRAL, *The Cochrane Library*), Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R), Embase Classic+Embase (OvidSP), three other sources, clinical trials registers, and we also screened reference lists.

Selection criteria

All completed, published or unpublished, randomised, controlled trials with a placebo or no treatment arm, where snake antivenom was administered for venom induced consumption coagulopathy in humans with snake bites.

Data collection and analysis

Two authors reviewed the identified trials and independently applied the selection criteria.

Main results

No studies met the inclusion criteria for this review.

Authors' conclusions

Randomised placebo-controlled trials are required to investigate the effectiveness of snake antivenom for clinically relevant outcomes in patients with venom induced consumption coagulopathy resulting from snake bite. Although ethically difficult, the routine administration of a treatment that has a significant risk of anaphylaxis cannot continue without strong evidence of benefit.

PLAIN LANGUAGE SUMMARY

Snake antivenoms for treating people who have been bitten by a snake, and have developed abnormal blood clotting

Many snake venoms cause coagulopathy in humans. Coagulopathy is a condition in which the person's blood is unable to clot because the venom causes decreased levels of clotting factors. Coagulopathy increases the risk of bleeding. Antivenom is a treatment used to neutralise venom in people who have been bitten by a snake. There is some evidence from observational studies in humans which suggest that snake antivenom is helpful to people who have been bitten by a snake. However, the use of antivenom has some risks, and can cause allergic reactions.

Antivenom is made by injecting venom into either horses, sheep or goats, and then collecting the animal blood and separating out the specific antibodies to the snake venom. The antivenom is put into a person's vein, so that it can mix with the blood in their body.

The authors of this Cochrane review investigated whether there was evidence that antivenom helped people who had been bitten by a snake and had developed coagulopathy. The authors looked for studies where antivenom was used as a treatment for people who developed coagulopathy after a snake bite, regardless of the type of snake.

The type of study eligible for inclusion in the review was the randomised controlled trial, and the control group needed to receive either a placebo or no antivenom. The review authors did not find any trials meeting this criteria, despite searching all the major international medical reference databases. The databases were searched on 30 January 2015.

Since no relevant randomised controlled trials were identifed, this systematic review provides no evidence to help doctors decide if and when to use antivenom for snakebite coagulopathy. The authors say that trials of antivenom are urgently needed so that doctors and patients can fully understand the benefits and risks of antivenom. At the moment doctors make decisions about when to use antivenom based on the results of observational studies, which may not fully describe the effects of antivenom.

BACKGROUND

Description of the condition

Snake envenoming is a major medical problem in tropical areas. The estimated burden of snake bite is approximately 421,000 cases of envenoming with 20,000 fatalities annually, although there may be as many as 1,841,000 envenomings and 94,000 deaths (Kasturiratne 2008).

Venom induced consumption coagulopathy is one of the major clinical manifestations of snake envenoming and may be complicated by fatal haemorrhage (Isbister 2010a). Venom induced consumption coagulopathy has previously been referred to by a number of different terms, including disseminated intravascular coagulation, defibrination syndrome and procoagulant coagulopathy (Isbister 2010b). Venom induced consumption coagulopathy results from the action of snake procoagulant toxins on human coagulation factors causing consumption of these clotting factors leading to multiple factor deficiencies (Isbister 2009a). There are many examples of procoagulant snake toxins that cause venom induced consumption coagulopathy, including prothrombin activators in *Echis carinatus*, *Pseudonaja textilis, Notechis scutatus* venoms (Rosing 1992; Joseph 2001; Rosing 2001), factor X activators in *Dabois russelii, Bothrops atrox, Cerastes cerastes, Bungarus, Ophiophagus* venom (Tans 2001), factor V activators in *Bothrops atrox, Naja naja oxiana* venom (Rosing 2001), thrombin-like enzymes in *Agkistrodon contortrix contortrix* venom (Swenson 2005), and plasminogen activators in *Trimeresurus stejnegeri* venom (Sanchez 2006). Venom induced consumption coagulopathy can result in bleeding if there is trauma, or spontaneous haemorrhage in cases where the venom also contains a haemorrhagin (e.g. *E. carinatus*).

Major haemorrhage in vital organs, such as intracranial haemorrhage, is the most serious issue and is often fatal.
A number of laboratory clotting times and clotting factor studies are used to diagnose and monitor venom induced consumption coagulopathy, including the prothrombin time/international normalised ratio, the activated partial thromboplastin time, and the 20-minute whole blood clotting test. These play a major role in diagnosis, assessment and treatment of venom induced consumption coagulopathy (Isbister 2010a).

Description of the intervention

Antivenom is the primary treatment for snake envenoming (Lalloo 2003; Isbister 2010c). Antivenoms contain polyclonal antibodies raised against one or more snake venoms. They may contain whole immunoglobulins, but more commonly, pepsin or papain digested fragments of immunoglobulins such as F(ab')2 or Fab. They are made by injecting venom into either horses, sheep or goats, and then collecting blood and separating out the specific antibodies to the snake venom. Intravenously administered antivenom in patients with snake envenoming binds to circulating snake toxins which aims to neutralise or eliminate the toxins and thereby prevent or reverse the clinical effects of envenoming. Monovalent antivenoms are raised against a single snake species, while polyvalent antivenoms are raised against more than one species.
Immediate hypersensitivity reactions to the foreign proteins (immunoglobulins) in snake antivenoms are the major adverse effect of antivenom treatment, including life threatening anaphylaxis (Nuchprayoon 1999; Lalloo 2003; Gawarammana 2004; de Silva 2011; Isbister 2012). Manufacturing protocols and methods of snake antivenoms are different in various regions in the world and the standardisation of snake antivenom production remains problematic.

How the intervention might work

Antibodies in the antivenom bind to the toxic components in snake venom. Early administration of antivenom will bind the circulating procoagulant snake toxins and potentially prevent, delay or lessen the severity of venom induced consumption coagulopathy. In the majority of patients who have already developed venom induced consumption coagulopathy, antivenom is used to neutralise circulating toxins and allow recovery of venom induced consumption coagulopathy. However, the effectiveness and mechanism of action of antivenom in already developed venom induced consumption coagulopathy remains unclear (Isbister 2009b). Recovery of the coagulopathy depends on re-synthesising clotting factors which is not directly affected by antivenom administration (Isbister 2010a).

Why it is important to do this review

Even though snake antivenom is the mainstay of the treatment for snake envenoming, there is controversy regarding the effectiveness of antivenom for venom induced consumption coagulopathy (Isbister 2010a). It is unlikely that antivenom can be administered early enough to prevent venom induced consumption coagulopathy because the procoagulant toxins in snake venoms act rapidly (Isbister 2010a). The more important question is whether the administration of antivenom will speed the recovery of venom induced consumption coagulopathy by inactivating the active toxins to allow re-synthesis of clotting factors (Isbister 2010a). Thus only if further factor consumption is occurring due to significant amounts of circulating pro-coagulant venoms, would antivenom be expected to speed recovery.
Recent observational clinical studies on Australian elapid envenoming indicated that neither early (versus late) antivenom nor higher doses of antivenom (> one vial) were associated with more rapid recovery in venom induced consumption coagulopathy (Allen 2009; Isbister 2009b). In contrast, in *Echis* envenoming in Africa, the use of antivenom does appear to speed the recovery of the coagulopathy (Mion 2013). We aim to examine the clinical trial evidence regarding effectiveness of snake antivenom for venom induced consumption coagulopathy from all snake species.

OBJECTIVES

To assess the effects of antivenom for the recovery from venom induced consumption coagulopathy in people with snake envenoming.

METHODS

Criteria for considering studies for this review

Types of studies

All randomised controlled trials (RCTs) in humans.

Types of participants

People of any age with snake envenoming who have already developed snake venom induced consumption coagulopathy. Diagnosis of venom induced consumption coagulopathy must be based on abnormal results from the 20-minute whole blood clotting test or an elevated international normalised ratio of >2.

Types of interventions

Intravenous administration of snake antivenom regardless of the type of antivenom or the dose. People who were not treated with antivenom were the comparison group.

Types of outcome measures

Primary outcomes

- Mortality

Secondary outcomes

- Major haemorrhages
- Time to improve clotting studies (e.g. time to international normalised ratio <2; time to improve 20-minute whole blood clotting test)
- Immediate systemic hypersensitivity reactions
- Serum sickness

Calculation of information size requirements

Snakebite mortality is very variable and has contributors other than venom induced consumption coagulopathy such as neurotoxicity, myotoxicity and acute renal injury. However, for simplicity we have taken the mortality rate from Kasturiratne 2008, which estimates an overall case-fatality of around 5%. Using G*Power (http://www.gpower.hhu.de/en.html), the estimated sample size required in order to show this rate could be halved would require 2504 people in total.

Search methods for identification of studies

In order to reduce publication and retrieval bias we did not restrict our search by language, date or publication status.

Electronic searches

The Cochrane Injuries Group's Trials Search Co-ordinator searched the following:

1. Cochrane Injuries Group Specialised Register (30/01/2015);
2. The Cochrane Central Register of Controlled Trials (*The Cochrane Library*) (issue 1 of 12, 2015);
3. Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R) 1946 to 30/01/2015;
4. Embase Classic + Embase (OvidSP) 1947 to 30/01/2015;
5. ISI Web of Science: Science Citation Index Expanded (1970 to 30/01/2015);
6. ISI Web of Science: Conference Proceedings Citation Index-Science (1990 to 30/01/2015);
7. Toxicology Literature Online (TOXLINE) (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?TOXLINE) (30/01/2015);
8. ClinicalTrials.gov (https://clinicaltrials.gov/) (30/01/2015);
9. WHO International Clinical Trials Registry Platform (ICTRP) (http://apps.who.int/trialsearch/) (30/01/2015);
10. OpenGrey (http://www.opengrey.eu/) (30/01/2015).

We adapted the MEDLINE search strategy illustrated in Appendix 1 as necessary for each of the other databases. We also added search filters, and a modified version of the 'Cochrane Highly Sensitive Search Strategy, for identifying randomised trials in MEDLINE and Embase' (Lefebvre 2011).

Searching other resources

We searched the reference lists of all relevant studies and contacted experts in the field in order to identify ongoing and completed studies. We also ran a search on Google and Google Scholar restricting the search results from 1947 to present, and reviewed over 500 results to find relevant studies (Appendix 1).

Data collection and analysis

We performed this systematic review according to the instructions given in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011), and our protocol (Maduwage 2014).

Selection of studies

Two authors (KM and GI) independently screened the titles and abstracts of all articles identified by the search strategy. When either or both authors identified the article as possibly being a report that meets the inclusion criteria, we obtained the full text version of the published article. Both authors reviewed the full text of each article to determine if the article meets the inclusion criteria. There were no disagreements between the two authors about the inclusion of studies. We provided details of the included and excluded studies in the appropriate tables within the review.

The two authors independently reviewed each article that met the inclusion criteria, and extracted data from the article onto a standard data extraction form. We then compared these data forms, which were consistent with each other.

Data extraction and management

Two authors (KM and GI) extracted data on the following items onto a standard form.

- General information about the article (title of the article, source, publication year, years the study was conducted, language of publication, etc.).

- Clinical trial characteristics: design, diagnostic ascertainment, standard care provided, randomisation, allocation concealment, interventions, drop-out and lost to follow up rates, definitions of outcomes, and methods of outcome assessment.
- Patients: inclusion and exclusion criteria, sample size, baseline characteristics (e.g. age of the patients, past history of bleeding, anticoagulant therapy or coagulation disorders, clinical severity on enrolment, etc.).
- Interventions: type of antivenom (polyvalent or monovalent), manufacturer, dose of antivenom (number of vials or mg), duration of administration, timing of administration of antivenom after the bite.
- Outcomes: mortality, major haemorrhage (according to the definition by the International Society on Thrombosis and Haemostasis), time to improved clotting function defined as either the time to international normalised ratio <2 or time until a negative result of the 20-minute whole blood clotting test, length of hospital stay, systemic hypersensitivity reactions.

Assessment of risk of bias in included studies

In the future if studies are included in this review, two authors (KM and GI) will independently assess the included studies for risk of bias in the following areas. We will assess risk of bias using the suggested domains and guidance provided in the Cochrane Collaboration's tool for assessing risk of bias (Higgins 2011). We will assess random sequence generation (selection bias), allocation concealment (selection bias), blinding of participants and personnel (performance bias), blinding of outcome assessment (detection bias), incomplete outcome data (attribution bias), selective reporting (reporting bias), and other sources of bias (in particular, funding source). If there is insufficient information we will initially judge domains as "unclear risk" and will attempt to clarify the risk of bias by contacting the study authors. We plan to include all studies irrespective of the risk of bias; however, we plan to perform a sensitivity analysis. If the sensitivity analysis shows substantial differences, we will present alternative estimates that exclude studies with high or unclear risk of bias.

Sequence generation of the randomisation process

- Low risk: using random number tables, computer random number generation, coin tossing, stratified or block randomisation, shuffling cards or envelopes, throwing dice, drawing lots or other valid methods
- High risk: "quasi" randomisation, date of birth, day of visit, identification number or record results, alternate allocation
- Unclear risk: not described or not enough information to make a clear judgment

Allocation concealment

- Low risk: allocation concealment is described and would not allow either the investigator or participants to know or influence treatment group assignment at the time of study entry
    - Acceptable methods include central randomisation (phone, web, pharmacy) or sequentially numbered, opaque sealed envelopes
- High risk: the method of allocation is not concealed (e.g. random sequence known to staff in advance, envelopes or packaging without all safeguards or a non-randomised and predictable sequence)
- Unclear risk: trial either did not describe the method of allocation concealment or reported an approach that clearly was not adequate

Blinding of participants and personnel

- Low risk: blinding, and unlikely that the blinding could have been broken, or no blinding, or incomplete blinding but outcome unlikely to be influenced
- High risk: no blinding, incomplete or broken blinding and outcome likely to be influenced
- Unclear risk: not described or not enough information to make a clear judgment

Blinding of outcome assessment

- Low risk: blinding of outcome assessors was clearly maintained, or no blinding but measurements unlikely to be influenced
- High risk: no blinding, or broken blinding, and measurements likely to be influenced
- Unclear risk: not described or not enough information to make a clear judgment

Intention-to-treat analysis

- Low risk: specifically reported that intention-to-treat analysis was undertaken by the authors, or report that makes it unmistakable that intention-to-treat was undertaken for the primary analysis
- High risk: no report of an intention-to-treat analysis being conducted
- Unclear risk: not described or not enough information to make a clear judgment

Incomplete outcome data

- Low risk: no missing data, reasons for missing data not related to outcomes, missing data balanced across groups and proportion missing or plausible effect size not enough to have clinically relevant effects
- High risk: reasons related to outcome and imbalance in number or reasons, proportions missing or plausible effect size

enough to have clinically relevant effect, "as treated" analysis with substantial departure from allocation, inappropriate use of imputation

- Unclear risk: not described or not enough information to make a clear judgment

Selectiveness of outcome reporting

- Low risk: method is available and all pre-specified outcomes of interest are reported in the pre-specified way, protocol not available but it is clear that all pre-specified and expected outcomes of interest are reported
- High risk: outcomes not reported as pre-specified or expected e.g. missing, added, subset, unexpected measurement or methods. Outcomes reported are incomplete and cannot enter a meta-analysis
- Unclear risk: not described or not enough information to make a clear judgment

Reporting bias

We will interpret our results cautiously and with an awareness of the likelihood of reporting bias. We will consider using funnel plots.

Other sources of bias

- Low risk: studies appear to be free of other sources of bias such as imprecision (e.g. small sample size), diversity (e.g. inadequate dose, unusual population)
- High risk: baseline imbalance, non-randomised studies, recruitment bias in cluster-randomised trials, inadequate power and/or implausible sample size calculation, early stopping of trial (based on interim analysis of efficacy)
- Unclear risk: not described or not enough information to make a clear judgment

Measures of treatment effect

Dichotomous data

We planned to present dichotomous data outcomes as risk ratios (RRs) with 95% confidence intervals (CIs) for individual trials.

Continuous data

We planned to present continuous data outcomes with mean differences (MDs) and 95% CIs. We planned to calculate the mean difference if possible as these results are easier for clinicians and readers to interpret; and use standardised mean differences (SMDs) when different scales are used in the trials.

Ordinal data

We planned to report the types of adverse events and complications.

Unit of analysis issues

Individual participants are the unit of analysis. To answer our primary question (does antivenom improve venom induced consumption coagulopathy compared to no antivenom treatment) we planned to initially simply combine all active intervention groups of the study into a single group and compare their outcomes to the control groups(s) not receiving antivenom. We may also explore comparison of doses or types of antivenom (post-hoc).

Dealing with missing data

In the future if studies are included in this review, we will contact the authors of the original studies if essential data are missing from their trial reports. If we receive no reply after eight weeks, we will extract the available data from the published reports. We will assess the missing data and attrition rates for each of the included studies and report the number of participants who are included in the final analysis as a proportion of all participants in the study.

Assessment of heterogeneity

In the future if studies are included in this review, we will evaluate statistical heterogeneity using the Chi^2 test to assess for heterogeneity between trials, and the I^2 statistic for quantifying heterogeneity across studies (roughly interpreted as follows: 0 to 30%: probably not important; 31 to 60%: may represent moderate heterogeneity; 61 to 75%: may represent substantial heterogeneity; 76 to 100%: very considerable heterogeneity) as outlined in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011). We expect considerable heterogeneity due to considerable variation across trials in setting, snake, intervention and outcomes. We intend to use a random-effects model to account for this heterogeneity in any summary estimates of effect. We may also (post-hoc) look for plausible explanations of heterogeneity. We will discuss the implications of heterogeneity and how they relate to external validity in the discussion.

Assessment of reporting biases

Systematic difference between reported and unreported findings are referred to as reporting bias. We will include selective outcome reporting assessment as part of the 'Risk of bias table' and also under 'Intention-to-treat analysis'.
We will assess publication biases by using funnel plots when there are at least 10 studies included in the meta-analysis.

Data synthesis

In the future if studies are included in this review, we will analyse the data using the Cochrane Collaboration statistical software Review Manager. We will express results for dichotomous outcomes as RRs with 95% CIs and continuous outcomes as MDs. We will present data in a 'Summary of findings' table according to Grading of Recommendations Assessment, Development and Evaluation (GRADE) guidelines as well as the method described in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011). The table will include mortality, major haemorrhages, time to improved clotting (e.g. time to international normalised ratio <2 or time to normalised result of the 20-min whole blood clotting test), immediate systemic hypersensitivity reactions and serum sickness as outcomes.

We planned to present dichotomous outcomes such as mortality, number of haemorrhages, number of immediate type hypersensitivity reactions, and number of cases of serum sickness as RRs with 95% CIs for individual trials. For dichotomous data meta-analysis we planned to use a Mantel-Haenszel random-effects model. For continuous outcomes (e.g. time to improve clotting studies) that have been recorded as MDs, SMDs or standard deviations (SDs) with 95% CIs, we planned to use an inverse variance random-effects model. If we were to find two or more studies assessing the same outcomes we will perform meta-analysis. If meta-analysis is not possible we will write a narrative summary of the study findings and follow alternative methods as described in the *Cochrane Handbook for Systematic Reviews of Interventions* (Higgins 2011).

Subgroup analysis and investigation of heterogeneity

Where possible (if sufficient data and information are available) we will perform subgroup analysis based on the following factors, which are thought to affect outcomes after venom induced consumption coagulopathy:

1. type of snake envenoming (elapids and viperids);
2. type of snake antivenoms;
3. dose of antivenom.

Sensitivity analysis

In the future if studies are included in this review, we will restrict sensitivity analyses to include studies with both adequate allocation concealment and blinded outcome assessment.

RESULTS

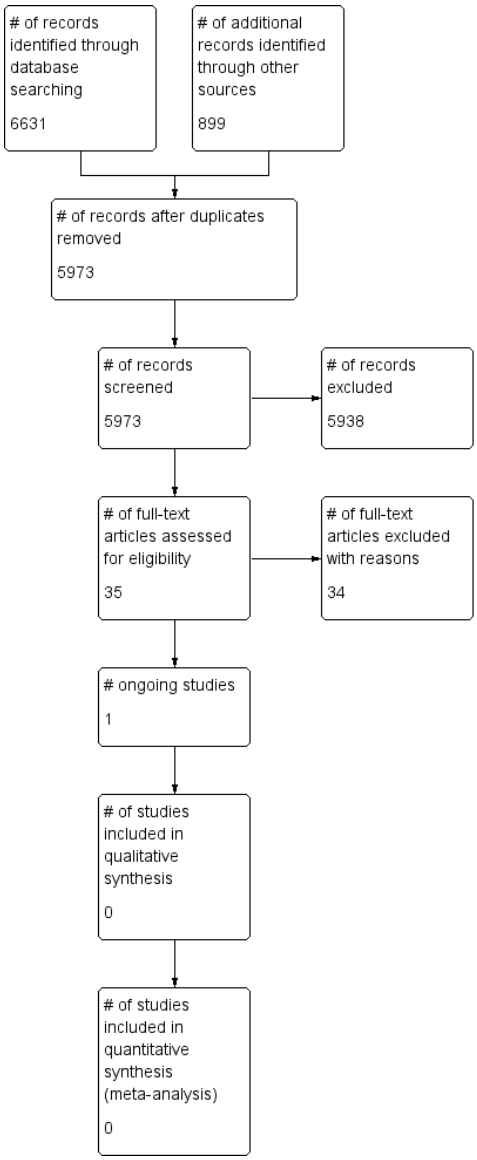
Description of studies

We did not find any studies for inclusion in this review. There is one ongoing trial (NCT01864200).

Results of the search

The search retrieved 7530 records and after duplicates were removed we screened 5973 records (Figure 1). The search identified one ongoing study, and the results will be included in the review when they become available.

Figure 1. Study flow diagram.



Included studies

There are no studies included in this review.

Excluded studies

We excluded 34 of 35 studies after reviewing the full text report. See Characteristics of excluded studies. One ongoing study was identified.

Risk of bias in included studies

There are no studies included in this review.

Effects of interventions

There are no studies included in this review.

DISCUSSION

Summary of main results

We were unable to identify any placebo randomised controlled trials of snake antivenom for venom induced consumption coagulopathy meeting the inclusion criteria. We identified one ongoing trial. There were 32 published and two ongoing studies comparing two or more different antivenoms or comparing different doses of antivenoms for venom induced consumption coagulopathy. Few non-randomised trials including comparison groups without antivenom showed that antivenom was effective for envenoming by some snakes (e.g. *Echis* species in Africa), but not others (e.g. Australasian elapids) (Isbister 2010a; Mion 2013).

Overall completeness and applicability of evidence

There is a lack of evidence to support or refute a benefit of antivenom for venom induced consumption coagulopathy.

Quality of the evidence

There was no evidence to assess the quality of the evidence.

Potential biases in the review process

There are no studies included in this review.

Agreements and disagreements with other studies or reviews

The results of this review agree with the conclusion of a systematic review article that also examined RCTs comparing the effects of different antivenoms (n=14) and different doses of antivenom (n=5) (Maduwage 2014a). If antivenom was always highly effective in shortening the duration of VICC it might be expected that differences would also be seen commonly in such trials. In nine of 14 studies, the authors concluded equal effectiveness (or ineffectiveness) of two or three antivenoms, and four of five studies investigating different doses or dosing regimens concluded equal effectiveness. Even the six RCTs that concluded a difference between doses or antivenoms, lacked a strong statistical basis for this conclusion (i.e. the difference was not based on intention to treat analysis; three of these trials were very small and lacked the statistical power to support the significance of the differences observed (Warrell 1974; Warrell 1980; Dart 2001), and the others did a post-hoc analysis (a selected time point, sub-group, outcome, or statistical technique that had not been pre-specified) (Ariaratnam 2001; Smalligan 2007; Abubakar 2010).

AUTHORS' CONCLUSIONS

Implications for practice

There are no completed placebo randomised controlled trials of antivenom for venom induced consumption coagulopathy and therefore nothing from this systematic review provides evidence to help clinicians in deciding to use antivenom for venom induced consumption coagulopathy. The effectiveness of administration of antivenom for venom induced consumption coagulopathy will continue to be based on observational studies until placebo randomised controlled trials are undertaken.

Implications for research

Significant mortality and morbidity is associated with snake envenoming (Kasturiratne 2008) so effective treatments are desperately required. Antivenom was introduced for the treatment of snake envenoming over a century ago and its clinical use has been based on in vitro and in vivo animal studies of efficacy, small observational studies and clinical experience. As confirmed in this review there has never been a placebo randomised controlled trial

to demonstrate clinical effectiveness for venom induced consumption coagulopathy. This raises some difficult clinical questions regarding the use of a treatment known to have significant adverse effects (e.g. severe anaphylaxis; Nuchprayoon 1999) where there is no good evidence demonstrating benefit.

Undertaking placebo randomised controlled trials of snake antivenom is a challenge to clinical research and regarded as potentially highly unethical by many clinicians and experts (Gerardo 2014). Such a suggestion would be regarded by some as similar to doing a placebo controlled trial of insulin for diabetes mellitus, such is the overwhelming belief in the benefit of antivenom therapy. Therein lies the inescapable ethical dilemma. How do we undertake the appropriate placebo controlled trial of antivenom to demonstrate clinical effectiveness, if it is regarded as unethical to not give antivenom to some patients.

Well designed observational studies have demonstrated that for some snakes there appears to be a clear benefit of antivenom, speeding the recovery of coagulopathy in *Echis ocellatus* envenoming (Mion 2013), but for others there is little or no benefit, such as Australasian elapids (Isbister 2009b). There is substantial *in vitro* and *in vivo* evidence that antivenom binds toxins and that antivenom can neutralise the procoagulant and anticoagulant effects of venoms (Isbister 2009a). However, it is essential to translate pre-clinical efficacy studies into clinical effectiveness studies (Isbister 2010c), and understand that antivenom may be beneficial for some snake and some clinical syndromes, but not others.

There are a number of precedents where placebo randomised controlled trials have been commenced or completed for different antivenoms. The ongoing study identified in this review is a good example of such a trial. Details of the ethical considerations for this trial have been published (Gerardo 2014). A recently published placebo randomised controlled trial of antivenom for redback spider bite showed no benefit despite decades of belief that it was effective (Isbister 2014). Again, this study required sufficient evidence to justify ethically undertaking a study with a placebo. In envenoming that causes coagulopathy, the safety of this approach could be ensured by first performing observational studies that demonstrate the time to recovery of clotting factors is not strongly influenced by time to antivenom or dose for a particular snake.

The way forward for developing evidence for antivenom treatment in venom induced consumption coagulopathy will be to use novel study designs to introduce placebo arms. For example, undertaking a placebo controlled trial of early antivenom, where all patients will get antivenom at some stage. Such studies will be challenging but are essential to providing sufficient evidence of benefit for a treatment with severe adverse reactions.

A parallel way forward for developing evidence for antivenom treatment in venom induced consumption coagulopathy might be to examine the use of early (on arrival) versus delayed (after blood test results are returned) antivenom. Such studies will be challenging, not least because of the considerable heterogeneity of clinical features of envenoming by a particular species, but are essential to providing evidence that there is a benefit for this treatment that outweighs the considerable risk of severe adverse reactions.

ACKNOWLEDGEMENTS

We would like to acknowledge the Cochrane Injuries Group editors for their advice and support.

This project was supported by the UK National Institute for Health Research, through Cochrane Infrastructure funding to the Cochrane Injuries Group. The views and opinions expressed are those of the authors and do not necessarily reflect those of the Systematic Reviews Programme, NIHR, NHS or the Department of Health.

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* *Indicates the major publication for the study*

CHARACTERISTICS OF STUDIES

Characteristics of excluded studies *[ordered by study ID]*

Study	Reason for exclusion
Abubakar 2010	No placebo control group. Compared two different antivenoms.
Ariaratnam 2001	No placebo control group. Compared two different antivenoms.
Boyer 2013	No placebo control group. Compared two different antivenoms.
Bush 2014	No placebo control group. Compared two different antivenoms.
Cardoso 1993	No placebo control group. Compared three different antivenoms
Cherian 1998	No placebo control group. Compared two different doses of antivenom
Dart 2001	No placebo control group. Compared two different doses of antivenom
Isbister 2013	No placebo control group. Compared antivenom versus antivenom with fresh frozen plasma
Jorge 1995	No placebo control group. Compared two different doses of antivenom
Karnchanachetanee 1994	No placebo control group. Compared two different doses of antivenom
Kothari 2001	No placebo control group. Compared two different doses of antivenom
Meyer 1997	No placebo control group. Compared two different antivenoms.
Myint-Lwin 1989	No placebo control group. Compared antivenom versus antivenom with heparin
NCT00639951	No placebo control group. Compared two different doses of antivenom. Ongoing study
NCT00868309	No placebo control group. Compared two different antivenoms. Ongoing study
Otero 1996	No placebo control group. Compared two different antivenoms.
Otero 1999	No placebo control group. Compared two different antivenoms.
Otero 2006	No placebo control group. Compared two different antivenoms.
Otero-Patino 1998	No placebo control group. Compared three different antivenoms
Otero-Patino 2012	No placebo control group. Compared two different antivenoms.
Pardal 2004	No placebo control group. Compared two different antivenoms.

