Eastern brown snake (*Pseudonaja textilis*) envenomation in dogs and cats: Clinical signs, coagulation changes, brown snake venom antigen levels and treatment with a novel caprylic acid fractionated bivalent whole IgG equine antivenom

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This report describes the diagnosis and treatment of 16 confirmed cases of snakebite from the Australian eastern brown snake (*Pseudonaja textilis*) in dogs and cats. The clinical signs, brown snake venom antigen concentrations, coagulation parameters, and treatment outcomes following administration of an experimental caprylic acid fractionated bivalent whole IgG antivenom are documented. A brown snake venom antigen specific sandwich ELISA was used to retrospectively quantify venom levels in serum and urine. The characteristic clinical signs of envenomation in all cases were neurotoxicity to a variable extent and coagulation disturbances. The median serum venom concentration at presentation was 122 ng/mL and ranged from 1.9 to 3607 ng/mL. The median urine venom concentration at presentation was 55 ng/mL and ranged from 3.3 to 2604 ng/mL. Mechanical ventilation was used to successfully support respiration in three severely paralysed cases for 1–30 h. In four cases where serum samples were available post-antivenom treatment, venom was no longer detectable. Coagulation parameters measured on citrated plasma samples collected prior to antivenom from each case were abnormally prolonged to variable degrees in all cases. Three cases (2 dogs; 1 cat) were euthanized within four hours of presentation for either cost based reasons (2) or poor prognosis (1). One dog developed massive and potentially fatal pulmonary haemorrhage and was euthanized.

In vitro testing of the venom procoagulant neutralising efficacy of the experimental antivenom demonstrated it was 9.6–72 times more effective when compared to two other commercial veterinary antivenom products. This is the first detailed report of a case series of *P. textilis* envenomation in dogs and cats. The envenomation syndrome in dogs and cats differed to that reported in humans, dominated by neurotoxicity and coagulopathy; unlike in humans, where coagulopathy is of primary clinical significance.

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1. Introduction

The Australian eastern brown snake (*Pseudonaja textilis*) is considered one of the most dangerous and venomous snakes in the world, with bites to domestic animals common (Padula et al., 2016). This snake is also responsible for the majority of snakebite cases in humans in Australia (Allen et al., 2012); however, the total number of envenomed humans per year is small (~10%) compared to estimates for dogs and cats. A mailout survey of veterinarians in 1996 found that annually, approximately 6200 cases of snake envenomation are treated by Australian veterinarians and 76% of these were for brown snakes (Mirtschin et al., 1998). Another survey of veterinarians in New South Wales, Australia, found that 40.6% of snakebites were due to brown snakes (Heller et al., 2005). Despite the large number of cases diagnosed and treated by veterinarians, surprisingly, there has been no detailed description of the clinical syndrome, venom concentrations and response to various forms of treatment described in animals.

The lethal effect of *P. textilis* venom in the cat was first demonstrated over 90 years ago by the subcutaneous injection of...
graded doses of venom into ten cats (Kellaway, 1931). A dose of 0.5 mg/kg resulted in death within 90 min whilst 0.1 mg/kg caused hind limb paralysis in 20 h, with death occurring the following day (Kellaway, 1931). From this work, a dose of 0.1 mg/kg was deemed lethal to the cat; and, was comparatively similar in toxicity in the cat to tiger snake venom (Notechis scutatus) (Kellaway, 1929).

Further studies on the effect of P. textilis in mice and guinea pigs found that guinea pigs required a four-fold lower dose per body-weight than mice; the lethal dose in mice was similar to the lethal dose in cats (Kellaway, 1931). More recent studies of the venom of P. textilis found that it was highly toxic to mice with an LD50 of 0.053 mg/kg (Broad et al., 1979). Controlled experimental envenomation by subcutaneous injection of domestic cats with 0.1–0.5 mg/kg doses of whole brown snake venom induced severe clinical signs in cats including paralysis within 24 h (Moisidis et al., 1996). A dose of 0.1 mg/kg was considered fatal, which resulted in a peak serum venom concentration of 50–100 ng/mL and urine 100 to 1000 ng/mL (Moisidis et al., 1996).

The brown snake (Pseudonaja sp.) family presently contains nine different species found on the Australian continent (Cogger, 2014). However, due to the geographical distribution of P. textilis overlapping primarily with the most populated areas of Australia, P. textilis is the principal species responsible for human and animal envenomation. P. textilis has wide distribution across the eastern half of Australia and is found from the lower Cape York Peninsula in Queensland to south-eastern South Australia; it is not found in Tasmania (Cogger, 2014). The snake is agile, nervous, fast moving and varies in colour with horizontal banding present in some young snakes tending to solid colour with increasing age. Typical length is 1.5–2 m. The snake is preferentially found in dry areas, avoiding the swamp wet areas typical for tiger and red-bellied black snakes.

This study was undertaken to describe the clinical syndrome of natural eastern brown snake envenomation in dogs and cats, quantify serum and urine venom antigen concentrations, and assess response to treatment with an experimental bivalent whole IgG caprylic acid fractionated antivenom.

