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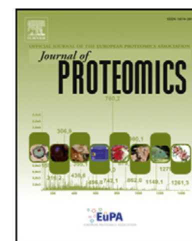

Vintage venoms: Proteomic and pharmacological stability of snake venoms stored for up to eight decades☆

Clémence Jesupret[a,1], Kate Baumann[a,1], Timothy N.W. Jackson[a,b,1], Syed Abid Ali[a,c,1], Daryl C. Yang[e,1], Laura Greisman[d,e,1], Larissa Kern[d,1], Jessica Steuten[d], Mahdokht Jouiaei[a], Nicholas R. Casewell[a,f,g], Eivind A.B. Undheim[a,b], Ivan Koludarov[a], Jordan Debono[a], Dolyce H.W. Low[a], Sarah Rossi[a], Nadya Panagides[a], Kelly Winter[e], Vera Ignjatovic[h,i], Robyn Summerhayes[h], Alun Jones[b], Amanda Nouwens[j], Nathan Dunstan[k], Wayne C. Hodgson[e], Kenneth D. Winkel[d], Paul Monagle[h], Bryan Grieg Fry[a,b,*]

[a]Venom Evolution Lab, School of Biological Sciences, University of Queensland, St. Lucia, Queensland 4072, Australia
[b]Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland 4072, Australia
[c]HEJ Research Institute of Chemistry, International Centre for Chemical and Biological Sciences (ICCBS), University of Karachi, Karachi 75270, Pakistan
[d]Australian Venom Research Unit, Department of Pharmacology and Therapeutics, University of Melbourne, Victoria 3010, Australia
[e]Monash Venom Group, Department of Pharmacology, Monash University, Clayton, Victoria 3800, Australia
[f]Alistair Reid Venom Research Unit, Liverpool School of Tropical Medicine, Liverpool L3 5QA, UK
[g]Molecular Ecology and Evolution Group, School of Biological Sciences, Bangor University, Bangor LL57 2UW, UK
[h]Department of Paediatrics, Faculty of Medicine, Dentistry and Health Sciences, University of Melbourne, Parkville, Victoria 3000, Australia
[i]Murdoch Childrens Research Institute, Parkville, Victoria 3052, Australia
[j]School of Chemistry and Molecular Biosciences, University of Queensland, St. Lucia, Qld 4072, Australia
[k]Venom Supplies, Tanunda, SA, Australia

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ABSTRACT

For over a century, venom samples from wild snakes have been collected and stored around the world. However, the quality of storage conditions for "vintage" venoms has rarely been assessed. The goal of this study was to determine whether such historical venom samples are still biochemically and pharmacologically viable for research purposes, or if new sample efforts are needed. In total, 52 samples spanning 5 genera and 13 species with regional variants of some species (e.g., 14 different populations of *Notechis scutatus*) were analysed by a combined proteomic and pharmacological approach to determine protein structural stability and bioactivity. When venoms were not exposed to air during storage, the proteomic results were virtually indistinguishable from that of fresh venom and bioactivity was equivalent or only slightly reduced. By contrast, a sample of *Acanthophis antarcticus* venom that was exposed to air (due to a loss of integrity of the rubber stopper) suffered significant degradation as evidenced by the proteomics profile. Interestingly, the

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* *Corresponding author at:* Institute for Molecular Bioscience, University of Queensland, St. Lucia, Queensland 4072, Australia.
E-mail address: bgfry@uq.edu.au (B.G. Fry).

[1] Contributed equally to the work.

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Death adder
Coastal taipan
Snake venom detection

neurotoxicity of this sample was nearly the same as fresh venom, indicating that degradation may have occurred in the free N- or C-terminus chains of the proteins, rather than at the tips of loops where the functional residues are located. These results suggest that these and other vintage venom collections may be of continuing value in toxin research. This is particularly important as many snake species worldwide are declining due to habitat destruction or modification. For some venoms (such as *N. scutatus* from Babel Island, Flinders Island, King Island and St. Francis Island) these were the first analyses ever conducted and these vintage samples may represent the only venom ever collected from these unique island forms of tiger snakes. Such vintage venoms may therefore represent the last remaining stocks of some local populations and thus are precious resources. These venoms also have significant historical value as the *Oxyuranus* venoms analysed include samples from the first coastal taipan (*Oxyuranus scutellatus*) collected for antivenom production (the snake that killed the collector Kevin Budden), as well as samples from the first *Oxyuranus microlepidotus* specimen collected after the species' rediscovery in 1976. These results demonstrate that with proper storage techniques, venom samples can retain structural and pharmacological stability. This article is part of a Special Issue entitled: Proteomics of non-model organisms.

Biological significance

- These results show that with proper storage venoms are useful for decades.
- These results have direct implications for the use of rare venoms.

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1. Introduction

Biodiversity is adversely affected by numerous external pressures including climate change and habitat fragmentation [1]. Habitat fragmentation, caused by agricultural practices and logging, has many impacts on the demographic characteristics of populations and communities and can ultimately lead to complete habitat loss [2,3]. Invasive species are also a major threat to biodiversity [2]. In general, threatened species are affected by more than one pressure source [2] and human activities result in the reduction of the range of native species and the isolation of populations, which may lead to local and even global extinction [4–6].

Reptiles are not exempt from these threats and a recent study estimates that 19% of the world's reptile species are currently threatened [2]. This figure is likely to increase if the relevant anthropogenic processes are not controlled. Among snakes, many species are declining around the world and the limited coverage of current snake population data hampers conservation initiatives [7]. Moreover, the processes threatening snakes are poorly known [8]. It has been estimated that 12% of snakes are threatened with extinction [2], although this figure may underestimate the severity of the problem due to difficulty in attaining population data (e.g., the challenges of detecting and surveying cryptic species). Of particular note, the biodiversity of oceanic islands is often underestimated [9]. In addition to the ecological impact of a decrease in snake species richness, these extinctions have economic consequences: venoms have applications in many fields including medicine, pharmacology and immunology [10–14].

In consideration of the potential applications of snake venom research, it is of paramount importance that venom samples are stored correctly after collection. Proper storage may limit the need to collect additional samples from wild snakes. This would save time and money for researchers and also spare the snakes unnecessary disturbance and stress (this is especially important when working with venoms from endangered or particularly dangerous species). Furthermore, as some snake species are rare and infrequently encountered, it might be impossible to collect additional samples in the future. As a result, field collection of venom should be conducted efficiently and with an emphasis on endangered species in order to preserve a sub-section of venom biodiversity that is declining from existence.

