Haemostatic function of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*)

by

Salomé Nagel

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Pretoria, October 2012
Omnium artium medicina nobilissima est.

"Medicine is the noblest of all arts."

(Latin proverb)
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS ...................................................................................... V

LIST OF FIGURES .......................................................................................... vi

LIST OF TABLES .............................................................................................. viii

LIST OF ABBREVIATIONS .............................................................................. ix

SUMMARY ...................................................................................................... xi

CHAPTER 1 LITERATURE REVIEW ...................................................................... 1

1.1 INTRODUCTION ......................................................................................... 1

PUFFADDER (*BITIS ARIETANS*) .................................................................... 3

SNOUTED COBRA (*NAJA ANNULIFERA*) .................................................... 6

1.2 SNAKE VENOM AND INFLAMMATION ...................................................... 7

1.3 HAEMOSTASIS ........................................................................................... 8

THE CASCADE MODEL ................................................................................... 8

THE CELL-BASED MODEL OF FIBRIN FORMATION ....................................... 10

COAGULOPATHY IN SNAKE ENVENOMATION ............................................. 14

THROMBOELASTOGRAPHY ............................................................................ 16

CHAPTER 2 STUDY OBJECTIVES ...................................................................... 20

2.1 HYPOTHESES ............................................................................................ 20

2.2 STUDY OBJECTIVES ................................................................................ 20

2.3 STUDY BENEFITS .................................................................................... 20

CHAPTER 3 MATERIALS AND METHODS ....................................................... 21

3.1 MODEL SYSTEM ........................................................................................ 21

3.2 EXPERIMENTAL DESIGN ....................................................................... 21

3.3 EXPERIMENTAL PROCEDURES .............................................................. 22

3.4 OBSERVATIONS ....................................................................................... 23

3.5 STATISTICAL ANALYSES ....................................................................... 24

CHAPTER 4 RESULTS ...................................................................................... 26

4.1 SIGNALMENT (BREED, AGE, GENDER) AND BODY WEIGHT ............... 26

4.2 TIME-LAG FROM ENVENOMATION, TREATMENT, OUTCOME AND POST MORTEM FINDINGS ............................................................. 27
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LIST OF FIGURES

**Figure 1** A diagrammatic presentation of the cascade model of fibrin formation from fibrinogen 9

**Figure 2** A diagrammatic presentation of the cell-based model of coagulation 13

**Figure 3** Diagrammatic presentation of a thromboelastogram 18

**Figure 4** Box plot of the thromboelastography R-time at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 30

**Figure 5** Box plot of the thromboelastography K-value at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 31

**Figure 6** Box plot of the thromboelastography angle (α) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 32

**Figure 7** Box plot of the thromboelastography maximal amplitude (MA) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 33

**Figure 8** Box plot of the thromboelastography global clot strength (G) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 34

**Figure 9** Box plot of the platelet count (Plt) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 35

**Figure 10** Box plot of the haematocrit (Ht) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 36
**Figure 11** Box plot of the white blood cell count (WBC) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls

**Figure 12** Box plot of C-reactive protein (CRP) concentration at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls

**Figure 13** Box plot of antithrombin (AT) activity at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls

**Figure 14** Box plot of fibrinogen (Fib) concentration at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls

**Figure 15** Box plot of prothrombin time (PT) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls

**Figure 16** Box plot of activated partial thromboplastin time (aPTT) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls

**Figure 17** A hypocoagulable thromboelastogram at presentation of a dog envenomed by an African puffadder (*Bitis arietans*)

**Figure 18** A hypercoagulable thromboelastogram at presentation of a dog envenomed by a snouted cobra (*Naja annulifera*)
LIST OF TABLES

Table 1 A list of snake venom toxins and their effects on haemostasis 15

Table 2 Signalment and body weight of dogs envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls 27

Table 3 Haematologic and haemostatic variables in dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and control groups at presentation and 24 hours post envenomation 29
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AChBP</td>
<td>Acetylcholine binding protein</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANCOVA</td>
<td>Analysis of Covariance</td>
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<tr>
<td>aPTT</td>
<td>Activated partial thromboplastin time</td>
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<td>AT</td>
<td>Antithrombin</td>
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<tr>
<td>CBC</td>
<td>Complete blood count</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>DIC</td>
<td>Disseminated intravascular coagulation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine-tetra-acetic acid</td>
</tr>
<tr>
<td>FDP</td>
<td>Fibrin(ogen) degradation products</td>
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<td>FFP</td>
<td>Fresh frozen plasma</td>
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<tr>
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<tr>
<td>TEG</td>
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<tr>
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<td>Description</td>
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<tr>
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<td>----------------------------------</td>
</tr>
<tr>
<td>TF</td>
<td>Tissue factor</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue factor pathway inhibitor</td>
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<tr>
<td>t-PA</td>
<td>Tissue plasminogen activator</td>
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<tr>
<td>VICC</td>
<td>Venom-induced consumption coagulopathy</td>
</tr>
<tr>
<td>WBC</td>
<td>White blood cell count</td>
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<td>WHO</td>
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SUMMARY

Haemostatic function of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*).

Nagel, S.S. University of Pretoria, October 2012

Snake envenomations are often medical emergencies and occur regularly in dogs. Snake venom contains hundreds of enzymes, proteins and peptides that assist in paralysing, killing and digesting prey, or as a defence against predators. Multiple snake venom components affect haemostasis in the victim. Inadvertent activation of coagulation may also result from expression of large amounts of tissue factor (TF) from injured tissues at the envenomation site, especially with potent cytotoxic venoms. The purpose of this study was to investigate the haemostatic functions in dogs envenomed by two South African snakes (*Bitis arietans* and *Naja annulifera*) using thromboelastography and traditional plasma-based coagulation assays.

This prospective study included 18 client-owned dogs, of which nine dogs were envenomed by African puffadder and nine by snouted cobra. Blood was collected at presentation and at 24 hours post-envenomation. Complete blood count, thromboelastography (TEG), prothrombin time (PT), activated partial thromboplastin time (aPTT), antithrombin (AT) activity and C-reactive protein (CRP) and fibrinogen (Fib) concentrations were measured. Ten healthy client-owned dogs served as controls. These dogs were presented for routine ovariohysterectomy, castration or blood donation. Haematologic and haemostatic assay results at presentation were compared between groups using ANCOVA (analysis of covariance), and results over time between the puffadder and cobra groups were compared using linear mixed models at 5% significance. At presentation, the mean TEG R-time was significantly prolonged in the puffadder group when compared to the cobra and control groups (*P*=0.01 and 0.05, respectively). Visual appraisal of the thromboelastograms at presentation revealed that 5/9 (56%) of puffadder-envenomed dogs had hypocoagulable thromboelastograms as was demonstrated by prolonged R-time and decreased Angle (α), maximal amplitude (MA) and global clot strength (G). Despite this observation of hypocoagulability, none of the other TEG parameters (α, MA or G) were significantly decreased when compared to the cobra and control groups. This finding of hypocoagulability was surprising, because puffadder venom is cytotoxic, often inducing severe tissue necrosis...
and potentially leading to limb loss and disability in people. It therefore seems that certain components in puffadder venom affect the thromboelastograph by either interfering with or consuming coagulation factors, resulting in a hypocoagulable tracing. It is also possible that this is a dose-dependent effect, with only dogs with a significant amount of envenoming demonstrating this phenomenon. This effect appears to be transient, as 6/8 dogs (one fatality) envenomed by puffadders reverted to a severely hypercoagulable state at 24 hours post-envenomation. One dog was still hypocoagulable and one dog that was hypocoagulable became normocoagulable but still had a prolonged R-time. In the cobra-envenomed group hypercoagulable thromboelastograms were observed in 5/9 (56%) dogs at presentation as was demonstrated by increased MA and G. At 24 hours post-envenomation all cobra-envenomed dogs demonstrated hypercoagulable thromboelastograms. This hypercoagulability at presentation and 24 hours post-envenomation was not statistically significant between groups. This hypercoagulable state was likely due to tissue factor-activated coagulation promoted by inflammation at the envenomation site. At presentation, marked thrombocytopenia was evident in the puffadder-envenomed dogs when compared to the cobras and controls (P=0.04 and 0.001, respectively). Thrombocytopenia following puffadder envenomation has been reported in dogs and baboons. Components have been identified in puffadder venom that interfere with platelet function either by inhibiting or promoting aggregation. At 24 hours post-envenomation mean platelet count (Plt) was mildly increased compared to its value at presentation in the puffadder-envenomed dogs. There were Plt abnormalities in the cobra-envenomed dogs at presentation or at 24 hours post-envenomation. Marked leucocytosis was detected in the puffadder-envenomed dogs at presentation when compared to the cobras and controls (P=0.003 and 0.001, respectively) and was more severe at 24 hours post-envenomation when compared to the cobra group (P=0.01). Leucocytosis has been reported in different types of snake envenoming including puffadder-envenomed dogs. C-reactive protein (CRP) concentration at presentation was below the lowest detection limit for most dogs (14/18) in this study. At 24 hours post-envenomining all but two dogs (one each in the puffadder and cobra groups) had severely elevated CRP. This increase in CRP was statistically significant in both puffadder and cobra-envenomed dogs when compared to its concentration at presentation (P=0.04 and 0.001, respectively). Fibrinogen (Fib) concentration was not elevated in any envenomed dogs at presentation, but increased 24 hours post-envenomining. Although this increase was not statistically significant, an increase would suggest activation of the inflammatory response, as both Fib and CRP are positive
acute phase proteins. Elevated CRP, neutrophilic leucocytosis and increases in cytokine IL-6 and IL-8 has been documented in four human patients bitten by Bothrops and Crotalus snakes (two each) in Brazil. CRP levels were low immediately post-envenoming, peaked at two days post-envenoming and dropped to within normal limits four days post-envenoming. These findings demonstrated a typical acute-phase response and it is likely that a similar acute phase response occurred after puffadder and cobra envenoming in our study. Mean antithrombin (AT) activity was mildly decreased in both the puffadder- and cobra-envenomed dogs compared to the controls ($P=0.002$ and 0.004, respectively), suggesting that the activation of haemostasis led to some AT consumption. Mean PT and mean aPTT were prolonged in the cobra-envenomed dogs compared to the controls ($P=0.03$ for both), but were within their reference intervals (RI). At 24 hours post envenomation mean haematocrit (Ht) was significantly decreased in the puffadder group compared to the cobra group ($P=0.01$), but was within RI. The Ht was significantly lower at 24 hours post envenomation compared to presentation values in both these groups ($P<0.001$ and 0.02, respectively).

At presentation, marked thrombocytopenia, leucocytosis and prolonged clot initiation were common features in puffadder-envenomed dogs and were likely venom-induced. Snouted cobra-envenomed dogs were normo- to hypercoagulable at presentation. Both puffadder- and cobra-envenomed dogs equally showed hypercoagulability at 24 hours post-envenomation and this was more pronounced compared to their coagulability at presentation. TEG proved to be a useful tool to detect abnormal haemostasis in all envenomed dogs in this study. TEG also provided additional insights into certain aspects of snake envenomation (such as hypercoagulability) that has not been reported on previously and cannot be assessed using traditional coagulation assays. TEG may serve as a differentiating tool in early envenomation between these two types of snake envenoming in scenarios where the identity of the snake species involved is not known.
CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Snake envenomation is a common occurrence in both people and animals in many world regions, especially in rural areas, and is often classified a true medical emergency. Women, children and agricultural workers are most frequently affected. Urbanization and deforestation have led to an increase in the annual number of envenomation. Snake venom has been estimated by the World Health Organization (WHO) as the cause of approximately 100 000 human deaths annually and as many as 300 000 people annually suffer permanent disabilities after amputation, paralysis or renal failure. Farm animals are commonly envenomed, mostly accidentally when stepping on a snake. Many dogs will attack and kill snakes and are commonly presented at veterinary practices with early signs of envenomation. In Australia more than 6000 animals are envenomed by snakes annually, including cats, (52%) dogs (44%) and other species (4%). The WHO has issued guidelines for prevention and clinical management of snake envenoming in people, but there are no universally-accepted guidelines for snake envenomation in animals.