(*Continued*)

Paul 2003	No placebo control group. Compared antivenom versus antivenom with heparin
Paul 2004	No placebo control group. Compared two different doses of antivenom
Paul 2007	No placebo control group. Compared antivenom versus antivenom with heparin
Sellahewa 1994	No placebo control group. Compared antivenom versus antivenom with intravenous immunoglobulin
Shah 1986	No placebo control group. Compared antivenom versus antivenom with heparin
Smalligan 2004	No placebo control group. Compared three different antivenoms
Srimannarayana 2004	No placebo control group. Compared three different doses of antivenom
Tariang 1999	No placebo control group. Compared two different doses of antivenom
Thomas 1985	No placebo control group. Compared two different doses of antivenom
Warrell 1974	No placebo control group. Compared two different antivenoms.
Warrell 1976	No placebo control group. Compared antivenom versus antivenom with heparin
Warrell 1980	No placebo control group. Compared two different antivenoms.
Warrell 1986	No placebo control group. Compared two different doses of antivenom

Characteristics of ongoing studies *[ordered by study ID]*

NCT01864200

Trial name or title	A Randomized, Double-Blind, Placebo-Controlled Study Comparing CroFab® Versus Placebo With Rescue Treatment for Copperhead Snake Envenomation (Copperhead RCT)
Methods	A Randomized, Double-Blind, Placebo-Controlled Study
Participants	Inclusion Criteria: • Envenomation by a copperhead snake. A snake identified by one of the following means: i. Snake or photograph of snake brought to Emergency Department; ii. Patient chooses copperhead from an array of snake photographs; iii. Patient envenomated in an area where only copperheads are endemic; iv. Patient envenomated by a captive copperhead snake • Completion of informed consent and eligibility confirmation within 24 hours of envenomation • Envenomation on only one extremity, distal to the elbow or knee • Clinical evidence of mild or moderate venom effect (limb swelling and/or tenderness) is present (Venom effects need not be progressing.) • Patient willing and able to complete follow-up schedule of assessments • Patient is able to read, comprehend and sign the IRB approved consent document(s)

NCT01864200 (*Continued*)

	• Patient is able to read and comprehend the written assessment tools (e.g. DASH, SF-36, etc.) • Patient is ≥14 years of age • Patient is sober, competent, and able to complete verbal and written informed consent Exclusion Criteria: • Patient has clinical evidence of severe venom effect as defined by meeting any one of the following parameters: i. Swelling to an entire extremity (all major joints affected). Lower extremity: i. swelling crossing hip joint. Upper extremity: swelling crossing shoulder joint; ii. INR > 2.0; iii. Platelets <50,000 cells/μL; iv. Fibrinogen <50 mg/dL Compartment syndrome; vi. Systolic Blood Pressure <90 mmHg; vii. More than minimal bleeding; viii. Investigator's clinical discretion • Patient has already received antivenom for the management of the current envenomation • Patient is pregnant or breastfeeding • Patient is a prisoner • Patient has a distracting injury or condition with acute pain or functional impairment, and/or is unable to make a reliable self-report of functionality status based solely on the condition of interest • Patient had a previous snake envenomation to any body area in the 30 days prior to screening/ enrolment, regardless of whether antivenom was administered for the previous envenomation • Patient had an acute traumatic event, surgery, an acute medical event, or exacerbation of a pre-existing medical or surgical condition affecting the envenomated extremity within the 30 days prior to screening/ enrolment • Patient has participated in a clinical study involving an investigational pharmaceutical product or device within the 3 months prior to screening that may have impact on clinical outcomes of snakebite • Patient has previously participated in this clinical study • Patient has a known history of hypersensitivity to any components of CroFab®, or to papaya or papain • Patient is otherwise unsuitable for inclusion in this study, based on the opinion of the investigator
Interventions	Crotilidae polyvalent immune fab (ovine) and placebo
Outcomes	Patient Specific Functional Scale at Day 14
Starting date	July 2013
Contact information	Anna Temu: anna.temu@btgplc.com
Notes	This study completed in March 2015. Its results will be included in this review when published

DATA AND ANALYSES

This review has no analyses.

APPENDICES

Appendix 1. Search strategies

Cochrane Injuries Group Specialised Register & Cochrane Central Register of Controlled Trials (*The Cochrane Library)*
#1MESH DESCRIPTOR Snakes
#2MESH DESCRIPTOR Boidae
#3MESH DESCRIPTOR colubridae EXPLODE ALL TREES
#4MESH DESCRIPTOR elapidae EXPLODE ALL TREES
#5MESH DESCRIPTOR Viperidae EXPLODE ALL TREES
#6(snake* or viper*):TI,AB,KY
#7MESH DESCRIPTOR Snake Venoms EXPLODE ALL TREES
#8MESH DESCRIPTOR Elapid Venoms EXPLODE ALL TREES
#9MESH DESCRIPTOR Viper Venoms EXPLODE ALL TREES
#10(snake venom*):TI,AB,KY
#11((venom* or bite*) adj3 snake*):TI,AB,KY
#12((envenomation or venom-induced or antivenom* or antivenin*)):TI,AB,KY
#13(snake adj3 poisonous):TI,AB,KY
#14MESH DESCRIPTOR Antivenins EXPLODE ALL TREES
#15#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14
#16* NOT INMEDLINE NOT INEMBASE
#17#15 AND #16

Ovid MEDLINE(R), Ovid MEDLINE(R) In-Process & Other Non-Indexed Citations, Ovid MEDLINE(R) Daily and Ovid OLDMEDLINE(R)
1. exp snakes/ or exp boidae/ or exp colubridae/ or exp elapidae/ or exp viperidae/
2. (snake* or viper*).ab,ti.
3. exp snake venoms/ or exp elapid venoms/
4. snake venoms.mp.
5. ((venom* or bite*) adj3 snake*).ab,ti.
6. (envenomation or venom-induced or antivenom* or antivenin*).ab,ti.
7. (snake adj3 poisonous).ab,ti.
8. exp Antivenins/
9. exp Viper Venoms/
10. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9
11. randomi?ed.ab,ti.
12. randomized controlled trial.pt.
13. controlled clinical trial.pt.
14. placebo.ab.
15. clinical trials as topic.sh.
16. randomly.ab.
17. trial.ti.
18. Comparative Study/
19. 11 or 12 or 13 or 14 or 15 or 16 or 17 or 18
20. (animals not (humans and animals)).sh.
21. 19 not 20

22. 10 and 21

Embase Classic + Embase (OvidSP)

1. exp snakes/ or exp boidae/ or exp colubridae/ or exp elapidae/ or exp viperidae/
2. (snake* or viper*).ab,ti.
3. exp snake venoms/ or exp elapid venoms/
4. snake venoms.mp.
5. ((venom* or bite*) adj3 snake*).ab,ti.
6. (envenomation or venom-induced or antivenom* or antivenin*).ab,ti.
7. (snake adj3 poisonous).ab,ti.
8. exp Antivenins/
9. exp Viper Venoms/
10. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 8 or 9
11. exp Randomized Controlled Trial/
12. exp controlled clinical trial/
13. exp controlled study/
14. comparative study/
15. randomi?ed.ab,ti.
16. placebo.ab.
17. *Clinical Trial/
18. exp major clinical study/
19. randomly.ab.
20. (trial or study).ti.
21. 11 or 12 or 13 or 15 or 16 or 17 or 18 or 19 or 20
22. exp animal/ not (exp human/ and exp animal/)
23. 21 not 22
24. 10 and 23

ISI Web of Science: Science Citation Index Expanded & Conference Proceedings Citation Index-Science

#11#10 AND #7 AND #6
#10#9 OR #8
#9TS=(envenomation or venom-induced or antivenom* or antivenin*)
#8TS=(venom* or bite* or poisonous or poison)
#7TS=(snake* OR boidae OR colubridae OR elapidae OR viperidae OR viper*)
#6#5 AND #4
#5TS=(human*)
#4#3 OR #2 OR #1
#3TS=((singl* OR doubl* OR trebl* OR tripl*) SAME (blind* OR mask*))
#2TS=(controlled clinical trial OR controlled trial OR clinical trial OR placebo)
#1TS=(randomised OR randomized OR randomly OR random order OR random sequence OR random allocation OR randomly allocated OR at random OR randomized controlled trial)

Toxnet (toxicology data network)

antivenom* OR antivenin* OR venom-induced OR envenomation

OpenGrey

(antivenom* OR antivenin* OR venom-induced OR envenomation) AND (poison* OR bite* OR venom* OR venom-induced)

WHO International Clinical Trials Registry Platform (ICTRP)

Title:(poison* OR bite* OR venom* OR venom-induced)
AND
Condition:(antivenom* OR antivenin* OR venom-induced OR envenomation)
AND
Recruitment status: ALL

Clinicaltrials.gov

(antivenom* OR antivenin* OR venom-induced OR envenomation) AND (poison* OR bite* OR venom* OR venom-induced)
Google and Google Scholar
snake envenoming OR snake envenomation OR coagulopathy OR venom induced OR coagulation abnormalities OR snake antivenom OR snake antivenin AND (randomized controlled trial or randomised controlled trial).

CONTRIBUTIONS OF AUTHORS

All authors contributed to this protocol.

DECLARATIONS OF INTEREST

All authors: none known.

SOURCES OF SUPPORT

Internal sources

- Library services, University of Newcastle, NSW, Australia.

Support to find the references for the review.

External sources

- No sources of support supplied

Chapter Nine

Current Treatment for Venom-Induced Consumption Coagulopathy Resulting from Snakebite

Introduction to Chapter Nine "Current Treatment for Venom-Induced Consumption Coagulopathy Resulting from Snakebite" to the overall thesis

Antivenom is the mainstay treatment for snake envenoming. Venom-induced consumption coagulopathy is the commonest systemic effect of snake envenoming often resulting in a fatal outcome. Antivenom is a drug which has a high risk of severe anaphylaxis reactions after administration. Therefore, the use of antivenoms should be supported by clear scientific evidence for its effectiveness especially for venom-induced consumption coagulopathy. Moreover, some studies have suggested giving fresh frozen plasma for VICC. I aimed to explore the published evidence of different treatment modalities for venom-induced consumption coagulopathy, to ascertain the level of evidence of using antivenom for VICC. Therefore, the systematic review titled "Current Treatment for Venom-Induced Consumption Coagulopathy Resulting from Snakebite" is highly relevant under the subtitle of efficacy and the effectiveness of antivenom in my thesis.

Statement of Contribution of Others

I hereby certify that other researchers have collaborated and contributed to the work embodied in this Chapter.

This original article was published in PLoS Neglected Tropical Diseases on 23th October 2014. I, Maduwage Kalana Prasad, was the first author of this manuscript, and the work embodied in this Chapter was performed by myself, with the exception of the following:

Overall supervision of this review was done by G. K. Isbister.

The work embodied in this Chapter attests and clearly identifies the nature of intellectual input to be included as part of the overall thesis. Therefore, this work will be discussed in Chapter Nine.

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PLOS | NEGLECTED TROPICAL DISEASES

Review

Current Treatment for Venom-Induced Consumption Coagulopathy Resulting from Snakebite

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Abstract: Venomous snakebite is considered the single most important cause of human injury from venomous animals worldwide. Coagulopathy is one of the commonest important systemic clinical syndromes and can be complicated by serious and life-threatening haemorrhage. Venom-induced consumption coagulopathy (VICC) is the commonest coagulopathy resulting from snakebite and occurs in envenoming by Viperid snakes, certain elapids, including Australian elapids, and a few Colubrid (rear fang) snakes. Procoagulant toxins activate the clotting pathway, causing a broad range of factor deficiencies depending on the particular procoagulant toxin in the snake venom. Diagnosis and monitoring of coagulopathy is problematic, particularly in resource-poor countries where further research is required to develop more reliable, cheap clotting tests. MEDLINE and EMBASE up to September 2013 were searched to identify clinical studies of snake envenoming with VICC. The UniPort database was searched for coagulant snake toxins. Despite preclinical studies demonstrating antivenom binding toxins (efficacy), there was less evidence to support clinical effectiveness of antivenom for VICC. There were no placebo-controlled trials of antivenom for VICC. There were 25 randomised comparative trials of antivenom for VICC, which compared two different antivenoms (ten studies), three different antivenoms (four), two or three different doses or repeat doses of antivenom (five), heparin treatment and antivenom (five), and intravenous immunoglobulin treatment and antivenom (one). There were 13 studies that compared two groups in which there was no randomisation, including studies with historical controls. There have been numerous observational studies of antivenom in VICC but with no comparison group. Most of the controlled trials were small, did not use the same method for assessing coagulopathy, varied the dose of antivenom, and did not provide complete details of the study design (primary outcomes, randomisation, and allocation concealment). Non-randomised trials including comparison groups without antivenom showed that antivenom was effective for some snakes (e.g., Echis), but not others (e.g., Australasian elapids). Antivenom is the major treatment for VICC, but there is currently little high-quality evidence to support effectiveness. Antivenom is not risk free, and adverse reactions can be quite common and potentially severe. Studies of heparin did not demonstrate it improved outcomes in VICC. Fresh frozen plasma appeared to speed the recovery of coagulopathy and should be considered in bleeding patients.

Introduction

Venomous snakebite is considered to be the single most important cause of human injury from any kind of venomous or poisonous animal worldwide. Envenoming and deaths resulting from snakebite are a particularly important public health problem in the tropical world, with the highest burden in rural areas of South Asia, Southeast Asia, and sub-Saharan Africa [1]. Coagulopathy is the commonest important, systemic clinical syndrome caused by snake envenoming in the world, and venom-induced consumption coagulopathy (VICC) is the most clinically important coagulopathy, because it can be complicated by serious and life-threatening haemorrhage [2].

Methods

We searched MEDLINE from 1946 and EMBASE from 1947 to September 2013 and included any clinical studies of snake envenoming with VICC which provided information on treatment, including antivenom. The following keywords were used: "snakebite", "snake envenoming/envenomation", "coagulopathy", "bleeding", "haemorrhage", "antivenom", "heparin", and "treatment". Reference lists of identified articles were searched to find additional publications. Only articles in English were reviewed. The UniPort database (www.uniport.org) was also used for information on isolated toxins from snake venoms with coagulant actions. We identified a total of 1,355 studies of which 95 were included for review. There were 25 randomised comparative trials, 13 non-randomised comparative trials, and a large number of observational clinical studies which discussed the effectiveness of treatments for VICC.

Citation: Maduwage K, Isbister GK (2014) Current Treatment for Venom-Induced Consumption Coagulopathy Resulting from Snakebite. PLoS Negl Trop Dis 8(10): e3220. doi:10.1371/journal.pntd.0003220

Editor: Janaka de Silva, Faculty of Medicine, University of Kelaniya, Sri Lanka

Published October 23, 2014

Funding: GKI is supported by an National Health and Medical Research Council (NHMRC) Senior Research Fellowship (1061041) and the review was partly funded by an NHMRC Project Grant ID 631073. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Venom-Induced Consumption Coagulopathy (VICC)

Various terms have been used to refer to the consumption coagulopathy following snake envenoming, including disseminated intravascular coagulation (DIC), defibrination syndrome, and procoagulant coagulopathy [3]. More recently, the term "venom-induced consumption coagulopathy" has been introduced because it provides a more general description of the coagulopathy [4]. VICC can occur in envenoming by Viperid snakes, certain elapids, including Australian elapids [2], and a few Colubrid (rear fang) snakes [5]. A list of the major snake species that cause VICC is included in Table 1.

VICC results from the activation of the clotting pathway by procoagulant toxins in the venom. The snake venom components that act on the coagulation system are classified according to the part of the coagulation pathway where they act and include factor V activators, factor X activators, prothrombin activators, and thrombin-like enzymes (TLEs) or fibrinogenases (Figure 1) [6]. The severity, duration, and type of consumption coagulopathy differ depending on the type of procoagulant toxin. Table 1 provides more detailed information on the clotting factor deficiencies in VICC for different snake groups. Almost all of these toxins cause activation of one or more clotting factors and lead to low or undetectable concentrations of fibrinogen following envenoming [2]. Thrombin-like enzymes or fibrinogenases generally cleave either the α-chain or the β-chain of fibrinogen to give fibrinopeptide A or B, which results in the consumption of fibrinogen without forming fibrin [4]. Therefore these toxins do not strictly activate the entire clotting pathway, but result in low or undetectable fibrinogen concentration, often with normal levels of the other clotting factors. In contrast, toxins that act higher up the clotting pathway, such as factor X activators or prothrombin activators, result in multiple factor deficiencies such as those occurring with Australian elapids [2], Russell's viper [7], and *Echis* spp. [8].

The diagnosis and monitoring of VICC requires coagulation studies and clotting times [9]. The majority of these tests are rarely available where most cases of snake envenoming occur. Internationally, the most commonly used test is the 20-minute whole blood clotting test (WBCT20) [8,10–15]. However, the reliability of the WBCT20 as a diagnostic test has come into question for the diagnosis of Russell's viper envenoming [16]. There is no standardisation of the WBCT, including the duration of the test, the type of glass tube used for the test, and the procedure. The duration of the WBCT ranges from 10 minutes [17] in some studies to 30 minutes in others [18–20]. In other parts of the world more routine clotting tests, such as the prothrombin time (PT; international normalised ratio, INR), activated partial thromboplastin time (aPTT), and thrombin clotting time (TCT), are used [2,21–23].

Many patients with VICC may exhibit minimal clinical features other than bleeding from the bite site or cannula site. However, some patients develop bleeding from the gums, gastrointestinal tract bleeding (clinically manifesting as melaena or haematemesis), and haematuria [7]. This is usually caused by snakes with venoms that also contain haemorrhagins such as *Echis* spp. [22,24] and *Bothrops* spp. [25]. More severe bleeding includes intracranial haemorrhage, which is often fatal, and bleeding associated with trauma. Bleeding into the pituitary gland has also been reported following viper envenoming and produces subtle or delayed clinical signs compatible with Sheehan's syndrome [26,27].

This review will focus on the effectiveness of various treatments for VICC based on the evidence from clinical studies, but will not cover venom-induced thrombocytopenia, [28,29], snakebite-associated thrombotic microangiopathy [2], or anticoagulant coagulopathy [30].

Antivenom

Antivenoms and their mechanism of action

Antivenom is the recommended standard treatment for snake envenoming. Antivenoms consist of polyclonal antibodies to the toxins in snake venoms [31]. They may be whole immunoglobulins (IgG) or fractionated IgG, either $F(ab')_2$ or Fab [32]. The antibodies are produced in animals, including horse, sheep, goats, and rabbits. The animals are injected with the snake venom so that they mount an immune response and produce antibodies to that venom [33,34]. The polyclonal nature of antivenom means that they are able to neutralise multiple venom components [32]. Monovalent antivenoms are raised against one species of snakes, while polyvalent snake antivenoms are produced by immunizing with venoms from more than one species of snake [33].

The efficacy of antivenom is best defined as its ability to bind venom components or toxins [35], while the effectiveness of antivenom is its ability to prevent or reverse the effects of envenoming in humans. There are a number of proposed mechanisms by which the binding of antivenom to venom results in prevention or reversal of envenoming. Antivenom can potentially block the active site of a toxin or bind to a toxin to prevent it interacting with its substrate (steric hindrance) to neutralise the toxin. Antivenom-venom complex formation in the central compartment may prevent the distribution of toxins to the target tissues (e.g., nervous system) or cause the redistribution of toxins from their target tissues back to the vascular compartment [32,36–38]. Finally, antivenom can increase the elimination of toxins from the circulation and body. In the case of VICC, the toxins act in the central compartment, so antivenom must either bind to the toxins in the blood, and therefore prevent the action of the toxins, or increase the elimination of toxins.

Numerous studies have demonstrated that antivenom can bind to procoagulant toxins and prevent their effects in vitro if the antivenom and venom are pre-mixed [39–43]. Despite antivenom being efficacious and binding to the multiple toxins in the venom, there are a number of reasons that it may not be effective [35]. The most important being that irreversible toxic effects cannot be reversed by antivenom binding to toxins after the damage has occurred, such as clotting factor deficiencies resulting from VICC [9]. For antivenom to be effective against such irreversible effects, it must be administered early, so it can bind with toxins before they distribute to their target sites and cause irreversible toxicity.

Procoagulant toxins act in the central compartment (circulation), making their onset of action relatively rapid. Once they have activated the clotting pathway and clotting factors have been consumed, this process is irreversible until further clotting factors can be re-synthesized. Antivenom can only be effective in preventing the onset of VICC if it binds the procoagulant toxins prior to the clotting pathway being activated. A semi-mechanistic systems model of the coagulation cascade has been used to simulate the effects of Australasian elapid venoms on the clotting pathway and shows that antivenom needs to be administered within 15 to 30 minutes to prevent or even partially prevent VICC occurring [44–46]. However, the duration of VICC can be days for some snakes such as *Echis* spp., so the administration of antivenom will potentially bind the active procoagulant toxins, allowing the clotting factors to recover. Antivenom will therefore be clinically effective in shortening the duration of VICC and reducing the risk of bleeding.

Table 1. Summary of snakes known to cause venom-induced consumption coagulopathy, the procoagulant toxin, and the factor deficiencies that have been reported (with permission from WikiToxin).

Snake species	Common name	Distribution	Procoagulant Toxins	VICC Testing	Factor Deficiencies	References
Daboia russelii	Russell's viper	Asia	FX, FV activators	WBCT20, CT, fibrinogen, clotting factor studies	Fibrinogen, FV, FX	Phillips [7], Isbister [16]
Daboia russelii siamensis	Eastern Russell's viper, Siamese Russell's viper	Asia	FX, FV activators	PT, non-clotting blood	Fibrinogen, FV, FX	Than [88], Tin Na [78]
Hypnale hypnale	Hump-nosed pit vipers	Asia	Unknown? TLE	WBCT20, PT, aPTT, clotting factor studies, D-Dimer	Fibrinogen, FVIII	Maduwage [13]
Echis carinatus	Saw scaled viper	Asia	PTA	WBCT20	NR	Kularatne [11]
Calloselasma rhodostoma	Malayan pit viper	Asia	TLE	VCT >30 minutes, fibrinogen, FDP, clotting factor studies	Fibrinogen	Kulapongs [89], Warrell [70]
Trimeresurus albolabris	White-lipped green pit viper	Asia	TLE	Fibrinogen, FDP, fibrinopeptide A, plasminogen	Fibrinogen	Hutton [90], Rojnuckarin [91]
Trimeresurus macrops	Large-eyed pitviper (green pitviper)	Asia	TLE	Fibrinogen, FDP, fibrinopeptide A, plasminogen	Fibrinogen	Rojnuckarin [91]
Trimeresurus stejnegeri	Bamboo pitviper, Chinese tree viper	Asia	TLE, plasminogen activator	Fibrinogen, FDP, AT-III	Fibrinogen	Li [92]
Rhabdophis subminiatus	Red-necked keelback	Asia	?	Fibrinogen, FDP	Fibrinogen	Li [92]
Rhabdophis tigrinus	Tiger keelback	Asia	?	PT, aPTT, Fibrinogen, FDP	Fibrinogen	Mori [93]
Pseudonaja spp.	Brown snake	Australia	PTA	PT, aPTT, clotting factor studies, D-dimer	Fibrinogen, FII, FV, FVIII	Isbister [3]
Notechis scutatus	Tiger snake	Australia	PTA	PT, aPTT, clotting factor studies, D-dimer	Fibrinogen, FII, FV, FVIII	Isbister [3]
Tropidechis carinatus	Rough-scaled snake	Australia	PTA	PT, aPTT, clotting factor studies, D-dimer	Fibrinogen, FII, FV, FVIII	Isbister [3]
Hoplocephalus spp.	Broad-headed snakes	Australia	PTA	PT, aPTT, clotting factor studies, D-dimer	Fibrinogen, FII, FV, FVIII	Isbister [3]
Oxyuranus scutellatus	Coastal taipan	Australasia	PTA	PT, aPTT, clotting factor studies, D-dimer	Fibrinogen, FII, FV, FVIII	Isbister [3], Lalloo [94]
Bothrops atrox	Common Lancehead	South America	TLE, FX, FV, activators	PT, aPTT, D-dimer, FDP	Fibrinogen	Pardal [60]
Bothrops asper	Lancehead, Terciopelo	South America	TLE, PTA	PT, aPTT, clotting factor studies, D-dimer	Fibrinogen, FII, FV	Barrantes [95], Otero-Patino [59]
Bothrops jararaca	Jararaca	South America	TLE, PTA, FX activator	Fibrinogen, clotting factor studies	Fibrinogen, FII, FV, FVIII	Kamiguti [96], Jorge [71]
Lachesis spp.	Bushmasters	Central America	TLE	Fibrinogen, D-dimer, α2-antiplasmin, FDP	Fibrinogen	Pardal [60]
Crotalus durissus	South American rattlesnake	Central and South America	TLE	PT, aPTT, clotting factor studies, D-dimer, FDP	Fibrinogen, FII, FV	Sano-Martin [97], Kamiguti [98]
Crotalus atrox	Western diamondback rattlesnake	North America	TLE	PT, aPTT, Fibrinogen	Fibrinogen	Budzynski [99]
Crotalus adamanteus	Eastern diamondback rattlesnake	North America	TLE	PT, aPTT, fibrinogen, D-dimer, FDP, antiplasmin III	Fibrinogen, D-dimer (normal)	Kitchens [100]
Crotalus molossus molossus	Black-tailed rattlesnake	North America	? TLE	PT, fibrinogen, FDP	Fibrinogen	Hardy [101]
Crotalus horridus	Timber rattlesnake	North America	TLE	Fibrinogen, FDP	Fibrinogen	Hasiba [102]
Crotalus helleri	Southern Pacific rattlesnake	North America	TLE	PT, fibrinogen	Fibrinogen	Bush [103]
Vipera aspis	European asp/Asp viper	Europe	FX activator	PT, aPTT, fibrinogen, D-dimer	Fibrinogen	Boels [104], Petite [105]
Vipera berus	Common European viper	Europe		PT, aPTT, fibrinogen, D-dimer	Fibrinogen	Boels [104]
Vipera ammodytes ammodytes	Horned viper	Europe		PT, aPTT, fibrinogen, D-dimer	Fibrinogen	Luksic [106]
Atheris squamigera	Green bush viper	Africa	TLE	aPTT, fibrinogen	Fibrinogen	Mebs [107]

Table 1. Cont.

Snake species	Common name	Distribution	Procoagulant Toxins	VICC Testing	Factor Deficiencies	References
Atheris chlorechis	Western bush viper	Africa	TLE	PT, aPTT, fibrinogen	Fibrinogen	Top [108]
Atheris nitschei	Great lakes bush viper	Africa	TLE	PT, aPTT, fibrinogen, D-dimer	Fibrinogen	Hatten [109]
Cerastes cerastes	Saharan horned viper	Africa/Middle East	TLE	PT, aPTT, fibrinogen, D-dimer, factor V	Fibrinogen, FV	Lifshitz [110], Schneemann [111]
Cerastes vipera	Sahara sand viper	Africa/Middle East	TLE (cerastobin)	PT, aPTT, fibrinogen, D-dimer	Fibrinogen	Lifshitz [112]
Proatheris superciliaris	Lowland viper	Africa		PT, aPTT, fibrinogen, D-dimer	Fibrinogen	Valenta [113]
Bitis arietans	African puff adders	Africa	TLE	Fibrinogen, PT, clotting factor studies	Fibrinogen	Jennings [114], Warrell [115], Lavonas [116]
Bitis gabonica	Gaboon viper	Africa	TLE (Gabonase)	Fibrinogen, PT, clotting factor studies	Fibrinogen	McNally [117]
Echis coloratus	Painted carpet viper	Africa	PTA	Fibrinogen, FDP, PT	Fibrinogen,? FII, FV, FVIII	Porath [118] Mann [73]
Echis ocellatus	West African carpet viper	Africa	PTA	WBCT20, fibrinogen, clotting factor studies	Fibrinogen, FII, FV, FVIII	Warrell [8]
Echis pyramidum	Northeast African carpet viper	Africa	PTA	Fibrinogen, PT, clotting factor studies	Fibrinogen, FII, FV, FVIII	Mion [22], Gillissen [119]
Dispholidus typus	Boomslang	Africa	SVMP*	PT, aPTT, fibrinogen, FDP, thromboelastography	Fibrinogen	Aitchison [5]

aPTT – activated partial thromboplastin time, CT – clotting time, VCT – venous clotting time, FDP – fibrinogen degradation products, PLA_2 – phospholipase A_2, PT – prothrombin time, TLE – thrombin like enzymes, WBCT – whole blood clotting time, WBCT20 – 20 minutes whole blood clotting time, FII – factor II, FV – factor V, FX – factor X, FDP – fibrinogen degradation products; PTA – prothrombin activator; SVMP – snake venom metalloproteinase; NR – not reported;
* A SVMP has been isolated from *D. typus* venom but its function (? PTA, FX activator, TLE) is unclear and only fibrinogen has been measured in patients.
doi:10.1371/journal.pntd.0003220.t001

Antivenoms are not without risk because administration of foreign proteins in the antivenoms can cause systemic hypersensitivity reactions (SHR) [47]. Early SHR include skin-only SHR, and anaphylaxis and severe anaphylaxis have been reported. Delayed reactions can also occur, and are referred to as serum sickness.

Clinical studies of antivenom

Our literature review did not identify any placebo randomised control trials of snake antivenom for VICC. There were 25 randomised comparative trials [24,48–71] of antivenom for VICC, which compared two different antivenoms (ten studies), three different antivenoms (four studies), two or three different doses or repeat doses of antivenom (five studies), heparin treatment and antivenom (five studies), and intravenous immunoglobulin treatment and antivenom (one study) (Table 2). There were a further 13 studies [8,9,20–22,72–79] which compared two groups in which there was no randomisation, including studies with historical controls (Table 3). There have been numerous observational studies of patients with VICC given antivenom, but with no comparison group.

Unfortunately there were major design issues with most of the randomised controlled trials, including lack of definition of a primary outcome or post-hoc definition of the primary outcome, no information on allocation concealment, no information on randomisation, no information on antivenom dose, or varying doses given to patients, and all but two studies [48,66] were underpowered with no sample size calculation (Table 2). The primary assessment of coagulopathy in these studies varied with different whole blood clotting tests and times (12, 15, 20, or 30 minutes) and measurement of the PT, aPTT, fibrin degradation products (FDP), D-Dimer, or fibrinogen, making comparison between studies difficult and reliability of the WBCT outcomes questionable. Many of the studies used a restoration of "coagulable blood" based on the WBCT as the major outcome, which is problematic because the reliability of WBCT20 for VICC has been recently questioned [16].

The greatest limitation of the randomised controlled trials was the absence of placebo controlled trials, so none of the trials could effectively address the question as to whether antivenom was beneficial in treating VICC. In nine of 14 studies, the authors concluded equal effectiveness of two or three antivenoms, and four of five studies of different doses or dosing regimens concluded equal effectiveness. The commonest interpretation of these studies is that antivenoms are equally effective. However, these studies actually provide no evidence for antivenom effectiveness and can be interpreted as two antivenoms being equally ineffective. All that can be concluded from these studies is that using any one of the two or three antivenoms is similarly effective.