Studies have investigated the identification of proteins in stored formalin-fixed, paraffin-embedded in a diversity of tissue samples (*c.f.* [15–21]). Due to the harsh chemicals involved in the preparation of such samples, the proteins would not be expected to be intact or active, thus such studies are limited to protein identification. Similarly, a recent study showed that MS/MS could be used to identify the venom proteins in 26 year-old dried SDS gels [22] but this study did not determine the protein stability of protein, rather the study was restricted to simple protein identification. However, some data is available from previous studies examining the stability of venoms following storage, freeze–thawing cycles and sonication or dilution. Importantly, over the short term, a variable approach to preparation and storage procedures for venoms did not appear to result in marked degradation [23,24]. In regards to immunological stability, venoms also retain this over variable conditions throughout short periods of time [25] or longer if lypholised [26]. The effect of long-term storage conditions has been much less intensively investigated due to the unique logistics involved. In 1951, Schoettler published an article on the stability of desiccated snake venoms [27]. In this study, venoms from four different genera of venomous snake (*Agkistrodon*, *Bitis*, *Naja*, *Vipera*,) were tested over a period of 13 years and the influences of storage on the venom toxicity

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and antigenicity were assessed [27]. The experiment revealed that the stability of desiccated venoms depended on two factors: the type of venom (non-hemorrhagic, non-enzymatic venoms maintained their toxic potencies far better) and the method of storage. Venom exposed to sunlight in a vacuum desiccator was considered a less favourable method of storage, as opposed to being contained within cork stoppered glass tubes in a dark environment. Furthermore, the toxic and antigenic properties of venoms respond differentially to storage, as venom may exhibit a reduction in toxicity following storage, whilst its antigenicity appears unaltered. This study therefore concluded that such venoms could still be used for antivenom titration. A later study examined the effect upon toxicity of rattlesnake venom stored in closed containers at 42 °F to 82 °F in the dark for 26 to 27 years [28]. This study concluded that even after such long periods of time, venoms retained most of their lethality and physiological/pharmacological effects.

The first significant venom collection in Australia was that of George Britten Halford at the University of Melbourne in the 1860–70's [29]. Halford's controversial germ theory of snakebite poisoning stimulated demand for Australian snakes and their venoms from physician scientists in many parts of the world — most notably from Fayrer in India and Mitchell in Philadelphia. This was followed by the collection of Charles James Martin at the same institution in the late 1890's. Martin focused on assessing the efficacy of Calmette's purportedly 'universal' antivenom against Australian venoms and on characterizing the key actions of the constituent toxins [29]. Australian venom research was largely in abeyance thereafter until the late 1920's. This period brought together the Walter and Eliza Hall Institute of Medical Research and the then Commonwealth Serum Laboratories (known as CSL) in a Melbourne collaboration to develop Australia's first commercial antivenoms. By late 1930 Australia had its first commercial antivenom — against the mainland tiger snake, *Notechis scutatus* [30]. By 1934, most of this venom extraction was undertaken by CSL as part of antivenom production. The urgent need for antivenoms produced against Australia's most dangerous snakes motivated the so-called 'snake men' to catch medically-relevant species such as tiger snakes (*Notechis* spp.) and brown snakes (*Pseudonaja* spp.), and later taipans (*Oxyuranus* spp.). Once caught, snakes were either lodged at CSL and milked there or kept elsewhere, such as the Australian Reptile Park in NSW, to assist antivenom research and/or production. Some of the most important herpetologists of this period were Donald Thomson, Charles Tanner, Eric Worrell and David Fleay, who caught most of the snakes used in the early days of the antivenom project [31].

The present study compared dried venom samples stored for up to 80 years with freshly collected samples, observing differences in the proteomics profile, bioactivity and immunogenicity of the venom. The results are potentially significant for two reasons. If old samples retain biochemical and pharmacological viability, they can continue to be used for scientific research thus limiting the need to collect new material in the field. In cases where snake populations have undergone localized extinctions due to habitat degradation or impact of feral animals such as cane toads, these venoms may represent the last remaining stocks. Proteomic investigations were first used to examine if any breakdown had occurred in any of the vintage venoms. Bioactivity testing of the major venom types (*Acanthophis*, *Notechis*, *Oxyuranus* and *Pseudechis*) investigated whether functional activities could be preserved despite long-term storage conditions. Antigenic integrity was then assessed to examine if such venoms may be of use in antivenom studies or production. This study differed from previous venom stability studies [22–28] in both the age of the samples, the taxonomical diversity present and the conductance of bioactivity studies. This study is therefore novel in examining the relative retention of biological activity, and therefore their relative usefulness, of venoms stored for very long periods of time.

2. Materials and methods

2.1. Venom collection

The late Struan Sutherland subsequently maintained a collection of these historic venoms as well as developing a bank of his own samples that together represented milkings occurring between 1935 and 1986. Fifty two of the venoms from this long-term national reference collection, housed at the Australian Venom Research Unit, were investigated in this study. Venom types included the full molecular diversity of Australian snake venoms [32]: those rich in 6–10 kDa peptides (*Acanthophis* species); those rich in 14 kDa PLA_2 enzymes (*Pseudechis* species); those containing 50 kDa factor Xa enzyme (*N. scutatus* variants, *Oxyuranus* species and *Pseudonaja* species); as well as factor Va (*Oxyuranus* species and *Pseudonaja* species). To investigate the hypothesis

Fig. 1 – Representative of the vintage venom collection analyzed in this study: 1935 tiger snake venom.

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that lyophilized venoms stored in anhydrous conditions retain their structural integrity and bioactivity, a combined proteomic and pharmacological approach was used.

Dried venoms from the Sutherland collection had been stored in the dark at room temperature in small rubber stoppered glass tubes inside larger rubber stoppered glass tubes filled with desiccating beads (Fig. 1). The date and locality of collection for the specific venoms analysed in this study are displayed in Table 1. Historical venom samples were compared against freshly milked venoms from the same species (and from the same locality if the location of the vintage venom was known). Fresh venoms were milked by authors BGF and ND.