The Onderstepoort Veterinary Academic Hospital (OVAH) provides emergency treatment to approximately 50 to 60 dogs annually with either confirmed or suspected snake envenomation. Analysis of OVAH records from January 2006 to December 2008 indicates that in 55 of 173 (31.8%) dogs treated for snake envenomation there was a definitive identification of the snake. Of these, 43 cases (78%) were puffadder (Bitis arietans) envenomations and 12 (21.8%) included cobra (Naja spp.) envenomations. A retrospective study performed in 2004 at the OVAH recorded that the two most commonly encountered snake envenomations in dogs in this geographical area included, in order of frequency, puffadders and snouted cobras (Naja annulifera).

Snake venoms contain many enzymes, proteins and peptides, assisting in paralysing, killing and digesting its prey or defending the snake against predators. These venom components damage tissues, and can induce a clinically significant inflammatory response in
the victim through cytokine release.\textsuperscript{13,14} In vipers (adders) and vipers (rattlesnakes), inflammation is a major characteristic of the envenomation.\textsuperscript{14} Leucocyte infiltration, and severe oedema due to capillary and venule leakage at the envenomation site are common findings, often leading to tissue ischaemia and neural compression.\textsuperscript{13,15} The link between inflammation and haemostasis has been established.\textsuperscript{16-18} Tissue factor (TF) overexpression occurs in multiple inflammatory disease states,\textsuperscript{19} resulting in hypercoagulability and potentially thrombosis.\textsuperscript{20} The inflammatory response and haemostasis are known to inadvertently activate each other.\textsuperscript{21} Therefore, abnormalities in both systems may potentially coexist in snake envenomation.

There are many pathological consequences following snake envenomation, of which coagulopathy is the most unpredictable and complex\textsuperscript{22} and is potentially fatal.\textsuperscript{23} Several snake venom components can cause bleeding in a number of different ways.\textsuperscript{22,24-29} These venom components include: procoagulants (activating prothrombin and coagulation factors V, IX and X, thereby inducing fibrin formation); anticoagulants (e.g., phospholipase A\textsubscript{2}, thrombin inhibitor and protein C activators); fibrinolytic toxins (plasminogen activators and formation of fibrin(ogen) degradation products); vessel wall interactive toxins (haemorrhagins); toxins affecting platelet function (platelet aggregation inducers and inhibitors).\textsuperscript{23}

Determining the type of coagulopathy present in an envenomation can be diagnostically and therapeutically important,\textsuperscript{23} especially when mostly monovalent antivenom therapy is employed, such as in Australia,\textsuperscript{7} or when snake venom detection kits or a definite identification of the snake by other means are unavailable.\textsuperscript{1,2,7,23} As an example, two separate types of coagulopathy (defibrination and anticoagulation) are seen in Australia following snake envenomation.\textsuperscript{23} Defibrination is a manifestation of envenomation by brown snakes (\textit{Pseudonaja spp.}), tiger snakes (\textit{Notechis spp.}), rough scaled snakes (\textit{Tropidechis carinatus}), taipans (\textit{Oxyuranus spp.}) and broad headed snakes (\textit{Hoplocephalus spp.}). Anticoagulant coagulopathy is seen after envenomation by mulga snakes (\textit{Pseudechis spp.}). This distinction between coagulopathies is so important that it is considered a cardinal separator in Australian envenomation diagnostic algorithms.\textsuperscript{23} Plasma-based traditional assays are often limited, as they do not assess platelet and endothelial function nor do they assess the contribution of TF-bearing cells and the endothelium in the process of haemostasis.\textsuperscript{30} Thromboelastography (TEG) is currently believed to be a closer approximation of haemostasis as it occurs \textit{in vivo}.\textsuperscript{2}
and has been successfully used in the assessment of haemostasis in people since its first description in 1948.\textsuperscript{30,31}

The role of TEG has been evaluated in the clinical management of children with snake-envenomation in southern Africa.\textsuperscript{32} Abnormal thromboelastograms were recorded in 35/51 (69\%) patients, of which 17 (49\%) patients developed a severe clinical picture, concluding that 50\% of envenomed patients with abnormal thromboelastograms will develop a severe clinical diathesis, whilst if the thromboelastogram is normal, the clinical course is likely to be benign (predictive value of 94\%). Patients with abnormal thromboelastograms should therefore be closely monitored for development of coagulopathy.\textsuperscript{32} To the author’s knowledge haemostatic dysfunction occurring in snake envenomation has been reported in a single study of 39 dogs envenomed by \textit{Vipera palaestinae}. Prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT) were observed in 21 (54\%) and 17 (44\%) dogs, respectively. Hyper- and hypofibrinogenaemia were observed respectively in 17 (46\%) and 7 (19\%) of 37 dogs, respectively. The D-dimer concentrations were positive (> 250 ng/ml) in 28/31 dogs (90\%) at least once during the study. The study concluded that haemostatic abnormalities were the most common clinicopathological finding in this envenomation, and that careful monitoring of haemostasis is necessary to promote early intervention.\textsuperscript{33}

The African puffadder (\textit{B. arietans}) and snouted cobra (\textit{N. annulifera}) are typically found in Sub-Saharan Africa and envenomations by both snakes commonly occur in both people and animals.\textsuperscript{34,35} Although the haematological and biochemical findings in dogs envenomed by the African puffadder and snouted cobra have been documented,\textsuperscript{9} the haemostatic functions in such dogs were never reported, to our knowledge, and recognizing changes in haemostasis may be of diagnostic, therapeutic and prognostic importance in dogs envenomed by these snakes.

**PUFFADDER (BITIS ARIETANS)**

Snakes are classified into the phylum \textit{Chordata} (vertebrates), class \textit{Reptilia}, suborder \textit{Serpentes}. Three families are considered to have clinically significant haemostatic effects, namely \textit{Colubridae} (including the boomslang and vine snake), \textit{Elapidae} (including cobras
and mambas) and Viperidae (including rattlesnakes and true vipers). The African puffadder (*B. arietans*) belongs to the family Viperidae. It is the most widely distributed snake in Africa. A puffadder is typically 60-100 cm in length with a thick body, a triangular-shaped head and a short tail. The striking black and yellow markings are a well-known characteristic of this snake. Puffadders are heavy-bodied and lazy, extremely well camouflaged and remain motionless in order to catch prey or to escape detection. An animal or human frequently encounters a puffadder by either accidentally stepping on one or passing close by.

**Puffadder (** *Bitis arietans**)**

The venom of puffadders is cytotoxic and exerts cytolitic, haemorrhagic and thrombotic effects, which may lead to severe tissue lysis and necrosis, endothelial cell lysis, with secondary haemorrhage and coagulopathy. These pathological effects are attributed to the multiple toxic components present in puffadder venom. The venom proteome of *B. arietans* has been analysed using a combinatorial peptide ligand approach. Approximately 43 different proteins from 9 toxin families have been identified in puffadder venom. The major toxins are snake venom metalloproteinases (SVMPs), serine proteases, C-type lectin-like proteins (CLPs), phospholipase A₂ (PLA₂), disintegrin bitistatin and cystatin.

SVMPs cause rapid local haemorrhage following experimental intradermal injection, but systemic haemorrhage has also been observed in envenomed human patients. Local
haemorrhage at the envenomation site is likely due to SVMPs’ ability to degrade extracellular matrix proteins, particularly type IV collagen, which is a major component of the basement membrane. The integrity of blood vessels become compromised and local haemorrhage occurs. SVMPs are also shown to degrade large haemostatic proteins such as fibrinogen, as well as adhesion proteins. Systemic haemorrhage occurs by more complex mechanisms and the main target of SVMPs appears to be platelets. SVMPs are able to inhibit platelet interaction with collagen and/or von Willebrand Factor (vWF) through either the ligands or the platelet receptors, undoubtedly contributing to systemic bleeding. Serine proteases are not only fibrin(ogen)olytic enzymes, but are also platelet activators (acting through protease activated receptors) and prothrombin activators. CLPs are able to bind to a wide range of coagulation factors, other haemostatic proteins and platelet receptors. Some CLPs have been shown to either inhibit platelet functions by binging to GPIb, while others are able to activate platelets through the same receptor. Most CLPs function by inducing thrombocytopenia through various routes. PLA₂ is an esterolytic enzyme which causes hydrolysis of glycerophospholipids leading to the release of lysophospholipids and fatty acids. A large number of isozymes are present in snake venom. PLA₂ binds to target proteins and exhibit a wide range of effects (anticoagulant, neurotoxic, cardiotoxic, myotoxic, antiplatelet and tissue damaging effects) PLA₂ specifically binds to factor X and interferes with the formation of the prothrombinase complex. Disintegrins are polypeptides that selectively block the function of platelet integrin receptors, thus inhibiting platelet aggregation induced by thrombin, ADP, collagen and platelet-activating factor.

Clinical signs observed with puffadder envenomation result from the cytotoxic effects of the venom. The puffadder is responsible for causing severe local effects at the envenomation site, with swelling that varies from mild to severe. Swelling is usually noticed within 2 hours post-envenomation, peaks within 12-24 hours and then starts to regress by 72 hours, usually with no antivenom administration. Swelling at the envenomation site is due to local haemorrhage. However, this bleeding does not initially affect the haematocrit. Pain is variable, depending on the envenomation site. Bacterial infection of snake envenomation wounds is relatively uncommon in the dog since snake venom is antibacterial in action and very few bacteria are found in snake mouths. However, necrosis with secondary bacterial infection can occur, and thus, dogs should be monitored. Laryngeal, oral and neck swelling can result in airway obstruction. Such cases require aggressive treatment. Management of puffadder
envenomations is mostly symptomatic. The current treatment recommendations are presented in Appendix A.

**SNOUTED COBRA (NAJA ANNULIFERA)**

The snouted cobra (*N. annulifera*) belongs to the family Elapidae. It is commonly found in savannah, particularly bushveld and lowveld. Adults are commonly 1.3 meters in length, but can grow up to 3 meters. Its body is slender as it tapers to the tail. The cervical ribs are capable of expansion to form a hood when threatened. The head is broad and flattened. Most snouted cobras have a characteristic dark brown or black band over the throat area. Snouted cobras are non-spitters. They are nocturnal, emerging to hunt at dusk. The snake is commonly encountered when it is basking in the early morning sun, and will feign death or will raise its forebody and spread its hood when confronted.

*Snouted cobra (Naja annulifera)*

![Snouted cobra](image)

The snouted cobra’s venom is mainly neurotoxic, but has some cytotoxic effect. The neurotoxic venom exerts pre- and post-synaptic effects at the neuromuscular junction by blocking neurotransmitters (i.e., acetylcholine), usually resulting in progressive paralysis. The venom profile of *N. annulifera* towards nicotinic receptor affinity has recently been elucidated by assessing bioactivity towards snail-derived (*Lymnaea stagnalis*) acetylcholine binding protein (AChBP) expressed from a *Baculovirus* as an analytical platform mimicking...
neuronal acetylcholine receptor binding. Two novel acetylcholine binding protein ligands were identified (α7 acetylcholine receptor antagonist and α1 acetylcholine receptor inhibitor), as well as cardiotxin and orphan toxin. An orphan toxin is so named because of unknown function and protein targets.

The clinical signs associated with snouted cobra envenomation result from a combination of the venom's cytotoxic and neurotoxic effects. Usually only a mild swelling develops at the envenomation site, but occasionally swelling may be more severe, and necrosis may occur. The neurotoxic effects are of major concern. The onset of life-threatening collapse is quick, invariably within less than one hour and in severe cases, within less than 30 minutes. The swallowing reflex is lost first, followed by tongue and jaw paralysis with dysphagia resulting in hypersalivation. This is followed by limb weakness, and terminating with flaccid skeletal muscle paralysis, including respiratory muscle paralysis. The most important aspects of treating a neurotoxic snake envenomation include artificial ventilation and specific antivenom administration. The treatment recommendations for snouted cobra envenomation are presented in Appendix A.