In five of the 14 studies comparing different antivenoms, the authors concluded that one antivenom was superior to the other(s). However, on reviewing these studies, there were problems with study design or dose was confounded with antivenom type (i.e., the antivenom was less effective because an insufficient dose was given [49,68,69]). One of the better clinical trials of antivenom for VICC was the randomised comparative trial of EchiTAb Plus equine antivenom and EchiTAb G ovine antivenom for *Echis ocellatus* envenoming, which concluded that EchiTAb Plus was slightly more effective than the other [48]. However, this study was designed as a non-inferiority study, and therefore the authors can only conclude that neither antivenom was inferior to the other, based on the primary outcome. It is incorrect to then use a

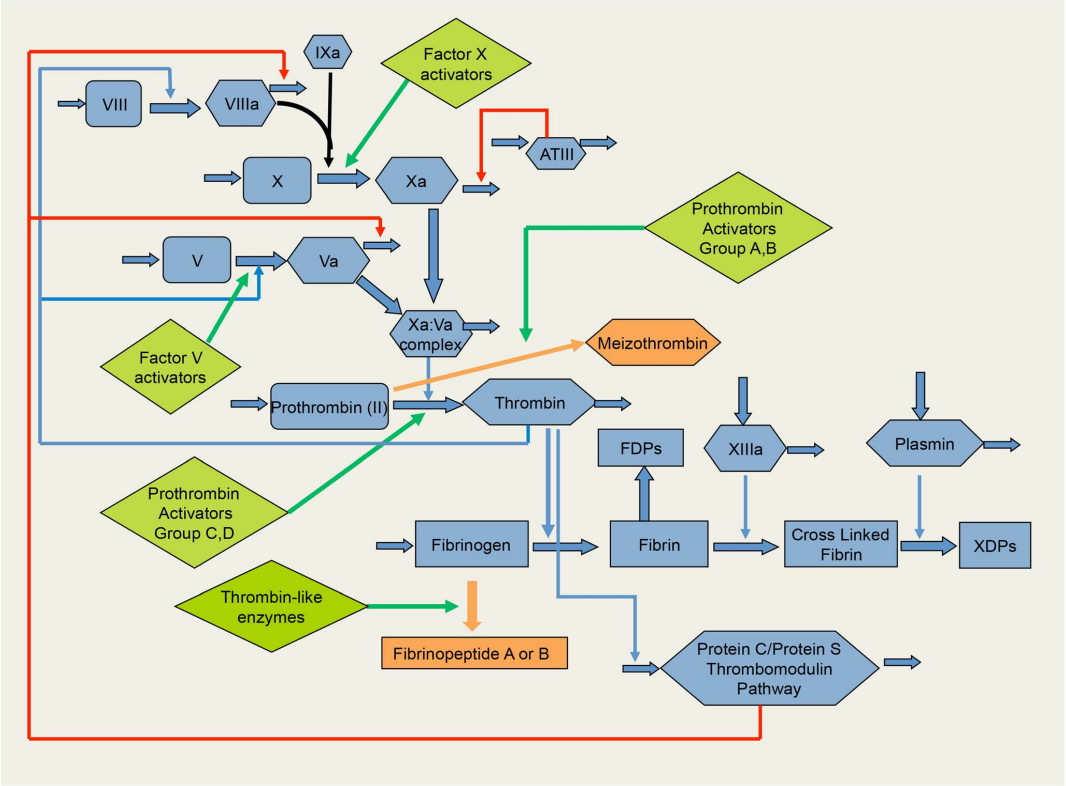

Figure 1. Diagram of the clotting pathway showing the major clotting factors (blue) and their role in the activation of the pathway and clot formation. The four major groups of snake toxins that activated the clotting pathway are in green and the intermediate or incomplete products they form are indicated in dark red. There are four major types of prothrombin activators, which either convert thrombin to form the catalytically active meizothrombin (Group A and B) or to thrombin (Group C and D).
doi:10.1371/journal.pntd.0003220.g001

one-sided p-value to suggest that one antivenom was superior to the other. Any conclusion on positive secondary outcomes is also questionable in a non-inferiority trial design. The study by Ariaranee et al., in 2001, concluded that Haffkine antivenom was more effective than Polonga TAb and that a larger dose of Polonga TAb was required [49]. They based this on only 74% of patients having coagulable blood 6 hours after Haffkine antivenom compared to 41% after Polonga Tab. However, 12 hours after antivenom, 95% of patients receiving Haffkine antivenom had coagulable blood compared to 86% receiving Polonga Tab, which was unlikely to be significant. The authors did not define a primary outcome, so the study can be interpreted differently based on whether a 6-hour or 12-hour outcome is used. The study by Smalligan et al. also concluded that one antivenom was more effective, but the study was underpowered (210 recruited versus 300 required for the sample size) and the result was not significant ($p = 0.054$) for the primary outcome at 6 hours if only patients given the standard initial dose were included (the outcome that the sample size appeared to be based on) [66]. Warrell et al., in 1974, compared S.A.I.M.R. antivenom and Behringwerke antivenom, but their outcomes included the dose of antivenom required and the proportion with coagulation restored. The study showed that larger amounts of Behringwerke antivenom were required, and not necessarily that it was less effective [68]. Warrell et al., in 1980, conclude in another underpowered study of 14 patients with no clearly defined primary outcome that Behringwerke antivenom was unreliable [69]. The trial was too small to show any significant difference between the two antivenoms.

In contrast to the randomised controlled trials, some non-randomised comparative studies do provide evidence for and against the effectiveness of antivenom for VICC. A number of Australian studies and one study of Papuan taipan bites found that antivenom does not prevent or speed the recovery of VICC in Australian elapid envenoming [3,9]. This was supported by computer modelling of the coagulation pathway that showed that antivenom needed to be given almost immediately to prevent VICC in Papuan taipan and Australian elapids [44,45]. One Australian study found that early (<6 hours after the bite) and late (>6 hours after the bite) administration of antivenom resulted in the same recovery rate of VICC with 3% and 33% recovering to an INR of 2 or less after 6 and 12 hours for early antivenom, compared to 3% and 27% for late antivenom [9]. Trevett et al. also showed that early antivenom (<4 hours) versus late antivenom (>4 hours) in Papuan taipan did not result in a more rapid

Table 2. Summary of the randomised comparative trials of treatment for VICC, including antivenom and heparin with details of study size, design, and outcomes.

Study	Numberin each arm	Snake species	Trial Arms	Blinded	Randomisation method	Allocation concealed	AV dose defined	Primary outcome	VICC measures	Conclusion†
Abubakar, 2010	194/206	*Echis ocellatus*	2 AV	Yes	Yes	Good	Yes	Yes	WBCT20	No difference between antivenoms (neither inferior)
Ariaratnam, 2001	23/20	*Daboia russelii*	2 AV	No	Yes	Good	Yes	No	WBCT20	No difference but multiple outcomes
Meyer, 1997	22/17	*E. ocellatus*	2 AV	No	Yes	Nil	Yes	No	WBCT20	No difference in restoration of clotting function
Otero, 1999	25/28	*Bothrops Porthidium* sp.	2 AV	Yes	Nil	Nil	Yes Varied*	Partially, defines 2	WBCT15/30	No difference for either outcome
Otero, 1996	20/19	*B. atrox*	2 AV	Yes	Nil	Good	Yes Varied*	No	WBCT15/30	No difference but no clearly defined outcomes
Otero, 2006	34/33	*B. asper*	2 AV	Yes	Nil	Good	Yes Varied*	No	WBCT20, fibrinogen	No difference but no outcomes and variable dosing
Otero-Patino, 2012	38/34	*B. asper*	2 AV	Yes	Nil	Good	Yes Varied*	No	WBCT 20, fibrinogen	No difference
Pardal, 2004	38/36	*Bothrops Lachesis*	2 AV	Yes	Nil	Good	Unclear	No	WBCT20, fibrinogen, D-dimer,	No difference
Warrell, 1974	23/23	*E. ocellatus*	2 AV	No	Nil	Nil	Yes Varied*	No	WBCT20, fibrinogen	Unclear difference in outcomes. Dose and AV confounded.
Warrell, 1980	7/7	*E. ocellatus*	2 AV	No	Nil	Nil	Yes Varied*	No	WBCT20, fibrinogen, factor II, X, XIII	Study too small for any conclusion
Cardoso, 1993	39/41/41	*B. jararaca*	3 AV	Yes	Nil	Nil	Yes Varied*	No	WBCT20, fibrinogen, D-dimer	Similar effectiveness of all three antivenoms
Otero-Patino, 1998	30/27/22	*Bothrops*	3 AV	Partial	Partial	Nil	Yes Varied*	No	WBCT30, fibrinogen	Similar effectiveness of all three antivenoms
Smalligan, 2007	82/87/41	*Bothrops Lachesis*	3 AV	Yes	Yes	Good	Yes Varied*	Proportion with clotting blood at 6 hr	WBCT 20	No statistically significant difference in the primary outcome or 24-hour outcome
Warrell et al., 1986	15/15/16	*C. rhodostoma*	3 AV	No	Nil	Nil	Yes Varied*	No	WBCT20, fibrinogen	Equally effective based on outcomes of restoration of coagulation
Dart, 2001	16/15	North American Crotalid	2 doses	No	Yes	Good	Yes	Yes	Fibrinogen, PT	Both dosing regimens were equally effective
Jorge, 1995	88/82	*Bothrops jararaca*	4 vs. 2 vials	Yes	Yes	Nil	Yes	No	WBCT10/30, fibrinogen, FDP	Both dosing regimens were equally effective
Karnchanachetanee, 1994	13/11	*D. russelii*	Low vs. high dose	No	Nil	Nil	Yes	No	WBCT20	No difference but no clear information on clotting outcomes

Table 2. Cont.

Study	Numberin each arm	Snake species	Trial Arms	Blinded	Randomisation method	Allocation concealed	AV dose defined	Primary outcome	VICC measures	Conclusion[†]
Paul, 2004	50/50	*D. russelii E. carinatus*	6 vs. 12 vials AV	No	Nil	Nil	Yes	No	WBC time, PT	No difference
Thomas and Jacob, 1985	26/27	Probably *E. carinatus D. russelii*	High vs. low dose	No	Nil	Nil	Yes	No	WBCT15	No difference. Unusual dosing regimens.
Myint-Lwin, 1989	14/14	*D. russelii*	AV vs. heparin+AV	No	Nil	Nil	Yes	No	fibrinogen, factor V, X	No difference with the addition of heparin
Paul, 2003	57/65	*D. russelii*	AV vs. heparin+AV	No	Nil	Nil	Yes	No	WBCT30, PT, fibrinogen	No difference with the addition of heparin
Paul, 2007	40/40	Probably *D. russelii E. carinatus*	AV vs. deltaparin+AV	No	Nil	Nil	Yes	No	WBCT30, PT, fibrinogen	No difference with the addition of deltaparin
Shah, 1986	25/25	*E. carinatus*	AV vs. heparin+AV	No	Nil	Nil	Yes	No	Undefined clotting time, PT, fibrinogen	More rapid improvement in haematological parameters
Warrell, 1976	7/7	*E. carinatus*	AV vs. heparin+AV	No	Nil	Nil	Yes	No	WBCT20, factor V, VIII, II	No difference with the addition of heparin
Sellahewa, 1994	8/7	Probably *D. russelii E. carinatus*	AV vs. IVIG+AV	No	Partial	Nil	Yes	No	WBCT12	No statistically significant differences and patients were given further antivenom

* Varied based on the clinical assessment of the severity on admission;
[†]May differ from the author's conclusion, see text. Abbreviations: AV – antivenom; WBCT20 – 20-minute whole blood clotting test (or 12-, 15-, or 30-minute); WBC time – whole blood clotting time; IVIG – intravenous immunoglobulin; PT – prothrombin time;
doi:10.1371/journal.pntd.0003220.t002

Table 3. Summary of the non-randomised studies if VICC comparing two groups.

Authors	Type of study	N	Snake species	Study Arms	Primary Outcome	Design Problems	VICC measures	Study conclusions[†]
Bregani, 2006	Prospective (comparison over time)	130/98/60	*E. ocellatus*	3 AV	No	Multiple outcomes without correction for multiple testing	WBCT30	Sii Polyvalent is not effective compared to two other antivenoms.
Warrell, 1977	Observational	48/65/4	*E. ocellatus*	3 AV	No	Unbalanced groups, patients re-treated with different antivenom	WBCT20	Suggests that one antivenom was inferior.
Abubakar, 2010	Dose finding study	24	*E. ocellatus*	3 AV	Yes	Nil major	WBCT20	Established the dose for two antivenoms for a clinical trial
Visser, 2008	Prospective	278(48)/66	*E. ocellatus*	2 AV	No	Only 114 had WBCT20 done, and not all positive. Multiple outcomes.	WBCT20	One antivenom was superior to the other based on death rate and antivenom dose
Isbister, 2009	Prospective cohort	112/29	Australian elapids	Early (<6 h) vs. late (>6 h) AV; [AV vs AV+FFP]	Yes	Primary analysis was a time to event analysis, secondary analysis compared late vs. early	INR	No difference between early and late AV group on VICC recovery. More rapid recovery with FFP.
Suchithra, 2008	Prospective cohort	142/127 [102/25]	Probably *D. russelii*, *E. carinatus*	Early (<6 h) vs. late (>6 h) AV	No	Subgroup analysis. Error in comparison of early and late AV for WBCT – not parametric and not significant ($p = 0.15$)	WBCT20, PT, aPTT	No difference between early and late AV. However, incorrect statistical analysis may have missed significant difference for WBCT20.
Trevett, 1995	Prospective observational study	33/31	*O. scutellatus*	Early (<4 h) vs. late (>4 h) AV	No	Small subgroup analysis.	WBCT20	No difference between early and late AV in time to recovery of WBCT20.
Mann, 1978	Retrospective study	9/9	*E. coloratus*	AV vs. no AV	No	Sample size too small.	Fibrinogen	No difference in recovery of fibrinogen with AV.
Mion, 2013	Prospective observational study (47 vs. 13)	47/13	*E. pyramidum*	AV vs. no AV	No	Multiple outcomes	FibrinogenaPTT, PT	Significant difference in recovery of all coagulation parameters with AV compared to no AV.
Brown, 2009	Retrospective/ Prospective study (106 vs. 21)	106/21	Australian elapids	AV vs. no AV; FFP vs. no FFP	Yes	Two separate studies amalgamated.	INR, aPTT, Fibrinogen	FFP given within 4 hours of antivenom is associated with a more rapid recovery of the INR.
Win-Aung, 1996	Prospective, observational study	34/82	*D. siamensis*	IM AV vs IV AV	No	IM groups less severely envenomed, multiple outcomes and unclear if all patients given IM included	WBCT20	Authors report a significant difference ($p = 0.03$) but poor design and unbalanced groups suggest this may not be a significant difference.
Srimannarayana, 2004	Prospective with two arms randomised	30/30/30	Probably *D. russelii*, *E. carinatus*	Three dose levels	No	Randomised controlled trial but included one non-randomised arm	WBCT30	No difference
Tin, 1992	Prospective two arms	10/10	*D. russeli*	AV vs. heparin+AV	No	Small study with multiple outcomes	Fibrinogenfactor V, X	No difference when adding heparin

[†]May differ to the author's conclusion, see text. Abbreviations: AV – antivenom; WBCT20 – 20 minute whole blood clotting test (or 12, 15 or 30 minute); PT – prothrombin time; aPTT – activated partial thromboplastin time; INR – international normalised ratio; IM – intramuscular; IV - intravenous.

doi:10.1371/journal.pntd.0003220.t003

recovery of the coagulopathy [75]. These studies suggest that there is a limited role for antivenom in the treatment of VICC resulting from Australasian elapid envenoming. However, other studies have shown that antivenom can prevent other clinical effects of envenoming such as neurotoxicity and myotoxicity [75,80], so evidence of VICC and therefore envenoming remains an indication for antivenom. However, in brown snake (*Pseudonaja*) envenoming, where the major clinical syndrome is VICC [81], it could be argued that antivenom does not improve outcomes, and it might be ethical to undertake a placebo controlled trial of antivenom.

Different to Australian antivenom, studies of *Echis* species have demonstrated an important role for antivenom in the treatment of VICC, because antivenom greatly shortens the duration of the coagulopathy. A recent study of *Echis* envenoming by Mion et al. showed that there was a much more rapid recovery of the PT, aPTT, and fibrinogen levels in patients given antivenom compared to those not treated [22]. The mean recovery times to fibrinogen >1 g/l was 7.5 days versus 40 hours; to a PT>50% was 5.8 days versus 25 hours, and to an aPTT<1.5 times normal was 4.7 days versus 9 hours, for untreated and antivenom treated patients respectively [22]. This supports earlier work that found the mortality from *Echis* envenoming was reduced in patients treated with specific antivenoms in Nigeria and the time to the restoration of clotting was much more rapid [8]. The study by Visser et al. reports an increased mortality for patients envenomed by *E. ocellatus* given Asna Antivenom C (Bharat Serum and Vaccines Ltd.) compared to FAV-Afrique (Aventis-Pasteur) and significantly more doses required until the WBCT20 normalised [76]. The failure of Indian antivenom is likely due to the fact that a different *Echis* spp. is used to produce it. There is therefore sufficient evidence from non-randomised studies that doing a placebo controlled trial would be considered unethical.

One study investigated whether the time of antivenom post-bite affected the time to recovery of the coagulopathy in Russell's viper and carpet vipers (*E. carinatus*) in India [74]. The study reported that early antivenom (<6 hours after bite) compared to late antivenom (>6 hours) resulted in a more rapid recovery of the WBCT20, but not the time to recovery of standard coagulation studies (INR, aPTT). This result is difficult to interpret because it included two snake types with different types of procoagulant toxins and did not clearly define outcomes a priori. In addition, there is an error in the statistical analysis because comparison of the values reported in Table 4 in [74] does not give a significant difference in recovery of the WBCT20 between early and late antivenom administration.

The failure of antivenom for VICC in Australia and success of antivenom for VICC from *Echis* spp. in Africa demonstrates that studies of one snake (and therefore one procoagulant toxin) cannot be generalised to other snakes. Studies are required for each major group of snakes or toxins in different parts of the world, although understanding the mechanisms of the procoagulant toxins should inform empirical studies of different antivenoms. The prothrombin activators in Australasian elapids (Group C and D) [4] are similar to the prothrombinase complex in humans and therefore likely to be removed rapidly by pathways that eliminate human prothrombinase. However, the prothrombin activators in the venoms of *Echis* spp. (Group A and B) are metalloproteinases, which differ from the human clotting factors, and therefore are unlikely to be removed by normal elimination pathways.

The study by Win-Aung et al. reported the effectiveness of intramuscular antivenom and is the only study of intramuscular antivenom [77]. This finding is not consistent with pharmacokinetic studies of intramuscular antivenom, which suggest very slow and delayed absorption [82]. The study by Win-Aung did not have an appropriate control group, which was clearly shown by the fact that "test" patients had significantly lower venom concentrations ($p<0.001$) than the control patients and so were more mildly envenomed. Considering the statistical significance in the number of patients with coagulopathy between groups was $p = 0.03$, it is likely that this is accounted for by the "test" group being less severely envenomed. Another concern with the study is that the authors do not report how many patients actually got intramuscular antivenom; they only included those cases in which venom was detected in blood [77]. Contrary to the authors' conclusions this study does not support the effectiveness of intramuscular antivenom, and this route of administration should not be used.

The remaining non-randomised studies of antivenom for VICC were of poor quality (Table 3). Bregani et al. undertook a study of three different antivenoms for *Echis* in Africa and suggested that one was ineffective and was associated with a higher mortality and slower return of clotting function [20]. However, the study compared three groups and multiple outcomes without correcting

Key Learning Points

- Venom-induced consumption coagulopathy (VICC) results from the activation of the clotting pathway by procoagulant snake toxins and consumption of clotting factors.
- Investigation of VICC requires laboratory-based clotting studies until accurate and cheap bedside tests are available.
- There are no placebo controlled trials of antivenom, and effectiveness is not supported by numerous clinical trials comparing antivenoms.
- Non-randomised observational studies with control groups suggest that antivenom may be effective for some snakes but not others.
- There is little evidence to support the use of heparin, and fresh frozen plasma is likely to be beneficial only in actively bleeding patients.

Top Five Papers

1. Isbister GK, Scorgie FE, O'Leary MA, Seldon M, Brown SG, et al. (2010) Factor deficiencies in venom-induced consumption coagulopathy resulting from Australian elapid envenomation: Australian Snakebite Project (ASP-10). J Thromb Haemost 8: 2504–2513.
2. Isbister GK (2009) Procoagulant snake toxins: laboratory studies, diagnosis, and understanding snakebite coagulopathy. Semin Thromb Hemost 35: 93–103.
3. Mion G, Larreche S, Benois A, Petitjeans F, Puidupin M (2013) Hemostasis dynamics during coagulopathy resulting from Echis envenomation. Toxicon 76: 103–109.
4. Isbister GK, Duffull SB, Brown SG (2009) Failure of antivenom to improve recovery in Australian snakebite coagulopathy. QJM 102: 563–568.
5. Abubakar IS, Abubakar SB, Habib AG, Nasidi A, Durfa N, et al. (2010) Randomised controlled double-blind non-inferiority trial of two antivenoms for saw-scaled or carpet viper (Echis ocellatus) envenoming in Nigeria. PLoS Negl Trop Dis 4: e767.

for this, so not all of these differences may have been significant. Mann et al. undertook a small retrospective study of no antivenom versus antivenom for envenoming by *E. colorata*. Although it suggests the recovery in fibrinogen was similar, the study was small and there was no clear definition of recovery [73]. Two studies not included in the 15 simply compared the occurrence of coagulation abnormalities in the envenomed patients and not the recovery of coagulation [23,83]. This meant that severity of envenoming was confounded with antivenom treatment, making it difficult to assess the effect of antivenom.

Another important issue is determining the effective dose of antivenom. There has been significant contention in Australia regarding the dose of antivenom. Recent studies have demonstrated that one vial of antivenom is as effective as two or more vials of antivenom for VICC resulting from snake envenoming [81]. However, it is clear from many of the studies that some antivenoms are not effective because an insufficient dose of the antivenom has been given [49,76]. It is essential that future studies do not confound dose and type of antivenom, and that the optimal dose of antivenom is determined in pre-clinical studies or small dose-finding studies prior to larger controlled trials of antivenom effectiveness.

There are numerous observational studies that report the effectiveness of antivenom, but they simply report the restoration of coagulability for a single group of patients that are all given antivenom. Clearly, further studies of antivenom for VICC are required, but conducting placebo controlled trials is challenging if not impossible due to ethical issues. Good observational studies and historical control studies will hopefully help provide better evidence for the role of antivenom in VICC from different snakes.

Other Treatments

Clotting factor replacement

VICC is characterised by low or undetectable levels of one or more clotting factors, most commonly fibrinogen. Antivenom will only stop the consumptive process so once it has been given it still takes 24 to 48 hours for full recovery of the clotting factors [3]. While the clotting factors are being re-synthesized by the liver there is a period of time during which the patient remains at risk of haemorrhage. For this reason, clotting factor replacement has been suggested as an adjunct treatment for VICC. The most commonly used factor replacement is fresh frozen plasma because it is the most widely available and contains almost all the important factors, such as fibrinogen, factor V, factor VIII, and factor X.

Clotting factor replacement for VICC is controversial because of the concern that it may worsen VICC by providing more clotting factors (substrate) for the procoagulant toxins [84,85]. However, it has been assumed that once antivenom has been given and the toxins are bound, clotting factor replacement is likely to speed the rate of recovering. Two observational studies from Australia support this [9,21] but a more recent randomised controlled trial in Australia only partly supports the use of fresh frozen plasma (FFP) and raises concern about the early use of FFP [86].

The recent randomised controlled trial of FFP for treating VICC in Australian snake envenoming shows that the administration of FFP within 4 hours of antivenom results in more rapid restoration of clotting function in the majority of patients [86]. In a study of 65 patients, 30 of 41 patients (73%) randomised to FFP had an INR of <2 six hours after antivenom compared to only six of 24 patients (25%) not given FFP (absolute difference 48%; 95% confidence interval (CI): 23%–73%; $p = 0.0002$). However, there was no difference in time to discharge and the study was too small to detect any different in major haemorrhage between FFP and no FFP. An interesting finding of the study was that non-responders in the FFP arm were given FFP significantly earlier post-bite (not post-antivenom) than those who responded to FFP (4.7 hours versus 7.3 hours; $p = 0.002$) [86]. The reason for this finding is not completely clear, but clotting factor studies done in a subgroup of patients demonstrated that those receiving early FFP had evidence of consumption after the FFP was given with increasing D-Dimer and decreasing fibrinogen. However, all these patients had received antivenom prior to the FFP, suggesting that the active clotting factors were endogenous ones activated in the initial consumptive process and not the procoagulant toxin.

Reactions to FFP are well recognised, but are relatively uncommon [87]. There were no adverse reactions in the randomised controlled trial by Isbister et al. in 2013 that could be directly attributed to the FFP. However, the study was small and uncommon complications such as transfusion-related acute lung injury (TRALI) and anaphylaxis must be considered when balancing the risk of FFP versus the benefit. The current evidence would suggest that FFP should be administered in patients with acute bleeding and is more likely to be effective if given more than 6 hours after the bite. However, there is much less evidence to support FFP in patients with VICC without active bleeding, and larger studies are required to better define this patient group. Nevertheless, in life-threatening bleeding from VICC, such as with intracranial haemorrhage, delaying the use of FFP based on these findings is not recommended.

There is far less information on other forms of factor replacement, including cryoprecipitate, prothrombinex, or single factor concentrations. It would seem theoretically useful to give cryoprecipitate to patients bitten by snakes with thrombin-like enzymes, who mainly have a fibrinogen deficiency. However, there is little evidence to support this and patients only need low levels of fibrinogen to have close-to-normal clotting function. One retrospective study included patients given cryoprecipitate, but the numbers were too small for any analysis [21].

Heparin

Heparin has been suggested for the treatment of VICC resulting from viper envenoming, but its use is controversial and there is little evidence to support its effectiveness. Three of five randomised comparison studies concluded that heparin and antivenom are more effective than antivenom alone [24,53,61,63,65]. There was one non-randomised study that concluded no benefit [78]. However, all these studies were poorly designed with poor definition of primary outcomes, blinded allocation, and clotting tests.

Shah et al. reported in 1986 that the addition of heparin resulted in a great proportion of patients with normalised haematological parameters of four different time points compared to antivenom alone [65]. This result needs to be interpreted with caution because it is unclear which parameters had to normalise and multiple time points were used [65]. Paul et al reported two studies investigating the effect of heparin that showed no significant difference despite the conclusion heparin was effective. The first study showed no statistical benefit of the addition of heparin, including no effect on the clotting times, which were the same between antivenom alone and antivenom and heparin [61]. The authors incorrectly suggest that heparin resulted in an improved morbidity and mortality, based on very small and non-significant differences in some outcomes [61]. In the second, smaller study the authors found no significant difference between the antivenom and antivenom with heparin on all outcomes [63].

The three trials that concluded heparin was not effective were all too small (N = 14, 20, 28) to detect any but the largest difference between treatments, due to type II errors. In addition, all these studies had poorly defined outcomes, including no pre-specified primary outcome [24,53,78]. There is insufficient evidence to support the use of heparin, but well-designed, large studies are required to confirm that there is no effect.

Conclusions

VICC is one of the most important clinical syndromes that occurs with snake envenoming and it includes a broad range of factor deficiencies depending on the particular procoagulant toxin in the snake venom. Diagnosis and monitoring of the coagulopathy is problematic, particularly in resource-poor countries where the only clotting test available is the whole blood clotting test. Research is required to develop more reliable cheap clotting tests to be used for the diagnosis and treatment of VICC. Antivenom is the major treatment for VICC, but there is little high-quality evidence to support its effectiveness. Observational studies have suggested that it may be highly effective for some snakes (e.g., *Echis* spp.) and ineffective for other snakes (e.g., Australasian elapids). Antivenom is not risk free and adverse reactions can be quite common and potentially severe. There is evidence to support the use of FFP in bleeding patients with VICC. There is no evidence to support the use of heparin. In all cases it is important to observe for signs of external and internal bleeding. Patients should be observed in hospital until clotting function has normalised.

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Chapter Ten

Discussion and future directions

Discussion and future directions

This thesis has investigated venom-induced consumption coagulopathy (VICC) following snake envenoming, with a focus on data from Russell's viper envenoming, and the effectiveness of antivenom for VICC. In addition, the efficacy of antivenom has been investigated for coagulopathy, as well as other clinically important effects. The systematic reviews highlight the absence of good evidence to support the use of antivenom in VICC. The clinical laboratory studies of VICC in Russell's viper and hump-nosed viper bite improve our understanding of the pathophysiology of VICC in humans. Further studies of antivenom treatment will require animal studies using appropriate animal models and *in vitro* studies.

The first two chapters demonstrate the time course, severity and factor deficiencies in two different vipers, a true viper (Russell's viper) and a pit-viper (hump nosed viper). The coagulopathy seen with Russell's viper envenoming is more severe and results in severe deficiencies of factors V, X and fibrinogen, with unrecordable clotting times (PT and aPTT). It appears to respond to antivenom, although there is limited information in the recovery of clotting factors in Russell's viper envenoming (Phillips et al., 1988, Myint et al., 1985)

In contrast, hump-nosed viper envenoming resulted in incomplete clotting factor consumption mainly of fibrinogen and factor VIII, with only mildly elevated PT and aPTT. The study also showed that the coagulopathy was so mild it was not diagnosed by the commonly available bedside test, the 20 minute whole blood clotting test (WBCT20). This means that detection of coagulopathy in hump-nosed viper envenoming will be a challenge, but also may not be clinically important. Laboratory clotting time tests PT/INR, aPTT, fibrinogen and D-dimer are necessary to detect mild VICC. Although no major systemic bleeding has been reported in our study, previous studies have described life threatening bleeding manifestations in hump-nosed pit viper

envenoming (Kularatne and Ratnatunga, 1999b, Maduwage et al., 2011, Ariaratnam et al., 2008a, de Silva et al., 1994). The pattern of factor deficiencies suggests that venom contains a thrombin-like enzyme, similar to other pit-vipers (see Table 1, chapter nine).

As shown very clearly by the work on the hump nosed viper coagulopathy, definite envenoming can result in very modest clinical effects. In an area of limited resources, combined with the expense and potential adverse effects of antivenoms, a public health decision has to be made about whether to treat subclinical envenoming. The answer will probably very different in a country like Australia with high quality expensive healthcare and high quality expensive antidotes. It may then be appropriate to treat all patients with definite envenoming, whether clinical or subclinical. In Nigeria, or Sri Lanka, the public health decision will probably be very different due to the scarcity of antivenom, its cost and adverse effects, and the resources available for healthcare. In this situation, it is absolutely essential that antivenom is reserved for patients with significant clinical envenoming who are at highest risk of major complications. It is likely to be quite inappropriate to treat patients with modest perturbations of the clotting pathway or other subclinical envenoming, whether antivenom is available or not.