2.2. Proteomics

RP-HPLC, *1D-electrophoresis*, *LC/MS and MS/MS analyses* were as per described by us previously [33].

Table 1 – Vintage venoms examined in this study.

#	Species	Date	Locality	Collector
1.	*Acanthophis antarcticus*	1961	Not recorded	Not recorded
2.	*Acanthophis praelongus*	1960	Cooktown, Queensland	Not recorded
3.	*Acanthophis rugosus*	1953	Papua New Guinea	Not recorded
4.	*Acanthophis rugosus*	1955	Papua New Guinea	Not recorded
5.	*Acanthophis rugosus*	1955	Papua New Guinea	Not recorded
6.	*Notechis scutatus*	1935	Not recorded	Not recorded
7.	*Notechis scutatus*	1950	King Island	Worrell
8.	*Notechis scutatus*	1950	King Island	Worrell
9.	*Notechis scutatus*	1954	Kangaroo Island	Tanner
10.	*Notechis scutatus*	1954	Kangaroo Island	Tanner
11.	*Notechis scutatus*	1954	New Year Island	Not recorded
12.	*Notechis scutatus*	1954	Tasmania	Not recorded
13.	*Notechis scutatus*	1954	Lake Alexandrina, South Australia	Worrell
14.	*Notechis scutatus*	1955	Flinders Island	Worrell
15.	*Notechis scutatus*	1955	Flinders Island	Worrell
16.	*Notechis scutatus*	1955	Barmah, New South Wales,	Not recorded
17.	*Notechis scutatus*	1955	Franklin, Victoria	Not recorded
18.	*Notechis scutatus*	1955	Werribee, Victoria	Not recorded
19.	*Notechis scutatus*	1956	St. Francis Island	Not recorded
20.	*Notechis scutatus*	1957	Babel Island	Not recorded
21.	*Notechis scutatus*	1960	Chappell Island	Worrell
22.	*Notechis scutatus*	1960	King Island	Worrell
23.	*Notechis scutatus*	1961	Western Australia	Tanner
24.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
25.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
26.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
27.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
28.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
29.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
30.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
31.	*Oxyuranus microlepidotus*	1976	Not recorded	Not recorded
32.	*Oxyuranus s. canni*	1953	Papua New Guinea	Not recorded
33.	*Oxyuranus s. scutellatus*	1950	Cairns, Queensland	Not recorded
34.	*Oxyuranus s. scutellatus*	1952	Cairns, Queensland	Worrell
35.	*Oxyuranus s. scutellatus*	1956	Cairns, Queensland	Ram Chandra
36.	*Oxyuranus s. scutellatus*	1960	Cairns, Queensland	Not recorded
37.	*Oxyuranus s. scutellatus*	1960	Cairns, Queensland	Cook
38.	*Oxyuranus s. scutellatus*	1960	Cairns, Queensland	Cook
39.	*Oxyuranus s. scutellatus*	1960	Cairns, Queensland	Not recorded
40.	*Oxyuranus s. scutellatus*	1961	Cairns, Queensland	Cook
41.	*Pseudechis australis*	1958	Not recorded	Not recorded
42.	*Pseudechis guttatus*	1953	Not recorded	Not recorded
43.	*Pseudechis guttatus*	No date	Not recorded	Not recorded
44.	*Pseudechis papuanus*	1966	Not recorded	Not recorded
45.	*Pseudechis porphyriacus*	1964	Not recorded	Not recorded
46.	*Pseudonaja affinis*	1960	Not recorded	Tanner
47.	*Pseudonaja inframacula*	1960	Not recorded	Tanner
48.	*Pseudonaja inframacula*	1960	Not recorded	Tanner
49.	*Pseudonaja textilis*	1976	Cuddapan, Queensland	Tanner
50.	*Pseudonaja textilis*	1977	Cuddapan, Queensland	Tanner
51.	*Pseudonaja textilis*	1978	Cooktown, Queensland	Tanner
52.	*Pseudonaja textilis*	1979	East coast, New South Wales	Tanner

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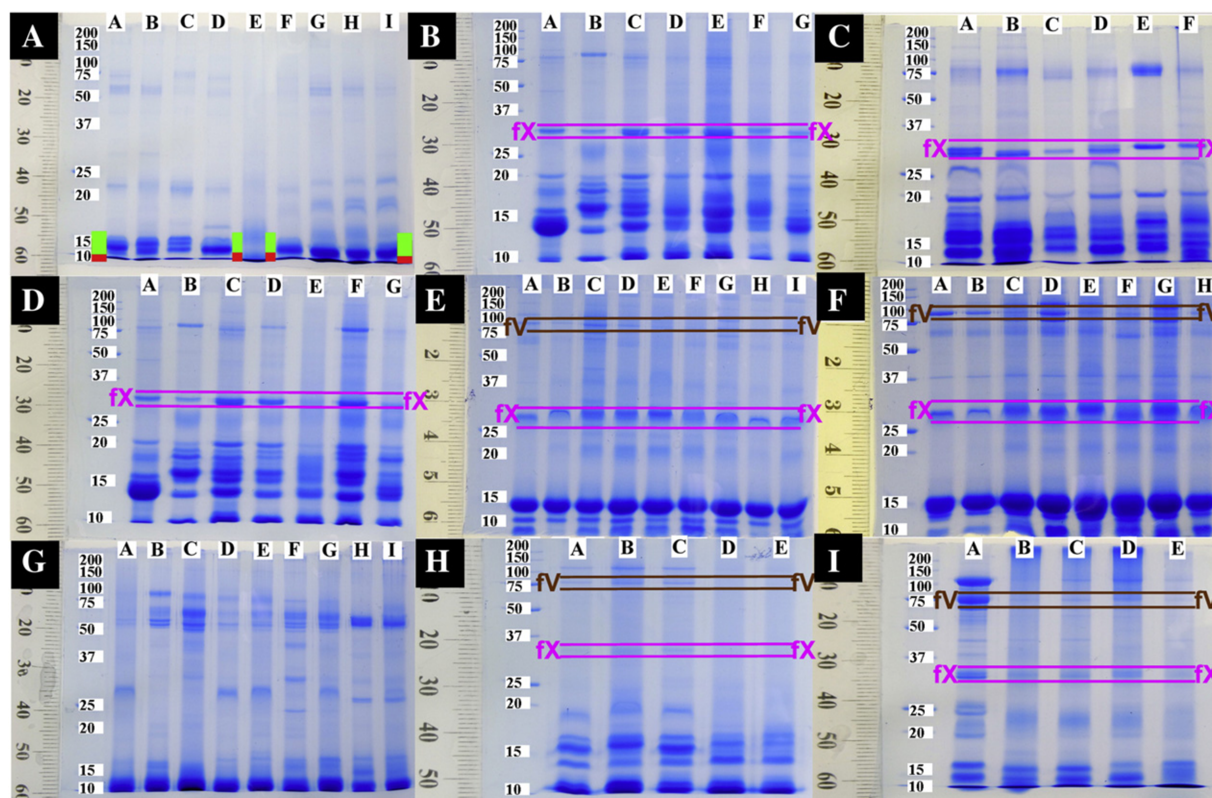