2. Materials and methods

2.1. Diagnosis and treatment

All clinical cases were treated at a specialist veterinary referral hospital or the emergency after hours service in the same facility located in Brisbane, Queensland, Australia (Veterinary Specialist Services and Animal Emergency Service, Underwood, Qld). Critical cases requiring close monitoring or mechanical ventilation (MV) were admitted to the Pet Intensive Care Unit, Underwood, Qld. Cases were prospectively enrolled during 2016–2017 as either primary presentations or referrals from other veterinary practices. Cases were handled by multiple veterinarians and the following general procedures were performed.

Depending upon the animal’s condition at the time of presentation a triage procedure was performed by a veterinarian. Cases were scored for gait and respiration at initial presentation using the following system. Gait Score: 0 = no clinical signs; 1 = mild paresis, able to ambulate; 2 = able to stand/sit unaided but can’t walk; 3 = unable to stand but can maintain sternal recumbency; 4 = unable to maintain sternal recumbency. Respiratory Score: A = no compromise; B = mild increase in effort and or RR; C = moderate, RR < 16 or > 40, minimal excursions, abdominal component; D = dyspnoea, cyanosis, unsustainable respiratory pattern, respiratory arrest, imminent death.

In cases presenting with severe respiratory distress an intravenous catheter was placed and the animal was immediately intubated and positive pressure ventilation commenced (Fig. 4). A sample of citrated plasma and serum was frozen within 4 h of collection prior to antivenom administration and at various time-points for different cases throughout the hospitalisation period. A clinical diagnosis of brown snake envenomation was made based on the history, presenting signs and a urine or serum snake venom detection kit (SVDK; Seqirus, Parkville, Australia) was used where appropriate in an attempt to confirm the diagnosis of snakebite.

Antivenom was used from a single production batch. Immediately prior to use it was diluted to a volume of between 20 and 50 mL depending upon recipient bodyweight, and infused intravenously either manually or via a syringe pump so that the total volume was received within 20 min. In some cases, where a rapid effect was desired, the antivenom was diluted and administered intravenously as a bolus in less than two minutes. All cases received supportive intravenous fluids; either, Hartmann’s solution or a mixed electrolyte solution (Plasma-Lyte 148, Baxter, Australia) was given during the period of hospitalisation, potassium chloride was supplemented if indicated. In some cases, sedation was administered to the animal to reduce stress; butorphanol (0.1–0.2 mg/kg; iv) initially, followed by a constant rate infusion (0.02–0.1 mg/kg/hour). Following antivenom administration, envenomed animals were monitored either intermittently or continuously depending upon clinical severity for mucous membrane colour, respiratory rate and effort, heart rate, blood pressure, blood oxygen saturation (SpO2) and rectal temperature. In cases where the respiratory rate and effort were deemed abnormal, venous blood gas measurements were performed for venous blood carbon dioxide tension (PvCO2).

During the period of hospitalisation, intensive nursing care was provided including soft bedding, sternal positioning with change from left to right every 2–4 h, eye care every 1–4 h case dependant, and application of protective contact lenses in mechanically ventilated or severely paralysed patients. Urine output was measured 4 hourly if the patient required an indwelling urinary catheter. Clinical chemistry tests were performed using in-house test equipment (VetScan2, REM Systems, Australia) or an external veterinary diagnostic laboratory (QML, Brisbane, Australia). Packed cell volume measurements were made using a centrifuge and micro-haematocrit tubes. Total plasma protein was measured using a handheld refractometer. Whole blood activated clotting time (ACT) was performed by collecting 2 mL of whole blood and rapidly transferring it to a commercial ACT tube (Actalyke, Helena Laboratories, Mt Waverly, Australia). The tube was immediately placed into a single well whole blood coagulation monitoring instrument designed to accurately measure ACT in seconds (Hemochron 401, Edison, New Jersey) The maximum clotting time for blood collected from normal healthy dogs and cats was 85 s (See et al., 2009).

MV was performed according to the Pet Intensive Care Unit protocol using total intravenous anaesthesia (TIVA). MV was recommended as an essential treatment if any of the following criteria were met: (i) hypoxaemia, defined as PaO2 <60 mmHg or pulse oximetry (SpO2) values <90% despite supplemental oxygen therapy; (ii) hypoventilation, defined by partial pressure of carbon dioxide in venous blood (PCO2) >60 mm Hg; (iii) respiratory arrest (cessation of thoracic excursions); or, (iv) unsustainable respiratory effort was observed and respiratory arrest appeared imminent.