SNAKE ENVENOMATION AND INFLAMMATION

The mechanisms of the acute inflammatory response to injury, changes in vascular flow, role of inflammatory mediators and chemotaxis of leucocytes and macrophages to the injury site are well documented. Inflammation is a major characteristic of envenomation by viperine (adders) and crotaline (rattlesnakes) species. Severe oedema due to capillary and venule leakage at the envenomation site is a common finding in victims, often leading to tissue ischaemia and neural compression. A considerable inflammatory leucocyte infiltrate was demonstrated in mice injected with Bothrops asper (Fer-de-lance viper) venom. Certain venom components such as phospholipase A₂ and metalloproteinases also appear to play a role in the development of inflammation. Some inflammatory mediators even contribute to the development of envenomation-associated dermal necrosis in knockout mice deficient in tumour necrosis factor and interleukin-6 (IL-6) receptors.
HAEMOSTASIS

Normal physiologic haemostasis is a balance between the vital protective mechanism preventing blood loss and the need to prevent excessive intravascular coagulation. Blood vessel wall damage results in vasoconstriction and platelet aggregation and formation of an insoluble fibrin mesh (or clot) at the injury site. Efficient haemostasis therefore involves rapid activation of blood coagulation, with localization of the clot to the injured blood vessel, and rapid termination of the process once the clot is formed.

Haemostasis was initially thought to be initiated after exposure of blood to air. In the mid-19th century the concept that haemostasis actually was dependent on a component of blood was proposed. In the early 20th century, the terminal portion of haemostasis was described, where fibrinogen is converted to fibrin by thrombin. It was only later (during the 1940s and 1950s) that the discovery of coagulation factors responsible for generating thrombin occurred. These coagulation factors were named after their discoverers, or after the first patient that was described with a deficiency of that factor, and were numbered in order of their discovery. The plasma components of coagulation were gradually sorted into specific pro-enzymes that could be converted to enzymes, and pro-cofactors that could be converted to cofactors, but were without enzymatic activity.

THE CASCADE MODEL

In the 1960s the cascade model was proposed as a series of steps, where activated enzymes with their cofactors cleaved proenzymes (or zymogen substrates) to generate the next enzyme in the cascade. The majority of the steps occur on phospholipid membrane surfaces (i.e., platelets) and require calcium. The cascade model was divided into intrinsic and extrinsic pathways, both of which terminate in the common pathway. The extrinsic pathway consisted of TF (extrinsic to the blood) and activated factor VII (FVIIa). The intrinsic pathway includes factors present within the blood and is initiated through the contact activation of factor XII (FXII) on negatively charged surfaces (e.g., subendothelial collagen). Activation of factor X (FX) to FXa could occur through either pathway. Activation of the common pathway then
follows, where FXa (with its cofactor FVa) then activates prothrombin to thrombin, resulting in fibrinogen cleavage to fibrin monomers.  

![Diagram of the coagulation cascade](image)

**Fig. 1** A diagrammatic presentation of the cascade model of fibrin formation from fibrinogen. Many of the enzyme complexes require calcium (Ca^{2+}) and binding to active membrane surfaces for full activity. Feedback activation of pro-cofactors to cofactors and the many inhibitors of the various enzymes have been omitted.  

The cascade model has proved extremely valuable in understanding plasma-based coagulation in vitro. The discovery that coagulation is calcium-dependent has facilitated the use of calcium chelators to prevent coagulation in blood samples. Various haemostatic function assays have since been developed to investigate and monitor haemostatic disorders. Deficiencies in the extrinsic and common pathways are identified using PT, while deficiencies in the intrinsic and common pathways are identified using aPTT. Less common assays, such as Russell’s viper venom time, thrombin time and specific coagulation factor assays further localize coagulation defects.  

Although the cascade model successfully separates the various enzymatic processes of coagulation into a Y-like cascade in vitro, the model does not adequately explain the haemostatic process as it occurs in vivo. The cascade model suggests that the intrinsic and
extrinsic pathways operate independently, while clinical manifestations of individual factor deficiencies clearly contradict this concept.\textsuperscript{19,21} Previous studies have reported that deficiencies in the initial components of the intrinsic pathway (factor XII, high molecular weight kininogen or prekallikrein) caused marked prolongation of aPTT, but were not associated with a bleeding tendency in mice or humans.\textsuperscript{21} Furthermore, FXII is clearly not required for normal haemostasis, and is absent in some mammalian species (whales and dolphins).\textsuperscript{19,21} Deficiency of factor XI (hemophilia C) is associated with variable haemostatic deficits in humans. Deficiency of either factor VIII or factor IX results in serious bleeding tendencies (hemophilia A and B, respectively), despite the fact that these patients have an intact extrinsic pathway. Deficiency of the primary enzyme of the extrinsic pathway (factor VII) is associated with bleeding, although the intrinsic pathway is intact.\textsuperscript{21,46}

**THE CELL-BASED MODEL OF FIBRIN FORMATION**

The cell-based model of haemostasis that was introduced during the past decade incorporates numerous procoagulant and anticoagulant factors other than those in blood plasma. This model of haemostasis also takes into account the role of cells \textit{in vivo}, particularly cells bearing TF and platelets. The current theory of haemostasis consists of 3 stages involving initiation, amplification and propagation phases that result in thrombin generation and thrombus formation.\textsuperscript{21}

**The role of TF**

All evidence to date supports the finding that TF is the sole relevant initiator of coagulation \textit{in vivo}.\textsuperscript{19,21} Cells that express TF are mostly localized outside the vasculature which prevents the initiation of haemostasis when blood flow is normal and the vascular endothelium is intact. Tissue factor is expressed in relatively high concentrations in the adventitia of the blood vessels, epidermis, mucosae, organ connective tissue capsules, and in other vital tissues such as the placenta, brain, heart, kidneys and lungs. Subendothelial cells such as fibroblasts and macrophages also express TF.\textsuperscript{19} Very low concentrations of free TF are present in circulating blood.\textsuperscript{19} Endothelial cells, smooth muscle cells, neutrophils and monocytes are able to express TF due to a variety of stimuli, including cytokines (e.g., IL-1, tumour necrosis factor-\textalpha{}), viruses, endotoxin, thrombin, immune complexes, phorbol esters and mitogens (e.g., platelet-derived growth factor, epidermal growth factor and insulin). Some circulating
cells such as monocytes and tumour cells may express TF on their membrane surface, but this TF is believed to be inactive under normal conditions. Haemostasis is initiated when a blood vessel is injured and the flowing blood is exposed to a cell bearing TF.\textsuperscript{19,21}

**The role of TF pathway inhibitor (TFPI)**

Tissue factor pathway inhibitor is the main inhibitor of the TF pathway and occurs in two forms: TFPI-1 and TFPI-2. TFPI-1 inhibits the TF pathway, while TFPI-2 inhibits trypsin, plasmin, plasma kallikrein and FXIa-amidolytic activity. TFPI is mainly produced by the microvascular endothelium and is present in three different pools; circulating free TFPI and lipoprotein-bound TFPI, platelet cytoplasmic TFPI, endothelium-bound (the largest pool).\textsuperscript{19}

**Initiation**

Haemostasis is initiated when endothelial injury exposes cells bearing TF to FVII and FVIIa. Plasma FVII exists in both an active and inactive state, but only 1\% of FVII occurs in the active state. FVIIa then rapidly binds to exposed TF, thereby initiating the extrinsic pathway. The TF-FVIIa complex activates both FX and FIX on the subendothelial surface. FXa activates plasma FV and will combine with FVa (prothrombinase) to produce small amounts of thrombin (FIIa), which play an important role in platelet activation during the amplification phase. Any FXa that dissociates from the protected cell surface microenvironment is immediately inactivated by TFPI and AT.\textsuperscript{21,44}

**Amplification**

Once the initial thrombin has been formed, amplification can occur. Thrombin has strong procoagulant properties and amplifies the initial procoagulant signal by enhancing platelet activation and aggregation, as well as activating co-factors V and VIII on the platelet surfaces. Thrombin binds to the platelet surface and causes shuffling of membrane phospholipids. A procoagulant membrane surface is created, which leads to degranulation of the platelets. Alpha granules released from the platelets contain a large amount of procoagulant proteins (e.g., FV) and other substances (e.g., calcium). Calcium can induce a clustering of phosphatidyl serine (PS) and can stimulate binding of coagulation proteins to this membrane surface.\textsuperscript{21} Von Willebrand’s factor (vWF; carrier protein of FVIII) binds platelets and is cleaved by thrombin to release FVIII, and at the same time, thrombin activates FVIII. Thrombin also leads to activation of FXI (intrinsic pathway).\textsuperscript{44}
Propagation

More platelets are recruited to the injury site through the release of procoagulant alpha granules. Ligands are expressed on the cell surface of the platelets allowing for cell-to-cell interaction, leading to the aggregation of platelets. A large amount of thrombin is generated on platelet surfaces, termed "thrombin burst". This occurs through the tenase complex (FVIIIa/FIXa) activation of FX to FXa. Since FIXa, activated during initiation, is not rapidly inhibited by AT or other plasma proteases it can diffuse to the platelet surface from its site of activation on TF-bearing cells. FXIa on platelet surface can also provide additional FIXa through the initiation of the intrinsic pathway. Thrombin cleaves fibrinogen to fibrin monomers. FXIII is activated by thrombin (requiring calcium as cofactor) to FXIIIa and then acts on fibrin to form cross links between molecules. Platelets also bind fibrinogen, fibrin and vWF, thereby promoting fibrin clot formation. The result is polymerisation of the fibrin clot.

Termination

Physiological inhibitors exist that terminate and control every aspect of haemostasis. Endothelial cells release ADPase and prostacyclin, which inhibit platelet activation and aggregation, controlling primary haemostasis. TFPI inhibits the TF-FVIIa-FXa complex during secondary haemostasis. AT is seen as a general inhibitor, with special affinity for FIXa, FXa, FXIa and thrombin. Free thrombin is bound by endothelial thrombomodulin. The thrombin-thrombomodulin complex activates protein C and its cofactor protein S, which cleave and inactivate FVα and FVIIIa. All these actions result in the termination of coagulation. Fibrinolysis is the process whereby dissolution of a fibrin clot occurs through plasmin (a serine protease), resulting in formation of soluble fibrin degradation products (FDP). Plasmin is formed from plasminogen by tissue-type and urinary-type plasminogen activators (t-PA and u-PA) and by FXIa, FXIIa and kallikrein. t-PA is a serine protease that is released from vascular endothelium and is the main agent responsible for initiating fibrinolysis. Inhibitors of fibrinolysis exist that regulate the enzymes that become activated during fibrinolysis (α-2-antiplasmin, alpha-2-macroglobulin, plasminogen activator inhibitor type 1 (PAI-1) and thrombin activatable fibrinolysis inhibitor). PAI-1 is an important regulator of fibrinolysis. All these actions are responsible for maintaining normal haemostasis.
The role of TF and TFPI in inflammation

1. **Sepsis**: The TF pathway becomes activated during various pathological conditions, including sepsis, where TF is one of several secondary inflammatory mediators involved in propagation of sepsis and septic shock. TF expression is stimulated by tumour necrosis factor-α, IL-1, IL-6, activated complement, immune complexes, and lipopolysaccharide. Studies in human patients have shown that during sepsis TF production exceeds that of TFPI, which remains normal, or may be slightly increased. This altered balance between TF and TFPI levels promote a prothrombotic state. Tissue plasminogen activator is also decreased in septic patients and PAI-1 is increased, which leads to suppression of fibrinolysis.