Fig. 2 – Representative 1D gels: A A) *Acanthophis antarcticus* FRESH Middleback ranges, South Australia, B) *A. praelongus* FRESH Cairns, Queensland, C) *A. rugosus* FRESH Camooweal, Queensland, D) *A. laevis* FRESH Merauke, West Papau, E) VV1, G) VV3 I) VV4; *B* A) *Notechis scutatus* FRESH Lake Alexandria, South Australia, B) *N. scutatus* FRESH Chappell Island, C) *N. scutatus* FRESH Melbourne, Victoria, D) VV7, E) VV8, F) VV22, G) VV13; *C* A) *N. scutatus* FRESH Victoria, Melbourne), B VV16), C) VV17, D) VV18, E) *N. scutatus* FRESH Western Australia, F) *N. scutatus* 1961 Western Australia; *D* A) *N. scutatus* FRESH Lake Alexandria, South Australia, B) *N. scutatus* FRESH Chappell Island, C) *N. scutatus* FRESH Melbourne, Victoria, D) VV6, E) VV20, F) VV21, G) VV15; *E* A) *Oxyuranus microlepidotus* FRESH Lake Eyre, South Australia; B) VV24, C) VV25, D) VV26, E) VV27, F) VV28, G) VV29, H) VV30, I) VV31; *F* A) *Oxyuranus scutellatus scutellatus* FRESH Cairns, Queensland, B) VV33, C) VV34, C) VV35, D) VV36, E) VV37, F) VV38, G) VV39 H) VV40; *G* A) *Pseudechis australis* FRESH Alice Springs), B) VV41 C) *P. guttatus* FRESH no locality for founding stock, D) VV42, E) VV43, F) *P. papuanus* FRESH no locality for founding stock, G) VV44, H) *P. porphyriacus* FRESH no locality for founding stock, I) VV45; *H* A) *Pseudonaja affinis* FRESH Perth, Western Australia, B) VV46, C) *P. inframacula* FRESH no locality for founding stock, D) VV47, E) VV48; *I* A) *Pseudonaja textilis* FRESH Brisbane, Queensland, B) VV49, C) VV50, D) VV51, E) VV52. VV = vintage venom and numbers refer to values in Table 1. Mass spectrometry results for the green zone of Panel A, Lane A and green and red zones of Panel A, Lane E are given in Supplementary Table 1. fX = blood coagulation factor Xa, fV = blood coagulation factor Va. fX and fV identity were confirmed through the MS/MS analysis of digested representative spots: Lane D (VV18) in Panel C and Lane C (VV25) in Panel E. Mass spectrometry results for these two spots are given in Supplementary Table 1.

2.3. Pharmacological investigations

2.3.1. Haemotoxicity

The venoms were diluted with distilled water to a final concentration of 100 μg/ml, 10 μg/ml and 1 μg/ml. Plasma preparation blood samples were collected into freshly prepared polypropylene tubes containing 1:7 (w/w) Sodium citrate/Glucose/Citric Acid anticoagulant (pH 4.5). Following centrifugation at 180 *g* for 10 min at +23 °C, the Platelet Rich Plasma (PRP) was removed and centrifuged for an additional 15 min at 1100 *g* and +23 °C (as per Mustard et al., 1972). The Platelet Poor Plasma (PPP) was removed and stored at −80 °C in 2 ml aliquots. On the day of performance, the samples were thawed in a water bath at +37 °C for 10 min and three plasma pools prepared, each containing six different individual samples. A modification of the Activated Partial Thromboplastin Time (APTT) was set up for this experiment which added 50 μl saline and 50 μl venom to 50 μl plasma pool and 50 μl $CaCl_2$. The modified APTT was set up to test the effect of snake venom as an activator or inhibitor. As a control 50 μl APTT reagent and 50 μl CaCl2 were added to 50 μl pooled plasma. The measurements were performed on automated coagulation analyser, STA-R (Diagnostica Stago, France).

2.3.2. Neurotoxicity

Neurotoxicity protocol was as described by us previously [34].

2.4. Snake venom detection kit

Snake venom detection kit protocol was as per [35] with each venom tested at a concentration of 10 ng/ml.