To initiate MV, patients were anaesthetised using an intravenous bolus injection of alfalone (2–5 mg/kg; Alfaxan, Jurox, Australia) to enable orotracheal intubation. Patients were maintained on TIVA to provide a light plane of anaesthesia and provide anxiolysis. All patients received a combination of butorphanol (0.1–0.2 mg/kg/h; Ililum Butorberic Injection, Troy Laboratories, Australia) and midazolam (0.1–0.5 mg/kg/h; Midazolam Sandoz, Sandoz, Australia). Dogs received propofol (0.05–0.4 mg/kg/min) and cats received alfaxalone (1–4 mg/kg/h) administered by
constant rate intravenous infusion. MV was performed with an intensive care ventilator (Dräger Evita Ventilator Model 2&4; Lubeck, Germany) using pressure-controlled modes of support. Either the assist control mode or synchronised intermittent mandatory ventilation was used at the preference of the clinician and guided by the results of blood gas analysis SPO2 and end tidal carbon dioxide (ETC02). Arterial catheters were only placed in dogs, not cats, and only after resolution of the coagulopathy. The aim of mechanical ventilation was to maintain PaO2 >60 mmHg (or SPO2 >90%) and PC02 <60 mmHg, while applying minimal ventilator settings and fraction of inspired oxygen (FiO2). Initial settings were, FiO2 100%, inspiratory pressures of 10–12 cm H2O, respiratory rate of 20 breaths/min and positive end-expiratory pressure of 5 cm H2O. These were then adjusted to maintain minimal acceptable oxygenation and ventilation. When the patient was maintaining acceptable blood gas values with sustainable respiratory effort, mechanical ventilation was discontinued. Whilst ventilated, patients received continuous ETC02, ECG and temperature monitoring in addition to previously described monitoring. Additional patient ventilator care included strict asepsis, oral care, airway humidification and suction, eye care and passive range of motion physiotherapy with regular recumbency repositioning.

2.2. ELISA for serum and urine venom concentration

The concentration of brown snake venom (BSV) antigen in frozen-thawed serum and urine samples was retrospectively determined using a previously reported 3-step venom sandwich ELISA (Padula and Winkel, 2016a, b). The BSV antigen ELISA was also run in a 1-step assay format in an attempt to shorten the run time and increase sensitivity. Previous comparisons of 1-step, 2-step and 3-step biotin labelled sandwich ELISA formats found good correlation between all formats, improved sensitivity, but potential for the ‘hook-effect’ at high antigen concentrations (Vilja, 1991). Both 1-step and 3-step formats of the BSV antigen ELISA were evaluated for the hook effect by measuring the absorbance at 450 nm for doubling dilutions of whole BSV in PBS ± 0.5% BSA diluent. The hook effect was found to be relevant to samples with BSV concentrations greater than 100 ng/mL; hence all samples were assayed at eight serial dilutions to account for this. Briefly, for the 1-step ELISA a ‘pre-mix’ was prepared by combining the biotin labelled alpaca anti-BSV IgG (0.3 µg/mL) with streptavidin-horseradish peroxidase (Pierce, USA; 1:40,000) in PBS-T20 ± 0.5% BSA. This pre-mix solution was used as the assay diluent for all test samples, controls and calibrators. Test samples (urine or serum) were initially diluted 1:10 in the pre-mix solution, aliquots of 100 µL were then applied to a 96-well polystyrene high binding microplate (Maxisorp, Nunc, USA) in eight doubling dilutions (1:10 to 1:1280). Microplates were coated with alpaca anti-BSV antibody at 10 ng/mL by adding 100 µL per well, incubating at room temperature for 3 h and then overnight at 4 °C. Plates were washed three times with PBS-T20 prior to applying samples. Free BSV antigen could bind to the adsorbed capture antibody during incubation for 30 min at 37 °C on a microplate shaker at 600 rpm. Unbound BSV antigen was removed by washing the plate six times in PBS-T20. Detection of bound labelled secondary antibody was achieved with addition of 100 µL of TMB, reaction stopped with 10% H2SO4 and the well absorbance read immediately at 450 nm using a reference wavelength of 620 nm in an automated microplate reader (Sunrise, Tecan, Australia). A standard curve was applied to each plate in triplicate in eight doubling dilutions using a 10 ng/mL BSV antigen diluent. The hook effect was found to be relevant to samples with high antigen concentrations (Vilja, 1991). Unknown sample absorbance values were interpolated against a 5-point sigmoidal curve fitted to the standard curve data using software (Magellan 7.2, Tecan, Australia). Internal negative control samples consisting of normal dog urine and serum diluted in pre-mix were run on each plate. To account for non-specific absorbance of normal urine and serum the absorbance of the control samples was used as the lowest acceptable absorbance value of the standard curve, which determined the sensitivity of the ELISA.

2.3. Serum and urine antivenom concentration

Where serum and urine samples were available post-antivenom treatment, antivenom concentration was measured by indirect ELISA as described previously (Padula and Winkel, 2016a). Briefly, microplates were coated with whole BSV at 10 µg/mL in carbonate coating buffer pH 9.6 and allowed to adsorb overnight at 4 °C. Following plate washing, the test samples were applied to the plate in eight doubling dilutions in PBS-T20 ± 0.5% BSA starting at 1:100. Plates were incubated at 37 °C for 30 min. Unbound antibody was removed by washing the plate three times in PBS-T20. A standard curve was prepared using serial dilutions of the antivenom (1:50,000). Detection of BSV specific equine IgG was achieved with rabbit anti-horse IgG (Sigma A-6917; 1:20,000). TMB was used as the chromogen and absorbance read at 450 nm with a reference wavelength of 620 nm. A standard curve was constructed using eight doubling dilutions of the whole IgG antivenom diluted appropriately in PBST20 + 0.5% BSA to result in a maximum absorbance at 450 nm of approximately 2.0.