Although there is no placebo randomized controlled evidence to support antivenom for VICC, observational studies provide reasonable evidence that antivenom is effective for VICC in *Echis* envenoming (Mion et al., 2013), but not in VICC due to Australasian elapids (Isbister et al., 2009). There are no good clinical studies that demonstrate that antivenom prevents or speeds the recovery of VICC in Russell's viper envenoming. Recovery of VICC in Russell's viper envenoming takes 24 to 48 hours after antivenom treatment, irrespective of whether repeated doses of antivenom are given. This recovery time is similar to Australian elapids (Isbister et al., 2010) suggesting that after antivenom is given, recovery begins. However, it is difficult to assess the effectiveness of antivenom treatment without a comparison with patients who

are not given antivenom. It will therefore be important to assess the recovery of VICC with and without antivenom in an animal model.

The thesis has also investigated the efficacy of antivenom, focusing on Indian antivenoms for Sri Lankan snakes, but also exploring the problems with lethality studies as an assessment of efficacy and dose for antivenoms. The study on antivenom efficacy found that one Indian antivenom appeared to be superior to the other. More importantly it found that *in vitro* studies of the effects relevant to human envenoming differed to lethality and effective dose studies. The *in vitro* studies appeared to be more in line with binding studies and protein content, suggesting that death in a rodent (e.g. mouse) may not be as relevant for snake envenoming in humans. This raises an important question for future studies – "What is the relevance of death in a rodent to snake envenoming in a human?" This was further supported by the study of the effect of various procoagulant venoms in seven different animal plasmas, demonstrating that human plasma was far more susceptible to the procoagulant venom, compared to rats and other animals, except rabbits. Studies of procoagulant venoms need to be undertaken in rabbits to develop a better animal model of VICC where the effect of antivenom can then be tested.

The assessment of the efficacy of antivenom using clinically relevant venom concentrations (i.e. concentrations reported in clinical envenoming) is essential to make sure that the *invitro* experiments correspond to human envenoming. This is in contrast to lethality studies where the venom dose is based on what kills the mouse (i.e. the LD_{50}) which may be far in excess of the dose seen in human envenoming, or conversely far lower than a dose that causes clinical effects in humans. Therefore, antivenom doses based on ED_{50} may not be appropriate for the treatment of human envenoming.

An important way to determine if an appropriate dose of antivenom has been given is to measure the venom concentration before and after antivenom. However, it was found that for Russell's viper envenoming, venom was detected in over one fifth of post-antivenom patients but they all appeared to

still recover the same and often without repeat antivenom doses. In this thesis we have shown that the re-detection of Russell's viper venom after antivenom is not associated with worsening of VICC, and that fibrinogen continued to recover despite venom being detected. The reason for this finding is that the traditional venom enzyme immunoassay will also detect bound venom (venom-antivenom [VAV] complexes), which has recently been demonstrated (O'Leary and Isbister, 2014, O'Leary et al., 2015). Our study suggests that repeated doses of antivenom for Russell's viper envenoming are unnecessary. This is quite different to a recently described case of Asian pit viper envenoming (Isbister et al., 2014) where undetectable levels of fibrinogen (worsening VICC) occurred simultaneously with venom re-detection (Isbister et al., 2014). A similar phenomenon has been described in North American pit viper envenoming (Seifert et al., 1997, Seifert and Boyer, 2001, Boyer et al., 2013). The worsening of VICC following its initial recovery may be explained by slow absorption of venom from the bite site while antivenom is eliminated more rapidly. This is commonly described in American pit viper envenoming with Fab fragments which are rapidly eliminated compared to $F(ab')_2$ fragments (Boyer et al., 1999, Boyer et al., 2013). Investigation of the pharmacokinetics of venom and antivenoms will be important to understand the true recurrence of venom in the circulation after various types of antivenom treatment (Isbister et al., 2015).

The inability to diagnose envenoming early leads to delayed or inappropriate antivenom administration in snake envenoming. All treatment guidelines have been formulated based on clinical evidence of envenoming or abnormal laboratory tests results which happens after the development of specific systemic effects of envenoming. Administration of antivenom after the development of systemic envenoming is too late to reverse or prevent already developed toxicities. In this thesis we demonstrated, in a preliminary study, that early PLA_2 detection in serum correlates well with the presence of venom and may be a possible early diagnostic test. Investigation of the time course of

clinical effects of envenoming, concentrations of venom and PLA_2 levels will help to understand the usefulness of the PLA_2 test as a diagnostic test.

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Appendix

APPENDIX

List of Other Publications during PhD Candidature (not included as a part of the thesis)

1. 2015. Isbister GK, **Maduwage K**, Saiao A, Buckley NA, Jayamanne SF, Seyed S, et al. (2015) Population Pharmacokinetics of an Indian F(ab')2 Snake Antivenom in Patients with Russell's Viper (Daboia russelii) Bites. *PLoS Neglected Tropical Diseases* 9(7): e0003873. doi:10.1371/journal.pntd. 0003873.

2. 2015. O' Leary MA, **Maduwage K**, Isbister GK. Detection of venom after antivenom administration is largely due to bound venom. *Toxicon*. 93: 112-115.

3. 2014. Isbister GK, **Maduwage K**, Page CB. Antivenom cross neutralisation in a suspected Asian pit viper envenoming causing severe coagulopathy. *Toxicon* 90: 286-90.

4. 2013 Isbister KG, **K. Maduwage**, S. Shahmy, F. Mohamed, C. Abeysinghe, H. Karunathilake, CA Ariaratnam, NA Buckly. Diagnostic 20-min whole blood clotting test in Russell's viper envenoming delays antivenom administration. *Quarterly Journal of Medicine* doi:10.1093/qjmed/hct102.

5. 2013 O'Leary M, **Maduwage K**, Isbister, GK. Use of immunoturbidimetry to detect venom-antivenom binding using snake venoms. *Journal of Pharmacology and Toxicological Methods* 67: 171-181.

6. 2013. **Maduwage K**, Isbister GK, Silva A, Bowatta S, Mendis S, Gawarammana I. Epidemiology and clinical effects of Hump-nosed pit viper (Genus: *Hypnale*) envenoming in Sri Lanka *Toxicon* 61: 11-15.

7. 2016. **Maduwage K**, O'Leary M, Silva A, Isbister GK. Detection of Snake Venom in Post-Antivenom Samples by Dissociation Treatment Followed by Enzyme Immunoassay *Toxins 8*, 130; doi:10.3390.

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Population Pharmacokinetics of an Indian F(ab')$_2$ Snake Antivenom in Patients with Russell's Viper (*Daboia russelii*) Bites

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OPEN ACCESS

Citation: Isbister GK, Maduwage K, Saiao A, Buckley NA, Jayamanne SF, Seyed S, et al. (2015) Population Pharmacokinetics of an Indian F(ab')$_2$ Snake Antivenom in Patients with Russell's Viper (*Daboia russelii*) Bites. PLoS Negl Trop Dis 9(7): e0003873. doi:10.1371/journal.pntd.0003873

Editor: José María Gutiérrez, Universidad de Costa Rica, COSTA RICA

Received: April 4, 2015

Accepted: June 2, 2015

Published: July 2, 2015

Data Availability Statement: Data are available on NOVA, the University of Newcastle's institutional digital repository at the URL http://hdl.handle.net/1959.13/1063469

Funding: The study was supported in part by NHMRC Project Grant 631073. GKI is funded by an NHMRC Senior Research Fellowship ID1061041. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Background

There is limited information on antivenom pharmacokinetics. This study aimed to investigate the pharmacokinetics of an Indian snake antivenom in humans with Russell's viper bites.

Methods/Principal Findings

Patient data and serial blood samples were collected from patients with Russell's viper (*Daboia russelii*) envenoming in Sri Lanka. All patients received Indian F(ab')$_2$ snake antivenom manufactured by VINS Bioproducts Ltd. Antivenom concentrations were measured with sandwich enzyme immunoassays. Timed antivenom concentrations were analysed using MONOLIXvs4.2. One, two and three compartment models with zero order input and first order elimination kinetics were assessed. Models were parameterized with clearance (CL), intercompartmental clearance(Q), central compartment volume(V) and peripheral compartment volume(V_P). Between-subject-variability (BSV) on relative bioavailability (F) was included to account for dose variations. Covariates effects (age, sex, weight, antivenom batch, pre-antivenom concentrations) were explored by visual inspection and in model building. There were 75 patients, median age 57 years (40-70y) and 64 (85%) were male. 411 antivenom concentration data points were analysed. A two compartment model with zero order input, linear elimination kinetics and a combined error model best described the data. Inclusion of BSV on F and weight as a covariate on V improved the model. Inclusion of pre-antivenom concentrations or different batches on BSV of F did not. Final model

Competing Interests: The authors have declared that no competing interests exist.

parameter estimates were CL,0.078 Lh^{-1}, V,2.2L, Q,0.178Lh^{-1} and V_P,8.33L. The median half-life of distribution was 4.6h (10-90%iles:2.6-7.1h) and half-life of elimination, 140h (10^{th}-90^{th} percentilesx:95-223h).

Conclusion

Indian $F(ab')_2$ snake antivenom displayed biexponential disposition pharmacokinetics, with a rapid distribution half-life and more prolonged elimination half-life.

Author Summary

Snake envenoming is a neglected tropical disease that affects hundreds of thousands of people in the rural tropics. Antivenom is the main treatment for snake bites but there is limited information on the pharmacokinetics and appropriate dosing regimen. Most studies have been done in animals and dosing guidelines are based on arbitrary and often irreversible clinical signs. In this study we measured serial antivenom concentrations in patients with Russell's viper envenoming given antivenom. Using this data we modelled the pharmacokinetics of antivenom in the population and showed that antivenom concentrations had a bi-exponential decay with an initial decrease over 12 hours and then a slow decrease over days. There was significant variability in the dose given which was not affected by the particular antivenom batch given. The presence of venom did not appear to modify the pharmacokinetics of antivenom. Understanding the time course of antivenom in patients with snake envenoming will provide a better basis for antivenom dosing.

Introduction

Snake envenoming is a major health issue in South and South-eastern Asia [1]. Although antivenom is the most important treatment for snake envenoming, it can cause early systemic hypersensitivity reactions [2, 3], and there is limited evidence to support currently practiced dosing schedules. Dosing and assessment of the effectiveness of antivenom in human envenoming remains controversial and treatment protocols are not based on the kinetics of venom or antivenom. There are few studies of the pharmacokinetics of antivenom, and most of these are in animals [4].

Snake envenoming is a common problem in Sri Lanka and large amounts of antivenom are used throughout the country each year. A number of different Indian antivenoms are currently used and the initial dose ranges from 10 to 20 vials [5–7]. The initial dose is based on ED50 studies and clinical experience by titrating dose against the resolution of coagulopathy and neurotoxicity. However, the clinical effects of envenoming in these species are generally irreversible so determining if enough antivenom has been given and deciding to re-dose is often arbitrary and not based on whether all venom has been bound, or on the pharmacokinetics of antivenom. Measurement of venom and antivenom concentrations in patients with snake bite is required to improve effective initial and repeat dosing [8].

The pharmacokinetics of antivenom are expected to be similar to other intravenous drugs being delivered to the central compartment with zero order input kinetics (constant rate of infusion). Antivenom is then distributed throughout the body and is eliminated by the kidneys and/or the reticuloendothelial system [4]. Decreasing antivenom concentrations in the central compartment are therefore due to both distribution and elimination. Different types of

antivenom have different pharmacokinetics due to the difference in their molecular masses [4]. Fab antivenoms have much larger volumes of distribution (V_D) than $F(ab')_2$ or whole IgG [5, 9]. Most studies of antivenom pharmacokinetics show a biphasic (two-compartment) decline after intravenous administration of whole IgG and $F(ab')_2$ antivenoms, as a result of an initial rapid decline (distribution phase) and a slower decline (terminal elimination phase) [4, 9].

Most studies of the pharmacokinetics of antivenom are in animals [4, 10], and the pharmacokinetics appear to differ between animals making animal models problematic for defining the pharmacokinetics of antivenom in humans [10]. Although there have been several publications of antivenom concentrations in snake envenoming, there are only a few studies of the pharmacokinetics of antivenom in human snake envenoming [4, 5, 9, 11–14]. These studies were all in small numbers of patients using a classic two phase approach, without including input processes (i.e. delivery of the antivenom, usually via an infusion to the central compartment as a zero order process) and providing limited information on the pharmacokinetics and variation between patients.

A population approach to pharmacokinetic analysis is increasingly being used to define the pharmacokinetics of drugs in humans because it provides information about population variability and the need for individualisation of drug treatment. The traditional approach to pharmacokinetic analysis (two stage analysis) estimates the pharmacokinetic parameters for each individual patient and then provides summary statistics which only give a population average and standard error. In contrast the population approach estimates the typical value of each parameter for the population and the variability of the parameters simultaneously. This provides an estimate of unexplained random variation and allows the effects of covariates to be accounted for in the model (e.g. weight, renal function). There are no previously published population pharmacokinetic analyses of antivenom in humans or animals.

The aim of this study was to investigate the pharmacokinetics of antivenom in patients with snake bites using a population based analysis, including an investigation of the covariates that may influence the pharmacokinetics of antivenom.

Methods

This was a population pharmacokinetic analysis of an $F(ab')_2$ antivenom using data and serial antivenom concentrations collected in snake-bite patients admitted to a single hospital in Sri Lanka. The patients were recruited from within a large cohort of snakebites admitted to the Base Hospital Polonnaruwa in Central Eastern Sri Lanka.

Ethics statement

The study was approved by the Ethical Review Committee, Faculty of Medicine, University of Peradeniya, Sri Lanka. All patients gave written and informed consent for the collection of clinical data and blood samples.

Patients

All patients (>15 years old) from October 2010 to March 2012 with a suspected snake bite who presented to the Base Hospital Polonnaruwa were recruited to a prospective cohort study. Those with coagulopathy were then entered in a dose finding randomised clinical trial of fresh frozen plasma. The entry criteria for the trial was a suspected Russell's viper (*Daboia russelii*) bite with coagulopathy defined as an abnormal 20 minute whole blood clotting test (20WBCT). This resulted in a small number of patients being recruited where Russell's viper (*D. russelii*) venom was not detected and on further testing, hump-nosed viper (*Hypnale* spp.)

venom was detected (in some *Hypnale* bites the 20WBCT and coagulation studies may be abnormal [15, 16]).

In this pharmacokinetic study, patients were only recruited from the clinical trial and were included if they had serial serum collection for antivenom measurement and complete demographic details (including weight). All patients received the Indian polyvalent snake antivenom intravenously manufactured by VINS Bioproducts Limited (batch numbers: 1060 [MFD 2008], 1096 [MFD 2009], 1102 [MFD 2009], 01015/10-11 [MFD 2010], 01AS11112 [MFD 2011]). For a dose of antivenom, each of 10 vials of antivenom are reconstituted in 10ml of normal saline for a total of 100ml of antivenom. From a 500ml bag of normal saline 100ml volume is removed and replaced by the 100ml of antivenom so the 10 vials are administered in a total of 500ml of normal saline. This is given over 1 hour.

Data collection

The following data were collected prospectively in all cases: demographics (age, sex and weight), time of the snake bite, clinical effects (local envenoming, coagulopathy, bleeding and neurotoxicity) and antivenom treatment (dose, time of administration and antivenom batch number). Blood samples were collected for research on admission and regularly throughout each patient admission. Blood was collected in serum tubes for venom-specific enzyme immunoassay (EIA) and antivenom EIA. All blood samples were immediately centrifuged, and then the serum aliquoted and frozen initially at -20°C, and then transferred to -80°C within 2 weeks of collection.

Enzyme immunoassays for venom and antivenom

A sandwich enzyme immunoassay was used to measure antivenom in serum samples as previously described [8, 17]. The plate was first coated with Russell's viper venom and then stored and blocked overnight. Serum was then added to the plates. The detecting antibodies were conjugated with horseradish peroxidase. Russell's viper (*D. russelii*) and hump-nosed (*Hypnale* spp.) viper venoms were measured in samples with a venom specific enzyme immunoassay as previously described [6, 8, 17]. Briefly, polyclonal IgG antibodies were raised in rabbits against Russell's viper (*D. russelii*) and hump-nosed viper (*Hypnale* spp.) venom. The antibodies were then bound to microplates and also conjugated to biotin for a sandwich enzyme immunoassay using streptavidin-horseradish peroxidase as the detecting agent. All samples were measured in triplicate, and the averaged absorbance converted to a concentration using a standard curve made up with serial dilutions of antivenom and using a sigmoidal curve. The limit of quantification for the antivenom enzyme immunoassay assay was 40μg/ml and for the venom enzyme immunoassay was 2ng/mL for Russell's viper and 0.2ng/ml for hump-nosed viper.

Pharmacokinetic analysis

Patient data was analysed using MONOLIX version 4.2 (Lixoft,Orsay, France. www.lixoft.com). MONOLIX uses the Stochastic Approximation Expectation Maximization algorithm (SAEM) and a Markov chain Monte-Carlo (MCMC) procedure for computing the maximum likelihood estimates of the population means and between-subject variances for all parameters [18]. One, two and three compartment models with zero order input and first order elimination kinetics were assessed and compared to determine the best structural model. Proportional and combined models were evaluated for the residual unexplained variability. Method M3 was used to deal with antivenom concentrations below the limit of quantification (BLQ) [19]. Between-subject variability (BSV) was included in the model and assumed to have log-normal distribution.

Models were parameterized in terms of volume of distribution (V_D; V, V_P, V_{P2}), clearance (CL), inter-compartmental clearance (Q; Q1, Q2) and relative bioavailability (F) for either 1-, 2- or 3-compartment models. Initial estimates of parameters were taken from a previous pharmacokinetic study of anti-venom [9].

Uncertainty in antivenom dose was included in the model by allowing BSV on F to account for batch to batch variation in antivenom (five different batches) and for variation within batches. F was fixed to 1 and the BSV was estimated for each patient similar to including uncertainty on dose as previously described [18]. The BSV on F was plotted for each batch to determine if there was a difference between batches.

The effect of covariates, including age, sex, weight, and pre-antivenom concentrations in patients with detectable venom, were explored by visual inspection of the individual parameter estimates versus the covariate of interest. Age, sex and pre-antivenom concentrations were not included in the final model evaluation due to the absence of an association visually. The influence of weight (wt) on volume was included in the modelling process. Weight was assumed to be related to V by a power function. The covariate was centred to the average weight. Thus in the model the estimation of the effect of weight on volume is:

$$V = \theta_V \times (wt/wt_{av}) \wedge f_{wt}$$

Where θ_V is the typical value of volume of distribution, wt is the individual patient weight, wt_{av} is the average weight and f_{wt} accounts for the influence of wt on volume.

Model selection decisions were based on a decrease in the objective function (OFV), a decrease in residual error, clinical relevance of the pharmacokinetic parameters and goodness of fit plots. The log likelihood was computed for each model and used to discriminate through the difference in log likelihood (−2LL). A p-value of 0.05 was considered statistically significant, equivalent to a drop in OFV by 3.84.

Simulations

From the final model we simulated 1000 patients using the individual predicted patient parameters from the final model with MatLab to explore different initial doses and repeat doses. The following scenarios were explored:

1. One dose (10 vials) of antivenom given with infusions rates of 20 minutes, 1 hour and 2 hours.
2. Two doses of antivenom given, each over 1 hour and 6 hours apart.
3. Two doses of antivenom given, each over 1 hour and 12 hours apart.

The median antivenom concentration versus time was plotted with 10% and 90% percentiles.

Results

Patients

There were 75 patients with a median age of 38 years (16 to 64y) and 64 were male. Seventy one were Russell's viper envenoming cases and 52 of these had detectable venom prior to the administration of antivenom. Four patients had hump-nosed viper envenoming (confirmed by detectable hump-nosed viper venom). In all four patients with hump-nosed viper envenoming there was a steady decline of venom concentrations despite the administration of antivenom consistent with the antivenom not being raised against this snake venom. In nineteen patients meeting the inclusion criteria venom was not detected prior to antivenom, most likely because the blood was collected prior to envenoming. The demographics of the patients are listed in Table 1.

There were 510 antivenom concentration data points but only 411 had detectable antivenom, the other 99 were serial samples after the disappearance of antivenom. There were 54 patients who had a single dose of antivenom who had 265 antivenom concentration measurements with a median of five antivenom concentrations in each patient (Range: 2 to 10), and a median antivenom concentration of 1607μg/ml (Range: 40 to 13673μg/ml). There were 21 patients who had multiple doses of antivenom who had 146 antivenom concentrations with a median of seven antivenom concentrations in each patient (Range: 3 to 11) and a median antivenom concentration of 2293μg/ml (Range: 40 to 12599μg/ml). The observed concentration versus time data is shown in Fig 1.

Pharmacokinetic analysis

A two compartment model with zero order absorption and linear elimination kinetics and a combined error model best described the data. The final model incorporated BSV on F, which was fixed to 1 to allow variability between patients in dose. The model also incorporated weight as a covariate with a power effect on central volume, V. The inclusion of pre-antivenom concentrations on BSV of F did not improve the model. Plots of the BSV on F versus the batch number showed no relationship between the batch and BSV on F (S1 Fig). The final model

Table 1. Demographics and clinical information of 75 patients who were administered Indian antivenom, including clinical features of envenoming, treatment and outcomes.

	Median (range)	Number (%)
Age (years)	38 (16 to 64)	75 (100)
Male	-	64 (85)
Weight (kg)	57 (40 to 70)	
Clinical Effects		
Local envenoming		73 (97)
Coagulopathy (20WBCT+)		75 (100)
Systemic Bleeding		26 (35)
Neurotoxicity (ptosis)		32 (43)
Antivenom batch		
1060 (MFD 2008)		1 (1)
1096 (MFD 2009)		8 (11)
1102 (MFD 2009)		12 (16)
01015/10-11 (MFD 2010)		40 (53)
01AS11112 (MFD 2011)		14 (19)
Antivenom dose (vials)	18 (8 to 40)	
Repeat antivenom dose		21 (28)
Median pre antivenom venom concentration ng/ml (range)	169 (2 to 2805)†	52 (69)
Fresh Frozen Plasma (FFP)		
2 Units FFP		44 (59)
4 Units FFP		14 (19)
8 Units FFP		1 (1)
Length of hospital stay (days)	2 (1 to 10)	
Antivenom concentrations (μg/ml)	1916 (27 to 13673)	392 samples*

* Samples with antivenom measured in them from a total of 510 samples, 128 had no detectable antivenom;

† Russell's viper venom only detectable in 53 patients; 20WBCT– 20 minute whole blood clotting test.

doi:10.1371/journal.pntd.0003873.t001

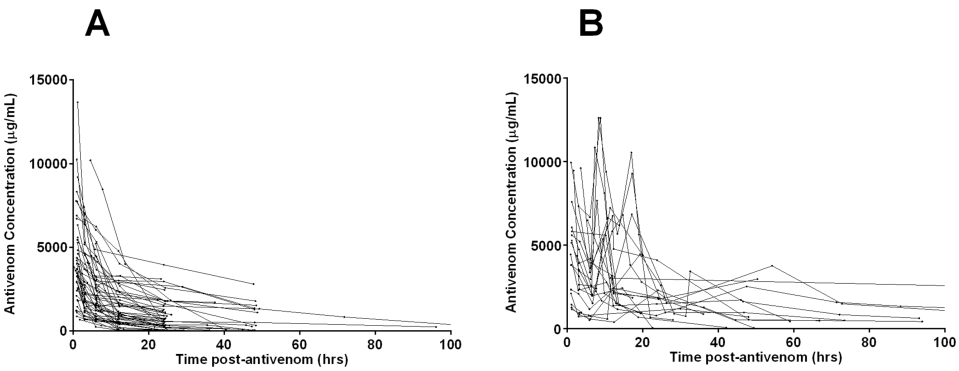

Fig 1. Plots of the observed antivenom concentration (µg/ml) versus time for patients given a single dose of antivenom (A), and for patients given multiple antivenom doses (B).

doi:10.1371/journal.pntd.0003873.g001

parameter estimates were CL, 0.078 Lh^{-1}, V, 2.2L, Q, 0.178Lh^{-1} and V_P, 8.33L. The median half-life of distribution was 4.6h (10^{th}-90^{th} percentiles: 2.6 to 7.1h) and the half-life of elimination, 140h (10^{th}-90^{th} percentiles: 95 to 223h). There was no difference in the parameter estimates between those with Russell's viper envenoming with detectable venom prior to antivenom (52), those with Russell's viper envenoming and no detectable venom prior to antivenom (19) and those with hump-nosed viper envenoming (S2 Fig). S3 and S4 Figs shows the goodness-of-fit plots for the final model. The individual PK parameter estimates from the base models with modelling decisions and final model parameters are described in Table 2. There

Table 2. Parameter estimates using Monolix version 4.2.

Mean value (rse%)	Base model	Model 1 Including F	Model 2 Including weight on V	Model 3 (Final) Including F and weight on V
Structural model (θ)				
CL (Lh^{-1})	0.0445 (50)	0.038 (65)	0.129 (23)	0.0779 (34)
V (L)	2.23 (9)	1.98 (11)	2.12 (11)	2.16 (10)
Q (Lh^{-1})	0.171 (21)	0.271 (20)	0.107 (38)	0.178 (31)
Vp (L)	17.6 (50)	14.1 (43)	3.27 (121)	8.33 (52)
f_{wt}	-	-	0.137 (96)	0.132 (84)
F	-	1 (-)	-	1 (-)
Between subject variance (ω)				
Cl	1.64 (47)	0.451 (81)	0.886 (30)	0.715 (46)
V	0.42 (22)	0.0627 (74)	0.419 (30)	0.188 (126)
Q	0.738 (33)	0.237 (71)	2.57 (84)	0.533 (57)
Vp	1.24 (57)	1.62 (56)	0.782 (282)	0.836 (125)
F	-	0.309 (23)	-	0.197 (42)
Objective Function (*-2 log-likelihood value)*	6840.81	6823.54	6835.96	6820.75

CL = clearance, V = volume of the central compartment, Q = intercompartmental clearance, Vp = volume of the peripheral compartment, fwt = effect of weight on V, F = relative bioavailability.

doi:10.1371/journal.pntd.0003873.t002

was also no difference in parameter estimates between patients given 1 dose of antivenom and those given 2 doses, or between patients with different initial venom concentrations (S5 Fig).

Simulations for one dose (10 vials) of antivenom given over 20 minutes, 1 hour and 2 hours shows there is a slightly lower and later peak antivenom concentration with slower infusions (Fig 2). Simulations for two doses of antivenom shows that antivenom concentrations decrease rapidly after each dose and there are low but persistent levels of antivenom after one dose and both two doses regimens (Fig 3).

Discussion

The study adds to the limited information available on the pharmacokinetics of antivenom in humans supporting previous studies [4, 9]. Indian $F(ab')_2$ snake antivenom displayed biexponential disposition pharmacokinetics, with a rapid half-life of distribution and a much longer half-life of elimination. Weight accounted for some of the variability in the central volume, and the volumes of the central and peripheral compartment were consistent with a large molecule which does not have a large volume of distribution. Including variability on F improved the model showing that there was significant random variability in dose. The plots in Figs 2 and 3 show the expected antivenom concentration profiles in the first 24 hours after administration.

Previous human and most animal studies have also shown a biexponential decay in antivenom concentrations [4, 9, 20, 21], with similar values for the distribution half-life of 2 to 4 hours and much longer elimination half-life of 90 to 230h. Previous studies have been small with 10 or less patients in each analysis (for different antivenoms) and a classic two phase approach has been undertaken. Such an approach will over-estimate the error and not account for true random variability or covariate effects. In this study we have undertaken a population approach, which provides information on the variability of the pharmacokinetics in the population and an improved model by including weight and variability in dose. Previous studies have not shown why they chose particular models (2-compartment versus 3-compartment), with no statistical criteria or goodness of fit plots.

Some previous animal models and one human study have described the pharmacokinetics with a tri-exponential decay in animals [14, 22, 23]. These analyses have not included an input process in the analysis which will bias the estimation of the disposition parameters, particularly with three or more compartments when the initial very short half-life is similar to the time of the input phase. Ismail et al. estimated the initial rapid half-life in animals to be 0.2h and Vazquez et al estimated it to be 0.25h, which are both similar to the usual infusion rate of antivenom over 10 to 30 minutes. It is possible that there is only 2-compartmental disposition kinetics in these studies, and future pharmacokinetic analyses need to include an input phase in the model. A possible limitation of our study was that there may have been insufficient sampling in the initial period after antivenom administration to detect a third compartment. In contrast to this, Vazquez et al were likely to have taken samples in the input phase, since the first sample was taken 5min after antivenom administration, although they do not report the infusion time or rate [14].

One animal study of a $F(ab')_2$ has shown that the pharmacokinetics of antivenom are the same in envenomed and non-envenomed rabbits [24]. This is consistent with this study demonstrating that pre-antivenom venom concentrations did not influence the pharmacokinetics of antivenom, including different initial venom concentrations (S5 Fig). However, this may be different for Fab antivenoms where high molecular weight toxins may change the route of elimination from renal (for free Fab antivenom) to phagocytosis/reticulo-endothelial system for Fab-toxin molecules. The latter has been shown in rabbits with anti-*Vipera* Fab antivenom [25].