2. **Disseminated intravascular coagulation (DIC)**: It has been reported that human patients are in a hypercoagulable state during the initial stages of DIC. Circulating inflammatory mediators increase the TF expression and coagulation inhibitor consumption. Down-regulation of thrombomodulin (responsible for binding free thrombin) occurs, leading to decreased protein C activation, inadvertently resulting in decreased FVa and FVIIIa inactivation on endothelial surfaces. TFPI concentrations,
however, remain at physiologic levels and are unable to prevent progression of thrombosis. TFPI is also cleaved by neutrophil elastase, decreasing TF/FVIIa and FXa neutralization.\textsuperscript{19}

3. Trauma: TF expression occurs in trauma and traumatic brain injury, inducing hypercoagulability due to vascular wall damage and increased inflammatory cytokine production by macrophages and monocytes, which enhance TF exposure. The brain contains large amounts of TF, and thrombosis commonly occurs following traumatic brain injury.\textsuperscript{19}

The proposed role of TF expression in snake-envenomation
Local necrosis, oedema, haemorrhage and inflammation commonly occur to a varying degree at the snake envenomation site in victims. These effects are worse in animals suffering cytotoxic (i.e., puffadder) compared to neurotoxic (i.e., cobra) envenomation.\textsuperscript{3,8,13,15} Abnormal haemostasis may result from inadvertent activation of coagulation due to the expression of large amounts of TF. The likely sources of TF expression in snake envenomation victims are injured blood vessels, skin epithelium, mucosae, and the leucocyte infiltrate at the envenomation site.\textsuperscript{13,15,43} Circulating inflammatory mediators also increase TF expression and consumption of coagulation inhibitors.\textsuperscript{16,17,20}

COAGULOPATHY IN SNAKE ENVENOMATION

A significant amount of work has been done on investigating the pathophysiology of coagulopathy of snake envenomation in human medicine. The coagulopathy induced in people by snake envenomation is attributed to the multiple venom components affecting haemostasis. The potential clinical problems include the following:

- Decreased blood coagulability (with increased bleeding tendency)\textsuperscript{23}
- Frank haemorrhage due to blood vessel wall damage\textsuperscript{23}
- Secondary effects of increased haemorrhage (e.g. hypovolaemic shock and secondary organ damage, such as intracranial and anterior pituitary haemorrhage or kidney injury)\textsuperscript{23}
- Direct pathologic thrombosis and its sequelae (e.g. pulmonary thromboembolism)\textsuperscript{23}
The effects of snake venom on the haemostatic system can be categorized based on toxin type (Table 1).  

Table 1 A list of snake venom toxins and their effects on haemostasis.  

<table>
<thead>
<tr>
<th>Toxin type</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Procoagulants</td>
<td>Factor V activation</td>
</tr>
<tr>
<td></td>
<td>Factor X activation</td>
</tr>
<tr>
<td></td>
<td>Factor IX activation</td>
</tr>
<tr>
<td></td>
<td>Prothrombin activation</td>
</tr>
<tr>
<td></td>
<td>Fibrinogen clotting</td>
</tr>
<tr>
<td>Anticoagulants</td>
<td>Protein C activation</td>
</tr>
<tr>
<td></td>
<td>Factor IX/X activating protein</td>
</tr>
<tr>
<td></td>
<td>Thrombin inhibitor</td>
</tr>
<tr>
<td></td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>Fibrinolytic</td>
<td>Fibrinogen degradation</td>
</tr>
<tr>
<td></td>
<td>Plasminogen activation</td>
</tr>
<tr>
<td>Vessel wall interactive</td>
<td>Haemorrhagins</td>
</tr>
<tr>
<td>Interference with platelet activity</td>
<td>Platelet aggregation inducers</td>
</tr>
<tr>
<td></td>
<td>Platelet aggregation inhibitors</td>
</tr>
<tr>
<td>Plasma protein activators</td>
<td>SERPIN</td>
</tr>
</tbody>
</table>

Haemorrhage in snake envenomations occurs as a result of abnormal coagulation factors, capillary endothelium or platelet function. Viperid and Crotalid venoms are rich in metalloproteinases (haemorrhagins), which are responsible for rapid local haemorrhage following intradermal or subcutaneous venom injection, attributed to their ability to degrade extra-cellular matrix proteins, particularly type IV collagen, which is a major component of the basement membrane. This disrupts endothelial cell adhesion to the basement membrane, thereby compromising the blood vessel wall integrity. However, human patients envenomed by Viperid or Crotalid snakes often suffer from systemic haemorrhage with absence of evidence of coagulation abnormalities. This observation suggests that systemic haemorrhage can be attributed to a venom-induced platelet disorder. Venom metalloproteinases inhibit platelet interaction with collagen and vWF by various mechanisms through targeting platelet receptors or their ligands. Zinc-dependent metalloproteinases from viperid snake venoms
were reported to be largely responsible for the haemorrhagic syndrome in snakebite envenomations.\textsuperscript{38}

Many snake venoms contain procoagulant toxins (e.g., thrombin-like enzymes, prothrombin activators and factor X activators; Table 1) that activate the coagulation cascade.\textsuperscript{22} The venom of the Brown snakes (\textit{Pseudonaja} spp.) and taipans (\textit{Oxyuranus} spp.) were shown to contain group C prothrombin activators closely resembling mammalian prothrombinase (Xa:Va) complex and activating coagulation, which leads to development of consumptive coagulopathy.\textsuperscript{27} More recently, this state has been referred to as a venom-induced consumptive coagulopathy (VICC). It is characterized by prolonged clotting times, fibrinogen and FV and FVIII depletion, and high FDP concentrations.\textsuperscript{49} The consequence of VICC has often been accepted to be DIC, supported by the findings of elevated D-dimer, prolonged PT, and hypofibrinogenaemia. A recent publication questioned this belief, stating that other important features of DIC, such as evidence of systemic microthrombosis and end-organ failure, features of DIC, are absent in VICC, and therefore, these two processes differ. A clinical syndrome consistent with thrombotic microangiopathy has however been reported in a small number of human patients with VICC. It was suggested that the presence of thrombotic microangiopathy and VICC in these patients is the likely reason for mistakenly diagnosing DIC in such cases.\textsuperscript{22}

\textbf{THROMBOELASTOGRAPHY}

TEG has been used successfully for assessing haemostasis in human patients since its first description in 1948. It is used to record the viscoelastic changes that occur during coagulation in a graphical form, demonstrating fibrin polymerisation. The overall clot strength, as well as the rate of fibrin polymerisation can then be assessed by studying the thromboelastogram.\textsuperscript{31} TEG has mainly been used to monitor blood component therapy during surgery in humans, especially when severe haemorrhage is a potential complication. In human trauma patients, TEG has proved valuable to predict early transfusion requirements, as well as assessment of fibrinolysis and the efficacy of anti-fibrinolytic therapy.\textsuperscript{31}

\textbf{TEG validation in dogs}

TEG was originally developed as a bedside monitoring test to determine the patient's haemostatic function within 4 minutes of sampling using unstabilized fresh whole blood. In
veterinary medicine, citrated plasma is often collected to determine platelet count, fibrinogen and D-dimer concentrations, aPTT and PT. It has been proposed that citrated whole blood be used for TEG in order to increase the time span from sampling to analysis. Recalcification of the citrated sample immediately before analysis is then required.\textsuperscript{50}

The use of human recombinant TF (rhTF)-activated TEG on citrated whole blood samples from healthy dogs was reported, and it was concluded that citrated canine whole blood could be successfully used for TEG using rhTF at both 30 and 120 minutes post sampling. However, if serial measurements are performed, a fixed sample to assay time-point should be employed to minimize interassay variance.\textsuperscript{50}

**The principle of TEG**

In TEG, a citrated whole blood sample is used to evaluate clot formation and its subsequent lysis. A minimum rest time of 30 minutes is required before the sample can be processed. The addition of CaCl\(_2\) is necessary before analysis. An activator, such as rhTF can be used. The blood sample is incubated at 37\(^\circ\)C in a heated cup. The cup contains a suspended pin connected to a detector system. The pin is lowered into the cup containing the re-calcified sample after which the cup and pin are oscillated one to another. The resultant clot that forms between the cup and pin causes a rotation from cup to pin which is detected at the pin and the graphic thromboelastogram is then generated by a recorder.\textsuperscript{31,50}

**The thromboelastogram**

Three zones can be distinguished on the thromboelastogram (see Figure 3 below):

- The first zone indicates precoagulation and is depicted as the first linear segment, representing the time-period from initiation of the assay to formation of the initial fibrin strands, which causes divergence of the line into two branches.
- The second zone represents coagulation, extending from the end of precoagulation to the maximal amplitude (which in the thromboelastogram is seen as the point at which the separation point of the two diverging branches is maximal), and represents global clot formation.
- The third zone represents fibrinolysis, extending from the maximal amplitude to the end of the test, or until the two lines converge into the baseline, representing clot lysis.\textsuperscript{30,50}
Ca, calcium; PT, prothrombin time; PTT, partial thromboplastin time; TF, tissue factor; V, VII, X, XIII are the respective coagulation factors; r, k α and MA are the individual TEG parameters (see text).

From the thromboelastogram the following values are measured and evaluated: R, K, α, MA, G, Ly30 and Ly60 values.

The R-value (R) is the reaction- or precoagulation time (measured in millimetres), representing the time from initiation of the TEG analysis to fibrin formation (pre-set amount). It is measured from the start of the tracing to the point where the two lines diverge from baseline by 1 mm. The R-value depends on activity of FVIIIa, FIXa, FXIa and FXIIa if kaolin is used as activator, and on FVIIa activity, if TF is used as activator.50

The K-value (K) is the clotting time (measured in millimetres) and measures the time it takes for the clot to form, from the visible phase of coagulation (the end of R), to a set level of clot strength. The K value corresponds to the maximal divergence obtained in normal platelet-poor plasma (20 mm between the two lines). The K-value is influenced by factor II (thrombin), and FVIIIa activity, as well as platelet count and function, thrombin formation, fibrin precipitation, fibrinogen concentration and haematocrit.50 The combination of the R- and K-values reflects the time from initiation of haemostasis to a predetermined clot strength.

The Angle (α) is the angle between the baseline and the tangent to the curve drawn from the 1 mm divergence from baseline point. This represents the rate of fibrin build-up and cross-
linking (clot formation). While the K-value is a measure of time, \( \alpha \) is a measure of rate, of the same process, and therefore is influenced by the same factors as K.

The **MA-value** (maximal amplitude) is the maximal distance (in millimetres) between the diverging branches; representing final clot strength, when it is completely formed. The MA is influenced by fibrinogen concentration, platelet count and function, thrombin concentration, FXIIIa and haematocrit.

The **G-value** (global clot strength) is a measure of overall clot stiffness and the overall coagulant state and is calculated as follows:

\[
G = \frac{5000 \times MA}{(100-MA)} \text{ dyn/cm}^2
\]

The interpretation of \( G \) is as follows:

- Hypocoagulability – \( G<3.2 \text{ dyn/cm}^2 \)
- Normocoagulability – \( G 3.2–7.2 \text{ dyn/cm}^2 \)
- Hypercoagulability – \( G>7.2 \text{ dyn/cm}^2 \).\textsuperscript{31,50}

**Interpretation:**

When R and K are decreased and MA is increased a hypercoagulable state is present, while when the opposite occurs, a hypocoagulable state is present. It has been reported that the \( G \) value of TF-activated TEG correctly identifies dogs with signs of haemorrhage with a positive predictive value of 89% and a negative predictive value of 98%.\textsuperscript{30} TF-activated TEG may therefore be of diagnostic value in dogs with suspected haemostatic disorders.

**The use of TEG in veterinary medicine**

TEG has been used successfully in evaluation of hypercoagulability in dogs with parvoviral enteritis\textsuperscript{51} and dogs with platelet dysfunction in hypothermia.\textsuperscript{52} TEG, using rhTF on citrated whole blood, was also used to evaluate dogs with DIC\textsuperscript{46}, immune-mediated haemolytic anaemia,\textsuperscript{53,54} protein-losing enteropathy\textsuperscript{55}, naturally-occurring hyperadrenocorticism\textsuperscript{56} and neoplasia.\textsuperscript{19}
CHAPTER 2: STUDY OBJECTIVES

2.1 HYPOTHESES

- Dogs naturally-envenomed by puffadders (a snake with a cytotoxic venom) and snouted cobras (a snake with a neurotoxic venom) will show a hypercoagulable state, which will be detected utilising TEG.
- Results of haemostatic assays, obtained at presentation and at 24 hours post-envenomation of dogs naturally-envenomed by puffadder or snouted cobra will differ.

2.2 STUDY OBJECTIVES

To describe the use of various haemostatic assays, specifically TEG, in dogs naturally envenomed by common South African snakes presented to the OVAH, South Africa.

A. To assess the overall haemostatic status and haemostatic abnormalities of dogs naturally-envenomed by puffadder or snouted cobra in South Africa using TEG and traditional haemostatic assays.