2.4. Coagulation studies on frozen plasma

Frozen (−20 °C) samples of citrated plasma collected from clinical cases were thawed at 37 °C and retrospectively analysed for coagulation parameters. A semi-automated coagulation analyser (CoaData 2000; American Labor, Durham, NC, USA) was used to measure the Prothrombin Time (PT) with 50 µL of pre-warmed citrated plasma and 100 µL thromboplastin reagent (Helena Laboratories, Australia) added to stimulate clot formation. The activated partial thromboplastin time (aPTT) was measured using the same analyser and specific commercial reagents (Helena Laboratories, Mt Waverly, Australia). Citrated plasma fibrinogen was assayed using a commercial reagent (Helena Laboratories, Australia) utilising the Claus method on the above analyser. A reference standard for canine fibrinogen was prepared from a normal dog citrated plasma and calibrated against a standard from a commercial diagnostic veterinary laboratory (Gribbles Veterinary Pathology, Clayton, Australia). The normal reference ranges for these coagulation parameters were determined by assaying citrated plasma samples collected from normal canines (n = 4) and felines (n = 2), frozen within 1 h and subsequently thawed in a water bath at 37 °C (Table 1).

2.5. Antivenom

A whole IgG experimental antivenom formulation was used in all cases. It was produced by progressively immunising horses with venom pooled from captive but geographically diverse specimens of both tiger snake (Notechis scutatus) and eastern brown snake (Pseudonaja textilis). The IgG fraction was purified using caprylic
acid method as previously described (Rojas et al., 1994), concentrated to 140 g/L and dialysed against PBS. The potency of the antivenom was determined in a mouse bioassay where 1 unit is the amount of antivenom required to neutralise the lethal effects of 0.01 mg of whole BSV. The product was formulated to contain no less than 4000 units of tiger snake (N. scutatus) and 4000 units of brown snake (P. textilis) antivenom per vial. The experimental antivenom was used under the conditions of a small-scale trial permit (PER 7250) from the Australian Pesticides and Veterinary Medicines Authority. Guidance on animal ethics was obtained from the Principal Veterinary Officer, Bureau of Animal Welfare, Department of Primary Industries, Victoria, Attwood. All veterinary medical procedures were performed by registered veterinarians. The use of horses and mice were approved by animal ethics permit issued by the Department of Primary Industries, Victoria.

The efficacy of neutralisation of procoagulant effects was evaluated using the procedures recommended for antivenom (WHO, 2010) and previously described (Padula and Winkel, 2017). Briefly, the clotting time of citrated canine plasma was measured using the analyser described in section 2.4 for the clinical coagulation tests. The minimum coagulant dose (MCD) of P. textilis venom was determined by recording the clotting time of serial dilutions of whole BSV in saline buffer following addition of re-calciﬁed plasma. The selected MCD resulted in a citrated canine plasma clotting time of approximately 30 s. The MCD was then added to eight serial dilutions of antivenom to provide a range of neutralisation ratios, incubated at room temperature for five minutes, and clotting times recorded. The ratio of venom to antivenom that resulted in a clotting time three times that of the MCD was deemed the effective dose. The procoagulant neutralisation results were expressed as mg of venom neutralised per mL of antivenom. The experimental antivenom used to treat clinical cases was compared to two commercially available brown snake antivenom products coded as A and B.

### 3. Results

#### 3.1. Clinical findings and outcomes

A total of 16 cases (n = 6 cats; n = 10 dogs) of conﬁrmed brown snake envenomation were enrolled for the study during the period October 2016 to March 2017. Envenomation was conﬁrmed in all (16/16) cases by either a positive urine test by SVDK (10/12 cases), or retrospective serum or urine detection of P. textilis venom antigen by ELISA. Tetraparesis was found at initial presentation in 7/16 of cases (Gait Score 4), and although all of these cases survived, 2/7 of these cases required MV despite receiving antivenom. All cases were treated with one to three vials antivenom at the discretion of the attending veterinarian. The duration of hospital stay was 6.2 ± 31 h for the 13 cases that survived and 3.3 ± 1.2 h for the three cases that were euthanased. A total of 4/16 (25%) cases required MV (n = 3 dogs; n = 1 cat) for 1.5–30 h.

Minor clinical bleeding abnormalities such as venepuncture site haematoma, bite site bleeding, and gingival bleeding were observed in 6/16 cases, although one case experienced a massive catastrophic and potentially fatal pulmonary haemorrhage within two hours of hospitalisation (Case No. 16; Fig. 3) and was euthanized. One dog (Case No. 5) had continued bleeding from the bite site on the lower lip which resulted in signiﬁcant blood loss and fall in PCV to 0.27 (normal range 0.37–0.55) prompting administration of fresh frozen plasma (FFP). Three cases (n = 3 dogs) were treated with 1–3 units of canine FFP in an attempt to improve coagulation. Two animals (n = 1 cat; n = 1 dog) expressed minor reactions to the antivenom administration including angioedema of the lips, muzzle and paws within 1 h of treatment which resolved following treatment with antihistamine injection.

#### 3.2. Coagulation parameters

Normal reference ranges for frozen-thawed citrated plasma using the previously described coagulation measurement system were established for dogs (n = 4) and cats (n = 2) (Table 1). Coagulation abnormalities were present to a variable extent in all cases (Table 2) manifest as prolongation of PT and/or aPTT. The ACT was prolonged (>90 s) at the time of initial presentation in all dogs and 5/6 cats. In most cases, no clot formed during the period of tube incubation (>1500 s) for ACT measurement. Restoration of coagulation parameters was generally observed within 8–24 h following antivenom treatment.