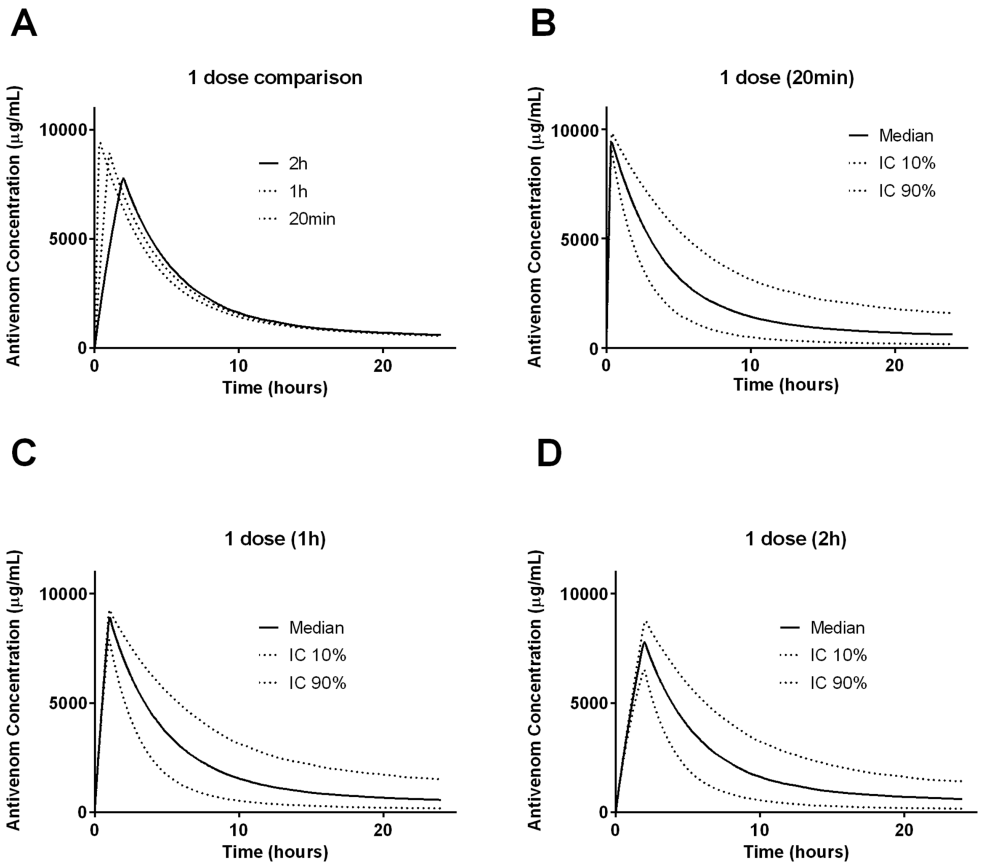

Fig 2. Plots of antivenom concentration versus time for 1000 patients simulated from the final model individual predicted patient parameters for 10 vials of antivenom given over 20min, 1h and 2h, comparing the median concentrations for all three regimens (A), and the median and 10% and 90% percentile concentrations for a 20 minute infusion (B), 1 hour infusion (C) and 2 hour infusion (D).

doi:10.1371/journal.pntd.0003873.g002

There has always been concerns about the variability between different batches of antivenom leading to potential differences in the dose administered between batches. The study did not support this concern and found that there was no difference in F on average between different batches (S1 Fig). However, the study found that including between subject variability on relative bioavailability did improve the model. This suggests there was random variability in the dose administered which is likely to be due to variable losses occurring during reconstitution of the individual freeze dried vials of antivenom. So, although there may be variability between batches, the variability in dosing errors appears to be larger than the differences between batches.

There are a number of limitations to the study including the fact that the sample collection was not optimally designed and sample times (windows) were based on timing of clinical samples and other research assays required for the clinical trial. This is unlikely to have a major

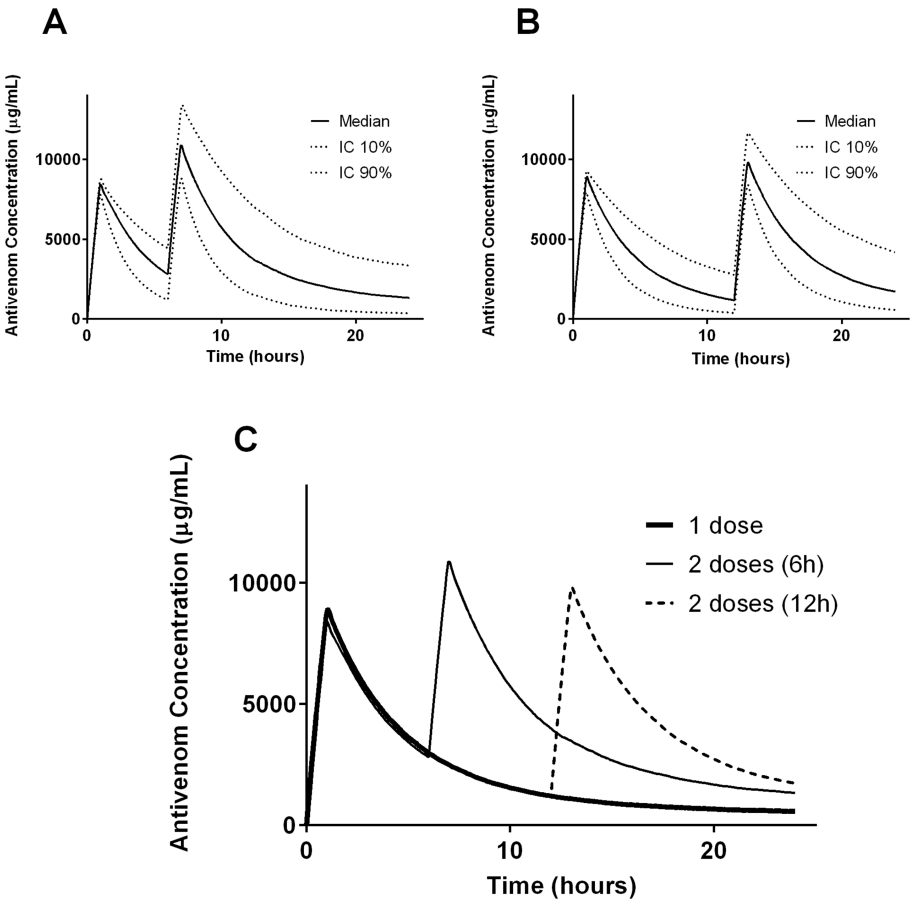

Fig 3. Plots of antivenom concentration versus time for 1000 patients simulated from the final model individual predicted patient parameters for patients given two doses of antivenom, the first given 10 vials over 1 hour and then repeated at 6 hours (A) and the second given 10 vials over 1 hour and then repeated at 12 hours (B), showing median, 10% and 90% percentile concentrations. The two regimens with repeat doses are compared to a single dose in Panel C.

doi:10.1371/journal.pntd.0003873.g003

influence on the analysis because a population approach will allow for both sparse and rich sampling in patients. Another issue is that antivenom is not a pure substance and consists of varying amounts of polyclonal antibodies to multiple toxins in the venom with varying affinities. However, the assay uses a single detecting antibody (anti-horse antibody), so will detect all antibodies against the snake toxins irrespective of their toxin target or affinity. Finally, the assay will only detect antibodies that bind to the snake toxins. In most antivenoms, specific antibodies to snake toxins make up only 10 to 20% of the total protein/immunoglobulin

content. This is unlikely to have affected the pharmacokinetic analysis because only immunoglobulins binding to snake toxins are relevant to the analysis.

This population pharmacokinetic analysis demonstrates that Indian $F(ab')_2$ antivenom has biexponential disposition kinetics and following an initial decline in antivenom concentrations in the first 12 hours, low concentrations are present for days after administration. The study demonstrates that the antivenom concentrations were not affected by the initial venom concentrations suggesting that sufficient antivenom in excess of the venom was being administered. Understanding the pharmacokinetics of antivenom may assist in improving antivenom dosing by matching antivenom pharmacokinetics to the neutralisation of venom (pharmacodynamics), as well as clinical effects.

Supporting Information

S1 Fig. Plots of the relative fraction absorbed (F) versus batch number.
(TIF)

S2 Fig. Plots comparing Russell's viper envenomed patients to hump-nosed viper envenomed patients for clearance (A), central volume (B), inter-compartmental clearance (C), peripheral volume (D), half-life of distribution (E), half-life of elimination (F) and the relative fraction absorbed (G).
(TIF)

S3 Fig. Goodness-of-fit plots including a visual predictive check (VPC) showing the observed data with the 10th, 50th, and 90th percentiles.
(TIF)

S4 Fig. A plot of observations versus individual predictions from the final model.
(TIF)

S5 Fig. Plots comparing patients receiving one versus two doses of antivenom and comparing 3 different pre-antivenom venom concentrations (Low– 2 to 60ng/ml; Medium– 61 to 300ng/ml; High—>300ng/ml) for clearance (A), central volume (B), inter-compartmental clearance (C), peripheral volume (D), half-life of distribution (E), half-life of elimination (F) and the relative fraction absorbed (G).
(TIF)

Acknowledgments

We acknowledge the assistance of the medical and nursing staff at Polonaruwa hospital for identifying patients for the study. We acknowledge Andrew Dawson and staff of the South Asian Clinical Toxicology Research Collaboration for assistance throughout the study.

Author Contributions

Conceived and designed the experiments: GKI KM AS NAB DGL HJdS. Performed the experiments: SS CA SFJ IG HK UC FM. Analyzed the data: GKI AS AM KM. Wrote the paper: GKI KM AS NAB DGL HJdS FM. Antivenom analysis: KM.

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Toxicon 93 (2015) 112–118

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

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

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ARTICLE INFO

Article history:
Received 16 September 2014
Received in revised form
31 October 2014
Accepted 5 November 2014
Available online 15 November 2014

Keywords:
Venom
Antivenom
Enzyme immunoassay
Recurrent envenoming
Russell's viper
Venom–antivenom complexes

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

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http://dx.doi.org/10.1016/j.toxicon.2014.11.221
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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

ULaRVV was made up to a total volume of 1 ml in suspension in PBS and stored at 4 °C.

2.5. Measurement of free venom using UltraLink resins

A series of venom–antivenom mixtures of 750 μL volume were prepared in 3% BSA/PBS with a constant venom concentration of 5 ng/mL and antivenom concentrations ranging from 0 to 25 μg/ml. The venom–antivenom mixtures were allowed to stand for one hour and then divided in half. To the first half was added ULaH (125 μL, 87 μg aH) in 3% BSA/PBS. To the second half was added 3% BSA/PBS alone. The venom–antivenom mixtures were put on a rotary mixer for one hour. Venom was then measured in the mixtures by using the venom specific ELISA as above. Standard curves were based on serial dilutions of the tubes containing no antivenom ± ULaH.

Patient samples (10 μL) were diluted to 800 μL with 2% BSA/PBS and then divided in half. To the first half was added ULaH suspension (50 μL) and to the second half was added PBS (50 μL). The samples were put on a rotary mixer for one hour and then allowed to settle. Venom and VAV were measured in the supernatant at a dilution of 50% in PBS. Antivenom was measured in the supernatant using ELISA at a dilution of 10% in PBS.

2.6. Measurement of free antivenom using UltraLink resins

Patient samples (10 μL) were again diluted to 800 μL with 3.5% BSA/PBS, and divided in half. To the first half was added ULaRVV suspension (100 μL), and to the second half PBS (100 μL). The samples were put on a rotary mixer for one hour and allowed to settle. Antivenom was measured in the supernatant using ELISA at a dilution of 7% in PBS. Venom and VAV were also measured in the supernatant at a dilution of 70% in PBS.

3. Results

3.1. Measurement of venom, antivenom and VAV in patient samples

Antivenom and venom concentrations were measured in 427 patients with Russell's viper envenoming. Of these there were 350 envenomed patients, but only in 255 were there four or more samples available. In 79 of the 255 (31%) venom was detected by the venom specific ELISA after the administration of antivenom (Fig. 1A,B). In these samples, antivenom was always present at concentrations sufficient to neutralise the venom *in vitro* based on measurement of venom in venom–antivenom mixtures (Fig. 2; see below). VAV was measureable in all cases where there was venom detected post-antivenom (Fig. 1A,B). In contrast, VAV was only measureable in some cases where venom was not detected (Fig. 1C). Fig. 1 shows the time course of venom, antivenom and VAV in three patients as examples of patients with and without recurrence of detectable venom after the administration of antivenom.

3.2. In vitro measurement of venom and VAV in venom–antivenom mixtures

To explore conditions in which both venom and antivenom can be detected in the same sample, we carried out a chequerboard titration, in which serial dilutions of venom were incubated with serial dilutions of antivenom. A plot of the measured venom concentration against the known antivenom concentration in each mixture with a different initial venom concentration produces a family of curves (Fig. 2A). These plots demonstrate that the antivenom concentrations measured in patient samples post-antivenom are sufficient to bind all venom *in vitro*, based on the patient's pre-antivenom venom concentrations. For example, in Fig. 1A, the antivenom and venom concentrations measured post-antivenom were approximately 1900 μg/ml and 600 ng/ml

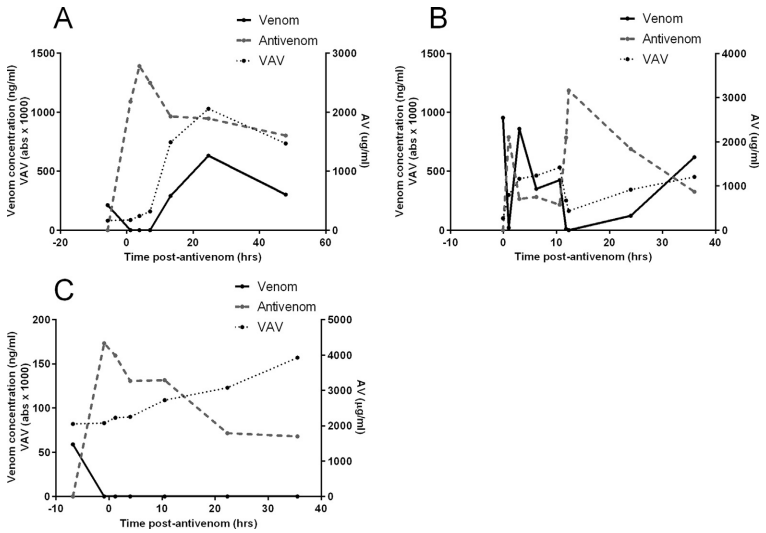

Fig. 1. Measurement of venom concentrations (ng/ml; thick black line), antivenom concentrations (μg/ml; dashed line) and venom–antivenom complexes (VAV; absorbance; dotted line) in a patient with recurrence given one dose of antivenom (A), a patient with recurrence given two doses of antivenom 11 h apart (B) and a patient without recurrence with measurable VAV (C).

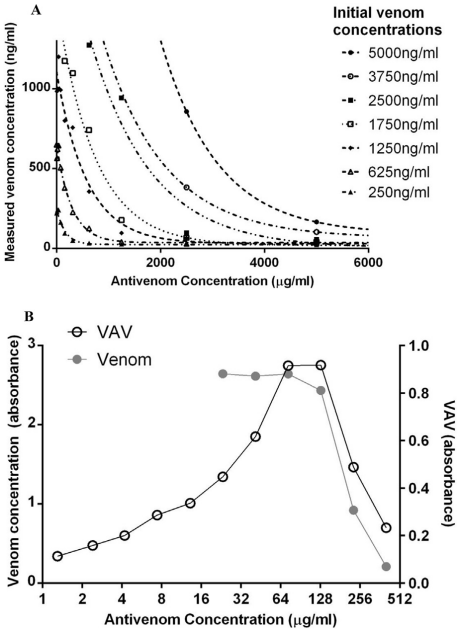

Fig. 2. A: The effect of increasing concentrations of antivenom on the measurement of venom in mixtures of venom and antivenom for different initial venom concentrations. The measured venom concentration is plotted against antivenom concentration for each initial venom concentration. Curves are fitted with a one phase exponential decay model. B: Measurement of venom (initial concentration 500 ng/ml) and VAV in serial dilutions of antivenom demonstrating that for certain antivenom concentrations both venom and VAV can be detected (9).

respectively. Reading these concentrations on Fig. 2A, shows that this would only be possible if the pre-antivenom concentration in this patient (i.e. without antivenom) was greater than 2500 ng/ml. However, the measured pre-antivenom concentration was only 200 ng/ml. This supports the contention that the venom measurement includes bound venom or VAV.

Venom and VAV were simultaneously measured using ELISA in incubated mixtures of venom and antivenom. Venom was added to serial dilutions of antivenom such that its concentration in the mixture was 500, 250 or 100 or 50 ng/ml. A plot of measured venom and measured VAV versus antivenom concentrations for a single initial venom concentration of 500 ng/ml is shown in Fig. 2B. Measureable venom did not begin to decline until the antivenom concentration was past the point at which VAV is at a maximum.

3.3. Measurement of venom in venom–antivenom mixtures treated with UltraLink resin

Venom concentrations were measured in venom–antivenom mixtures with and without the addition of ULaH, with an initial venom concentration of 5 ng/ml and serial dilutions of antivenom (0.39–25 μg/ml). Measurement of venom in the supernatant showed there to be significantly less free venom detected in mixtures to which ULaH had been added compared to those without (Fig. 3). Venom concentrations were also measured in samples containing venom and ULaH without antivenom and showed that non-specific absorption of venom by ULaH was relatively small. The

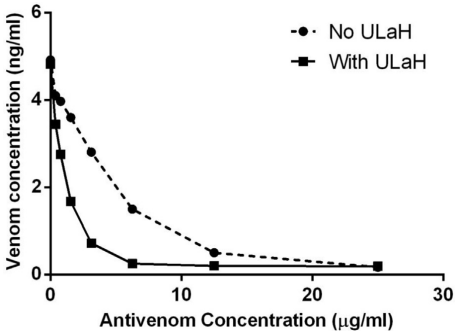

Fig. 3. Measurement of Russell's viper venom in pre-incubated mixtures of venom and antivenom (initial venom concentration of 5 ng/ml and serial dilutions of antivenom), with (thick line) and without (dashed line) added ULaH.

decrease in measureable venom with the addition of ULaH suggests that a proportion of the measurable venom is bound to antivenom.

3.4. Measurement of venom, antivenom and VAV in patient samples treated with UltraLink resins

ULaH was added to a series of 15 patient samples, which were then analysed for Russell's viper venom, antivenom and VAV (Table 1; Fig. 4). In nine 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. The reduction in venom measurement suggests both bound and free venom were present. Antivenom and VAV were no longer detectable in samples treated with ULaH.

ULaRVV was added to five patient samples, which were then analysed for Russell's viper venom, antivenom and VAV (Table 2).This material effectively removed venom and VAV from patient samples, but had no effect on measured antivenom concentrations (Table 2). This indicates that the measured antivenom is free, not bound to venom.

3.5. Measurement of venom, antivenom and VAV in brown snake envenoming cases

ULaH was added to five samples from three of 115 patients with brown snake envenoming where venom had been detected post antivenom (Allen et al., 2012). These samples were assayed for venom and compared to samples where ULaH was not added. This again showed that in post-antivenom samples the amount of venom detected was markedly reduced compared to no reduction in pre-antivenom samples.

4. Discussion

This study has shown that the venom specific ELISA measures both free and bound venom, and it cannot be assumed that venom detected post antivenom is free (i.e. potentially active). VAV was detected in post-antivenom patient samples which mirrored the venom measurement confirming that in these patients the greatest proportion of venom detected post-antivenom was bound venom. In addition, when ULaH resin was added to these samples the amount of venom measured was markedly reduced, demonstrating that the venom detected post-antivenom was mainly bound to antivenom, rather than free venom. This has significant

Table 1
Measurement of Russell's viper venom (RVV), antivenom (AV) and venom–antivenom complexes (VAV) in 15 samples from five different patients with and without anti-Horse IgG bound to UltraLink resin (ULaH).

Patient	Time post-AV (h)	RVV (ng/ml)		$\%\Delta_{RVV}$	AV (μg/ml)		VAV (absorbance)	
		Nil	ULaH		Nil	ULaH	Nil	ULaH
1	Pre-AV	521	428	18%	0	0	0.07	0.06
1	6	549	109	80%	972	20	0.24	0.07
1	12	769	136	82%	1416	32	0.28	0.08
1	24	510	88	83%	1112	39	0.26	0.07
1	48	249	31	88%	721	23	0.19	0.06
2	Pre-AV	322	204	37%	0	0	0.07	0.06
2	2	407	93	77%	1450	19	0.32	0.07
2	8[a]	1	4	–	4639	60	0.12	0.06
3	Pre-AV	1080	983	9%	0	0	0.07	0.06
3	0.25	1028	250	76%	1939	0	0.22	0.09
4	Pre-AV	798	628	21%	0	0	0.09	0.06
4	0.25	483	111	77%	2485	0	0.18	0.09
4	8	954	296	69%	1754	0	0.22	0.08
4	24	714	147	79%	2210	55	0.18	0.07
5	0.25	600	108	82%	4527	51	0.23	0.09

[a] After administration of a second dose of antivenom.

implications for viper envenoming where the detection of venom post-antivenom has been assumed to mean that insufficient antivenom has been given. The study also showed that the antivenom assay measures free antivenom.

Many previous reports of "venom recurrence" are likely to be a result of the venom ELISA detecting bound venom, and therefore not true recurrence. There is often no correlation with return of symptoms or laboratory abnormalities such as coagulopathy, which supports that the measured venom is mainly bound in the VAV form and therefore not active (Ariaratnam et al., 2001, 1999; Theakston, 1997; Phillips et al., 1988; Khin Ohn et al., 1984; Hanvivatvong et al., 1997; Otero et al., 2006).

The detection of venom post-antivenom has mainly been reported with viper envenoming where there are relatively high venom concentrations (i.e. venom load) compared to antivenom concentrations – i.e. low antivenom to venom ratio. When there are low antivenom concentrations, antivenom will bind to venom molecules in a one to one ratio and therefore form VAV complexes. The venom component of VAV will still have free binding sites which allows VAV to bind to the microplate of the venom specific ELISA [see Fig. 1 (O'Leary and Isbister, 2014)]. This can be seen

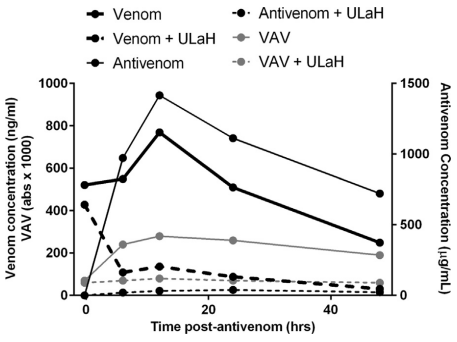

Fig. 4. Measurement of venom concentrations (ng/ml) alone (thick black line) and with ULaH (thick dashed black line), antivenom concentrations (μg/ml) alone (thin black line) and with ULaH (thin dashed black line) and venom–antivenom complexes (VAV; absorbance) alone (grey line) and with ULaH (dashed grey line) in a patient with measurable venom after antivenom.

in vitro in Fig. 2B where the venom specific assay still measures venom at the VAV peak. With increasing concentrations of antivenom all the binding sites on the venom molecules are occupied and then venom and VAV can no longer be detected (Fig. 2B).

In contrast venom recurrence is rarely reported for snakes that deliver smaller amounts of venom such as Australian elapids where there are high antivenom concentrations relative to venom concentrations. In studies of venom concentrations in Australian elapids venom is almost never detected post-antivenom (Isbister et al., 2013a; Allen et al., 2012). In this study we analysed three rare brown snake patients where there was venom detected post-antivenom which was shown also to be mainly bound venom (Table 3).

The interpretation of venom concentrations post-antivenom therefore appears to be more complex than previously believed. If no venom is detected then clearly more than sufficient antivenom has been administered, and that antivenom is likely to be in excess by more than a 2 to 1 ratio compared to venom. If venom is detected post-antivenom then in many cases sufficient antivenom has been given to ensure that each venom molecule is bound to at least one antivenom molecule on average (O'Leary and Isbister, 2014). Although the binding of one antivenom molecule to each venom molecule may not neutralise the toxin, it will trap toxins in the central compartment preventing them reaching their target site. In the case of toxins acting in the central compartment (e.g. procoagulant toxins), it may not immediately neutralise these, but it will lead to their rapid elimination. However, in some cases insufficient antivenom is given and free venom is still present and will result in persistent or recurrent clinical envenoming. In this case the addition of ULaH will not significantly reduce the venom

Table 2
Measurement of Russell's viper venom (RVV), antivenom (AV) and venom–antivenom complexes (VAV) in absorbance units for five samples from one patient samples after treatment with and without rabbit anti-RVV IgG bound to UltraLink resin (ULaRVV).

Patient	Time Post-AV (h)	RVV		AV		VAV	
		Nil	ULaRVV	Nil	ULaRVV	Nil	ULaRVV
6	Pre-AV	1.57	0.16	0.32	0.30	0.22	0.20
6	1.7	1.47	0.17	0.43	0.44	0.74	0.21
6	7.4	0.83	0.17	0.97	1.01	0.62	0.25
6	13.4	1.45	0.18	0.90	1.04	0.73	0.25
6	25.4	0.87	0.17	0.71	0.73	0.56	0.27

Table 3
Measurement of brown snake venom concentration and antivenom (AV) concentration in patient samples, followed by repeat venom measurement in absorbance units for five samples from three patients with and without anti-Horse IgG bound to UltraLink resin (ULaH).

Patient	Time Post-AV (h)	Venom (ng/ml)	AV (mU/ml)	Venom (absorbance)		Difference
		Nil	Nil	Nil	ULaH	
7	11	4.1	59	0.89	0.30	0.59
8	Pre-AV	3.9	0	0.46	0.45	0.01
8	10	1.2	38	0.28	0.16	0.11
9	Pre-AV	9.7	0	0.55	0.56	0.00
9	4	2	39	0.29	0.20	0.09

concentrations indicating that the venom is still unbound. Further quantitative studies are required to better define what a significant reduction in venom concentrations is.

It is important to note that the comparison of *in vitro* venom and antivenom concentrations in Fig. 2 to *in vivo* measurements in patients (Fig. 1) only applies for the particular antivenoms and batches of antivenoms used in this study. At least for some Indian antivenoms there is significant batch to batch variation and *in vitro* studies of venom–antivenom mixtures need to be undertaken for different antivenoms and batches.

In earlier *in vitro* work on the venom of *P. textilis* (O'Leary et al., 2006), we found VAV complexes could only be detected at venom concentrations greater than 50 ng/ml, a situation rarely encountered in patients. We have since systematically explored conditions under which VAV can be detected, and established conditions for its measurement, for a range of snake venoms (O'Leary and Isbister, 2014). With the high concentrations of venom found in Russell's viper envenomed patients, VAV is readily detectable.

Venom measurement should remain an important research tool in the diagnosis of envenoming and in determining the correct dose of antivenom to be administered. However, this study has found that the venom specific ELISA measures both free and bound venom (VAV complexes) making interpretation of the assay more problematic. The measurement of VAV and venom *in vitro* as well as in patient samples is required to determine if there is sufficient binding of venom after antivenom administration.

Ethics approval

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.

Funding

The study was supported in part by NHMRC Project Grant 631073. Geoff Isbister is funded by an NHMRC Senior Research Fellowship ID1061041.

Author contributions

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

Conflicts of interest

The authors declare that there are no conflicts of interest.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2014.11.221.

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Toxicon 90 (2014) 286–290

Contents lists available at ScienceDirect

Toxicon

journal homepage: www.elsevier.com/locate/toxicon

Antivenom cross neutralisation in a suspected Asian pit viper envenoming causing severe coagulopathy

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ARTICLE INFO

Article history:
Received 11 July 2014
Received in revised form 20 August 2014
Accepted 21 August 2014
Available online 30 August 2014

Keywords:
Antivenom
Cross-neutralisation
Venom
Pit viper
Snake envenoming

ABSTRACT

There is evidence of cross-neutralisation between common toxin groups in snake venoms and therefore the potential for antivenoms to be effective against species they are not raised against. Here we present a 49 year old female bitten by an unknown pit-viper in Nepal. She developed a venom induced consumption coagulopathy with an unrecordable international normalised ratio and undetectable fibrinogen. On return to Australia 5 days post-bite she was treated successfully with one antivenom raised against Malayan pit viper and green pit viper venoms (Haemato-polvalent antivenom from Thailand) and then subsequently with another antivenom raised against American pit-viper venoms (Antivipmyn). Presumed pit viper venom was detected in patient sera with an enzyme immunoassay against *Hypnale hypnale* venom. There was increased absorbance before antivenom compared to non-envenomed control samples, which then decreased after the administration of each antivenom. The recurrence of venom detected by enzyme immunoassay between antivenom doses was accompanied by a recurrence of the coagulopathy. Cross reactivity between the unknown venom and both antivenoms was supported by the fact that no venom was detected in the pre-antivenom samples after they were incubated in vitro with both antivenoms. This case and investigation of the venom and antivenoms suggest cross-neutralisation between pit vipers, including pit vipers from different continents.

1. Introduction

Snake envenoming is a neglected tropical disease causing significant mortality in morbidity in resource poor countries, mainly in the rural tropics (Kasturiratne et al., 2008). A major problem is the limited number of antivenoms available and many countries relying on other regions to manufacture antivenoms. With such a shortage of antivenom it is increasingly clear that other approaches are required to make antivenom more affordable, effective and accessible in many countries. One such approach is to focus on the cross-neutralisation of antivenoms and to develop antivenoms against common toxins in snake venoms (Wagstaff et al., 2006). Such cross-neutralisation has been demonstrated for Australian snake antivenoms (Isbister et al., 2010), for African snakes (Wagstaff et al., 2006), American snakes (Buschek et al., 2010) and Asian snakes (Leong et al., 2014; Tan et al., 2011). This potentially means that antivenoms developed in one country may be effective in other countries with snakes with similar toxin groups (Buschek et al., 2010).

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http://dx.doi.org/10.1016/j.toxicon.2014.08.071
0041-0101/

Many countries in Asia do not produce their own antivenoms and either have no antivenom available or rely on antivenoms made in other countries, principally Thailand, India, China and Taiwan. Although these antivenoms may be effective and it is more cost-effective to buy in antivenom from other countries, there needs to be pre-clinical and clinical studies to support the efficacy and effectiveness of antivenoms in different regions. A major issue in many parts of Asia is that antivenoms have not been raised against many of the Asian pit vipers, including hump-nosed viper (*Hypnale* spp.) and many *Trimeresurus* spp. A previous study has shown that antivenom raised against Malayan pit viper (*Calloselasma rhodostoma*) venom does cross-neutralise Hump-nosed viper venoms (Tan et al., 2011).

Here we present a case where a patient bitten by an unknown pit-viper in Nepal was treated successfully with one antivenom raised against Malayan pit-viper venom and then subsequently with another antivenom raised against American pit-viper venoms. Further in vitro studies demonstrated cross-reactivity and cross-neutralisation between the venom from this unknown pit viper from Asia, hump-nosed viper and Malayan pit-viper, and antivenoms made in Asia and the Americans.