B. To assess the prognostic value of TEG and traditional haemostatic tests in dogs naturally-envenomed by puffadder or snouted cobra in South Africa

2.3 STUDY BENEFITS

- Because TEG has never been used to investigate and document haemostatic function in dogs naturally-envenomed by puffadders or snouted cobras (common South African snake species), results of the present study will increase the knowledge of such envenomations in dogs. The results may prove to be of diagnostic, therapeutic and prognostic benefit
- The research conducted fulfils part of the requirements of the principal investigator’s MMedVet (Med) degree.
CHAPTER 3: MATERIALS AND METHODS

3.1 MODEL SYSTEM

This study is a prospective, descriptive, longitudinal, observational study of dogs naturally-envenomed by puffadders or snouted cobras in South Africa.

3.2 EXPERIMENTAL DESIGN

Dogs naturally envenomed by puffadders or snouted cobras and presented to the Outpatient Clinic, OVAH were included in this study. The study also included a control group of healthy age- and gender-matched dogs presented to the OVAH for routine ovariohysterectomy, castration or for blood donation. Owner consent was obtained for all the dogs included in the study (Appendix B). All dogs included in the study received treatment as was deemed necessary by the attending clinician, using a standard treatment protocol for snake envenomation in dogs, at the OVAH (Leisewitz et al.) (Appendix A).

Inclusion criteria of the study group dogs:

- Dogs of any breed or gender, with an age of at least 6 months and body weight of above 5 kg.
- Dogs that were envenomed by a puffadder (B. arietans) or a snouted cobra (N. annulifera), and presented to the OVAH no more than six hours after the incident. Snake species were identified based on either an accurate description provided by the witness, or based on accurate photos of snakes (compiled and kept at the Outpatient Clinic, OVAH) shown to the witness.
- Cases with complete medical records.

Exclusion criteria of the study group dogs:

- Any treatment administered prior to blood collection (e.g., antivenom, corticosteroids or antibiotics).
- Any treatment for a concurrent condition within the week prior to presentation.
Any treatment with medications known to interfere with haemostasis within a month prior to presentation (e.g., glucocorticoids, non-steroidal anti-inflammatory drugs or heparin products).

Concurrent *Babesia rossi* and/or *Ehrlichia canis* infection. A routine thin blood smear was examined, as well as a complete blood count performed.

Dogs diagnosed with a concurrent condition, unrelated to the snake envenomation, including wounds, infections, trauma and chronic disease (e.g., hepatic disease).

### 3.3 EXPERIMENTAL PROCEDURES

The dogs were divided into 3 groups:

**Puffadder group:** Dogs envenomed by puffadder (*B. arietans*).

**Cobra group:** Dogs envenomed by snouted cobra (*N. annulifera*).

**Control group:** Healthy gender- and age-matched dogs.

At presentation, the complete history was obtained from the owners, including a definite identification of the snake species involved and the time-lag from envenomation to presentation. All dogs underwent a complete physical examination; including recording the envenomation site, the presence of fang marks and the degree of local swelling (Appendix C). Mild swelling was recorded when oedema at the bite site was mild; moderate swelling was recorded when the oedema at the bite site was easily noticeable and palpable; severe swelling was recorded when the oedema at the bite site was marked and obscured normal structures and anatomic landmarks.

**Collection of samples**

The primary investigator was responsible for collecting all data, including obtaining a history and performing the physical examination. A quick peripheral blood smear was made from the ear upon presentation of the animal, stained with Diff Quick and examined by the primary investigator to rule out *B. canis* and *E. canis*. A second central blood smear was made from collected EDTA blood (stained by routine Diff-Quick) for evaluation by a qualified haematology technician. Blood smears were evaluated to rule out platelet clumping contributing to thrombocytopenia. Full urinalysis (specific gravity, dipstick and sediment
cytology) was performed for each animal at admission and repeated 24 hours post-envenomation. Urine was obtained by either cystocentesis or urinary catheterisation. Faecal flotation was performed at presentation for all dogs included in the study. A faecal sample was collected rectally and flotation was performed using zinc sulfate flotation solution allowing a 10 minute standing time before evaluation.

Because envenomed dogs were presented at different times post-envenomation, in order to standardise blood collection timing relative to the envenomation, dogs were only recruited if presented within 6 hours post-envenomation. This sample time at presentation therefore varied between dogs as the first samples could not be collected at exactly the same time post-envenomation. The 24 hour sample post-envenomation was therefore calculated according to the envenomation time as stated by the owner. Blood was collected at presentation, prior to any treatment, and at 24 hours post-envenomation. Whole blood samples were collected in plain tubes (3mL, Vacutainer), 3.2% tri-sodium citrate tubes (3mL Vacutainer) and EDTA tubes (3mL Vacutainer) with a 21-gauge venoject needle by careful venipuncture with minimum stasis. Sufficient blood was collected in the citrate tube to ensure a 1:9 ratio of 3.2% trisodium citrate and blood. The blood samples were collected in the order described above.

3.4 OBSERVATIONS

- The CBC was performed within 30 minutes from collection using a haematology analyser (ADVIA 2120, Siemens).
- Citrated whole blood was used for TEG analysis, using an analyser (TEG 5000 Thromboelastograph Hemostasis System, Hemoscope, Pro-Gen Diagnostics (Pty)). The TEG analysis was performed at 30 minutes from collection, to allow settling of the platelets’ activity. hrTF (Dade® Innovin®) was utilized as activator, previously validated for use in dogs. The thromboelastograms were performed for 120 minutes at 37°C and included R, K, angle, MA, Ly30 and Ly60. Standard thromboelastograms (Appendix E) were used as reference.

An additional citrated whole blood sample aliquot was centrifuged (2100 g for 8 minutes) within 30 minutes from collection, and plasma was harvested and stored at -80°C pending analyses. The coagulation profile analyses were performed as a batch within 4 months of collection, and included prothrombin time (PT), activated partial
thromboplastin time (aPTT), as well as Antithrombin (AT) activity and Fibrinogen (Fib) concentration. Studies in people and dogs have reported that coagulation proteins in frozen plasma remain stable between 6-24 months at -70º C. PT (Neoplastine® CI Plus reagent), aPTT (C.K. Prest® reagent) and Fib (Fibri-prest® Automate reagent) assays were performed on the STAT® 4 analyser (Kat Laboratory and Medical, Ansfrere, South Africa). The STAT® 4 analyser is a compact 4-channel coagulation instrument to run in vitro assays. It uses the principle of clot determination by measuring the variations of the ball oscillation amplitude through inductive sensors. AT (Precimat Chromogen® reagent) is a kinetic colorimetric test and was measured using a chromogenic assay on an automated spectrophotometric analyser (Cobas Integra 400 Plus, Roche, Randburg, South Africa). The Integra 400 Plus is a fully automated random access, software-controlled system for photometric, fluorescence polarization and electrolyte analysis.

- Blood samples obtained in plain tubes was allowed to clot, and then centrifuged (2100 g for 8 minutes) within 45 min from collection. The harvested sera were used to perform biochemical assays in order to rule out any underlying pre-existing conditions. The biochemical assays included: total protein, albumin, globulin, alanine aminotransferase, alkaline phosphatase, urea, creatinine, serum inorganic phosphate, sodium and potassium. The remainder of the sera was stored at -80º C pending C-reactive protein (CRP) analysis, performed within 6 months from collection. CRP concentration was measured using an automated turbidimetric immunoassay, calibrated with commercially available purified canine CRP (Randox Laboratories SA, Midrand, Johannesburg) to ensure species-specific measurement, with a heterologous assay (Cobas Integra 400 plus, Roche, Randburg, South Africa) previously validated in dogs. The lowest cut-off point that can be measured by this assay was 5.1 mg/dl. All results lower than this value will be recorded as < 5.1 mg/dl.

3.5 STATISTICAL ANALYSES

Data were typed in Excel spread sheets (Microsoft Excel 2007) (Appendix C) and made available to the statistician for analysis. Statistical analyses were performed using NCSS 2007 (NCSS, Kaysville, UT, U.S.A.) and Stata 12.1 (StataCorp, College Station, TX, U.S.A.). Descriptive statistics for all variables were computed. The normality assumption was
evaluated using the Shapiro-Wilk test. TEG variables R and K, as well as PT and aPTT, were log-transformed to normalise their distribution. A $P \leq 0.05$ was considered significant. Age and weight were compared between the three groups using Kruskal-Wallis analysis of variance and gender proportions were compared using the Fisher’s exact test.

Haematologic and haemostatic assay results at presentation were compared between groups using ANCOVA (analysis of covariance), and results over time between the puffadder and cobra groups were compared using linear mixed models. All results are reported in the text as mean. For the log-transformed variables (R, K, PT and aPTT) the geometric mean was used. The Ly30 and Ly60 were not included in the statistical analysis due to a large amount of zero values and incalculability of data by the TEG analyzer.

The TEG variables K, angle, MA and G were adjusted for the platelet count (Plt) and Fib concentration at presentation and at 24 hours post-envenomation, as well as for treatment administered (e.g., antivenom and/or blood products) at 24 hours. Since the R-value of the TEG is neither affected by Plt nor by Fib concentration, the R at 24 hours post-envenomation was adjusted only for treatment. Because mean haematocrit (Ht) remained within reference interval (RI) in all groups, it was not adjusted for Plt and Fib concentration. The remaining assay results were only adjusted for treatment for the 24 hours post-envenomation values.

The RI of the Clinical Pathology Laboratory, Faculty of Veterinary Science, Onderstepoort were used.
CHAPTER 4: RESULTS

The complete set of recorded data is included in Appendix E (raw data). Values that were absent for CRP concentration were due to insufficient serum samples available for analysis. Absent R and K-values were due to incalculable data as reported directly by the TEG analyser. One fatality in the study resulted in absence of data 24 hours post-envenomation (case no. 18).

4.1 SIGNALMENT (BREED, AGE, GENDER) AND BODY WEIGHT

Between November 2010 and April 2011, 23 envenomed dogs were presented to the OVAH, of which 18 dogs met the inclusion criteria and were enrolled in the study. Nine dogs were envenomed by puffadders and nine by snouted cobras. The five excluded dogs included a dog, referred to the OVAH for severe tetraparesis 18 hours post-envenomation by a snouted cobra, two dogs in which data were missing and two dogs envenomed by a Mozambique spitting cobra (*Naja mossambica*). The puffadder group included four females and five males and the median age and body weight of the dogs were 42 months (range 12-144) and 10.2 kg (range 7.0-33.6), respectively of the following breeds: smooth-haired dachshund (2), Jack Russell terrier (2), Boerboel (2), Rhodesian ridgeback and crossbreed (1 each). The cobra group included six females and three males and the median age and body weight of the dogs were 42 months (range 18-60) and 17.8 kg (range 5.8-43.2), respectively of the following breeds: smooth-haired dachshunds (3), foxterrier (1), Staffordshire bullterrier (1), bullmastiff (1), Boerboel (1), Rhodesian ridgeback (1) and crossbreed (1). The control group included four males and six females and the median age and body weight of the dogs were 52 months (range 16-96) and 17.6 kg (range 6.0-38.0), respectively of the following breeds: Beagle (3), Border collie (2), smooth-haired dachshund (1), Staffordshire bullterrier (1), German Shepherd Dog (1) and crossbreed (1). There were no significant differences in age ($P=0.60$), weight ($P=0.87$) or gender ($P=0.72$) between groups.
Table 2 Signalment and body weight of dogs envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Puffadder (n=9)</th>
<th>Snouted cobra (n=9)</th>
<th>Controls (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (months)</td>
<td>42 (12-144)</td>
<td>42 (18-60)</td>
<td>52 (16-96)</td>
</tr>
<tr>
<td>Males (N/I)¹</td>
<td>5 (2/3)</td>
<td>3 (0/3)</td>
<td>4 (1/3)</td>
</tr>
<tr>
<td>Females (N/I)¹</td>
<td>4 (1/3)</td>
<td>6 (3/3)</td>
<td>6 (3/3)</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>10.2 (7.0-33.6)</td>
<td>17.8 (5.8-43.2)</td>
<td>17.6 (6.0-38.0)</td>
</tr>
</tbody>
</table>

¹, N, neutered; I, intact.