#### 3.3. Serum and urine BSV antigen concentrations

A comparison of the 1-step BSV antigen ELISA format with the 3-step BSV antigen ELISA (Fig. 1) found both methods susceptible to the hook effect. However, the 1-step format appeared more affected, reporting low concentrations of BSV antigen when high concentrations were present. With the 1-step BSV antigen ELISA format, BSV (diluted in PBS-T20 + 0.5% BSA) concentrations from 10 ng/mL to 10,000 ng/mL produced absorbance values that could not be reliably determined by assay at a single sample dilution. In comparison, the 3-step BSV antigen ELISA format tended to produce an absorbance plateau at venom concentrations above 100 ng/mL. Consequently, the BSV concentrations were reported using only the values from the 3-step BSV antigen ELISA format. The limit of detection of the BSV antigen ELISA was 0.15 ng/mL, which was determined by the non-speciﬁc binding of normal serum and urine controls. Sensitive non-parallelism with serial dilutions of test samples was observed for some clinical samples. Progressive dilution of the sample resulted in increasing absorbance values and apparent free BSV antigen concentration. This appeared to be more of an issue in grossly hemolysed samples although the reasons were not explored in detail in this study. There was good agreement between the commercial urine SVDK test and the urine BSV antigen ELISA, with only one sample negative on SVDK (Case No. 12) but with measurable BSV antigen detected in the sandwich ELISA, although this was below the sensitivity of the SVDK of approximately 10 ng/mL (Moisidis et al., 1996).

At the time of initial presentation for veterinary treatment serum BSV antigen concentration ranged from 1.9 to 3607 ng/mL with a median value of 122 ng/mL (25% percentile 79 ng/mL; 75% percentile 479 ng/mL). For matched urine samples, BSV antigen concentration ranged from 3.3 to 2604 ng/mL with a median value of 55 ng/mL (25% percentile 10 ng/mL; 75% percentile 163 ng/mL).
High concentrations of brown snake antivenom were detected in the serum of antivenom treated dogs and cats 1–6 h following treatment (Table 3). BSV antigen was not detectable in the serum of 4/4 cases where post-treatment samples were available. Low concentration of antivenom was detectable in the urine in two cases where samples were collected more than five hours post-antivenom.

3.5. Comparative antivenom in vitro procoagulant neutralisation

In vitro testing of the neutralisation of the procoagulant effects of BSV demonstrated the experimental equine IgG antivenom was highly effective (Fig. 2). Whole BSV in saline was extremely potent for inducing clotting of citrated canine plasma. The MCD was determined to be 125 ng of venom in a volume of 100 μL. The procoagulant neutralising capacity varied with the experimental antivenom most effective (A = 2.9 mg/mL; B = 0.3 mg/mL; C = 0.04 mg/mL; expressed as mg of venom neutralised per mL of antivenom). The experimental antivenom was thus 9.7 times more potent than the CSL product, and 72 times more potent than the Summerland product on a volume basis (Fig. 2).

3.6. Treatment and outcomes

Euthanasia was performed in 3/16 cases (n = 1 cat; n = 2 dog) due to either cost constraints (n = 1 dog; n = 1 cat) where MV was required, or, massive and likely incompatible with life pulmonary haemorrhage (n = 1 dog; case No. 16).

4. Discussion

This report describes the clinical features, treatment and outcomes of a series of 16 confirmed clinical cases of envenomation in dogs and cats with the Australian eastern brown snake (P. textilis). Variation was observed in the initial clinical severity in respect of gait and respiration scores, serum and urine BSV antigen concentrations and eventual clinical outcomes. The majority (13/16) of envenomed animals were successfully treated; euthanasia was performed in the remainder (3/16), for either cost (2/3) or poor prognosis (1/3) reasons.

This case series highlights the risk of development of potentially fatal and generalised neuromuscular paralysis in dogs and cats following P. textilis envenomation, requiring in some cases, MV to support life. This observation contrasts markedly to human cases of P. textilis envenomation where clinically significant paralysis is rarely encountered and coagulopathy predominates (Allen et al., 2012). In a series of 149 brown snake envenomed human patients, only 1% developed neurotoxicity which was considered mild.