2. Case

A 49-year-old previously well Australian female holidaying in Pokhara, Nepal, was bitten by a snake on the dorsum of the right foot late in the evening while getting into a car. She subsequently attended two hospitals in Nepal over the next 24 h for bite site pain and right leg swelling. At the time of her presentations, there were no symptoms of neurotoxicity or evidence of spontaneous or abnormal bleeding. Her medical notes from the second hospital she visited documented fang marks on the dorsum of her right foot with swelling of the right lower leg but no bruising (Fig. 1). Her observations were within normal limits and her neurological examination was documented as normal. Investigations performed were a chest radiograph which was normal, full blood count [FBC] (white cell count, 13×10^{-9}; haemoglobin [Hb], 133 g/L; platelets, 215×10^{-9}), prothrombin time was unrecordable (no clot formed), biochemistry was normal, and urinalysis was normal. A diagnosis of snakebite with coagulopathy was made and she was advised admission to hospital and the need for antivenom, which was documented as not currently available in Nepal. She was given vitamin K, penicillin and analgesia before deciding to discharge herself and return home to Australia for treatment.

Upon return to Australia the patient noticed new bruising to her legs from minor trauma in addition to ongoing pain and swelling of the right leg. On day five post bite she attended her local doctor who ordered investigations including coagulation tests which demonstrated a severe coagulopathy with an international normalised ratio (INR) >10, activated partial thromboplastin time (aPTT) >200s, fibrinogen <0.2 g/L (reference range [RR]: 1.7–4.5 g/L) and an elevated D-Dimer 15.1 mg/L (RR < 0.5 mg/L). Her FBC was normal with a Hb, 133 g/L and platelets, 174×10^{-9}. Biochemistry including renal and liver function was normal. She was referred to hospital the following day and on admission, an unrecordable

Fig. 1. Photograph of the bite site taken on the day of the bite.

INR and undetectable fibrinogen were confirmed. Further coagulation factor studies (II, V, VII, VIII and X) were done and were within the normal limits suggesting a thrombin like enzyme effect. She remained systemically well but had new bruising from minor trauma as well as persistent pain around the bite site and lower leg swelling. The geographical location of the bite and the probable presence of a thrombin-like enzyme in the venom suggested an Asian pit-viper. The only antivenom against an Asian pit-viper available in Australia was Haemato-polyvalent snake antivenom (Thai Red Cross Society, Bangkok, Thailand). This was obtained from Monash Venom Group, Monash University in Melbourne on day 7 post bite and two vials were administered over 30 min. Prior to antivenom her coagulation profile was unchanged with unrecordable INR, aPTT and fibrinogen. Four hours post antivenom her INR was 1.4 and aPTT was 22s with a fibrinogen of 0.2 g/L. Her fibrinogen continued to rise over the next 24 h to 0.5 g/L. However, over the next 36 h the fibrinogen dropped to <0.2 g/L and the INR and aPPT became unrecordable again on day 10 post bite. Her clinical state remained unchanged (Fig. 2).

The recurrence of coagulopathy prompted another search for antivenom, but the only available antivenom against any pit-viper venom was Antivipmyn (Instituto Bioclon S.A. de C.V, Mexico) which was sourced from a reptile park in Gosford, New South Wales. Although this antivenom was raised against American pit-vipers, it was believed it might have some cross-reactivity and the potential benefit was felt to outweigh the risks. On day 10 post bite the patient was administered eight vials of Antivipmyn over 4 h. Prior to antivenom her INR, aPTT and fibrinogen were unrecordable. The following morning on day 11 post bite her INR was 1.3, aPTT 26s and fibrinogen 0.4 g/L. Her fibrinogen continued to rise to 1.3 g/L with an INR of 1.0 and aPTT of 23s on day 13 when she was discharged from hospital (Fig. 2). On phone

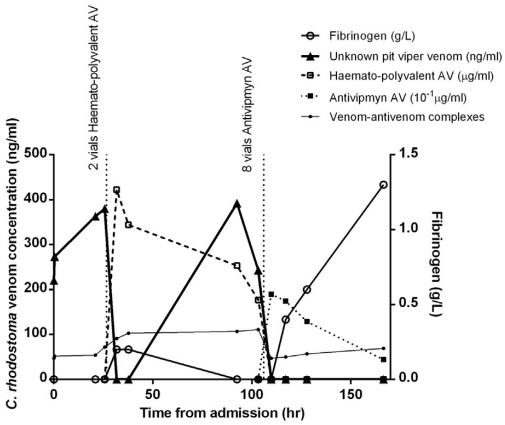

Fig. 2. Plots of pit viper venom (filled triangles), haemato-polyvalent antivenom (open squares), Antivipmyn antivenom (closed squares), venom-antivenom (VAV) complexes (small filled circles) and fibrinogen (open circles) against time after admission to hospital. The venom concentrations are in units of *C. rhodostoma* venom concentrations. The time that antivenom was given is marked with vertical dotted lines.

follow up one week later she remained well. Her bruising and right lower leg swelling had resolved and she was left only with some mild bite site pain.

3. Materials and methods

The venoms of Malayan pit viper (*C. rhodostoma*) and Eastern Diamondback rattlesnake (*Crotalus adamanteus*) were a gift from Monash Venom Group. Hump-nosed pit viper (*Hypnale hypnale*) and Russell's viper (*Daboia russelii*) venoms were collected in Sri Lanka. Rabbit anti-*H. hypnale* venom IgG was prepared at Rajarata University, Sri Lanka. Rabbit anti-*D. russelli* venom IgG was purchased from the West Australian Institute of Medical Research. Biotinylated anti-*H. hypnale* and anti-*D. russelli* venom IgG was prepared using Pierce EZ-Link Sulfo-NHS-LC-Biotin. Streptavidin conjugated with horseradish peroxidase was obtained from Chemicon/Millipore. Thai Red Cross Haemato-polyvalent antivenom™ raised against *C. rhodostoma*, *Cryptelytrops albolabris* and *D. siamensis* was purchased from the Queen Saovabha Memorial Institute, Bangkok, Thailand and Antivipmyn antivenom™ raised against *Crotalus* spp. and *Bothrops* spp. was purchased from Instituto Bioclon, Mexico, D.F., Mexico. Both antivenoms are equine $F(ab')_2$ and are supplied as desiccated powder. One vial of Haemato-polyvalent antivenom (batch# HP 00213, Exp August 2018) and one vial of Antivipmyn (batch# B-36-19, Exp July 2016) were used. Carbonate buffer is 0.05 M, pH 9.5. Bovine serum albumin (BSA) was purchased from Bovogen. Rabbit anti-Horse IgG labelled with horseradish peroxidase, and tetramethylbenzidine (TMB) were purchased from Sigma. The microplates used for the enzyme immunoassays (EIA) were Greiner high binding plates (#655061). Blocking solution is 0.5% BSA in phosphate buffered saline (PBS). Washing solution is 0.02% TWEEN 20 in PBS. All procedures were carried out at room temperature unless otherwise indicated. Plates were read in a BioTek Synergy HT plate reader at 450 nm.

3.1. Detection of pit viper venom by Enzyme Immune Assay

Detection of Asian pit viper venom in the patient samples was done by assuming that there would be cross reactivity between Asian pit viper venoms, including *Hypnale* spp. (hump nosed viper) and *C. rhodostoma* (Malayan pit viper). We chose to use rabbit anti-*H. hypnale* IgG antibodies because they were available and an assay has previously been published (Maduwage et al., 2013). The venom specific EIA methodology has been previously described (Kulawickrama et al., 2010; Maduwage et al., 2013). Briefly, plates were coated with anti-*H. hypnale* IgG (100 μl/well of 2 μg/ml in carbonate buffer) for 1 h at room temperature then at 4 °C overnight, washed once, then a blocking solution (300 μl/well) was applied. After 1 h, the plates were washed again, and a 10 times diluted patient sample solution (100 μl) was applied. After a further 1 h, the plates were washed 3 times and biotinylated anti-*H. hypnale* IgG (100 μl/well of 22 μg/ml in blocking solution) was applied. An hour later, the plates were again washed 3 times, and streptavidin horseradish peroxidase (100 μl/well of 0.1 μg/ml in blocking solution) applied. The plates were again washed 3 times, and TMB (100 μl) applied, followed by 1 M H_2SO_4 (50 μl). The concentrations of the pit viper venom were then interpolated from the standard curves made by serial dilutions of *C. rhodostoma* venom. Samples were also tested for Russell's viper venom using the same technique described above (Isbister et al., 2013).

3.2. Measurement of antivenom concentrations

Haemato polyvalent and Antivipmyn antivenom were detected in patient samples using EIA as previously described (Kulawickrama et al., 2010). Plates were coated with (100 μl/well of 2 μg/ml in carbonate buffer) *C. rhodostoma* venom for Haemato-polyvalent antivenom and *C. adamateus* venom for Antivipmyn antivenom respectively. Labelled anti-horse antibodies (100 μl/well of 1 μg/ml in blocking solution) were used to detect the antivenoms. The concentrations of Haemato polyvalent and Antivipmyn antivenom were interpolated from the standard curves made by serial dilutions of each antivenom.

3.3. Detection of venom–antivenom complexes (VAV)

The measurement of VAV has been previously described (O'leary and Isbister, 2014). In brief, the plates are prepared as for venom measurement by coating the plate with anti-*H. hypnale* IgG antibodies but the detecting antibodies are labelled anti-horse antibodies as per the antivenom assay. Patient samples were all measured at a dilution of 1:100 for the VAV assay.

3.4. Measurement of the binding capacity of the antivenoms against pit viper venom in patient samples

Diluted (1:10) patient's samples were incubated with standard doses (equivalent to the administration of one vial

of antivenom) of either Haemato-polyvalent or Antivipmin antivenom and left for 1 h at room temperature. 100 μl of this venom–antivenom mixture was then applied to the plate and venom was again measured using the venom specific EIA with biotinylated anti-*H. hypnale* IgG as described above.

4. Results

An unknown Asian pit viper venom was detected in the serum samples from the patient with increased absorbance before the administration of antivenom (Fig. 2). The venom concentrations were quantified as mass equivalents to *C. rhodostoma* venom because the venom was unknown and the values were interpolated from a standard curve derived from serial dilutions of *C. rhodostoma* venom (Fig. 2). This demonstrates that there is cross-reactivity between venoms from *H. hypnale*, *C. rhodostoma* and the venom of the snake that bit this patient. Russell's viper venom was not detected in any of the serum samples.

The measured venom concentration decreased after the administration of 2 vials of Haemato-polyvalent antivenom but then became detectable again after 24 h (Fig. 2). The recurrence of venom in the samples occurred at the same time the coagulopathy recurred with high INR and low fibrinogen levels. The recurrent high venom concentrations then decreased again after the administration of 8 vials of Antivipmyn antivenom (Fig. 2). This suggests that there is cross reactivity between the venom in the patient's samples and both types of antivenoms.

Cross reactivity between the unknown venom and both antivenoms was further supported by the fact that no venom was detected in the pre-antivenom samples after they were incubated in vitro with both antivenoms (Table 1). There were negligible concentrations of venom-antivenom complexes in the samples collected after antivenom treatment suggesting that the recurrence was of free venom in the circulation (Fig. 2).

5. Discussion

The cross reactivity on the venom specific assay between the venom in the patient's samples and both *H. hypnale* and *C. rhodostoma* suggests that the patient was bitten by one of the Asian pit vipers that occur in Nepal (see Table 2). This is supported by the colour of the snake and the geographical region. The decrease in venom concentrations after both antivenoms supports this cross-reactivity and suggests that there is significant cross-neutralisation between any antivenom raised to a pit viper (Asian or American) and other pit viper venoms. This is encouraging because it provides clinical evidence in human envenoming that polyvalent antivenoms can cover a broad range of snakes, in this case the potential for an antivenom that neutralises all pit viper venoms. However, further research is required and such antivenoms should only be used in the clinical setting when there are no other options.

An interesting feature of this case is that there was true venom recurrence based on the fact that the coagulopathy recurred (increased INR and decreasing fibrinogen) at the same time as venom was detected again in the samples, similar to reports of North American crotaline envenomings (Boyer et al., 2013). In addition, the venom detected was definitely free (or unbound) venom because there

Table 2
List of important venomous snakes in Nepal and the support for and against whether each snake or group of snakes is potentially the snake that bit the patient (David et al., 2011; Sharma et al., 2013).

Snake name	Potential to be implicated snake
Sub-Family: Viperinae Russell's Viper (*Daboia russelii*)	No Russell's viper venom detected in the patient's sample. No deficiency of factor V or factor X.
Sub-Family: Crotalinae Kramer's Pit Viper (*Trimeresurus septentrionalis*) Himalayan Pit Viper (*Gloydius himalayanus*) Tibetan Pit Viper (*Himalayophis tibetanus*) Mountain Pit Viper (*Ovophis monticola*)	All four species are potential candidates because they have venoms with thrombin like enzymes (isolated fibrinogen deficiency).
Family: Colubridae Red-necked Keelback (*Rhabdophis subminiatus*)	Possible, but highly unlikely to be cross-reactive with Malayan pit viper or hump-nosed viper

Table 1
Measured concentration of venom in the patient's samples interpolated from the standard curve of *C. rhodostoma* venom without antivenom, after the sample was incubated with Thai Haemato-polyvalent antivenom and after the sample was incubated with Antivipmin antivenom.

Time from hospital admission (h)	Venom concentration (ng/ml)[a] without antivenom	Venom concentration (ng/ml)[a] + Thai Haemato-polyvalent antivenom	Venom concentration (ng/ml)[a] + Antivipmyn antivenom
0.0	220	0	0
0.4	273	0	0
21.0	363	0	0
25.7	380	0	0
31.7	0	0	0
37.7	0	0	0
92.5	392	0	0
103.2	243	0	0
109.8	0	0	0
117.1	0	0	0
127.8	0	0	0
166.5	0	0	0

[a] All venom concentrations interpolated from a *C. rhodostoma* venom standard curve.

were negligible concentrations of bound antivenom (VAV) using a previously developed assay (O'leary and Isbister, 2014). The likely reason for recurrence in this case is that only a relatively small amount of antivenom binding the toxins was administered. Only two-thirds of the recommended dose of Thai Red Cross Haemato-polyvalent antivenom™ was administered because this was all that was available. In addition, there is unlikely to be complete cross-neutralisation, so only a proportion of the antibody fragments in the antivenom would have bound to the unknown pit viper venom. Similarly for the American antivenom, only a proportion of the antibody fragments would have cross-neutralised the pit viper venom. This suggests that if insufficient antivenom is administered then there will be recurrence of venom and clinical effects.

This case supports the idea that a single polyvalent antivenom could potentially be developed to cover the majority of medically important Asian snakes. A number of the available antivenoms cover all but one major group. Indian antivenoms cover cobra, krait, Russell's viper and saw-scaled viper, but not pit vipers. Thai antivenoms cover pit vipers, Malayan pit viper and have been shown to cross-neutralise at least hump-nosed vipers, cobra, krait and Russell's viper, but not saw-scaled vipers. Therefore countries such as Nepal where antivenoms are not produced could use one of these antivenoms, in this case, the Thai Haemato-polyvalent antivenom that appears to cross-neutralise Asian pit vipers and covers Russell's viper.

The patient had a prolonged coagulopathy that has been reported for a number of true vipers (e.g. *Echis* spp.) (Mion et al., 2013) and pit vipers (Seifert and Boyer, 2001), that may not resolve for days to weeks without antivenom treatment. There was an isolated deficiency of fibrinogen consistent with most pit viper venoms containing thrombin-like enzymes. Thrombin like enzymes consume fibrinogen but rarely cause a deficiency in any of the other clotting factors.

The clinical course and response to treatment in this case as well as the venom and antivenom measurements in the patient's sera support cross-neutralisation between pit vipers, including pit vipers from different continents. Further research is required to determine if antivenoms raised against pit vipers in one region are suitable for use in other areas with pit vipers.

Ethical statement

No approval was required because no animals were used and only human material was used in this study from one patient who consented to its use.

Funding

The study was supported in part by NHMRC Project Grant 631073. Geoff Isbister is funded by an NHMRC Senior Research Fellowship ID1061041.

Acknowledgements

We thank all the people and institutions involved in making available and transporting the antivenom to the patient, including Professor Wayne Hodgson and the Monash Venom Group for supplying the Thai antivenom, Dr Dino Druda for transporting the antivenom from Melbourne to Brisbane, Staff at the Gosford Reptile Park for supplying Antivipmyn antivenom and transporting it to Newcastle, and Dr Ingrid Berling for transporting the antivenom from Newcastle to Brisbane. We also thank Dr Margaret O'Leary for assistance with the laboratory assays.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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QJM Advance Access published May 14, 2013

Q J Med
doi:10.1093/qjmed/hct102

Diagnostic 20-min whole blood clotting test in Russell's viper envenoming delays antivenom administration

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Received 26 February 2013 and in revised form 31 March 2013

Summary

Background: The 20-min whole blood clotting test (WBCT20) is widely used for the identification of coagulopathy in snake envenoming, but its performance in practice has not been evaluated.
Aim: We aimed to investigate the diagnostic utility of the WBCT20 for coagulopathy in Russell's viper envenoming.
Design: Prospective observational study.
Methods: Adult patients with snake envenoming were recruited. Age, sex, bite information, clinical effects, serial WBCT20 and antivenom treatment were recorded. Definite Russell's viper envenoming was confirmed with venom specific enzyme immunoassay. We assessed sensitivity of admission WBCT20 to coagulopathy (international normalized ratio, INR > 1.5) in Russell's viper envenoming, the specificity of negative WBCT20 in non-envenomed patients and directly compared paired WBCT20 and INR.
Results: Admission WBCT20 was done in 140 Russell's viper bites with coagulopathy and was positive in 56/140 [sensitivity 40% (95% confidence interval (CI): 32–49%)]. A negative WBCT20 led to delayed antivenom administration [WBCT20−ve tests: median delay, 1.78 h (interquartile range (IQR): 0.83–3.7 h) vs. WBCT20 + ve tests: median delay, 0.82 h (IQR: 0.58–1.48 h); $P = 0.0007$]. Delays to antivenom were largely a consequence of further WBCT20 being performed and more common if the first test was negative (41/84 vs. 12/56). Initial WBCT20 was negative in 9 non-envenomed patients and 48 non-venomous snakebites [specificity: 100% (95% CI: 94–100%)]. In 221 paired tests with INR > 1.5, the WBCT20 was positive in 91(41%). The proportion of positive WBCT20 only increased slightly with higher INR.
Conclusions: In clinical practice, the WBCT20 has low sensitivity for detecting coagulopathy in snake envenoming and should not over-ride clinical assessment-based decisions about antivenom administration. There is an urgent need to develop a simple bedside test for coagulopathy in snake envenoming.

Introduction

Snake envenoming is being increasingly recognized as a major medical problem in tropical and subtropical countries. One of the most important effects of snake envenoming is coagulopathy, most commonly a venom-induced consumption coagulopathy (VICC).[1] The major treatment is antivenom administration that aims to neutralize the toxins in the venom.[2] However, antivenom is expensive, difficult to obtain in some parts of the world and associated with a significant risk of systemic hypersensitivity reactions.[3–5] It is therefore essential to rapidly and accurately determine which patients have envenoming (and will require antivenom), and which are non-envenomed, or have been bitten by non-venomous snakes.

The 20-min whole blood clotting test (WBCT20) has been used for decades in viper (and other snake) bites to determine if patients have a clinically significant coagulopathy.[6–8] The WBCT20 was not intended as a clotting test *per se* but as an indicator of envenoming (and need for antivenom) in patients bitten by snakes that cause coagulopathy. Despite the widespread reliance on this test and it being regarded as the standard of care for treatment of snake envenoming in resource poor settings,[9] there have been no studies that have determined the conditions under which the test can be performed accurately, validated it against standard tests or demonstrated that it is accurate in the field.[6] There is little standardization of the method for the WBCT20 and the original study describing it simply states that 'a few ml of blood were placed in a clean dry glass test tube and left undisturbed for 20 min and then tipped to discover whether the blood had clotted'.[7] Whether factors such as the size or type of tube (beyond that it should be glass), temperature and type of snake affect the test, has not been explored. Some studies even report a 30-min whole blood clotting test, indicating even the time of the whole blood clotting test is not universally standardized.[10,11] Coagulation studies are notoriously difficult to standardize. For example, laboratory measurement of clotting function in plasma has required significant standardization to make test results reproducible [e.g. the prothrombin time (PT) being standardized as the international normalized ratio (INR)].[12] It is therefore remarkable that no attempt has been made to standardize the WBCT20, or to demonstrate that this lack of standardization does not affect performance.

The study widely cited as demonstrating the reliability of the WBCT20 compared it with fibrinogen concentrations, rather than to conventional laboratory tests, such as a thrombin time (TT), PT or INR. These are the usual tests used and are more representative of global clotting function. Evaluation of the WBCT20 against such standard tests in the clinical setting is necessary to determine if it is an appropriate diagnostic test in snake bite coagulopathy or VICC. We have been conducting a prospective randomized controlled trial (RCT) of antivenom infusion rates for snake bite in Sri Lanka. A major feature of envenoming is coagulopathy from Russell's viper bites. Doctors in these hospitals routinely do a WBCT20 to determine if antivenom is required (as outlined in the National guidelines for Sri Lanka[13]). The determination of WBCT20 was, therefore, routine practice and not part of our study, however, we measured formal coagulation studies within the RCT. We compared the results of the WBCT20 done by the treating clinicians with an INR done on immediately frozen samples for definite Russell's viper envenoming cases and non-envenomed patients.

Methods

This was a prospective observational study of patients with snake envenoming in central western Sri Lanka conducted in conjunction with a RCT of different infusion rates of antivenom.[14] Our primary aim in this article is to determine the sensitivity and specificity of the WBCT20 in clinical practice under the usual conditions of its use for detection of coagulopathy in Russell's viper bites. The study had approval from the Ethical Review Committee, Faculty of Medicine, University of Colombo. All patients gave written and informed consent for the collection of data, and parents/guardians also gave consent for children.

Study patients

Patients were recruited from a secondary referral hospital in Chilaw, Sri Lanka, between 21 January 2007 and 31 July 2009. Every patient who was older than 13 years who presented with a snake bite was identified on arrival to the hospital. All patients then had baseline clinical data collected, including demographic features (age, sex), bite information (type of snake, time of bite), clinical features of envenoming, complications and treatment. A WBCT20 was done routinely in all patients by the treating hospital team, including repeat testing in patients with envenoming. A few milliliters of blood was placed in a small glass test tube. After 20 min, the tube was inverted to determine if a clot had formed. The test was negative if a clot formed and positive if no clot formed (incoagulable blood). We identified from review of INR and Russell's viper venom

specific enzyme immunoassay (EIA) results two groups: definite Russell's viper envenoming (venom concentration > 2.5 ng/ml) plus VICC (INR > 1.5) and suspected Russell's viper bites with no venom detected and no VICC (INR < 1.3). An INR cut-off of 1.5 was chosen because this was above the normal range and higher than any non-envenomed patients. An INR > 1.5 was present in all but one patient with detectable RV venom.

During the study, patients who were deemed to require antivenom were recruited to a clinical trial comparing different rates of antivenom infusion which is reported elsewhere.[14] All other decisions about treatment were made by the treating clinicians.

Patients had a 10-ml sample of blood collected at the time of initial assessment and close to the time the WBCT20 was done. In patients given antivenom, 5-ml samples were also collected 1, 4 and 12 h later, and thence once daily until discharge. Blood was collected in serum tubes for venom specific EIAs and citrated tubes for coagulation studies. All samples were immediately centrifuged, aliquoted and frozen at −20°C and then transferred to −80°C freezers within 2 weeks until the completion of the study.

Laboratory assays

Frozen samples were transported to Australia. Frozen serum was then thawed and used to measure Russell's viper venom concentrations using a previously described EIA.[15] In brief, polyclonal antibodies (IgG) to Russell's viper (*Daboia russelli*) were raised in rabbits.[16] These were conjugated to biotin and then used in a sandwich EIA with the detecting agent streptavidin-horseradish peroxidase. The limit of detection for the assay was 2.5 ng/ml.

Frozen citrate specimens were thawed to measure the INR. The INR was performed using a standard coagulometric method provided by the manufacturer on a Behring Coagulation System. An INR >1.5 was considered to be representative of coagulopathy in snake bite. The use of frozen citrate specimens to measure the INR in blood collected at a distant hospital, centrifuged, frozen and stored has previously been used in an Australian study of VICC.[17] INR was also measured in plasma from nine non-envenomed snake bite patients and the INR was ⩽ 1.4 in all cases.

Data collection

Demographic information (sex, age), bite site, clinical features of snake envenoming, WBCT20 results and treatments (antivenom) were all recorded on datasheets which were then entered into a relational database (Microsoft Access). The results of all WBCT20 and the time of each WBCT20, all INR results and the time they were tested and the time of antivenom administration were extracted from the datasheets.

Data analysis

We assessed the sensitivity of the admission WBCT20 in detection of the presence of VICC in Russell's viper bites defined as a peak or maximum INR > 1.5 on frozen citrate samples collected anytime during the admission. We also examined the proportion of negative WBCT20 in non-envenomed patients and made a direct comparison of paired WBCT20 and INR. The WBCT20 and the research INR were not collected at exactly the same time, so a paired comparison of a WBCT20 and INR was done for samples where the WBCT20 and INR were collected within 1 h of each other. We also examined how the time to antivenom was influenced by the result of the first WBCT20.

Statistical methods

In addition to visual analysis, sensitivity and specificity analyses were performed.[12] Sensitivity was defined as the proportion of envenomed cases with VICC where the WBCT20 was positive. Specificity was defined as the proportion of non-envenomed patients with a negative WBCT20. Ninety-five percent confidence intervals (95% CI) were calculated using a normal approximation. Continuous variables are summarized as medians and interquartile ranges (IQRs), and proportions are presented with 95% CIs. All analyses and graphs were done with GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Results

Of the 1004 patients presenting with suspected snakebites during the study period, there were 145 definite Russell's viper bites that developed coagulopathy based on the detection of Russell's viper venom in patient serum and an INR > 1.5. Thirty-one patients suspected to be Russell's viper bites were excluded because of a negative EIA for Russell's viper venom—21 were probable Russell's viper bites but no pre-antivenom blood was available, 6 were unknown and 3 were more likely to be hump-nosed viper (*Hypnale hypnale*) bite. Only one patient with detectable Russell's viper venom did not develop an INR > 1.5. The remaining 828 patients were bitten by other venomous snakes such

Table 1 Demographic features and clinical effects for patients in the study

	Number	%
Sex (male)	109	75
Age (median, IQR), years	39 (29–49)	
Activity		
Working in paddy fields	38	26
Walking outside/road	70	48
Working outdoors or in the garden	32	22
Other	4	3
Indoors	1	1
Bite site		
Foot or ankle	127	88
Lower leg	15	10
Forearm	1	1
Hand	2	1
INR		
1.5–5	51	35
5–12	42	29
>12	52	36
Neurotoxicity	68	47
Systemic symptoms	71	49

as hump-nosed vipers, krait and cobras, unidentified venomous snakes or non-venomous snakes.

The 145 patients with Russell's viper envenoming had a median age of 39 years (IQR: 29–49 years; range: 16–82 years) and 109 (75%) were male. The majority of bites occurred in people walking outdoors, working in the paddy fields or in the garden (Table 1). Only one patient was bitten while indoors. The bite site was most commonly the lower limb. The median peak venom concentration was 199 ng/ml (IQR: 70–480 ng/ml; range: 4.5–1952 ng/ml). The median maximum INR was 6.8 (IQR: 3.7 to >12; range: 1.7 to >12), and Figure 1 shows the association between pre-antivenom venom concentrations and the maximum INR for 123 patients who had pre-antivenom blood samples available. No patient had an initially normal INR that then became abnormal although the INR increased in a proportion of patients on the second test.

WBCT20 outcomes

On admission, a WBCT20 was done in 140 of the 145 Russell's viper bites with VICC. It was positive in 56/140 patients [sensitivity 40% (95% CI: 32–49%)] (Figure 2). A negative test result led to a delay in administration of antivenom [for WBCT20–ve tests: median delay, 1.78 h (IQR: 0.83–3.7) vs. WBCT20 + ve tests: median delay, 0.82 h (IQR: 0.58–1.48); $P = 0.0007$] (Figure 3). Delays in both

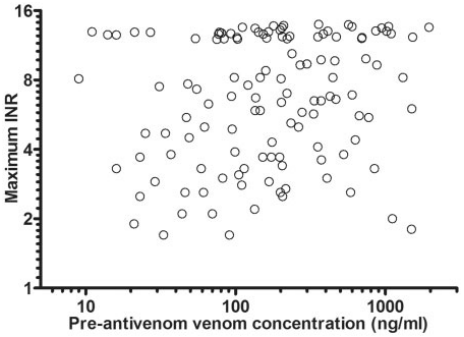

Figure 1. Relationship between pre-antivenom venom concentrations and the maximum INR in 123 of the 145 patients where pre-antivenom blood samples were available.

groups were often caused by further WBCT20 tests being performed (Figure 2). Even 12 of 56 positive WBCT20 patients had one or two further WBCT20 performed before antivenom (vs. 41 of 84 in WBCT20 negative tested patients).

WBCT20 testing

There were 232 paired WBCT20 and INR tests done within 1 h of each other during the admission for the 145 patients with Russell's viper envenoming. In 221 of the paired tests, the INR was >1.5, and the WBCT20 was only positive in 91 (41%) of these. Figure 4 shows the number of tests that were positive or negative based on the paired INR result. The proportion of WBCT20 that was positive only increased slightly with higher INR values (those indicating more severe coagulopathy). Figure 5 compares the INR values for negative vs. positive WBCT20. The median INR for WBCT20 + ve was 4.2 (IQR: 2.4–8.5; range: 1 to >12) compared with 3.0 (IQR: 2.1–6.3; range: 1.3 to >12; $P = 0.004$) for WBCT–ve.

There were nine non-envenomed patients with paired INR and WBCT20 tests available where the INR was $\leqslant 1.4$. All WBCT20 were negative in these patients. A further 48 patients bitten by non-venomous snakes (identified as cat or rat snakes) had a negative WBCT20. This indicates the WBCT20 had good specificity on admission in this setting [100% (95% CI: 94–100%)].