4.2 TIME-LAG FROM ENVENOMATION, TREATMENT, OUTCOME AND POST MORTEM FINDINGS

The median time-lag from envenomation to presentation in the puffadder and cobra groups was 1.5 hours (1-2) and 3 hours (1-4.5), respectively. Treatment of all dogs was supportive and additionally, specific treatment for the puffadder group included fresh frozen plasma (FFP) transfusion (1/9), whole blood transfusion (1/9), and polyvalent antivenom (2/9); and for the cobra group, polyvalent antivenom (8/9). Polyvalent antivenom was administered slowly intravenously over 10 minutes. In emergency cases (dogs that presented with respiratory paralysis) the antivenom was given as a fast bolus. No standard dosages for polyvalent snake antivenom are published. Administration in this scenario was based on what pet owners could afford and as indicated in the current suggested treatment protocol by Leisewitz et al (Appendix A). One to two vials were generally administered and the animal closely observed for clinical improvement or deterioration. Further antivenom was administered as deemed necessary according to owner affordability. One dog was treated with FFP (20ml/kg slowly intravenously over 2 hours) due to a severely hypocoagulable thromboelastogram at presentation with a markedly prolonged R time. Repeat TEG 12 hours post-envenomation showed an even more prolonged R compared to its value at presentation, which suggested that FFP had no immediate effect. Two dogs in the puffadder group died, and one dog in the cobra group was euthanized 72 hours post presentation due to a poor prognosis. The body weight of the two non-survivors envenomed by puffadder was below 10 kg. None of the two dogs received antivenom or blood products. They did not show antemortem macroscopic evidence of bleeding, with exception of mild to moderate soft tissue
swelling and oozing at the envenomation site. Post mortem examination demonstrated multiple haemorrhages in the subcutis and at the envenomation site musculature. Additionally, there were several small, paintbrush endocardial haemorrhages in the left ventricle in the one dog and moderate small intestinal and cecal enterorrhagia in the other.

4.3 HAEMOSTATIC ASSAY RESULTS AT PRESENTATION

Mean (standard deviation) (range) and median (interquartile range) for all haematologic and haemostatic analytes tested at presentation and at 24 hours post-envenomation in all three groups are depicted in Table 3.
Table 3 Haematologic and haemostatic variables in dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and control groups at presentation and 24 hours post-envenomation

<table>
<thead>
<tr>
<th>Variable (reference range)</th>
<th>Control group (n=10)</th>
<th>Puffadder group (n=9)</th>
<th>Snouted cobra group (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (range)</td>
<td>*Median (IQR)</td>
<td>Mean ± SD (range)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Presentation</td>
</tr>
<tr>
<td>Platelet count (×10^9/L)</td>
<td>328 ± 76 (218.455)</td>
<td>135.9 ± 154.3 (0.5143)</td>
<td>173.4 ± 97.2 (45.338)</td>
</tr>
<tr>
<td>(200-500)</td>
<td>*328 (269-377.3)</td>
<td>*76 (76-286)</td>
<td>*149.5 (100.8-257.5)</td>
</tr>
<tr>
<td>Haematocrit (L/L)</td>
<td>0.50 ± 0.05 (0.43-0.58)</td>
<td>0.47 ± 8.3 (0.34-0.55)</td>
<td>0.33 ± 0.1 (0.2-0.45)</td>
</tr>
<tr>
<td>(0.37-0.55)</td>
<td>*0.50 (0.47-0.53)</td>
<td>*0.53 (0.39-0.55)</td>
<td>*0.31 (0.22-0.45)</td>
</tr>
<tr>
<td>White blood cell count</td>
<td>8.8 ± 1.8 (6.0-11.1)</td>
<td>19.0 ± 10.1 (7.8-39.1)</td>
<td>19.1 ± 10.0 (8.3-41.6)</td>
</tr>
<tr>
<td>(6-15)</td>
<td>*8.8 (6.8-10.6)</td>
<td>*15.8 (11.9-26.9)</td>
<td>*17.9 (14.1-23.0)</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>5.7 ± 1.9 (5.1-11.1)</td>
<td>12.8 ± 15.7 (5.1-50.6)</td>
<td>50.9 ± 23.4 (5.1-84)</td>
</tr>
<tr>
<td>(&lt; 30)</td>
<td>*5.1 (5.1-13.2)</td>
<td>*5.1 (3.8-13.9)</td>
<td>*5.1 (3.8-13.9)</td>
</tr>
<tr>
<td>R-time (min)</td>
<td>8.2 ± 3.2 (4.2-13.8)</td>
<td>33.4 ± 38.3 (4.1-1119.9)</td>
<td>22.3 ± 40.0 (2.2-119.9)</td>
</tr>
<tr>
<td>(3-9)</td>
<td>*7.9 (5.5 – 11.2)</td>
<td>*21.9 (8.6 – 53)</td>
<td>*6.55 (3.6 – 19.3)</td>
</tr>
<tr>
<td>K-value (min)</td>
<td>3.4 ± 1.3 (1.4-5.4)</td>
<td>11 ± 18.8 (0-49.1)</td>
<td>4.6 ± 5.5 (0-16.2)</td>
</tr>
<tr>
<td>(2-8)</td>
<td>*3.8 (2.2 - 4.3)</td>
<td>*3.85 (1.2 - 18)</td>
<td>*2.4 (0.9 - 8)</td>
</tr>
<tr>
<td>Maximal amplitude</td>
<td>46.5 ± 13.3 (26.3-70.2)</td>
<td>21.2 ± 25.0 (0-67.8)</td>
<td>42.1 ± 29.4 (0-78.9)</td>
</tr>
<tr>
<td>(27-59)</td>
<td>*45.5 (36.5-58.4)</td>
<td>*4.8 (0.7-42.4)</td>
<td>*4.21 (17.0-71.3)</td>
</tr>
<tr>
<td>G-value (mm)</td>
<td>58.8 ± 6.4 (45.9-66.2)</td>
<td>32.1 ± 29.8 (0-74)</td>
<td>60.9 ± 26.3 (0-82.1)</td>
</tr>
<tr>
<td>(39-59)</td>
<td>*60.1 (33.6-63.8)</td>
<td>*12.1 (5.8-61.0)</td>
<td>*70.3 (54-73.9)</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>7.4 ± 1.8 (4.2-9.8)</td>
<td>4.1 ± 5.0 (0-14.2)</td>
<td>11.3 ± 6.8 (0-23)</td>
</tr>
<tr>
<td>(3.2K-7.2K)</td>
<td>*7.6 (5.8-8.9)</td>
<td>*0.7 (3.7-8.8)</td>
<td>*11.9 (6.3-14.2)</td>
</tr>
<tr>
<td>Antithrombin (%)</td>
<td>122.3 ± 14.2 (107.1-143.9)</td>
<td>96.7 ± 20.5 (66.2-138.3)</td>
<td>91.9 ± 28.2 (59.3-139)</td>
</tr>
<tr>
<td>(&gt; 80)</td>
<td>*116.6 (109.7-138.3)</td>
<td>*94.3 (83.0-108)</td>
<td>*91.4 (64.4-112.3)</td>
</tr>
<tr>
<td>Prothrombin time (sec)</td>
<td>2.5 ± 0.5 (1.69-3.42)</td>
<td>2.9 ± 0.5 (2.1-3.66)</td>
<td>3.8 ± 1.6 (0.64-5.88)</td>
</tr>
<tr>
<td>(2-4)</td>
<td>*2.49 (2.08-2.84)</td>
<td>*2.97 (2.61-3.19)</td>
<td>*3.9 (3.17-5.21)</td>
</tr>
<tr>
<td>Activated partial thromboplastin time (sec)</td>
<td>6.5 ± 1.6 (5.10-7)</td>
<td>7.6 ± 1.3 (6.6-10.4)</td>
<td>7.2 ± 0.8 (6.4-8.3)</td>
</tr>
<tr>
<td></td>
<td>*6.35 (5.5-6.7)</td>
<td>*7 (6.8-8.5)</td>
<td>*6.9 (6.5-8.1)</td>
</tr>
</tbody>
</table>
| 424 hours post-envenomation; 1Platelet count; 2Haematocrit; 3White blood cell count; 4 C-reactive protein; 5R-time; 6K-value; 7 Maximal amplitude; 8 G-value; 9Antithrombin; 10Prothrombin time; 11Activated partial thromboplastin time.
Of the TEG variables, at presentation, the mean R was significantly longer in the puffadder group compared to the cobra and control groups (P=0.01 and P=0.05, respectively); however, there was no significant difference between the cobra and the control groups (P=0.44) (Fig. 4). R-time was increased in 6/9 dogs in the puffadder-envenomed group and decreased in 1/9 dogs in the snouted cobra group. At 24-hours post-envenomation 6/8 dogs (one fatality) envenomed by puffadders reverted to a hypercoagulable state. One dog was still hypocoagulable and one dog that was hypocoagulable became normocoagulable but still had a prolonged R-time. All dogs in the cobra-envenomed group had either normal (4/9) or mildly increased R-time (5/9).

**Figure 4** Box plot of the thromboelastography R-time (min) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. The box represents the interquartile range and the line within the box represents the median. The whiskers extend to the minimum and maximum values, indicating the range. Outliers, (values above 1.5 times the interquartile range), are represented by dots.
There were no significant differences in mean K between groups (puffadder vs. cobra, $P=0.87$; puffadder vs. controls, $P=0.87$; and cobra vs. controls $P=0.99$) (Fig. 5). At presentation 1/9 dogs in the puffadder group had an increased and 1/9 a decreased K. In the cobra group 3/9 dogs had a decreased K. Values for 5/18 dogs could not be generated by the thromboelastograph analyser due to incalculable data. At 24 hours post-envenomation 5/8 (one fatality) dogs in the puffadder group had decreased and 2/8 increased K. In the cobra group 4/9 dogs had decreased K.

**Figure 5** Box plot of the thromboelastography K-value (min) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
There were no differences in the mean Angle ($\alpha$) between groups (puffadder vs. cobra, $P=0.14$; puffadder vs. controls $P=0.16$; and cobra vs. controls, $P=0.91$) (Fig. 6). At presentation 5/9 dogs in the puffadder group had decreased and 1/9 dogs an increased $\alpha$. In the cobra group 1/9 dogs had mildly decreased $\alpha$. At 24 hours post-envenomation 7/8 (one fatality) dogs had severely increased $\alpha$. In the cobra group all dogs had severely increased $\alpha$.

**Figure 6** Box plot of the thromboelastography Angle ($\alpha$) (degrees) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
The mean MA did not differ significantly between groups (puffadder vs. cobra, $P=0.16$; puffadder vs. controls, $P=0.18$; and cobra vs. controls, $P=0.91$) (Fig. 7). At presentation 5/8 (one fatality) dogs in the puffadder group had a decreased MA. In the cobra group 6/9 dogs had an increased MA. At 24 hours post-envenomation 6/8 (one fatality) dogs had a severely increased MA. In the cobra group all dogs had severely increased MA.

**Figure 7** Box plot of the thromboelastography maximal amplitude (MA) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
The mean G did not differ significantly between groups (puffadder vs. cobra, $P=0.17$; puffadder vs. controls, $P=0.23$; and cobra vs. controls $P=0.84$) (Fig. 8). At presentation 5/9 dogs in the puffadder group had decreased G. In the cobra group 5/9 dogs had increased G. At 24 hours post-envenomation 6/8 (one fatality) dogs had increased G. In the cobra group all dogs had increased G.

**Figure 8** Box plot of the thromboelastography global clot strength (G) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
The mean Plt was significantly lower in the puffadder compared to the cobra ($P=0.04$) and control groups ($P=0.001$), but there was no significant difference between the cobra and control groups ($P=0.17$) (Fig. 9). At presentation 6/9 dogs in the puffadder group and 1/9 dogs in the cobra group had thrombocytopenia. AT 24 hours post-envenomation 5/8 (one fatality) dogs in the puffadder group and 5/9 dogs in the cobra group had thrombocytopenia.

![Figure 9 Box plot of the platelet count (Plt) (10$^9$/l) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (Bitis arietans) or snouted cobra (Naja annulifera) and of negative controls. See figure 4 legend for explanation.](image-url)
The mean Ht did not differ significantly between groups (puffadder vs. cobra, \( P=0.12 \); puffadder vs. controls, \( P=0.36 \); and cobra vs. controls groups \( P=0.47 \)) (Fig. 10). All dogs had normal Ht at presentation, but at 24 hours post-envenomation 4/8 (one fatality) dogs in the puffadder group had mild to moderately decreased Ht.