Table 2
Clinical case summary data.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Animal Species</th>
<th>Weight (kg)</th>
<th>Serum BSV (ng/mL)</th>
<th>Urine BSV</th>
<th>Initial Urine SVDK</th>
<th>MV (h)</th>
<th>Hospital stay (h)</th>
<th>FFP (units)</th>
<th>Initial coagulation parameters</th>
<th>BPP to AV (h)</th>
<th>Initial Gait Score</th>
<th>Initial Resp Score</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dog</td>
<td>38.5</td>
<td>315 ND</td>
<td>- BSVA</td>
<td>ND</td>
<td>- BSV</td>
<td>ND</td>
<td>22 ND</td>
<td>No clot &gt;180 &gt;180 &lt;0.5</td>
<td>1.5</td>
<td>0</td>
<td>A</td>
<td>Lived</td>
</tr>
<tr>
<td>2</td>
<td>Dog</td>
<td>28</td>
<td>113 41</td>
<td>ND</td>
<td>24</td>
<td>96 ND</td>
<td>No clot &gt;180 &gt;180 &lt;0.5</td>
<td>&lt;12 ND</td>
<td>4</td>
<td>D</td>
<td>Lived</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Dog</td>
<td>24.8</td>
<td>534 163</td>
<td>ND</td>
<td>54 ND</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>14 4</td>
<td>C</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Dog</td>
<td>2.1</td>
<td>83 55</td>
<td>- BSVA</td>
<td>30 110 0.5</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>10 2</td>
<td>D</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>35.0</td>
<td>ND ND</td>
<td>- BSVA</td>
<td>62 3</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>8 2</td>
<td>B</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Cat</td>
<td>5.6</td>
<td>&lt;Min ND</td>
<td>ND</td>
<td>140 ND</td>
<td>ND</td>
<td>20 ND</td>
<td>193 &gt;180 &gt;180 &lt;0.5</td>
<td>12–18 2</td>
<td>B</td>
<td>Lived</td>
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<td></td>
</tr>
<tr>
<td>7</td>
<td>Cat</td>
<td>4.5</td>
<td>57 ND</td>
<td>ND</td>
<td>21 104 ND</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>12 4</td>
<td>C</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>Dog</td>
<td>10</td>
<td>181 936</td>
<td>ND</td>
<td>48 ND</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>1</td>
<td>0</td>
<td>A</td>
<td>Lived</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Dog</td>
<td>18.8</td>
<td>120 42</td>
<td>ND</td>
<td>80 ND</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>12 3</td>
<td>B</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>14</td>
<td>78 10</td>
<td>ND</td>
<td>78 ND</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>18 4</td>
<td>C</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Cat</td>
<td>6.0</td>
<td>ND ND</td>
<td>ND</td>
<td>ND 2</td>
<td>ND</td>
<td>194 20.9 23.3 0.5</td>
<td>24 4</td>
<td>C</td>
<td>Euthanize (cost)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>Cat</td>
<td>7.2</td>
<td>1.9 3.3</td>
<td>Negative ND</td>
<td>48 ND</td>
<td>174 &gt;180 &gt;180 &lt;0.5</td>
<td>12–24 2</td>
<td>B</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Cat</td>
<td>5.4</td>
<td>123 2604</td>
<td>- BSVA</td>
<td>ND 19 ND</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>4 1</td>
<td>A</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Dog</td>
<td>7.7</td>
<td>314 68</td>
<td>ND</td>
<td>ND 4</td>
<td>ND</td>
<td>118 18.5 22.2 0.65</td>
<td>14–20 4</td>
<td>D</td>
<td>Euthanize (cost)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>Cat</td>
<td>3.6</td>
<td>&lt;Min 7</td>
<td>ND</td>
<td>72 ND</td>
<td>86</td>
<td>28.5 91.4 0.65</td>
<td>24 4</td>
<td>C</td>
<td>Lived</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>Dog</td>
<td>30.0</td>
<td>3607 ND</td>
<td>ND</td>
<td>1.5 4 3</td>
<td>No clot</td>
<td>&gt;180 &gt;180 &lt;0.5</td>
<td>4 1</td>
<td>B</td>
<td>Euthanize (bled)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ND = not done. MV = mechanical ventilation. AV = antivenom. FFP = fresh frozen plasma (1 unit ~ 220 mL).

Table 3
Serum and urine brown snake antivenom (BSAV) and brown snake venom (BSV) antigen concentrations in four cases post-antivenom administration.

<table>
<thead>
<tr>
<th>Case No.</th>
<th>BSAV (units/mL)</th>
<th>BSAV antigen (ng/mL)</th>
<th>Time post-BSA</th>
<th>No. BSV vials</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>18.6</td>
<td>0.019</td>
<td>&lt;Min +6</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>64.8</td>
<td>0.125</td>
<td>&lt;Min +5</td>
<td>2</td>
</tr>
<tr>
<td>15</td>
<td>33.6</td>
<td>&lt;Min +1</td>
<td>&lt;Min +1</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>9.5</td>
<td>&lt;Min +2.5</td>
<td>&lt;Min +2.5</td>
<td>3</td>
</tr>
</tbody>
</table>

BSV antigen minimum detectable concentration 0.15 ng/mL. BSAV minimum detectable concentration 0.020 units/mL.
The difference in neurotoxic response to eastern brown snake venom between humans and mice has previously been termed the ‘brown snake paradox’ (Currie, 2000). Venom from *P. textilis* has been shown to contain the extremely neurotoxic pre-synaptic acting PLA2 enzyme called textilotoxin. Purified textilotoxin isolated from whole BSV had an intravenous LD50 in mice of 0.001 mg/kg (Coulter et al., 1983). Despite the presence of this extremely potent neurotoxin in BSV, the envenomation syndrome in humans is predominantly a venom induced consumption coagulopathy; although early and sudden cardiovascular collapse may occur and be potentially fatal (Allen et al., 2012). One hypothesis proposed to explain this is the relatively low total mass of textilotoxin present in BSV is insufficient to cause paralysis in larger bodyweight humans (Barber et al., 2012). However, in the case series described in this report, 7/16 cases presented tetraparetic and one dog weighing 28 kg (Case No. 2) required MV for 24 h to sustain life. This body-weight would likely be greater than found in many human paediatric brown snake cases - which are generally reported without neurotoxicity. We hypothesise that there may be neuromuscular junction receptor differences between animals (mice, dogs & cats) and humans that lead to the markedly reduced the susceptibility of humans to paralytic effects of BSV.