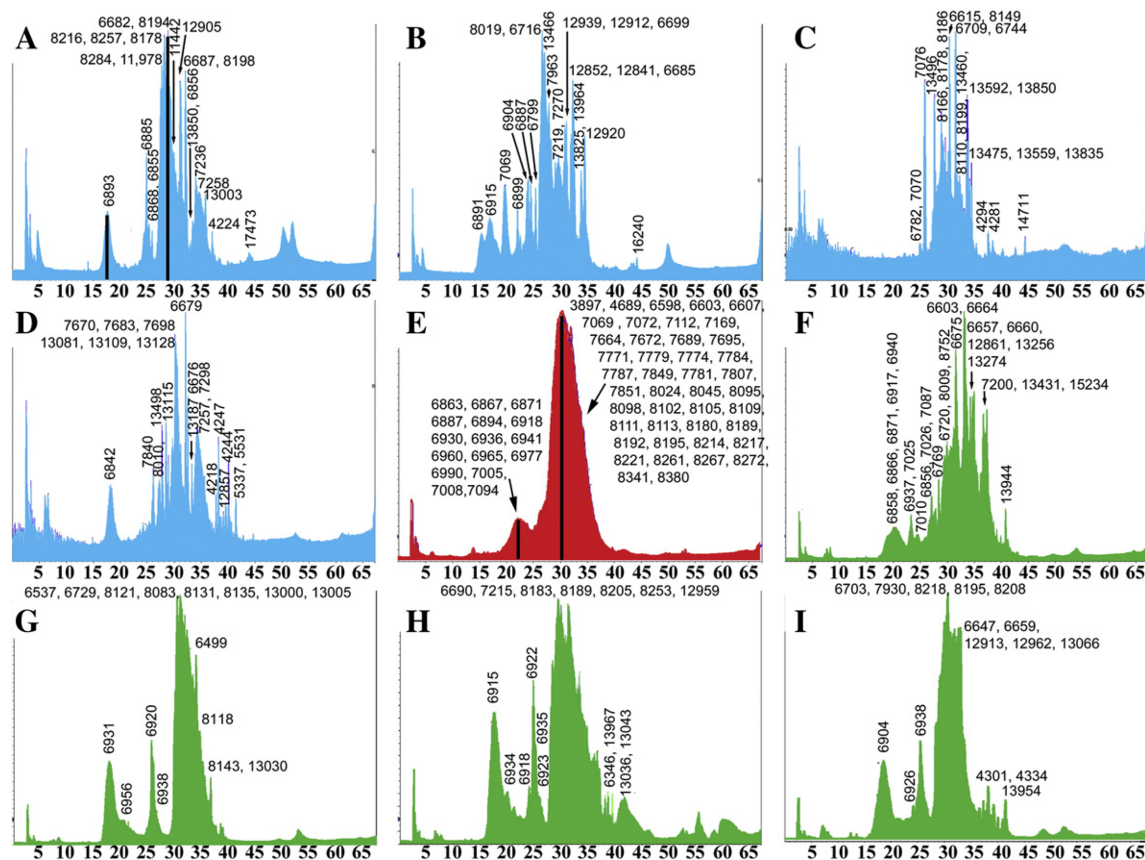

Fig. 3 – LC/MS comparison of vintage and fresh *Acanthophis* venoms: *A) Acanthophis antarcticus* FRESH Middleback ranges, South Australia, *B) A. praelongus* FRESH Cairns, Queensland, *C) A. rugosus* FRESH Camooweal, Queensland, *D) A. laevis* FRESH Merauke, West Papau, *E) A. antarcticus* 1961 locality not recorded, *F) A. praelongus* 1960 Cooktown, Queensland, *G) A. rugosus* 1953 Papua New Guinea, *H) A. rugosus* 1955 Papua New Guinea, *I) A. rugosus* 1955 Papua New Guinea. Y-axis is relative intensity. Reconstructed masses are given above each peak or subpeak. Black bars in A and E are the locations of the m/z presented in Fig. 4.

3. Results and discussion

Testing vintage *Pseudechis australis* and *Notechis scutatus* venoms in the snake venom detection kit revealed they were detectable at the same 10ng/ml concentration as fresh venom and in the appropriate well (Table 3). 1D-electrophoresis revealed little degradation of vintage venom samples, as few differences were evident between them and their fresh equivalents (Fig. 2). Only the *Acanthophis antarcticus* venom in lane E of panel A in Fig. 2 showed significant degradation, and this was also the venom for which both the external and internal rubber stoppers had degraded the most, allowing the entry of moisture. In the 6–14 kDa range (green zone in Fig. 2) there was an obvious difference, with an apparent disappearance of higher molecular weight components from the vintage sample. In-gel digestion followed by LC/MS/MS of a low molecular weight band present in this vintage venom (red zone in Fig. 2) that was absent in other vintage or fresh venoms showed the presence of toxins of higher molecular weight including: 3FTx [6–8 kDa]; kunitz [6–8 kDa]; lectin [18–20 kDa]; nerve growth factor [18–20 kDa]; and phospholipase A_2 [14–17 kDa] (Supplementary Table 1). However in gel digestion of the green zones of both Lane A and Lane E revealed the retention of comparatively similar components. RP-HPLC LC/MS comparison of this *A. antarcticus* vintage venom with other vintage venoms and fresh venoms showed a loss of peak resolution and complexity (Fig. 3). Mass spectrometry comparison of homologous peaks between fresh and vintage *A. antarcticus* venoms, showed an increase in m/z complexity in the vintage venom (Fig. 4), which is consistent with the degradation of this venom sample.

Surprisingly, our bioactivity studies showed the retention of significant neurotoxicity in this sample (Fig. 5). *A. antarcticus* venom from 1961 (3 and 10 μg/ml) and *Acanthophis praelongus* venom from 1960 (3 and 10 μg/ml) both caused a rapid blockade of nerve-stimulated twitches in the chick biventer cervicis nerve-muscle preparation. The time required to inhibit 90% of twitch contractions (t_{90}) for *A. antarcticus* (3 and 10 μg/ml) was 21 ± 1 min (n = 3) and 15 ± 1 min (n = 3) respectively. The time required to inhibit 90% of twitch contractions for *A. praelongus* (3 and 10 μg/ml) was 34 ± 2 min