Discussion

This study has shown that at the bedside in Sri Lanka, in a busy unit treating large numbers of snakebites for years with this method, the WBCT20 had an unacceptably high false negative

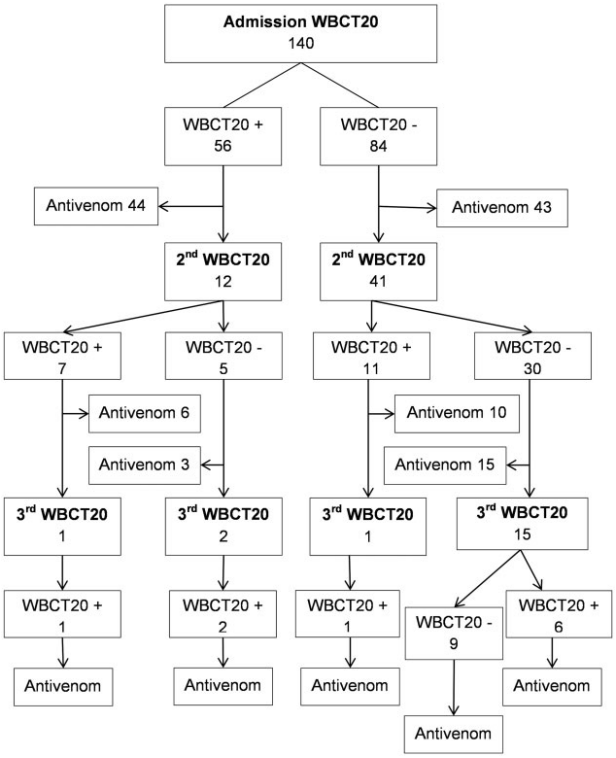

Figure 2. Flow diagram of all 140 patients with a definite Russell's viper bite and coagulopathy who had a WBCT20 on admission, showing all WBCT20 and when antivenom was administered.

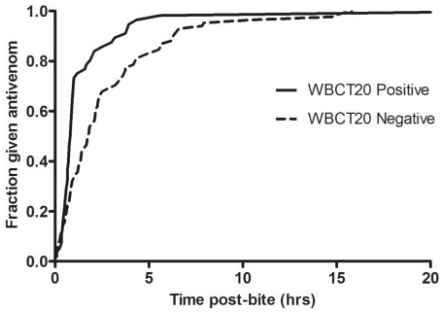

Figure 3. Time to antivenom in patients with Russell's viper coagulopathy comparing those with an admission WBCT20 positive and those with a negative WBCT20.

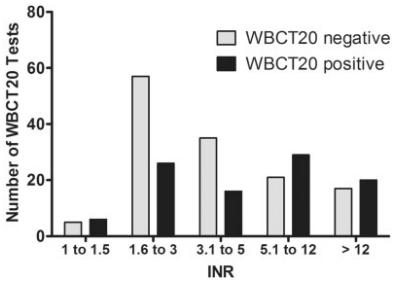

Figure 4. Number of WBCT20 tests that were positive or negative based on the INR done at the same time.

rate for the detection of coagulopathy in Russell's viper envenoming with a sensitivity of only 40%. The test performed only marginally better for those with severe coagulopathy and cannot be used to safely rule out coagulopathy. In contrast, the false positive rate in non-envenomed patients was zero suggesting a very high specificity of 100% (95% CI: 94–100%) in this setting. However, in patients with Russell's viper coagulopathy with normalizing

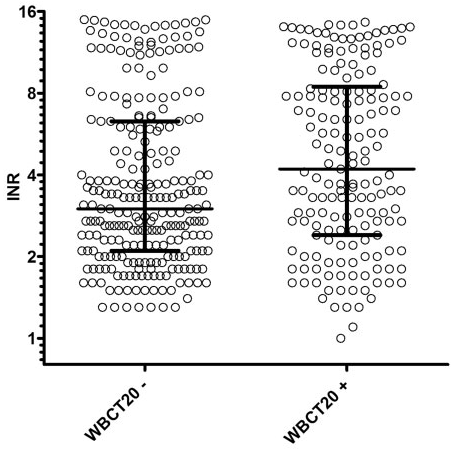

Figure 5. INR values for all positive WBCT20 compared with those for all negative WBCT20.

coagulation, there were some false positive WBCT20 (Figure 4). Despite the poor sensitivity of the WBCT20 to Russell's viper envenoming, all patients received antivenom, although just over one-third had delayed administration.

The WBCT20 was done by the clinical staff on the ward without any supervision or training by the investigators. A protocol was used but there was no standardization of the type of tube or whether tubes were re-used. This study assessed the WBCT20 in the clinical setting. Thus, the low sensitivity of the WBCT20 may have reflected the manner in which the test was conducted in these wards. However, this hospital has used WBCT20 in clinical practice and research for decades, including studies by the developer of the method.[18] Further studies using a standardized approach to the whole blood clotting test (i.e. use of one size of tube, the same volume of blood collection and trained operators undertaking the test) are needed to identify whether the WBCT20 can perform much better in this setting and whether it is simply poor standardization of clinical use of the test that is the problem.

The idea behind the development of the whole blood clotting test was inspired by an obvious clinical need in the developing world and a simple logic. However, the WBCT20 has moved into dozens of guidelines around the world without any translational research; there has been no systematic attempt at further refinement, standardization or evaluation of its strengths and limitations. Sri Lankan doctors are using the test because they have no alternative. However, it is clear that using the test can delay appropriate treatment (Figure 3) and that the doctors treat despite a negative result (Figure 2). In 43 patients, antivenom was given after one negative WBCT20 test on admission. A further 15 patients were given antivenom after a second negative WBCT20 and 9 after three consecutive negative WBCT20 (Figure 2). Therefore, antivenom was given in almost half of the envenomed patients without a prior positive WBCT20.

Although numerous studies have reported whole blood clotting test results,[7,8,10,11,18–24] they provide little information on the timing of the WBCT20 and its use as a diagnostic test of coagulopathy associated with envenoming—VICC. Many clinical trials have used abnormal WBCT20 as an inclusion criteria,[23] but provide no information on the diagnostic value of the test. One previous study in Russell's viper bites in Burma included 54 cases with systemic envenoming.[19] Only 32 patients (60%) had an abnormal WBCT20 on admission, and 16 patients had a delay of 30 min to 15 h until the WBCT20 was abnormal and presumably antivenom was given.[19] The proportion of abnormal WBCT20 on admission and the delay in abnormal WBCT20 is similar to our study. Another study of *Echis carinatus* in Sri Lanka does not report the admission WBCT20 but does find a similar delay in abnormal WBCT20 of 40 min to 18 h.[21] One study of *Echis ocellatus* and another of *Bothrops* species report a clotting test where 30 min is the cut-off for incoagulable blood.[10,11] Thus, these studies, while not designed to directly assess this issue, support the issues we highlight of low sensitivity in diagnosis of coagulopathy and envenoming, and lack of standardization.

Figure 4 shows that the WBCT20 was still not sensitive enough in patients with the most severe coagulopathy. The proportion of positive WBCT20 only increased slightly with increasingly severe coagulopathy based on the INR (Figure 4). Thus, the sensitivity of the WBCT20 to detect severe coagulopathy is not much better that its sensitivity to detect envenoming. In practice, the test could not even apparently be relied on to rule out an INR > 12. This, therefore, raises major concerns about the use of the WBCT20 for monitoring of ongoing coagulopathy (as used by some clinicians and recommended in some guidelines).

A limitation of our study was the use of frozen samples for the INR testing. However, this has been done previously[17] and meant that all of the tests were done during a short period of time. In addition, all samples were immediately centrifuged after collection and then aliquoted and frozen by trained clinical research assistants. Unfortunately, the WBCT20 was not always collected at the same time as the citrate sample for the INR because the

treating team collected blood for the WBCT20, while clinical research assistants collected the research bloods. Potential inaccuracies resulting from delayed testing may have implications for the quantitative INR–WBCT20 correlations (Figures 4 and 5), but this was only a secondary analysis in the study. An abnormal INR had more than 99% agreement with the diagnosis of Russell's viper envenoming based on detectable venom concentrations, indicating that the INR was highly sensitive. In contrast, the initial WBCT20 had 40% sensitivity for patients with Russell's viper envenoming. So any potential inaccuracies in the paired comparison do not affect our conclusions about the lack of sensitivity of the WBCT20. Future studies need to compare an INR done on-site with the WBCT20, but currently INR testing is not readily available in Sri Lankan hospitals.

It is clear that an inexpensive bedside whole blood clotting test for use in remote areas and developing countries could fill an important role. There also should be more attention given to standardizing the conditions, timing, use and interpretation of the current WBCT20. It is reassuring that clinicians eventually gave antivenom in all envenomed patients with a negative WBCT20 based on clinical features, indicating they recognize it has limited sensitivity. Further work is required to develop such a test that performs to an acceptable standard in the field, and delays to antivenom are minimized. To do this, it may be worth exploring a range of methodologies, an ideal test would be able to provide a result more rapidly than after 20 min.

Acknowledgements

We acknowledge the assistance of the medical and nursing staff at Chilaw hospital for identifying patients for the study. We acknowledge Andrew Dawson for assistance throughout the study.

Funding

The study was supported in by NHMRC Project Grant (631073) and by IRQUE Project Grant (C1A2 MBBS SPQEF).

Conflict of interest: None declared.

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Journal of Pharmacological and Toxicological Methods 67 (2013) 177–181

Contents lists available at SciVerse ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

Use of immunoturbidimetry to detect venom–antivenom binding using snake venoms☆,☆☆

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ARTICLE INFO

Article history:
Received 16 October 2012
Accepted 4 February 2013
Available online 13 February 2013

Keywords:
Antivenom
Snake venom
Immunoturbidimetry
Immunocomplexes
Antibody

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 increase 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 (100 μl) in water were placed in the wells of a microtitre plate and 100 μl of a venom solution (50 μg/ml in water) was added. Absorbance readings were taken at 340 nm 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 *Pseudonaja 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 predetermined 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 (*Daboia russelli*, *N. naja*, *Echis carinatus* and *Bungarus 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.

1. Introduction

Snake envenoming is now recognised as a global health issue and antivenom is the major treatment (Kasturiratne 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 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 et al., 2013). 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 (van der Weyden et al., 2000; Williams et al., 1994). 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 & Bentzon, 1987; Preneta-Blanc 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 the 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 phenomenon (Schottler, 1952). Solano et al. used a turbidimeter

☆ Funding: The study was supported in part by NHMRC Project Grant 631073. Geoff Isbister is funded by an NHMRC Clinical Career Development Award ID 605817.
☆☆ Author contributions: MAO designed the assay; MAO and KM undertook the experimental work; MAO and GKI undertook the analysis; MAO and GKI wrote the paper and all authors reviewed all; GKI takes responsibility for the manuscript.
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1056-8719/$ – see front matter.
http://dx.doi.org/10.1016/j.vascn.2013.02.004

to monitor the interaction between *Bothrops asper* venom and antivenom to determine how long it took for this interaction to be complete (Solano et al., 2010). They noted that turbidity continued to increase over the 30 minute incubation period, but that neutralisation was not dependent 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.

2. 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. *Echis 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 1 mg/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 (BlSAV; batch #0543-07301), Taipan (TAV; batch #0548-05601), and 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 (100 μl) 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 100 μl of a venom solution (50 μg/ml in water) was added. The final venom concentration was 25 μg/ml. Readings were taken at 340 nm 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.

3. Results

3.1. Snake venoms and Australian antivenoms

Experiments were undertaken in water because turbidity did not develop in PBS for with CSL antivenoms. Fig. 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.

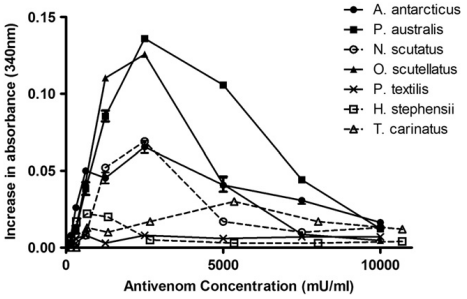

Fig. 1. Increase in absorbance resulting 30 min after the addition of seven snake venoms to their corresponding CSL Ltd. antivenoms. Tiger snake antivenom was used for *T. carinatus* and *H. stephensii*. Experiments were repeated in triplicate for *A. antarcticus*, *P. australis* and *O. scutellatus*.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

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 (Fig. 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.

3.2. Snake venoms and rabbit monovalent antibodies

Fig. 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. Fig. 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. Fig. 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. Fig. 3D shows

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

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

TSAV — tiger snake antivenom; BlSAV — black snake antivenom; TAV — taipan antivenom; BSAV — brown snake antivenom; DAAV — death adder antivenom.

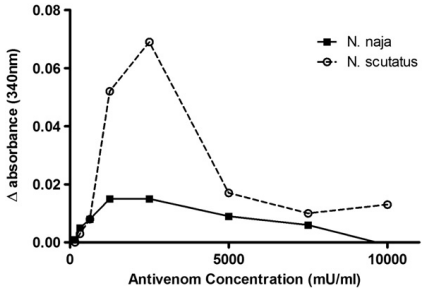

Fig. 2. Increase in absorbance 30 min after the addition of *N. scutatus* venom (25 μg/ml) and *N. naja* venom (25 μg/ml) to tiger snake antivenom (TSAV).

the cross-reactivity between *E. ocellatus* and *E. carinatus* venoms and antibodies to *E. ocellatus*.

3.3. 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*) (Fig. 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 detectable. Mixtures of RVVX with VINS antivenom showed a turbidity increase over a similar range of antivenom concentrations as for the whole venom.

4. 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 et al., 2010). As the concentration of

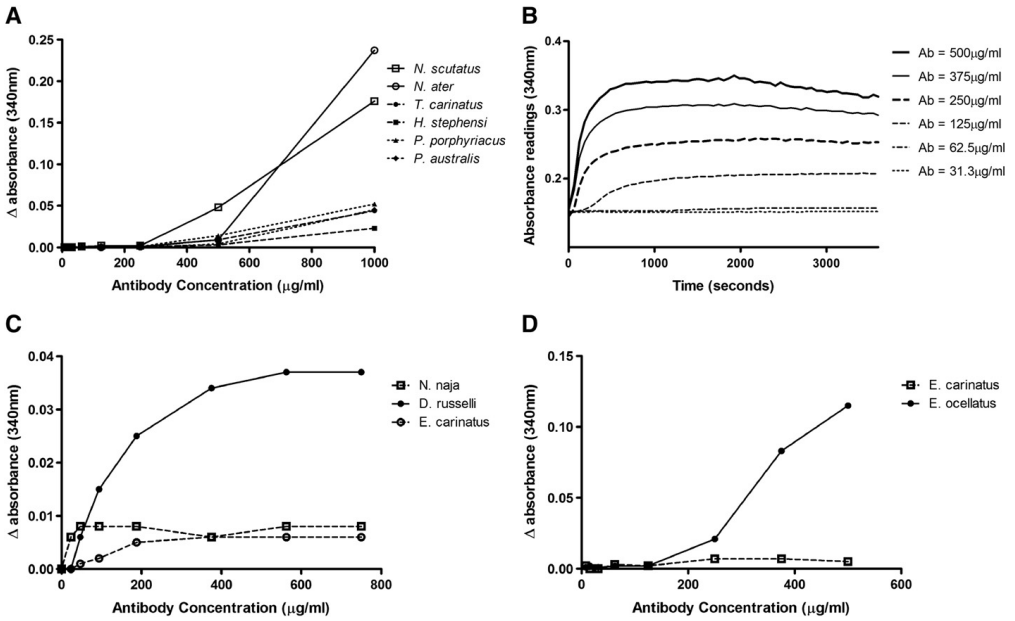

Fig. 3. A: Increase in absorbance 30 min after the addition of six different venoms to rabbit anti-*N. ater* IgG. All venoms were at 25 μg/ml. B: Development of turbidity over time, resulting from addition of *N. scutatus* venom to increasing concentrations (μg/ml) of rabbit anti-*T. carinatus* antibodies. Concentration of venom is 25 μg/ml. C: Increase in absorbance 30 min 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 μg/ml. D: Increase in absorbance 30 min after the addition of *E. ocellatus* venom (25 μg/ml; closed circle, thick line) and *E. carinatus* venom (25 μg/ml; open square, dashed line) to increasing concentrations of antibodies against *E. ocellatus*.

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

Antibody	Antibody concentration (μg/ml)	Venom	Δ Abs at 340 nm
Anti-*N. scutatus*	1000	*N. ater*	0.2
		N. scutatus	0.062
		P. porphyriacus	0.03
		T. carinatus	0.07
Anti-*N. ater*	1000	*H. stephensii*	0.02
		N. ater	0.24
		N. scutatus	0.18
		T. carinatus	0.04
		P. aust.	0.05
		P. porphyriacus	0.05
Anti-*T. carinatus*	700	*N. scutatus*	0.19
		T. carinatus	0.29
Anti-*H. stephensii*	500	*H. stephensii*	0.11
		N. scutatus	0
		T. carinatus	0
Anti-*P. porphyriacus*	750	*N. scutatus*	0
		P. porphyriacus	0.23
Anti-*P. textilis* IgY [a]	1500	*P. textilis*	0.08
Anti-*O. scutellatus*.	500	*O. scutellatus*	0.03
		P. textilis	0
Anti-*E. ocellatus*	500	*E. carinatus*	0
		E. ocellatus	0.11
Anti-*D. russelli*	750	*D. russelli*	0.04
		E. carinatus	0.01
		N. naja	0.01
		B. caeruleus	0

[a] IgY, not rabbit IgG.

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 2500 mU/ml — i.e. an AV:V ratio of 100 mU/μg or 1 U antivenom per 10 μg of venom. This corresponds to the CSL definition of 1 U of antivenom being the amount required to neutralise 10 μg 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 (Fig. 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 et al., 2000).

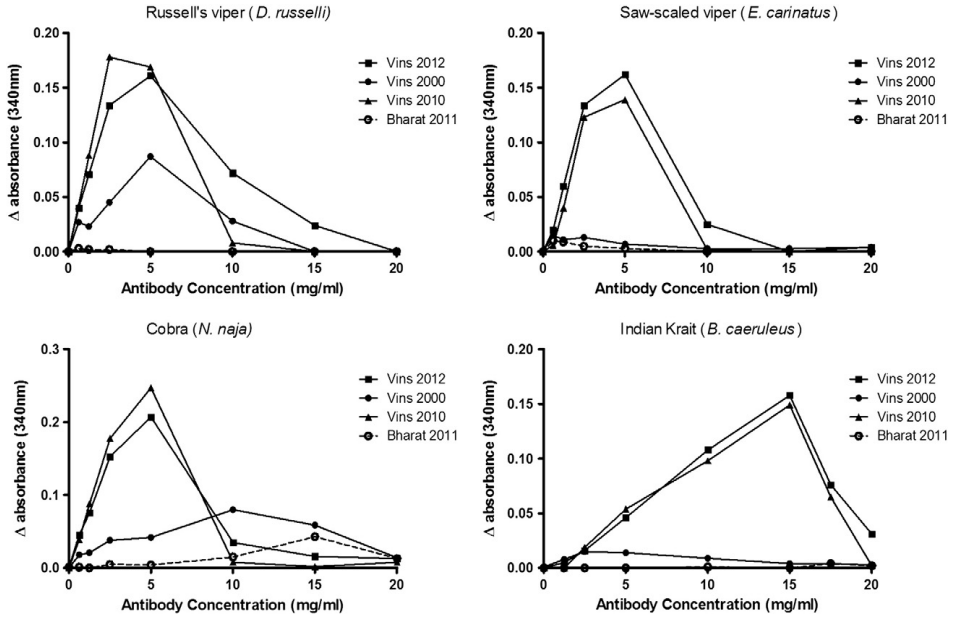

Fig. 4. Increase in absorbance 30 min after the addition of *D. russelli* venom, *N. naja* venom, *E. carinatus* venom and *B. caeruleus* to increasing concentrations of four different batches of Indian Polyvalent antivenom. All venoms were at a concentration of 25 μg/ml.

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 Fig. 3A show that 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 et al., 2009), whereas *Hoplocephalus* antibodies show no interaction with *T. carinatus* and *Notechis* spp. venom consistent with studies of this venom (Isbister 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 & Bentzon, 1987; Preneta-Blanc et al., 2008).

The interaction between Sri Lankan Cobra (*N. naja*) and Australian TSAV (Fig. 2), supports previous studies demonstrating cross-reactivity between *N. haje* (Egyptian cobra) venom and TSAV, using enzyme immunoassay and in vitro neurotoxicity studies (Kornhauser et al., 2013).

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 et al., 2011), but there was not sufficient interaction between *P. porphyriacus* venom and anti-*Notechis* antibodies for turbidity to develop (Fig. 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 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 whole 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.

5. 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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Toxicon 61 (2013) 11–15

Contents lists available at SciVerse ScienceDirect

Toxicon

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Epidemiology and clinical effects of hump-nosed pit viper (Genus: *Hypnale*) envenoming in Sri Lanka

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ARTICLE INFO

Article history:
Received 25 May 2012
Received in revised form 19 October 2012
Accepted 24 October 2012
Available online 2 November 2012

Keywords:
Envenoming
Sri Lanka
Hump-nosed viper
Hypnale
Snake bite

ABSTRACT

Hump-nosed pit vipers of Genus *Hypnale* are the commonest cause of snake bite in Sri Lanka. Although there are many reports of local effects, coagulopathy and acute kidney injury, it remains unclear how frequent these clinical effects are and therefore the medical importance of this snake genus. The genus has been recently revised to include *Hypnale hypnale* from Sri Lanka and Western Ghats of Southern India, and the two endemic species to Sri Lanka, *Hypnale zara* and *Hypnale nepa*. This was a prospective hospital-based clinical study of definite *Hypnale* spp. bites from July 2008 to July 2010 in six Sri Lankan hospitals. There were 114 patients included and all snakes were correctly identified by hospital staff as *Hypnale* spp. Of these, 93 snakes were identified as *H. hypnale* by an expert, 16 as *H. zara* and five as *H. nepa*. Most bites occurred on the lower limbs in the daytime. There was no difference in the clinical effects between the three species. Pain and fang marks were present in all patients, 101 had local swelling and only 16 (14%) developed extensive local swelling that spread proximally and involved more than half of the bitten limb. Systemic symptoms occurred in 18 patients; four patients had an abnormal 20 min whole blood clotting test and one patient developed an acute kidney injury that required haemodialysis. All patients were discharged alive with a median length of stay of 2 days. This study confirms that hump-nosed viper bites cause only minor effects in most cases. Future studies need to undertake formal coagulation studies and identify important early indicators of renal impairment.

1. Introduction

Snake bite is an important medical problem in the tropics with large numbers of envenomings and deaths per year. Although the burden of snake bite is high, the epidemiology remains unclear due to poor reporting of cases (Kasturiratne et al., 2008). In Sri Lanka Hump-nosed pit vipers (HNVs) are considered to be the commonest cause of snakebite (Kasturiratne et al., 2005; De Silva and Ranasinghe, 1983). However, the taxonomy of the HNV has been unclear until a recent revision. Anecdotal experience of clinicians suggests that most HNV bites only cause minor envenoming, but there are reports of more serious envenoming and most recently reports of acute kidney injury (Kularatne and Ratnatunga, 1999; Ariaratnam et al.,

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http://dx.doi.org/10.1016/j.toxicon.2012.10.013

2008; Maduwage et al., 2011a; Herath et al., 2012; de Silva et al., 1994).

The *Hypnale* genus was recently revised to contain three nominal species viz: *Hypnale hypnale* (Fig. 1a), *Hypnale nepa* (Fig. 1b) and *Hypnale zara* (Fig. 1c) and an un-named new species (*Hypnale* sp. "Amal"), described from a single specimen from Sri Lanka (Maduwage et al., 2009). *H. hypnale* is widespread throughout the lowlands (<600 m) of Sri Lanka but also occurs in the Western Ghats that border the west coast of the Indian peninsula. The other two species are restricted to Sri Lanka; *H. nepa* being restricted to the central highlands above ~1250 m, and *H. zara* being restricted to the rainforests of the island's south-western lowlands (Maduwage et al., 2009).

Although *H. hypnale* was one of the first snakes in which the pathophysiology of envenoming was studied (Davy, 1821), the clinical effects resulting from bites in humans still remain poorly defined. Coagulopathy, acute kidney injury and severe local effects have been documented following HNV bites in Sri Lanka. However, there is considerable variation in reported effects of *H. hypnale* bites (de Silva, 1989; Sellahewa and Kumararatne, 1994; Kularatne and Ratnatunga, 1999; Seneviratne et al., 2000; Ariaratnam et al., 2008; Wijewantha and Sellahewa, 2010). The medical importance of *H. hypnale* was also not recognized in India until the first case series of fatal envenoming appeared in literature (Joseph et al., 2007). There is limited information on the two *Hypnale* species endemic to Sri Lanka. Recent work on the venoms of the three *Hypnale* species suggests that local effects are likely to be the most common and important effects, although the venom has a mild procoagulant effect (Maduwage et al., 2011b).

The aim of this study is to investigate the clinical effects of envenoming by the three *Hypnale* species, to help determine the clinical importance of hump-nosed vipers.

2. Methods

A prospective hospital-based clinical study of definite bites by *Hypnale* spp. (hump-nosed vipers) was carried out from July 2008 to July 2010 in six Sri Lankan hospitals

Fig. 1. (a) Merrem's hump-nosed pit viper (*Hypnale hypnale*), Peradeniya (Central province), female 386 mm in total length; (b) High land hump-nosed pit viper (*Hypnale nepa*), Agarapathana, (Central province), male 368 mm in total length; (c) *Hypnale zara*, Galaha (Central province), female 406 mm in total length. *H. hypnale* is distinguished from *H. zara* by having the snout tip not raised (versus distinctly elevated in *H. zara*); 3–5 scales between rostral scale and internasals (versus 10–19 scales forming a wart-like protuberance at the snout tip in *H. zara*); 5–15 heterogeneous scales on the internasal-prefrontal region (versus 18–39 in *H. zara*); 4–5 scales (versus 3 in *H. zara*) surrounding the maxillary pit; and possessing a lacunal scale which is lacking in *H. zara*. *H. hypnale* is distinguished from *H. nepa* by lacking (versus possessing in *H. nepa*) a wart-like protuberance at the tip of the snout; lacking scales (versus possessing 1–3 scales in *H. nepa*) between the post-foveal and 3rd supralabial; having the lower postocular, 1st lower temporal, 4th and 5th supralabials in contact, with no scale between them (versus separated by a rhomboid scale in *H. nepa*); having the costal scales keeled (versus smooth in *H. nepa*) in mid-dorsal region; possessing 141–158 (versus 122–134 in *H. nepa*) ventrals; and having the lobes of hemipenes smooth, lacking spines (versus hemipenial lobes with spines in *H. nepa*).

representing different climatic zones inhabited by the three species of *Hypnale* (Maduwage et al., 2009) [Fig. 2].

All patients presenting following a suspected snakebite by a hump-nosed viper and who had brought the snake in were entered in the study. Patients were assessed on admission and then every 12 h until discharge according to the same protocol at all six hospitals. Demographic information, details of the bite, clinical effects, laboratory investigations were recorded on a clinical research form by the treating doctor or one of the investigators (KM). The clinical research form included pre-defined clinical information. Twenty minute whole blood clotting test (WBCT20) was carried out in all patients (Warrell et al., 1977; Sano-Martins et al., 1994) on admission and repeated in patients every 6 h if it was abnormal.

The severity of local pain was categorized as mild (only evident if patient was directly asked about pain), moderate (discomfort to the patient that is controlled by lying still, patient will either be asking for analgesia or happy to accept it if offered) and severe (sleep disturbance and calling for urgent pain relief) (Atkinson et al., 1993). The presence of secondary infection was based on the treating medical team's diagnosis. Specific systemic effects included coagulopathy, myotoxicity, neurotoxicity and acute kidney injury. Non-specific systemic symptoms included nausea, vomiting, headache, abdominal pain, diarrhoea and generalized diaphoresis which could not be attributed to a specific toxic effect.

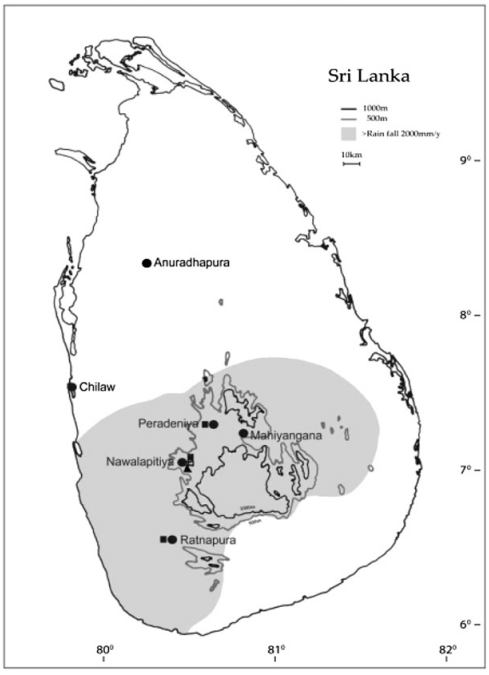

Fig. 2. Distribution of *Hypnale hypnale* (circle), *H. zara* (square) and *H. nepa* (triangle) cases collected in this study.

All snake specimens were labelled with patient's name and date of admission. They were provisionally identified in the ward and later transported to University of Peradeniya for definitive identification by one of the authors (KM). Dead snakes were transported preserved in 10% formalin while live snakes were transported in special containers for identification and were later released to their natural habitats. All snakes were identified using standard identification keys (Maduwage et al., 2009). Identified specimens were deposited in the Department of Biochemistry, Faculty of Medicine, University of Peradeniya.

3. Results

There were 114 snake bite patients who presented with either a live or a dead specimen of a *Hypnale* snake. All snakes were correctly identified as *Hypnale* species by the hospital staff. Of the 114 snake bite patients, 93 (81%) were of *H. hypnale*, 16 (14%) of *H. zara* and 5 (4%) of *H. nepa* bites. The median age of patients with all *Hypnale* bites was 39 years (IQR: 20–48; Range 4–74) and 75 (66%) were male. Most bites occurred in the daytime. The majority of bites were on the lower limb 78 (68%), and the remainder on the upper limb excepting one bite on the upper back. There was no difference in patient characteristics and the effect of bites between the three species, although there were small numbers of bites by *H. zara* and *H. nepa*. Most of *H. hypnale* bites were in home gardens whilst all *H. zara* bites occurred in the forests. Most *H. nepa* bites occurred in tea estates.

Fang marks were present in all patients associated with local pain (mild 42%, moderate 46% and severe 12%). One hundred and one (89%) patients had swelling but only 16 (14%) developed extensive local swelling that spread proximally to involve more than half of the bitten limb. There was no difference in the proportion of patients with swelling or the proportion with more extensive local injury, when comparing those who used a tourniquet or not. Other local effects are described in Table 1.