Figure 10 Box plot of the haematocrit (Ht) (l/l) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (\textit{Bitis arietans}) or snouted cobra (\textit{Naja annulifera}) and of negative controls. See figure 4 legend for explanation.
The mean WBC was significantly higher in the puffadder compared to the cobra and control groups ($P=0.003$ and $P=0.001$, respectively), but did not differ significantly between the cobra and control groups ($P=0.81$) (Fig. 11). At presentation and at 24 hours post-envenomation 5/9 dogs in the puffadder group had leucocytosis.

**Figure 11** Box plot of the white blood cell count (WBC) ($10^9/l$) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
There were no significant CRP concentration differences between groups (puffadder vs. cobra, \( P=0.21 \); puffadder vs. controls, \( P=0.14 \); and cobra vs. controls \( P=0.92 \)) (Fig. 12) CRP at presentation was below the lowest detection limit for all but 4 dogs in this study. Three dogs had CRP within normal reference limits and only one dog that was envenomed by a puffadder had an elevated CRP. At 24 hours post-envenoming all but two dogs (one each in the puffadder and cobra groups) had severely elevated CRP. This increase in CRP was statistically significant in both puffadder and cobra-envenomed dogs when compared to its concentration at presentation \( (P=0.04 \) and 0.001, respectively).

**Figure 12** Box plot of the C-reactive protein (CRP) concentration (mg/l) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (\( Bitis arietans \)) or snouted cobra (\( Naja annulifera \)) and of negative controls. See figure 4 legend for explanation.
The mean AT activity was significantly decreased in the puffadder and cobra groups compared to the control group ($P=0.002$ and $P=0.004$, respectively); however, there was no significant difference between the puffadder and cobra groups ($P=0.80$) (Fig. 13).

**Figure 13** Box plot of the antithrombin (AT) activity at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
There were no significant differences in mean Fib concentration between groups (puffadder vs. cobra, $P=0.72$; puffadder vs. controls, $P=0.32$; and cobra vs. controls, $P=0.53$) (Fig. 14).

**Figure 14** Box plot of the fibrinogen (Fib) concentration (mg/dl) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
Mean PT was significantly prolonged in the cobra group compared to the control group ($P=0.03$), but did not differ significantly between the puffadder and the cobra groups ($P=0.76$) and between the puffadder and control groups ($P=0.07$) (Fig. 15).

**Figure 15** Box plot of the prothrombin time (PT) (s) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
Mean aPTT was significantly prolonged in the cobra group compared to the control group ($P=0.03$), but did not differ significantly between the puffadder and the cobra groups ($P=0.53$) and puffadder and control groups ($P=0.11$) (Fig. 16).

**Figure 16** Box plot of the activated partial thromboplastin time (aPTT) (s) at presentation and at 24 hours post-envenomation of dogs naturally envenomed by the African puffadder (*Bitis arietans*) or snouted cobra (*Naja annulifera*) and of negative controls. See figure 4 legend for explanation.
Below are thromboelastograms included of two dogs at presentation after envenomation by African puffadder (Figure 17) and snouted cobra (Figure 18).

**Figure 17** A hypocoagulable thromboelastogram at presentation of a dog envenomed by an African puffadder (*Bitis arietans*). Dashed line indicates a normal tracing. Refer to text for discussion of the various TEG parameters depicted.

**Figure 18** A hypercoagulable thromboelastogram at presentation of a dog envenomed by a snouted cobra (*Naja annulifera*). Dashed line indicates a normal tracing. Refer to text for discussion of the various TEG parameters depicted.
4.4 HAEMOSTATIC ANALYTES OVER TIME IN THE PUFFADDER AND COBRA GROUPS, AND DIFFERENCES BETWEEN GROUPS AT 24 HOURS POST-ENVENOMATION

The mean MA and G were significantly increased in the puffadder group at 24 hours post-envenomation compared to their values at presentation ($P=0.05$ for both); however, these did not differ significantly in the cobra group ($P=0.98$ and $P=0.77$, respectively). In both groups, there were no significant changes between presentation and 24-hour post-envenomation in mean R ($P=0.27$ and $P=0.69$, respectively), mean K ($P=0.26$ and $P=0.27$, respectively) or mean angle ($P=0.11$ and $P=0.38$, respectively). At 24 hours all the dogs in the puffadder and cobra groups had hypercoagulable thromboelastograms. Treatment with polyvalent antivenom or blood products did not have any significant effect on the TEG variables (data not included).

In both the puffadder and cobra groups there were no significant differences between time-points in mean Plt ($P=0.69$ and $P=0.14$, respectively). The mean Ht was significantly lower at 24 hours post-envenomation compared to presentation values in both these groups ($P<0.001$ and $P=0.02$, respectively). There were no significant time-point related differences in mean AT activity ($P=0.63$ and $P=0.12$, respectively), mean PT ($P=0.65$ and $P=0.20$, respectively) or mean aPTT ($P=1.0$ and $P=0.83$, respectively); however, mean Fib concentration was significantly increased at 24 hours post-envenomation compared to presentation in the cobra group ($P<0.001$), but not in the puffadder group ($P=0.16$). No time-related differences were noted in mean WBC in both groups ($P=1.0$ for both); however, at 24 hours post-envenomation mean CRP concentration was significantly increased in both groups compared to its concentration at presentation ($P=0.04$ and $P=0.001$, respectively). Treatment with blood products had no effect on any variables. Dogs treated with polyvalent antivenom had a significantly lower mean AT activity ($P=0.05$) and longer mean aPTT ($P=0.01$) compared to their corresponding values at presentation.

The mean Ht was significantly lower and mean WBC was higher ($P=0.01$ for both) in the puffadder compared to the cobra group at 24 hours post-envenomation. There were no other significant differences between the puffadder and cobra groups at 24 hours post-envenomation.
CHAPTER 5: DISCUSSION

5.1 THE EVALUATION OF TEG VARIABLES BETWEEN GROUPS

This study demonstrated that a proportion of dogs envenomed by puffadders had a significantly prolonged clot initiation time (R), compared to dogs envenomed by snouted cobras and healthy controls. Visual appraisal of the thromboelastograms at presentation revealed that 56% of puffadder-envenomed dogs also had hypocoagulable thromboelastograms as was demonstrated by decreased $\alpha$, MA and G. Despite this unusual observation of hypocoagulable thromboelastograms in half of the puffadder-envenomed dogs at presentation, none of the other TEG parameters ($K$, $\alpha$, MA or G) were decreased enough to be statistically significant when compared to the cobra and control groups. This finding was surprising since envenomations by puffadders are considered to be severely cytotoxic, and therefore a hypercoagulable status was to be expected, secondary to inflammation-triggered activation of haemostasis.\(^3,^8,^9,^{16,17,20}\) This hypocoagulable state was transient and at 24 hours post-envenomation the R in the puffadder group was within RI in most of the dogs, even though no specific treatment was administered (e.g. polyvalent antivenom, FFP or stored whole blood). The small sample size could have affected the power of the study and contributed to this loss of significance of the TEG variables between groups.

Although there were no significant differences between the mean TEG variables of the cobra group compared to the control group at presentation, more than half of the cobra-envenomed dogs had mildly hypercoagulable thromboelastograms, with all nine dogs becoming severely hypercoagulable at 24 hours post-envenomation, based on the markedly increased MA and G compared to the laboratory reference values. The mild degree of hypercoagulability seen in these dogs at presentation is difficult to explain. One likely possibility would be that activation of haemostasis has occurred through release of large amounts of TF at the acutely inflamed envenomation site.\(^{14,15,19}\) Systemic inflammation would then be expected, which was not evident, as all cobra-envenomed dogs demonstrated normal WBC and low levels of CRP. Another possibility would be a direct venom effect promoting a hypercoagulable state through either activating coagulation factors or consuming or degrading them. The progressively hypercoagulable state observed in these envenomed dogs 24 hours post-
envenomation could still be attributed to unknown amounts of venom present in these dogs. However, CRP was now severely elevated in all these dogs and it is likely that the acute-phase response plays a major role in augmenting systemic inflammation and enhancing the hypercoagulable state. Although elapid snakes are mostly known for their neurotoxic effects, they also cause severe rhabdomyolysis (myotoxins)\textsuperscript{2,11} and occasionally, massive necrosis at the envenomation site.\textsuperscript{3} These effects will also potentially contribute towards the hypercoagulable state observed at 24 hours post-envenomation.

5.2 EFFECT OF TREATMENT ON TEG VARIABLES

Treatment with polyvalent antivenom and blood components were not associated with any changes in any of the TEG variables in this study. Only two dogs in the puffadder group were treated with a blood component. One dog was treated with FFP due to a severely hypocoagulable thromboelastogram at presentation with a markedly prolonged R time. Repeat TEG 12 hours post-envenomation showed an even more prolonged R compared to its value at presentation, suggesting that FFP had no immediate effect. Similarly, previous studies failed to show any beneficial effect of FFP in snakebite coagulopathy.\textsuperscript{23,60} In this dog, the follow-up TEG, at 24 hours post-envenomation, showed a markedly hypercoagulable thromboelastogram and the R was within RI.

A prolonged R time represents delayed initiation of haemostasis\textsuperscript{50,61} and supports the theory of a venom-induced phenomenon refractory to treatment with blood components. The haemostatic components involved during the initiation stage include TF, TF-presenting cells, and FVIIa, FVa and FXa.\textsuperscript{44} Snake venoms contain activators or inhibitors of haemostasis,\textsuperscript{23,49,62} such as phospholipases, fibrinogenolytic enzymes, and proteolytic enzymes that can degrade coagulation factors,\textsuperscript{29} assisting the snake in immobilizing, killing and digesting its prey.\textsuperscript{2,10} The coagulopathy and haemorrhage resulting from envenomations by snakes belonging to the family \textit{Viperidae} (\textit{B. gabonica, B. arietans}) in human patients is well described.\textsuperscript{23,35,63} The venom of the gaboon adder (\textit{B. gabonica}) contains components that interfere with platelet aggregation, as well as the blood thromboplastin system, resulting in defective thrombin generation and inhibition of thromboplastin formation and accelerated loss of formed thromboplastin.\textsuperscript{63} Clot formation is also impaired through direct fibrinogen proteolysis, releasing soluble fibrinogen breakdown products, with possible defective fibrin
polymerisation, resulting in a prolonged thrombin clotting time. The effects of puffadder venom on coagulation in baboons also demonstrated presence of dose dependant fibrinolysis and fibrinogenolysis, with evidence of consumption of blood coagulation factors and severe thrombocytopenia. In addition, normal human platelets were shown to be extremely sensitive to small doses of venom in vitro, with very rapid, irreversible aggregation, probably through adenosine diphosphate (ADP) action.

5.3 EVALUATION OF HAEMATOLOGICAL FINDINGS BETWEEN GROUPS

At presentation, in the puffadder group, the mean Ht was within RI. At 24 hours post-envenomation the Ht had decreased, although it was still within RI. This decrease likely resulted from marked acute subcutaneous haemorrhage at the bite site, with resultant fluid shifts due to capillary leakage. Puffadders have highly potent haemorrhagic and cytotoxic venom and animal victims usually develop marked swelling at the bite site that is progressive for up to 72 hours. The mean Ht also decreased in the cobra group at 24 hours post-envenomation compared to the corresponding value at presentation, but remained well within RI. Intravenous fluid administration probably also contributed to the decrease in the haematocrit at 24 hours post-envenomation due to haemodilution in both the puffadder and cobra groups.