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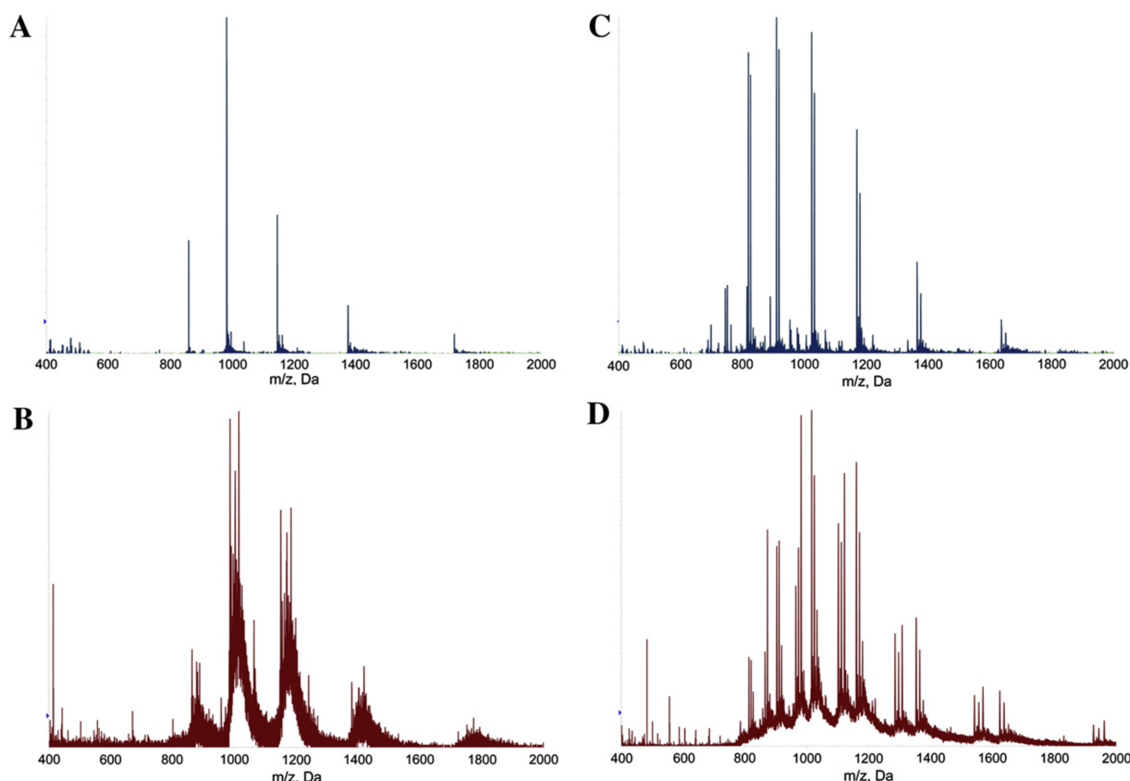

Fig. 4 – m/z from peaks with black bars in Fig. 3 from *Acanthophis antarcticus* FRESH Middleback ranges, South Australia and *A. antarcticus* 1961 locality not recorded. *A* and *B* are the first peaks selected for each venom and *C* and *D* are the second peaks respectively. Venom protein degradation is indicated by the increased complexity in *B* and *D* relative to *A* and *C*.

(n = 3) and 18 ± 4 min (n = 3) respectively. Both venoms (3, 10 μg/ml) produced significant inhibition of contractile responses to exogenous agonists (Fig. 5, $P < 0.0001$, n = 3) while not significantly affecting the response to KCl, which is indicative of post-synaptic neurotoxicity. Moreover, t_{90} values at 10 μg when compared against previously studied *Acanthophis* venoms confirmed the retention of significant amounts of neurotoxicity (Table 2). These results indicate that the degradation of the peptide neurotoxins may have been restricted to the functionally unimportant N- or C- terminus free-chains rather than having occurred in the loops containing functional residues, as indicated by the mass spectrometry results (Figs. 3 and 4). The other vintage venoms tested also showed significant retention of neurotoxic activities (Fig. 5) consistent with their relative proteomic stability (Fig. 2).

Hemotoxicity SVS testing of *N. scutatus* and *Oxyuranus scutellatus* venoms showed the vintage venoms to be only slightly less potent (*Notechis*) or equipotent (*Oxyuranus*) relative to fresh venoms (Fig. 5). Mass spectrometry analysis of the relevant bands (Supplementary Table 1) showed the preservation of the enzymes responsible for hemotoxic activity: factor Xa for *Notechis* venoms and to factor Xa in addition to factor Va for *Oxyuranus* and *Pseudonaja* venoms (Fig. 2). The coagulation profile of the *N. scutatus* and *O. scutellatus* venoms using a modified APTT assay showed the vintage venoms to be only slightly less potent (*Notechis*) or equipotent (*Oxyuranus*) relative to fresh venoms. Fresh venom at 10 μg/ml induced clot formation in 31 s, a 40% reduction in clotting time compared to the control. Similarly, vintage venom at a concentration of 10 μg/ml reduced APTT to 38 s. Fresh and vintage *Oxyuranus* venom at the higher concentration of 10 μg/ml both induced clot formation virtually immediately (prior to the minimum recorded time of 1 s).

The investigation of dried vintage snake venom samples with proteomic and pharmacological approaches revealed that degradation may occur over time if an airtight seal is not maintained. However, if the integrity of the seal is not compromised then venoms are remarkably stable, even over periods of greater than 50 years. Thus the use of vintage samples in contemporary research is feasible if the samples to be studied have been effectively stored. In addition, to prevent protein degradation during long-term storage, all freshly collected samples should be dried completely and stored in airtight containers, preferably in a temperature-controlled environment without exposure to sunlight.

Since some structural changes to proteins may occur during long-term storage, the use of historical desiccated venom samples for the production of antivenom may not be ideal. Nevertheless, these venoms remain a rich resource for evolutionary and biodiscovery studies — particularly in light of the global biodiversity crisis, with many snake species undergoing local extinctions. The *N. scutatus* samples analysed in this study (from Babel Island, Flinders Island, King Island and St Francis Island) are not only the only ones currently available for research, but this study also marks the first time analyses have been undertaken on the venoms from these isolated and