Pre-paralytic signs including collapse and apparent recovery were observed by the animal owner in some envenomed dogs and one cat in this case series, although it must be noted that in many veterinary cases no envenomation event is ever observed making diagnosis challenging. The early and dramatic collapse of human patients envenomed by *P. textilis* has similarly been described (Chaisakul et al., 2013). A rapid and massive reduction in blood pressure has been demonstrated in anaesthetised rats given whole BSV intravenously (Chaisakul and Hodgson, 2013) or subcutaneously (Skejic and Hodgson, 2013) and, in anaesthetised dogs injected intravenously with purified BSV prothrombin activator (Tibballs et al., 1992). This rapid pathological effect is considered important for rapid prey immobilisation by *P. textilis* (Skejic and Hodgson, 2013) and probably explains the ‘pre-paralytic’ signs observed in animals within minutes of envenomation. The dose and, or, rate of administration of BSV may be important in determining the pathological processes attributing to death. Mice injected with relatively low doses of BSV (5 × LD50) die slowly (3–24 h) from neurotoxicity (Broad et al., 1979), whilst typical doses injected in cases of natural snakebite (500–2000 × LD50) result in rapid death within minutes. The procoagulant toxin found in BSV shares similarities with that of the Australian Coastal Taipan (*Oxyuranus scutellatus*). Mice injected intravenously with 625 × LD50 of *O. scutellatus* venom died within...
3–6 min without signs of paralysis and post-mortem examination revealed widespread pulmonary thrombi suggesting the prothrombin activator has the principal role in prey immobilisation and death in that species (Herrera et al., 2012).

The clinical relevance of venom induced consumption coagulopathy in dogs is often considered of lesser importance by veterinarians than the paralytic signs. However, the massive and potentially fatal pulmonary bleed leading to euthanasia in case No. 16 highlights the potential for significant pathology to ensue. This case presented with only mild neurotoxicity; however, a soft cough was noted shortly after admission which was suspected to be due to pulmonary haemorrhage, this rapidly progressed to extensive haemorrhage, haemoptysis and hypoxaemia. Haemoptysis has been reported in cases of acute pulmonary thromboembolism in the human literature (Konstantinides et al., 2014; NATSPUTE, 2006; Reesink et al., 2007), the exact mechanism of haemoptysis in these patients is unclear, proposed mechanisms are due rupture of smaller vessels or bronchial arteries. To the authors’ knowledge this hasn’t been reported in the veterinary literature, we hypothesis that this could be the proposed mechanism of haemoptysis in this case due to massive acute pulmonary thromboembolism after activation of prothrombin C and the initial procoagulant effect of P. textilis. Further investigation of this small subset of P. textilis envenomation cases is warranted to gain a further understanding of the pathophysiology of the catastrophic pulmonary haemorrhage. This case had the highest serum BSV antigen concentration in this series, possibly suggesting a pathogenic prey immobilisation mechanism in action; similar to that observed in mice given massive doses of venom. An unusual complication of VICC in a dog resulting in extradural haematoma formation following envenomation by suspected Pseudonaja affinis has previously been described (Ong et al., 2009).

The finding of coagulation abnormalities in all clinical cases of P. textilis envenomation in both dogs and cats in this case series suggests that the assessment of coagulation as a highly useful clinical diagnostic test for veterinarians in suspected brown snake envenomation cases. There was also good agreement between the simple ACT test and more detailed laboratory assessment of coagulation parameters (PT, aPTT & fibrinogen). This information is of relevance to veterinarians who require rapid and cost effective diagnostic tests for snakebite. In contrast to a previous study of tiger snake (Notechis scutatus) envenomation in cats where coagulation abnormalities were rarely found (Holloway and Parry, 1989), this case series of P. textilis envenomation found most cats (5/6) presented with detectable coagulation abnormalities.

The experimental caprylic acid fractionated equine whole IgG antivenom proved highly effective in respect of clinical outcomes and binding of free circulating venom. Most antivenom appeared to remain in circulation with approximately 0.2% of the serum antivenom concentration detectable in urine up to 6 h post-treatment. The in vitro evaluation of the efficacy of neutralisation of procoagulant effects of BSV demonstrated high potency in contrast to two other antivenoms. It is difficult to explain the extremely poor in vitro procoagulant neutralising properties of the Summerland Serums antivenom product. The reduced efficacy of the Summerland product for P. textilis procoagulant neutralisation in canine plasma was previously demonstrated in a similar microplate based clotting assay (Jacoby, 2009). Similarly, a markedly reduced in vitro efficacy of procoagulant neutralisation of P. textilis venom was likewise shown for the CSL Brown Snake Antivenom product when compared to an experiment combined chicken and sheep antivenom (Madaras et al., 2005). The manufacturers of the Summerland antivenom state that they immunise their donor horses with a combination of three Australian brown snake species: P. inornata (dugite), P. textilis (eastern brown snake) and P. nuchalis (western brown snake or gwardar). However, there is no information available to support that this provides any greater benefit than using P. textilis venom alone, which is the most toxic (in respect of lethality in mice) of the Pseudonaja family. A previous comparison of the efficacy of an equine antivenom produced against P. textilis venom revealed it was four to five times more potent at neutralising the lethal effects of both P. nuchalis and P. affinis venoms (Sutherland, 1979). It is possible that there may be immunological interactions between venoms of the Pseudonaja species that may
depress the equine immune responses, similar to what has been described with co-immunization of horses with both Lachesis stenophrys and Bothrops asper (Arroyo et al., 2015). There may also be other factors that contribute to this reduced procoagulant efficacy including venom handling and downstream manufacturing.