Thrombocytopenia was noted on presentation in the puffadder group similar to previous reports in dogs and baboons. Snake venoms may inhibit platelets (thereby reducing their effectiveness in haemostasis), or may promote aggregation (thereby leading to thrombosis), or may even decrease platelet availability. Both platelet aggregators (e.g., bitiscetin) and inhibitors (e.g., bitistatin) have been identified in puffadder (B. arietans) venom. Bitiscetin, a disulphide-linked heterodimer, is a C-type lectin-like protein (CLP) that binds to the A1 domain of vWF and induces platelet aggregation through binding to platelet membrane glycoprotein Ib (GPIb). Bitistatin, on the other hand, is a disintegrin (a receptor antagonist) that binds to platelet surface integrin IIb-IIIa, thereby blocking fibrinogen binding to the receptor–glycoprotein complex on activated platelets. Platelet aggregation induced by ADP, thrombin, platelet-activating factor and collagen therefore becomes inhibited. None of the cobra-envenomed dogs demonstrated signs of thrombocytopenia.
5.4 DEGREE OF INFLAMMATION WITHIN THE GROUPS

A marked leucocytosis was recorded in the puffadder-envenomed dogs both at presentation and 24 hours post-envenomation as was described previously. The leucocytosis is likely induced by cytokines as a result of acute inflammation triggered by cellular damage at the envenomation site, but could also occur due to a stress or pain response. CRP concentration at presentation was below the lowest detection limit for most dogs in this study. At 24 hours post-envenomining all but two dogs (one each in the puffadder and cobra groups) had severely elevated CRP. This increase in CRP was statistically significant in both puffadder and cobra-envenomed dogs when compared to its concentration at presentation. Fib concentration was not elevated in any envenomed dogs at presentation, but increased 24 hours post-envenomining. Although this increase was not statistically significant, an increase would suggest activation of the inflammatory response, as both Fib and CRP are positive acute phase proteins. Elevated CRP, leucocytosis with neutrophilia and increases in cytokine IL-6 and IL-8 has been documented in four human patients bitten by Bothrops and Crotalus snakes (two each) in Brazil. CRP levels were low immediately post-envenoming, peaked at two days post-envenomining and dropped to within normal limits four days post-envenomining. These findings demonstrated a typical acute-phase response and it is likely that a similar acute phase response occurs after puffadder and cobra envenomining in our study.

5.5 COMPARISON OF PLASMA COAGULATION TEST RESULTS BETWEEN GROUPS

There were significant group differences in results of AT, PT and aPTT. Although AT was found to be significantly decreased at presentation in both the puffadder and cobra groups compared to the control group, it remained within RI (activity >80%) in most dogs. AT, a glycoprotein (α₂-globulin) produced by the liver, is the most important inhibitor of FIIa (thrombin) and FXa. It has a half-life of 1.7 days in dogs. Decreased AT usually results from consumption or loss (protein-losing nephropathy, enteropathy or haemorrhage). It therefore confirms that envenomation by both types of snakes initiate non-specific coagulation, with resultant consumption of AT. At 24 hours post-envenomation AT activity decreased further and more dogs were recorded with hypoantithrombinaemia (refer to Appendix E).
PT and aPTT were both significantly prolonged in the cobra-envenomed dogs compared to the controls at presentation and at 24 hours post-envenomation. The clinical relevance of this finding is questionable as the differences were small, with overlap between the groups and the results were also still within normal limits.

5.6 STUDY LIMITATIONS

Limitations to this study included the small sample size and therefore a high probability of type II error. Due to financial constraints, dogs were screened for \textit{B.canis} and \textit{E.canis} only by blood smear examination, which is an insensitive method for detection of these organisms. PCR examination of blood samples would have been the most reliable method to definitively exclude these blood-borne parasites. The statistical power of the study may also have been reduced due to the necessity of adjusting for covariates, including the low platelet count and high fibrinogen concentration that have been shown to affect certain TEG variables, as well as the disease-specific treatments provided (e.g., antivenom and blood products transfusion). The time-lag from envenomation to presentation could not be completely standardised, thereby potentially introducing variance. In an attempt to overcome this limitation the authors chose to include only cases that presented within 6 hours post-envenomation.
CHAPTER 6: CONCLUSIONS

Haemostatic changes occur in dogs envenomed by both puffadder and snouted cobra. The early hypocoagulable changes in dogs envenomed by puffadders are likely venom-induced and transient; most puffadder victims reverted to a hypercoagulable state within 24 hours post-envenomation possibly due to on-going local and systemic inflammation. We postulate that the hypocoagulable tracing seen with puffadder envenomation at presentation might be due to production of abnormal thrombin, or inhibition of certain coagulation factors.

Snouted cobra-envenomed dogs were found to have a tendency towards mild hypercoagulability at presentation. This hypercoagulability appears to be progressive, as follow-up thromboelastograms at 24 hours post-envenomation were more hypercoagulable than at presentation and could be attributed to the effect of certain venom components and the acute-phase response.

TEG proved to be a useful tool to detect abnormal haemostasis in all envenomed dogs in this study. TEG also provided additional insights into certain aspects of snake envenomation (such as hypercoagulability) that has not been reported on previously and cannot be assessed using traditional coagulation assays. TEG may serve as a differentiating tool in early envenomation between these two types of snake envenoming in scenarios where the identity of the snake species involved is not known. An abnormal thromboelastogram in a puffadder-envenomed dog should prompt the clinician to administer appropriate antivenom and to monitor for a bleeding diathesis. Unfortunately the use of TEG is currently limited to educational institutions due to expensive laboratory equipment and the need for undertaking regular quality control procedures.
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APPENDICES

Appendix A - Standardised treatment protocol for cytotoxic, neurotoxic and haemotoxic snake envenomation

The following treatment protocol was adapted from Leisewitz et. al. (2004)

Treating cytotoxic envenomation:
Most cases require no treatment, no antivenom administration and recover well. Antibiotics are unnecessary; unless necrosis of tissues has occurred (e.g. spitting cobra bites). Analgesics aren’t usually indicated, as pain seems to be minimal in most cases with swelling. Cases showing progressive swelling or deterioration however should be admitted for observation. The following treatment regimens may apply:

1. Start on maintenance rates of crystalloid fluid therapy (Shock rates may be necessary in patients showing hypovolaemic shock).
2. Synthetic colloids can be used (e.g. hetastarch).
3. Whole blood transfusions may be necessary if the haematocrit continues to fall. Decreased haematocrit may be due to haemorrhage at the bite site, or secondary immune-mediated haemolysis with evidence of a +ve ISA and haemoglobinaemia or haemoglobinuria.
4. Intravenous administration of as much antivenom as the owner can afford (as many as 8 vials may be given).
5. If upper airway obstruction occurs due to progressive swelling a tracheostomy tube must be placed.
6. Critically ill dogs should be placed on intravenous broad-spectrum antibiotic cover.

Treating neurotoxic envenomation:
Dogs suspected to have suffered a neurotoxic envenomation should be observed very closely and no treatment given until it is obvious that signs are present:

1. If signs of severe weakness or shallow breathing are noticed, an intravenous general anaesthetic needs to be administered and an ET tube placed. The patient should be
ventilated manually with an AMBU bag or via a closed circuit anaesthetic machine while the ventilator is set up.

2. A mechanical ventilator needs to be set up immediately. Ventilation will be required for at least 6-12hrs while the antivenom is reversing the paralysis. General anaesthesia is maintained for at least 6-12hrs with pentobarbitone or a constant rate infusion of propofol before attempts should be made to wean the dog off the ventilator.

3. Administer as much polyvalent antivenom as the owner can afford slowly intravenously over half an hour.

4. Adequate nursing care of a patient on a ventilator includes regular turning, maintaining fluid balance, ET tube cleaning via suction and good urinary catheter management.

5. The use of prophylactic antibiotics is controversial; however human ventilated patients are very susceptible to developing ventilator-induced pneumonia and are always placed on broad-spectrum antibiotics.

6. The venom of the snouted cobra may cause neurotoxic as well as local cytotoxic effects. These cases therefore need to be managed appropriately and effectively.

Treating haemotoxic envenomation:
The most important treatment is administering appropriate antivenom. A limited number of polyvalent antivenom is kept in the outpatient’s pharmacy. Monovalent antivenom is not kept routinely and will need to be obtained directly from the supplier (Edenvale, Johannesburg) via courier. Fresh whole blood or fresh plasma transfusion may also be necessary. Monitor urine output if renal failure is suspected. Thrombolytics are contraindicated (esp. heparin) as venom-induced thrombin is resistant to its action. Indications include:

- Any patient with an active bleed (internal or external).
- With laboratory evidence of significant coagulopathy (prothrombin time and partial thromboplastin time more than double the control).
- Where blood fails to clot in a test tube
Appendix B - Client consent form

I, ........................................................................................................ Owner/representative of the owner (please delete where not applicable) of ...........................................(name of animal) a ...................... old (age) ....................................................... (breed) hereby give permission for information (presenting abnormalities, test results, response to treatment) about my dog to be used in research that will enable veterinarians to better understand and treat snakebites in dogs.

I understand that a volume of blood (9 ml) will be collected from my dog in addition to what would normally be collected from him/her for the usual tests done at the Outpatient's clinic. I give permission for this to be done.

I understand that data about myself (name, suburb of residence) and my dog (name, patient number, age, sex, breed and clinical data) will be stored on a computer database. Personal information that could identify my dog or me will not be divulged to persons uninvolved in the research without my express consent.

Signature: ..........................................................................................
Place: ............................................................................................... 
Date: ...............................................................................................
**Appendix C - Examination findings and recording sheets**

Case no:                                                                                      Date:

Patient sticker

Identification of snake:

Method of identification:

Time and date of bite:

Time elapsed between envenomation and presentation:

Site of envenomation:

Number of fang marks:

Description of envenomation site (with emphasis on degree of swelling as mild, moderate or severe, bleeding/oozing, painful):

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<td>Pulse:</td>
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<td>Respiratory rate + depth:</td>
<td>Loss of swallowing reflex or any evidence of a neurological abnormality (explain):</td>
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<td>Mucous membrane colour:</td>
<td>Capillary refill time:</td>
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62
### Patient detail

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### Sample Collection procedure

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<td>Store remaining sample at -80°C</td>
</tr>
<tr>
<td>2. Citrate</td>
<td>2x 3ml vacutainer</td>
<td>TEG tracing</td>
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<td>Samples to be batched</td>
<td>D-Dimers</td>
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<td>Citrated plasma stored at -80°C</td>
<td>Fibrinogen</td>
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<td>3. EDTA</td>
<td>1x 3ml vacutainer</td>
<td>Ht</td>
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<td></td>
<td>PCR &amp; RLB – samples to be batched</td>
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<td></td>
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<td>4. Urine</td>
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Appendix D - Thromboelastogram analysis

- **Normal**
  - R;K;MA;Angle = Normal

- **Anticoagulants/hemophilia**
  - Factor Deficiency
  - R;K = Prolonged;
  - MA;Angle = Decreased

- **Platelet Blockers**
  - Thrombocytopenia/
  - Thrombocytopenia
  - R ~ Normal; K = Prolonged;
  - MA = Decreased

- **Fibrinolysis (UK, SK, or t-PA)**
  - Presence of t-PA
  - R ~ Normal;
  - MA = Continuous decrease
  - LY30 > 7.5%; WBCL30 < 97.5%;
  - Ly60 > 15.0%; WBCL60 < 85%

- **Hypercoagulation**
  - R;K = Decreased;
  - MA;Angle = Increased

- **D.I.C**
  - Stage 1
    - Hypercoagulable state with secondary fibrinolysis
  - Stage 2
    - Hypocoagulable state
### Appendix E – Complete data set at presentation

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C1-C10 are controls; 1 - puffadder; 2 - snouted cobra; Plt - platelet count (10⁹/L); Ht – haematocrit (1/L); WBC - white cell count (10⁹/L); CRP - C-reactive protein (mg/L); PT – prothrombin (s); aPTT - activated partial thromboplastin time (s); AT III – antithrombin activity (%); Fib – fibrinogen concentration (g/L). Missing CRP values were due to insufficient serum sample available for analysis. Missing R and K-values were due to incaclculable data as reported directly by the TEG analyser.
**Complete set of data at 24 hours post envenomation**

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Plt24 - platelet count (10^9/l) at 24 hours; Ht24 – haematocrit (l/l) at 24 hours; WBC24 - white blood cell count (10^9/l) at 24 hours; CRP24 - C-reactive protein (mg/L) at 24 hours; PT24 – prothrombin time (s) at 24 hours; aPTT24 - activated partial thromboplastin time (s) at 24 hours; AT III24 – antithrombin activity (%) at 24 hours; Fib24 – fibrinogen concentration (g/L) at 24 hours. Values for case no. 18 are missing, as the dog passed away soon after admission. Missing value for CRP for case no. 2 was due to inadequate sample available for analysis.