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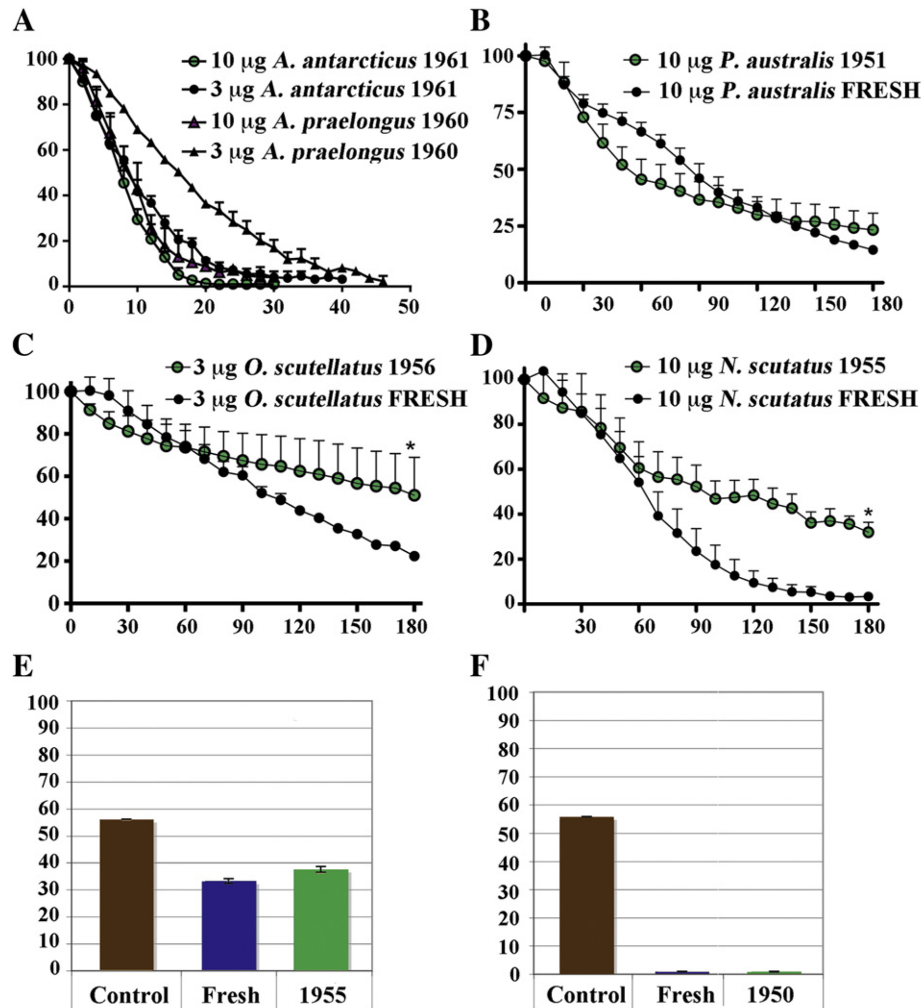

Fig. 5 – *A/B/C/D* venom effects upon nerve-mediated twitches of the chick biventer nerve-muscle preparation [*n* = 3, x-axis = time (mins), y-axis = % of initial twitch height]; 10 μg/ml action upon clot formation for *E) Notechis scutatus* FRESH Melbourne, Victoria and *N. scutatus* 1955 Werribee, Victoria *F) Oxyuranus s. scutellatus* FRESH Cairns, Queensland and *O. s. scutellatus* 1950 Cairns, Queensland; y-axis is time to clot formation.

unique populations. Some of these venoms are also of tremendous historical value, such as our analysis of the venom of the first *O. scutellatus* specimen collected for antivenom production (which infamously killed the young collector Kevin Budden) [31], as well as samples from the first *Oxyuranus microlepidotus* ever collected for research, after the rediscovery of the species in 1976 [36].

Table 2 – Comparisons of neurotoxicity (indicated by t_{90} values in minutes) induced by 10 μg doses of *Acanthophis* venoms in the chick biventer cervicis nerve-muscle preparation in this study and previous studies [37,38].

Species	Age	t_{90} [a]
Acanthophis antarcticus	FRESH	13.8 ± 1.3
Acanthophis antarcticus	1961	21 ± 1
Acanthophis praleongus	FRESH	19.4 ± 1.9
Acanthophis praelongus	1960	34 ± 2
Acanthophis pyrrhus	FRESH	13.6 ± 1.2
Acanthophis rugosus	FRESH	10.5 ± 0.5
Acanthophis wellsi	FRESH	13 ± 2

[a] t_{90} values represented as mean ± SEM.

This study demonstrates that vintage venom collections may still be viable for research purposes. It also demonstrates that venom samples collected now can be used as valuable reference bioresources for future scientific studies. It is therefore of paramount importance that the venoms of rare and endangered species, not currently represented in stock collections anywhere in the world, be collected and appropriately stored to preserve a declining bioresource. It goes without saying that collectors should seek to minimize the impact of their own collecting practices, by taking only the minimal number of specimens of each species required for venom production. It is also important that vintage venom collections be viewed as bioresources themselves and not merely as historical artefacts — they may represent the last opportunity to discover the potential wonder drugs hidden within the venoms of endangered species of snakes.

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Table 3 – sVDK (snake venom detection kit) testing of vintage venom (with strongest match shown in green) [35].

	Blank	Positive control	Negative control	Taipan	Death Adder	Black	Brown	Tiger
Notechis scutatus (1955)	0.007	2.655	0.026	0.06	0.121	0.173	0.043	0.203
Pseudechis australis (1958)	0.009	2.556	0.033	0.087	0.11	0.508	0.07	0.111
Pseudechis australis (1972)	0.005	2.419	0.027	0.081	0.112	0.535	0.055	0.109
Pseudechis australis (1983)	0.005	2.538	0.03	0.097	0.184	0.611	0.054	0.125

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2014.01.004.

REFERENCES

[1] Opdam P, Wascher D. Climate change meets habitat fragmentation: linking landscape and biogeographical scale levels in research and conservation. Biol Conserv 2004;117:285–97.

[2] Boehm M, Collen B, Baillie JEM, Bowles P, Chanson J, Cox N, et al. The conservation status of the world's reptiles. Biol Conserv 2013;157:372–85.

[3] Sarre SD. Demographics and population persistence of *Gehyra variegata* (Gekkonidae) following habitat fragmentation. J Herpetol 1998;32:153–62.

[4] Barnosky AD, Matzke N, Tomiya S, Wogan GOU, Swartz B, Quental TB, et al. Has the Earth's sixth mass extinction already arrived? Nature 2011;471:51–7.

[5] Dirzo R, Raven PH. Global state of biodiversity and loss. Annu Rev Environ Resour 2003;28:137–67.

[6] Essl F, Moser D, Dirnboeck T, Dullinger S, Milasowszky N, Winter M, et al. Native, alien, endemic, threatened, and extinct species diversity in European countries. Biol Conserv 2013;164:90–7.

[7] Cagle NL. Snake species distributions and temperate grasslands: a case study from the American tallgrass prairie. Biol Conserv 2008;141:744–55.

[8] Bonnet X, Guy N, Shine R. The dangers of leaving home: dispersal and mortality in snakes. Biol Conserv 1999;89:39–50.

[9] Brooks TM, Mittermeier RA, Mittermeier CG, da Fonseca GAB, Rylands AB, Konstant WR, et al. Habitat loss and extinction in the hotspots of biodiversity. Conserv Biol 2002;16:909–23.