The BSV antigen sandwich ELISA used in this study highlighted the difficulties with immunological assays for quantitation of venom concentrations in clinical samples rather than for qualitative use only. Saturation of passively adsorbed captured antibody binding sites is a problem with sandwich ELISA formats leading to paradoxical reductions in signal ("hook effect") when high concentrations of analyte are present. Both the 1-step and 3-step formats used suffered from this, more so with the 1-step; this has been described previously (Steuten et al., 2007). Concentrations of BSV antigen in clinical samples from dogs and cats in this study exceeded the concentration at which the hook effect would become a quantitation limiting issue if only a single dilution of sample is used. However, the simplicity and speed of the 1-step BSV antigen ELISA does offer potential to develop a rapid and simple qualitative point-of-care detection test. The commercial SVDK is an example of a 1-step enzyme immunoassay format whereby the conjugate is freeze dried in the microplate well, but this leads to reduced sensitivity compared to biotin labelled test with ELISA formats. We conclude that unless serial dilutions of test samples are assayed in the BSV antigen sandwich ELISA it is not possible to reliably quantify BSV antigen concentrations. Other workers have reported difficulties with high non-specific binding in venom antigen ELISA reducing the sensitivity, although this could be managed by pre-incubation of the sample with antivenom and assessing the difference in absorbance. Using a similar assay format to what is described in this report a sensitivity of 0.15 ng/mL for BSV was reported, similar to what was achieved with the camelid-type capture antibody used in this assay.

The serum concentration of antivenom appeared adequate in cases where post-treatment samples were available for indirect ELISA. Antivenom is a significant part of the cost of treating brown snake envenomation in dogs and cats. Based on in vivo mouse neutralising studies, the serum concentrations of antivenom measured suggest that the antivenom dosage is not limiting in respect of venom neutralisation and that much higher than measured concentrations of BSV could be effectively neutralised by antivenin in circulation. This situation is similar to what has been studied in human cases of brown snake envenomation (Ibister et al., 2007; O’Leary et al., 2006).

Geographical differences in venom components have been described for P. textilis specimens captured within Australia. Consequently, the use of a geographically diverse blend of P. textilis venom for immunization may be speculated to improve the neutralising capacity of the final experimental antivenom product. The milked venom yields from eastern brown snakes have been shown to vary depending upon geographical origin, age, and snake length. Milked brown snakes from Queensland yielded three times more venom as those from South Australia (11 v 3 mg) (Mirtschin et al., 2002). A strong correlation between brown snake length and total venom yield, was also found with Queensland snakes longer than South Australian (141 v 110 cm), although these larger snakes produced less concentrated venom (Mirtschin et al., 2002). A comparison of the procoagulant effects found that Queensland brown snakes contained significantly greater procoagulant activity than South Australian brown snakes (Flight et al., 2006). The dry yield of venom from captive brown snakes was shown to vary with location of origin and also between snakes. Six milked Queensland snakes yielded 30.2 ± 9.6 mg (17–76 mg) whilst six South Australian snakes 6.7 ± 1.4 mg (3–12 mg) (Flight et al., 2006). This variation in yield has implications for the total dose of antivenom required to neutralise the dry mass of venom. These data suggest that bites from Queensland brown snakes will be potentially more lethal than their southern counterparts.

In conclusion, this report describes the successful treatment of the majority of cases of eastern brown snake envenomation in dogs and cats presented for veterinary treatment. The use of mechanical ventilation for 1–30 h to support animals with respiratory muscle paralysis was considered essential in cases with severe hyperventilation. Significant neurotoxicity and respiratory muscle paralysis was present in approximately half of the cases at the time of presentation. VICT was detected in all cases to varying degrees manifest as prolongation of ACT, PT, aPTT and decreased plasma fibrinogen levels. Clinically significant bleeding was observed in a minority of cases although potentially fatal pulmonary haemorrhage was observed in one case resulting in euthanasia. The experimental antivenom was highly effective in binding circulating venom as measured by sandwich ELISA and serum concentrations appeared adequate.

Ethical statement

1) this material has not been published in whole or in part elsewhere;
2) the manuscript is not currently being considered for publication in another journal;
3) all authors have been personally and actively involved in substantive work leading to the manuscript, and will hold themselves jointly and individually responsible for its content.

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Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.toxicon.2017.08.015.

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