Non-specific systemic symptoms occurred in 18 (16%) of the 114 cases (Table 1). Specific systemic envenoming features occurred only in patients with *H. hypnale* bites. There were four patients with an abnormal WBCT20 and a single case of acute kidney injury. No patients had bleeding gums, haematemesis, haemoptysis or ptosis (Table 1). No patients developed neurotoxicity and myotoxicity.

Fifty seven percent of the patients received some form of first aid before their admission to hospital. The commonest first aid measures adopted were, washing the bite site and application of a tourniquet above the bite site. The majority of HNV bite victims were admitted to the hospitals within 2 h of the bite.

All HNV bites were managed symptomatically without the administration of antivenom. The patient with acute kidney injury was managed with haemodialysis. Local complications required treatment in 16 patients including, 14 who had wound toilet, one who required finger amputation due to severe local necrosis, and one patient who had a lower leg fasciotomy for regional swelling and local necrosis of the bite site. All victims recovered and were discharged from the hospital. The median length of hospital stay was 2 days (Range: 2–10 days).

Table 1
Clinical features in 114 patients with hump-nosed viper bites.

Clinical features	*H. hypnale* cases	*H. zara* cases	*H. nepa* cases
Local effects			
Local pain	93	16	5
Mild	43	4	1
Moderate	39	10	3
Severe	11	2	1
Local swelling	80	16	5
Swelling extend more than ½ of limb	10	3	3
Local bleeding (>10 min)	5	1	–
Fang marks	93	16	5
Blistering	10	3	–
Bruising	21	4	–
Lymph node enlargement	10	2	–
Local necrosis	12	4	–
Secondary infection at the site of bite	1	1	–
Non-specific systemic manifestations			
Fever	10	2	1
Headache	13	4	1
Nausea	13	4	1
Vomiting	9	3	1
Abdominal pain	5	1	1
Specific systemic manifestations			
Haematuria	1	–	–
Positive WBCT20	4	–	–
Acute kidney injury	1	–	–

4. Discussion

Development of local swelling and pain were the predominant local signs of the victims in this study. Severe systemic envenoming was present only among the victims of *H. hypnale*. However, non-specific symptoms and mild local envenoming were present among the victims of all three *Hypnale* species (Table 1). Hospital admissions of patients envenomed by the three species of HNV in this study were comparable with the geographical distribution of the each species as described in Maduwage et al. (2009) (Fig. 2). The study showed that doctors and hospital staff were able to correctly identify HNV to genus level.

H. hypnale prefers more anthropogenic habitats (Maduwage et al., 2009), which may explain the reason for most bites occurring in home gardens during daytime. All the envenoming by *H. zara* in this study occurred in forests which is the most likely reason for the low number of patients envenomed by this species compared to *H. hypnale*. This is consistent with the natural history of the snake as described by Maduwage et al. (2009).

In this study HNV envenoming caused local effects in the majority of patients and less frequently coagulopathy and acute kidney injury (Wijewantha and Sellahewa, 2010). Unfortunately more comprehensive coagulation studies were not available to define the frequency and severity of coagulopathy in HNV bites, and further studies should include at least an INR. However, Ariaratnam et al. (2008) reported coagulopathy as defined by a WBCT20 in 39% and acute kidney injury in 10% of the 302 cases of *Hypnale* envenoming from Sri Lanka. The difference between this study and ours in the frequency of coagulopathy may be due to the use of the WBCT20 to indicate coagulopathy.

Ariaratnam et al. (2008) reported the development of chronic renal failure in 2 patients after *H. hypnale* envenoming. We were not able to confirm this because follow up of patients after discharge from the ward was not possible in this study. A recent report of 13 cases of acute kidney injury following an HNV envenoming reported that six developed chronic kidney disease within 1 year of envenoming and histological findings revealed the development of focal segmental glomerular sclerosis, cortical necrosis and interstitial nephritis (Herath et al., 2012). Maduwage et al. (2011a) described a case report of *H. zara* envenoming causing coagulopathy and acute kidney injury. The mechanism of acute kidney injury in HNV envenoming remains unclear.

de Silva (1989) and Ariaratnam et al. (2008) reported two cases of *H. nepa* causing mild local pain and swelling consistent with this study. Although severe systemic envenoming has not been observed among the small number *H. nepa* and *H. zara* envenoming cases in this study, these two species are still likely to cause severe systemic envenoming in a small proportion of cases as reported in *H. zara* by Maduwage et al. (2011a).

Maduwage et al. (2011b), revealed that the three *Hypnale* venoms had similar chromatographic profiles in reverse phase high performance liquid chromatography and SDS-PAGE results. All three *Hypnale* venoms had potent cytotoxicity, mild procoagulant activity, and weak neurotoxic, myotoxic and the phospholopase A2 activity (Maduwage et al., 2011b). This is consistent with the clinical findings in this study with mainly local cytotoxic effects and coagulopathy in a small number of cases.

There were a number of limitations to the study. There were only a few cases of *H. nepa* and *H. zara* which reflects the frequency of bites by these less common species. This means that larger studies are required to better define severity and frequency of clinical effects in these two species, and whether they are similar to *H. hypnale*. Comprehensive laboratory testing is not routine in Sri Lanka for the management of snake envenoming, and specific tests such as creatinine and creatine kinase are only requested if clinically indicated and if available at the hospital. This lack of laboratory results in all patients and the fact that this study did not include additional blood collection from patients, limits our conclusions on coagulopathy, acute kidney injury and myotoxicity, which are dependent on laboratory investigations. Finally, interventions such as fasciotomy or surgical amputation were determined by the treating doctors and are simply reported in this study.

This study confirms that HNV bites cause only minor effects in the majority of cases. However, it is important that all HNV bites are observed and have investigations performed to detect the presence of coagulopathy or renal impairment. Future studies will need to define the timing and type of coagulation tests that are required and identify important early indicators of acute kidney injury.

Acknowledgements

We are grateful to administrators, consultant physicians and nursing staff of the 6 study hospitals for their support

in clinical data collection. We thank Andrew Dawson, Mohomad Fahim (South Asian Toxicology Research Collaborations) for providing support in hospital data collection for this study, Rohan Pethiyagoda (Australian Museum Natural History, Sydney), Dharma Sri Kandamby (National Maritime Museum, Galle), D.J. Weilgama (Rajarata University) and S. A. M Kularatne (University of Peradeniya) for their valuable advices and support.

Ethical approval

Ethics Committee of the Faculty of medicine, University of Peradeniya, Sri Lanka.

Funding

None.

Authors contributions

KM, IG, AS designed the study; KM, SB and SM carried out the clinical assessment and identified the species of snakes; KM, GI & IG carried out the analysis and interpretation of the data; KM, AS, IG and GI drafted the manuscript. All authors read and approved the final manuscript. KM, GI and IG are guarantors of the paper.

Conflicts of interest statement

None declared.

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Detection of Snake Venom in Post-Antivenom Samples by Dissociation Treatment Followed by Enzyme Immunoassay

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Academic Editor: R. Manjunatha Kini
Received: 11 March 2016; Accepted: 11 April 2016; Published: 28 April 2016

Abstract: Venom detection is crucial for confirmation of envenomation and snake type in snake-bite patients. Enzyme immunoassay (EIA) is used to detect venom, but antivenom in samples prevents venom detection. We aimed to detect snake venom in post-antivenom samples after dissociating venom-antivenom complexes with glycine-HCl (pH 2.2) and heating for 30 min at 950 °C. Serum samples underwent dissociation treatment and then Russell's viper venom or Australian elapid venom measured by EIA. In confirmed Russell's viper bites with venom detected pre-antivenom (positive controls), no venom was detected in untreated post-antivenom samples, but was after dissociation treatment. In 104 non-envenomed patients (negative controls), no venom was detected after dissociation treatment. In suspected Russell's viper bites, ten patients with no pre-antivenom samples had venom detected in post-antivenom samples after dissociation treatment. In 20 patients with no venom detected pre-antivenom, 13 had venom detected post-antivenom after dissociation treatment. In another 85 suspected Russell's viper bites with no venom detected pre-antivenom, 50 had venom detected after dissociation treatment. Dissociation treatment was also successful for Australian snake envenomation including taipan, mulga, tiger snake and brown snake. Snake venom can be detected by EIA in post-antivenom samples after dissociation treatment allowing confirmation of diagnosis of envenomation post-antivenom.

Keywords: venom; antivenom; dissociation; enzyme immunoassay; venom detection; snakebite

1. Introduction

Snake envenomation remains a neglected tropical disease with large numbers of cases in resource poor countries, many without antivenom [1]. In some cases snake envenomation syndromes are poorly defined and basic clinical research is required to better define human envenomation and the effect of interventions such as antivenom. Enzyme immunoassay (EIA) has been used to detect snake venom in envenomed patients' samples for the last four decades [2]. Detection and identification of snake venom is particularly crucial in clinical trials [3–5] as well as in prospective studies of definite snakebite cases to correctly define the effects of snake envenomation and the effect of different interventions. This avoids the need to collect and identify snakes that bite patients, which is only possible in a proportion of cases. Currently, this approach depends on the availability of a pre-antivenom serum sample to confirm snake venom by EIA. Lack of a pre-antivenom serum sample can lead to the exclusion of patients from some clinical studies. In addition, confirmation of envenomation in forensic cases is

important [6]. However, in such cases after antivenom is given there is currently no method to detect venom. Therefore, detection of snake venom in post-antivenom samples may increase the number of definite cases in clinical studies and confirmation of snake envenomation in forensic samples.

Specific venom EIAs rely on snake venom antigens in blood samples binding to anti-snake toxin antibodies bound to a microplate. These venom antigens are then detected by labelled anti-snake antibodies, a technique referred to as a sandwich EIA [5,7]. A recent study has shown that this venom specific EIA can also detect venom which is bound to antivenom [8,9]. However, this does not occur if there is excess antivenom present, which is usually the case after the administration of therapeutic antivenom. To be able to measure venom in the presence of excess antivenom the venom-antivenom complex needs to be dissociated and antivenom removed (destroyed) so that venom can then be detected.

In this study, we investigate a method to dissociate venom-antivenom complexes in post-antivenom samples, enabling detection of the venom by EIA. The method is tested in three series of post-antivenom samples from envenomed patients.

2. Results

2.1. Demonstration of Venom-Antivenom Dissociation

An initial trial of dissociation conditions, using buffers of pH 3.6 and 2.2, and temperatures of 37 °C or 95 °C, found that the most extreme conditions were more successful in recovering measureable venom from venom-antivenom complexes, without significant loss of free venom due to the harsh conditions. Solutions of Russell's viper (*Daboia russelii*) venom (RVV) at concentrations of 1000 ng/mL, 500 ng/mL, 250 ng/mL and 125 ng/mL with and without antivenom were treated with glycine-hydrochloric acid (HCl) buffer of pH 2.2 and heated at 95 °C for 30 min (dissociation treatment). The recovery of free venom in solutions without antivenom ranged from 70% to 86% (Figure 1; ratio of panel B to panel A). When the same solutions had antivenom (10 mg/mL) added and treated the same way, the recovery of free venom from venom-antivenom complexes ranged from 43% to 65% (Figure 1; ratio of panel D to panel A). From this, the limit of detection of RVV in samples with dissociation treatment was estimated to be 50 ng/mL. Measurement of antivenom in treated samples gave absorbance values not significantly different from samples to which no antivenom had been added, showing that antivenom (antibodies) is destroyed by the dissociation conditions.

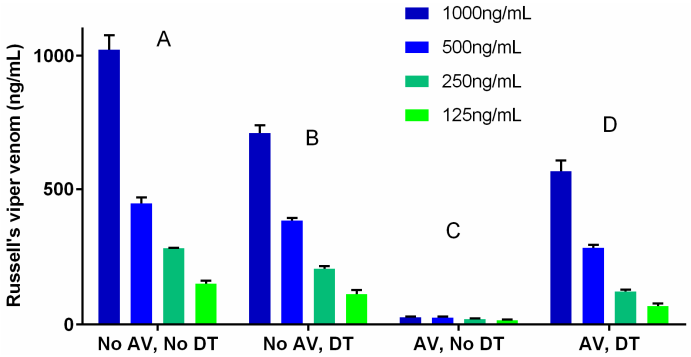

Figure 1. Effect of dissociation treatment (DT; treatment with glycine-HCl buffer of pH 2.2 and heated at 95 °C for 30 min) on the detection of free venom at four concentrations (mean ± SEM) of Russell's viper venom (RVV; ng/mL), with and without antivenom (AV; 10 mg/mL). The four sections of the graph represent solutions with no antivenom and no DT (Panel **A**), no antivenom with DT (Panel **B**), with antivenom but no DT (Panel **C**) and with antivenom and DT (Panel **D**).

Solutions of brown snake (*Pseudonaja textilis*) venom at a concentration of 50 ng/mL were prepared with increasing concentrations of brown snake antivenom (0 to 2500 mU/mL), and subjected to the dissociation treatment. Recovery of venom was near 100% in samples with no antivenom, declining to <40% at very high concentrations of antivenom (Figure 2).

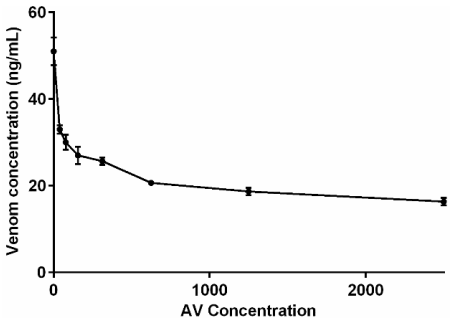

Figure 2. Plot of the measured venom concentration (mean ± SEM) versus antivenom concentration for venom-antivenom mixtures initially containing 50 ng/mL brown snake (*Pseudonaja textilis*) venom, after dissociation treatment.

In some cases of envenomation by Australian snakes, samples after dissociation treatment were measured for venom with and without the addition of antivenom. In the former case, venom was detected and then was not detectable after the addition of antivenom (data not shown). This ensures that the absorbance is not due to background, and is important for testing cases with low concentrations of venom near the limit of detection.

2.2. Detection of Venom after Venom-Antivenom Dissociation in Patient Samples

The venom-antivenom dissociation treatment was used in thawed serum samples collected from two series of Sri Lankan Russell's viper bites (Series 1 and 2) and one series of Australian snake bites (Series 3).

Series 1 included 143 patients recruited to a prospective cohort study of snakebites in north central Sri Lanka. The series consisted of 9 patients with venom detected pre-antivenom (positive controls), 104 non-envenomed patients (negative controls) and 30 patients with suspected Russell's viper bites who had either no pre-antivenom samples (10) or no venom detected in the pre-antivenom samples (20) (Figure 3). Venom was detected in all nine positive controls (False negative rate 0%; 95% Confidence intervals (CI): 0% to 37%) and was not detected in the 104 negative controls (False positive rate 0%; 95% CI: 0% to 4%). The latter demonstrates that the dissociation treatment does not produce false positives by measuring venom in samples without venom present. In all ten patients with no pre-antivenom samples venom was detected post-antivenom after dissociation treatment. Twenty patients had no venom detectable in their pre-antivenom samples, with or without dissociation treatment. In 13 of these 20, venom was detected in post-antivenom samples after dissociation treatment. There were seven patients for which venom could not be detected at all in any post-antivenom sample even after dissociation treatment. No particular bite site, time post-bite or number of antivenom doses given was associated with these seven patients.

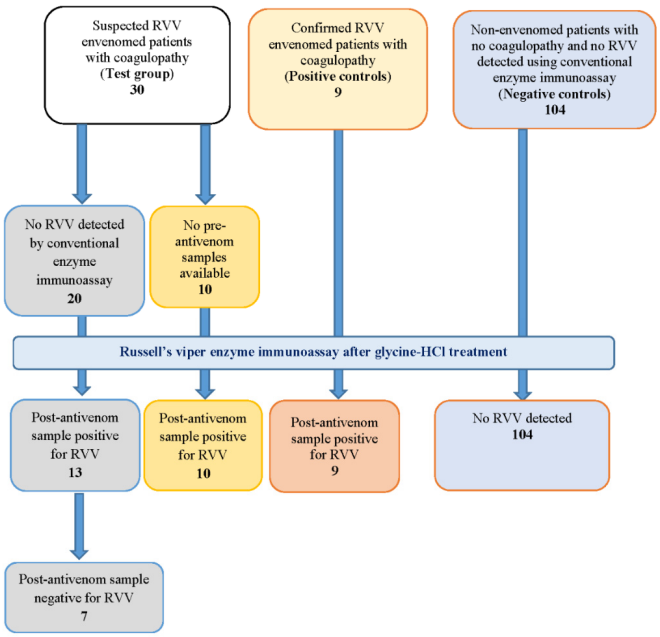

Figure 3. Detection of Russell's viper venom (RVV) by enzyme immunoassay after dissociation treatment of positive and negative controls, and patients with suspected Russell's viper envenomation.

Series 2 was a prospective cohort study of Russell's viper bites recruited to a study of neurotoxicity [10]. In this series, 85 of 216 patients had no venom detectable in their admission or pre-antivenom samples, and no snake had been properly identified. After dissociation treatment, at least one sample from 50 of these patients revealed the presence of RVV, enabling the inclusion of these additional patients in the study.

Series 3 included snake envenomation cases from the Australian Snakebite Project (ASP) which is a prospective study of snakebites across all Australia where serum samples are collected and frozen from recruited patients. Serum samples were subjected to dissociation treatment to confirm the type of snake in patients where pre-antivenom samples were not available. If no venom from the suspected snake type was detected after dissociation treatment, then the next most likely venom assay was done, based on geography and clinical effects. There were ten patients with suspected taipan (*Oxyuranus scutellatus*) envenomation. Four patients had taipan venom detected pre-antivenom and also after dissociation treatment in post-antivenom samples (positive controls; Figure 4). Six had no pre-antivenom samples of which four had taipan venom detected after dissociation treatment post-antivenom. In the two remaining cases no taipan venom was detected after dissociation treatment, but brown snake venom was detected in one of these being the next most likely snake in this region. There were four suspected mulga snake (*Pseudechis australis*) envenomations with no pre-antivenom samples. In two patients mulga snake venom was detected after dissociation treatment. In the remaining two, mulga snake venom was not detected, but in one brown snake venom was detected, again being the next most likely snake. Two cases of tiger snake envenoming were confirmed by subjecting post-antivenom samples to the dissociation treatment.

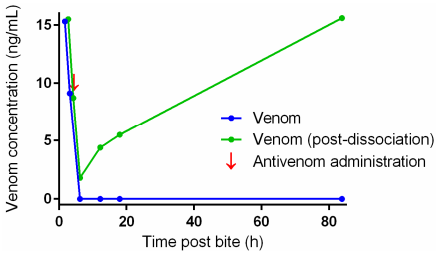

Figure 4. Plots of venom concentrations versus time post-bite before (filled circles and line) and after (filled squared and dashed line) dissociation treatment for a case of taipan envenomation. The arrow marks the time of the administration of antivenom.

3. Discussion

This study shows that venom can be detected in post-antivenom samples by dissociating venom and antivenom complexes with acid and heat. In three series of patients in which no pre-antivenom samples were available or in which venom could not be detected in pre-antivenom samples, venom was detected post-antivenom using this dissociation method. This allows the positive identification of cases for inclusion in research studies, as in series 2, or forensic identification of cases where only post-antivenom samples may be available. The measurement of venom in post-antivenom samples was shown not to be due to artefact by using negative control samples, and by the addition of antivenom to samples after dissociation, and re-measuring.

Dissociation of immune complexes with the aim of enabling antigen identification has been done with a number of other substances. Heating samples with ethylenediaminetetraacetic acid (EDTA) was found to improve the detection of *Histoplasma* and *Coccidioides* antigens [11,12], while sodium dodecyl sulfate (SDS) has been used in studies of autoimmune disease [13]. Heating in an acid such as HCl [14], acetic acid [15] or acid glycine [16,17] is a common approach for dissociating antigens and antibodies. Gustaw *et al*. used this technique to unmask anti-amyloid-β antibodies previously undetectable because of the presence of excess antigen [17]. Of these reagents, we found that glycine-HCl with heat gave the best recovery of venom from venom-antivenom complexes.

Antivenom was destroyed by heating (to 95 °C) in glycine-HCl and so was no longer detectable using a venom-coated plate. Antibodies have only two binding sites per molecule, and these are on the end on a chain, more susceptible to acid attack. Antigens have several epitopes, which, especially if linear, may be unaffected by hydrolysis elsewhere in the molecule.

An important limitation of the study was that RVV was not detectable in 7 out of 20 patient samples in series 1, which is most likely due to the concentration of RVV being below the limit of detection exacerbated by loss of venom during dissociation treatment. The reason for venom not being detected pre-antivenom (*i.e.*, <2 ng/mL) initially and then post-antivenom is best explained by a smaller delivered venom dose which is then slowly absorbed into the systemic circulation. These few negative cases after dissociation treatment need further investigation, in particular an improved understanding of the absorption kinetics of viper venoms. In addition, a larger study of patient samples needs to be undertaken to fully define the sensitivity and specificity of the assay.

Figures 1 and 2 demonstrate that both the venom concentration and the antivenom concentration change the recovery of the venom after venom dissociation. This means that venom measurements after venom dissociation are not quantitative and only determine if there is venom present or not. The low and essentially zero false positive rate supports the accuracy of detecting venom. Figure 4 shows that the venoms concentrations appeared to be increasing after the administration of antivenom, at least for the available samples assayed. There are a number of possible explanations for this including ongoing gradual absorption of venom into the circulation from the bite site or re-distribution of venom

from peripheral sites back into the circulation. It may also be simply that there is a changing ratio of venom to antivenom and therefore changes in venom recovery. Further work is required to investigate this phenomenon.

We have previously shown that venom, if detected in a post-antivenom sample, is largely in bound form, as venom-antivenom complexes [9]. However, for none of the patients in series 1 was venom detectable in untreated post-antivenom samples. Subjecting the samples in series 1 to dissociating conditions revealed the presence of venom in 32 of the 39 cases, and in 50 of 85 in series 2. The method was also successful in series 3 of Australian cases, despite the considerably lower venom concentrations after Australian elapid envenoming.

Dissociation of venom-antivenom complexes with dissociation treatment will be useful in all cases where no pre-antivenom sample is available and there is a question as to whether envenomation has actually occurred, or where the identity of the snake needs to be confirmed. This will be important for both clinical studies, confirmation of envenomation in patients and in forensic medicine.

4. Materials and Methods

4.1. Materials

Indian polyvalent snake antivenom was obtained from VINS Bioproducts Limited (Hyderabad, Andra Pradesh, India; Batch #01011/10-11). RVV was milked from snakes in Sri Lanka, pooled and then lypholised. Brown snake antivenom was obtained from CSL Ltd (Parkville, Australia, Batch #0559-11001; Expiry 05/13). Stock solutions of venom were prepared as 1 or 2 mg/mL in 50% glycerol and stored at −20 °C. Standard human serum (S7023) and tetramethylbenzidine (TMB) were purchased from Sigma (St Louis, MO, USA), Bovine serum albumin (BSA) from Bovogen (Keilor, Victoria, Australia), and Streptavidin-conjugated horseradish peroxidase (Streptavidin HRP) from Calbiochem (San Diego, CA, USA) (Cat#: OR03L). Blocking solution was 0.5% BSA in phosphate buffered saline (PBS). Washing solution was 0.02% TWEEN 20 in PBS. Polyclonal monovalent rabbit IgG to RVV was obtained by injection of rabbits with RVV followed by purification of the serum on a Protein G-Sepharose column and was carried out at the Western Australian Institute of Medical Research. Rabbit IgG antibodies were biotinylated using EZ-Link Sulfo-NHS-LC-Biotin (Pierce #21335).

4.2. Patients

Patients were recruited from three different prospective cohort studies, two studies of Russell's viper bites in Sri Lanka and one cohort of Australian snake bites. Approval for the collection of the blood samples in Sri Lanka was obtained from Human Research Ethics Committees of the University of Peradeniya (2012/EC/63; approved 2012), University of Rajarata (ERC 2013/019; approved 2013), Monash University (CF14/970–2014000404; approved 2014), University of New South Wales (HERC 10023; approved 2011) and the University of Newcastle (H-2010-1060; approved 2010). Approval for the collection of blood samples in Australia was obtained from Human Research Ethics Committees of the University of Newcastle (H-319-0502; approved 2003), Hunter New England Area Health Service (07/11/21/3.06; approved 2006) and Menzies School of Health Research (HR 03-802; approved 2004). Series 1 was a large cohort of snake-bite patients from two Sri Lankan hospitals [5,18], in which four groups of patients were selected to test the dissociative treatment. The first group was nine definite Russell's viper bites in which RVV was detected in pre-antivenom samples (positive controls). The second group was 104 non-envenomed patients (no RVV detected) with no coagulopathy or clinical features of systemic envenomation (negative controls). The third group was 30 suspected Russell's viper bites with coagulopathy that had either no pre-antivenom samples (10) or no venom detected in pre-antivenom samples (20). Coagulopathy or venom induced consumption coagulopathy was diagnosed based on increased prothrombin time (PT), increased activated partial thromboplastin time (aPTT), elevated D-Dimer and low fibrinogen concentrations.

Series 2 of snake bite patients was from a large study of neurotoxicity in Russell's viper bites [10]. In this study there were a group of patients where Russell's viper envenomation was suspected but no snake was collected for identification and either venom was not detected pre-antivenom or there was no pre-antivenom sample. Post-antivenom samples were tested after dissociation in 85 suspected Russell's viper bites.

Patients in Series 3 were selected from patients recruited to the Australian Snakebite Project to investigate the use of the dissociative treatment in other snakes, including taipan (10; *Oxyuranus scutellatus*), brown snake (2; *Pseudonaja textilis*), mulga snake (4; *Pseudechis australis*) and tiger snake (2; *Notechis scutatus*) [7,19,20]. The cases included both positive controls where a pre-antivenom sample was positive for the snake venom and suspected cases in which no pre-antivenom sample was available.

4.3. Venom Antivenom Dissociation

Solutions of RVV in standard serum were prepared, then diluted with equal volumes of Indian antivenom in water or with water only. The resulting mixtures had RVV concentrations of 1000 ng/mL, 500 ng/mL, 250 ng/mL, 125 ng/mL and 0 ng/mL, with or without antivenom at 10 mg/mL. These mixtures were allowed to stand at room temperature for an hour then at 4 °C overnight. For venom-antivenom dissociation, 50 μL of the mixture was removed and added to 50 μL of 0.1 M glycine-HCl buffer (pH 2.2) in a microplate. The plate was covered and placed in an oven at 95 °C for 30 min. As a control, a second 50 μL of the venom-antivenom mixture was added to 50 μL of water and not heated (*i.e.*, no dissociation treatment). To each well was then added 200 μL of blocking solution, then a portion was removed and further diluted in blocking solution to give a total dilution of 1:120 in the sample. This dilution was applied to a microplate to be assayed for venom, and then further diluted to 1:3120 to measure antivenom.

Solutions of brown snake venom in blocking solution (50 ng/mL) were mixed with increasing concentrations of brown snake antivenom (0 to 2500 mU/mL) and subjected to the dissociation treatment, similar to RVV. Dilutions of 1:14 were then applied to the microplates and assayed for venom using brown snake venom EIA. All dilutions were such that the maximum capacity of the assay was not exceeded (10 ng/mL in well). Dilutions were much greater for the higher venom concentrations seen with RVV, compared to the lower venom concentrations with Australian elapids.

4.4. Dissociation of Venom and Antivenom in Patient Samples

Thawed serum samples from patients were mixed with an equal volume of 0.1 M glycine-HCl buffer (pH 2.2) in a microtitre plate. The plate was covered and placed in an oven at 95 °C for 30 min. In a control series water was used instead of 0.1 M glycine-HCl buffer (pH 2.2) and the mixtures were not heated. Both treated and untreated samples were diluted 80 times in blocking solution before being assayed for RVV using the EIA. Samples from Australian snake bite patients were subject to the same conditions except were measured at less dilution. Again, sample dilutions were based on known venom concentrations which are much higher for RVV. In cases where RVV was not detected after venom dissociation, samples were re-run at a dilution of 1:10 to increase sensitivity.

4.5. Venom Enzyme Immunoassay

RVV was detected using a sandwich EIA as previously described [5,7,20] in thawed samples from the three series of patients. In brief, Greiner Microlon 96-well high-binding plates were coated with 100 μL of rabbit anti-RVV IgG (1 μg/mL) in carbonate buffer (50 mM, pH 9.6), kept at room temperature for 1 h and then at 4 °C overnight. The plates were then washed and 300 μL of blocking solution was applied. After 1 h the plates were washed again, and 100 μL of sample solution was applied. The plates were allowed to stand for 1 h and then washed three times. Next, 100 μL of biotinylated anti-RVV IgG (0.3 μg/mL in blocking solution) was added. After standing for a further hour the plates were washed again. Streptavidin-horseradish peroxidase (100 μL, 0.1 μg/mL in blocking solution) was added and

left for 1 h. The plate was then washed three times and 100 µL of TMB reagent added and colour allowed to develop for 3.5 min. The reaction was stopped by the addition of 50 µL of 1 M H_2SO_4. Plates were read on a BioTek Synergy HT instrument (BioTek Instruments Inc, Winooski, VT, USA) at 450 nm. All samples were measured in triplicate, and the averaged absorbance converted to a venom concentration by comparison with a standard curve based on eight serial dilutions of venom from 10 ng/mL to 0 ng/mL in blocking solution (concentration in the well). Data was fitted to a sigmoidal dose-response curve using GraphPad Prism software version 6.03 for Windows, GraphPad Software (San Diego, CA, USA).

Taipan, brown snake, mulga snake and tiger snake venoms were detected using a sandwich EIA, as previously described [7] and similar to RVV. For the Australian snake bite cases when venom from the suspected snake type was not detected, further snake venoms were then assayed based on the next most likely snakes to be involved considering geography, and clinical and laboratory effects.

Acknowledgments: We acknowledge all of the medical, nursing, laboratory and administrative staff in all of the hospitals involved in the snake studies.

Author Contributions: G.K.I. and M.A.O. conceived and designed the experiments; M.A.O., K.P.M. and A.S. performed the experiments; K.P.M., M.A.O. and G.K.I. analyzed the data; K.P.M., M.A.O. and G.K.I. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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