[10] Saez NJ, Senff S, Jensen JE, Er SY, Herzig V, Rash LD, et al. Spider-venom peptides as therapeutics. Toxins 2010;2:2851–71.

[11] Casewell NR, Wuster W, Vonk FJ, Harrison RA, Fry BG. Complex cocktails: the evolutionary novelty of venoms. Trends Ecol Evol 2013;28:219–29.

[12] Trookman NS, Rizer RL, Ford R, Ho E, Gotz V. Immediate and long-term clinical benefits of a topical treatment for facial lines and wrinkles. J Clin Aesthet Dermatol 2009;2:38–43.

[13] Zhang L, Falla TJ. Cosmeceuticals and peptides. Clin Dermatol 2009;27:485–94.

[14] McCleary RJ, Kini RM. Non-enzymatic proteins from snake venoms: a gold mine of pharmacological tools and drug leads. Toxicon 2013;62:56–74.

[15] Palmer-Toy DE, Krastins B, Sarracino DA, Nadol Jr JB, Merchant SN. Efficient method for the proteomic analysis of fixed and embedded tissues. J Proteome Res 2005;4:2404–11.

[16] Sprung Jr RW, Brock JW, Tanksley JP, Li M, Washington MK, Slebos RJ, et al. Equivalence of protein inventories obtained from formalin-fixed paraffin-embedded and frozen tissue in multidimensional liquid chromatography–tandem mass spectrometry shotgun proteomic analysis. Mol Cell Proteomics 2009;8:1988–98.

[17] Fowler CB, Cunningham RE, O'Leary TJ, Mason JT. 'Tissue surrogates' as a model for archival formalin-fixed paraffin-embedded tissues. Lab Invest 2007;87:836–46.

[18] Nirmalan NJ, Harnden P, Selby PJ, Banks RE. Development and validation of a novel protein extraction methodology for quantitation of protein expression in formalin-fixed paraffin-embedded tissues using western blotting. J Pathol 2009;217:497–506.

[19] Addis MF, Tanca A, Pagnozzi D, Crobu S, Fanciulli G, Cossu-Rocca P, et al. Generation of high-quality protein extracts from formalin-fixed, paraffin-embedded tissues. Proteomics 2009;9:3815–23.

[20] Addis MF, Tanca A, Pagnozzi D, Rocca S, Uzzau S. 2-D PAGE and MS analysis of proteins from formalin-fixed, paraffin-embedded tissues. Proteomics 2009;9:4329–39.

[21] Azimzadeh O, Barjaktarovic Z, Aubele M, Calzada-Wack J, Sarioglu H, Atkinson MJ, et al. Formalin-fixed

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paraffin-embedded (FFPE) proteome analysis using gel-free and gel-based proteomics. J Proteome Res 2010;9:4710–20.
[22] Righetti PG, Lomonte B, Calvete JJ. Resurrexit, sicut dixit, alleluia. Snake venomics from a 26-year old polyacrylamide focusing gel. J Proteome 2012;75:1074–8.
[23] Munekiyo SM, Mackessy SP. Effects of temperature and storage conditions on the electrophoretic, toxic and enzymatic stability of venom components. Comp Biochem Physiol B Biochem Mol Biol 1998;119:119–27.
[24] Egen NB, Russell FE. Effects of preparatory procedures on the venom from a rattlesnake (*Crotalus molossus molossus*), as determined by isoelectric focusing. Toxicon 1984;22:653–7.
[25] Morrison JJ, Pearn JH, Coulter AR, Halliday WJ. Immunological stability of an elapid venom, *Tropidechis carinatus*, and its relevance to the clinical detection of snake venom. Aust J Exp Biol Med Sci 1983;61(Pt 5):489–95.
[26] Aung K, Khin OL, Thant Z. Immunogenicity of toxoid of the Russellís viper venom. The Snake 1980;12:45–53.
[27] Schoettler W. On the stability of desiccated snake venoms. J Immunol 1951;67:299–304.
[28] Russell FE, Emery JA, Long TE. Some properties of rattlesnake venom following 26 years storage. Proc Soc Exp Biol Med 1960;103:737–9.
[29] Pearn J, Winkel KD. Toxinology in Australia's colonial era: a chronology and perspective of human envenomation in 19th century Australia. Toxicon 2006;48:726–37.
[30] Winkel KD, Mirtschin P, Pearn J. Twentieth century toxinology and antivenom development in Australia. Toxicon 2006;48:738–54.
[31] Mirtschin P. The pioneers of venom production for Australian antivenoms. Toxicon 2006;48:899–918.
[32] Fry BG. Structure–function properties of venom components from Australian elapids. Toxicon 1999;37:11–32.
[33] Ali SA, Yang DC, Jackson TN, Undheim EA, Koludarov I, Wood K, et al. Venom proteomic characterization and relative antivenom neutralization of two medically important Pakistani elapid snakes (*Bungarus sindanus* and *Naja naja*). J Proteome 2013;89:15–23.
[34] Brust A, Sunagar K, Undheim EA, Vetter I, Yang DC, Casewell NR, et al. Differential evolution and neofunctionalization of snake venom metalloprotease domains. Mol Cell Proteomics 2013;12:651–63.
[35] Steuten J, Winkel K, Carroll T, Williamson NA, Ignjatovic V, Fung K, et al. The molecular basis of cross-reactivity in the Australian Snake Venom Detection Kit (SVDK). Toxicon 2007;50:1041–52.
[36] Covacevich J, Wombey J. Recognition of *Parademansia microlepidotus* (McCoy) (Elapidae), a dangerous Australian snake. Proc R Soc Queensland 1976;87:29–32.
[37] Fry BG, Wickramaratna JC, Jones A, Alewood PF, Hodgson WC. Species and regional variations in the effectiveness of antivenom against the in vitro neurotoxicity of death adder (*Acanthophis*) venoms. Toxicol Appl Pharmacol 2001;175:140–8.
[38] Wickramaratna JC, Hodgson WC. A pharmacological examination of venoms from three species of death adder (*Acanthophis antarcticus*, *Acanthophis praelongus* and *Acanthophis pyrrhus*). Toxicon 2001;39:209–16.

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