Clinicopathological characteristics in canine *Vipera berus* envenomation

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Summary

Envenomation of dogs by the European adder (*Vipera berus* (*V. berus*)) is a common emergency in veterinary practice in Europe. Despite its common nature, little research exists regarding the clinical effects of this type of snakebite in dogs.

In this project, clinical and clinicopathological effects were prospectively and longitudinally assessed in dogs envenomated by *V. berus* with an emphasis on cardiac, renal and haemostatic systems. Whether severity of initial clinical signs might give an indication as to the subsequent development of effects on these body systems, was also of interest.

Dogs naturally envenomated by *V. berus* and presenting to the small animal clinics at NMBU Oslo, Evidensia Oslo Dyresykehus, Anicura Dyresykehus Oslo and Anicura Jeløy Dyresykehus, during 2017 and 2018, were recruited to the studies. A total of 60 envenomated dogs were included in different parts of the project and were assessed at five timepoints after bite, from presentation to the clinic to two weeks after bite. Data collection involved severity scoring of clinical signs, continuous 48-hour ambulatory electrocardiography (ECG), serum cardiac troponin I (cTnI, a cardiac injury biomarker) measurement, serum and urine biomarkers for kidney function and injury, and plasma coagulation parameters.

The main findings were:

- During the first 48 hours after envenomation, a large proportion of dogs developed myocardial injury, detected as a ventricular arrhythmia (12/21, 56%) or increased cTnI concentrations (17/21, 81%). Severe arrhythmias were detected in 6 of 21 (29%) dogs. Myocardial injury appeared to have resolved 14 days after bite (Paper I).

- There was evidence of mild, transient, non-azotaemic acute kidney injury (AKI) in dogs after *V. berus* envenomation, as measured by novel urinary AKI biomarkers and compared to a group of healthy control dogs. However, further assessment of many of the urinary biomarkers used in these studies is needed to elucidate their specificity for AKI , especially in the face of concurrent systemic inflammation (Papers II and III).

- Envenomated dogs in this cohort were hypercoagulable already at presentation and still at 15 days after *V. berus* envenomation, as measured using a thrombin generation assay, thrombin-antithrombin complexes and phosphatidylserine equivalents, and compared to a group of healthy controls. Dogs that that receive antivenom treatment might be less hypercoagulable than their non-antivenom treated counterparts (Paper IV).

- ❖ The severity of clinical signs at presentation was not generally a useful indicator for the subsequent development of clinical or clinicopathological effects in this cohort of envenomated dogs (Papers I-III).

This work provides new insights into the effects of *V. berus* envenomation in dogs and contributes to the generally sparse evidence base upon which treatment and monitoring decisions of these patients can be made. Based on the cardiac and renal effects observed in this cohort of envenomated dogs, prolonged ECG monitoring for ventricular tachycardia and hospitalisation with supportive intravenous fluid therapy, appear to be sensible recommendations after *V. berus* bites in dogs. The clinical significance of the hypercoagulable state detected is unclear, but coagulation parameters measured in paper IV may serve as laboratory endpoints in future randomised controlled trials of antivenom efficacy.

Introduction

A recent systematic review described envenomation by Vipera species in Europe as a neglected disease and highlighted the need for standardised protocols for snakebite management in humans (Paolino et al., 2020). A similar situation exists in veterinary medicine.

Accidental envenomation of dogs by the European adder (Vipera berus, *V. berus*) is a common seasonal emergency. Recording of snake envenomation is not mandatory in most European countries, including Norway, and the true incidence of *V. berus* envenomation in humans and dogs is therefore likely underestimated. However, data obtained from the recently developed national veterinary diagnosis register indicate that the incidence of *V. berus* envenomation could be as high at 150 cases per 100 000 dogs per year in Norway (private communication, Pyramidion, DyreID AS).

Despite the relative frequency of this emergency, studies investigating the clinical effects of this type of snakebite are sparse both in human and veterinary medicine, and evidence-based guidelines for the treatment of dogs bitten by *V. berus* are consequently lacking. Evidence-based treatment protocols and recommendations would allow clinicians to optimise the management of these patients and to better inform dog owners of the expected disease course and prognosis.

The theme for this project is broad: ***What are the effects of V. berus bites in dogs, and what implications do they have in terms of treating and monitoring envenomated dogs?***

Materials and methods

Study design

All studies included in this thesis are prospective longitudinal cohort studies, based on convenience sampling of dogs presenting for veterinary treatment after snakebite.

Animals

A total of 60 dogs bitten by *V. berus* and presenting to the small animal clinics at the Department of Companion Animal Clinical Sciences at the Norwegian University of Life Sciences (NMBU), Evidensia Oslo Dyresykehus, Anicura Dyresykehus Oslo and Anicura Jeløy Dyresykehus between 2017 and 2019, were included.

Forty healthy control dogs were included in papers II-IV. The control group was recruited through stratified sampling by weight and age to match the cases in papers II and III. Relationships between study populations are illustrated in Figure 1.

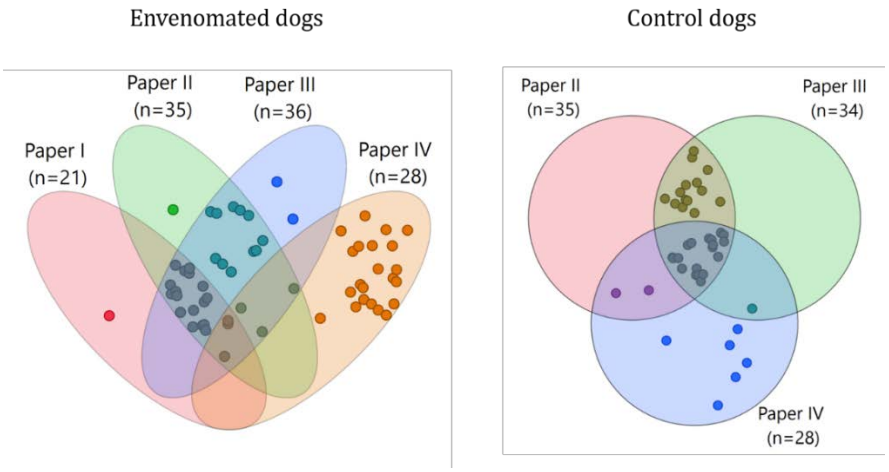

Figure 1. Venn diagrams illustrating the relationship between populations of envenomated dogs in papers I-IV, and control dog populations in papers II-IV. Paper I did not include a control group.

General inclusion and exclusion criteria

Diagnosis of snakebite and thus inclusion in each of the studies was based on a history and clinical signs compatible with snake envenomation (fang marks, local swelling or systemic signs of envenomation). Dogs were excluded if they presented more than 24 hours after a bite and if they lacked clinical signs of envenomation within 12 hours of the bite. Dogs were also excluded on the basis of any pre-existing conditions and medications, with a few exceptions. More detailed inclusion and exclusion criteria are described in the individual papers.

For the control dogs, healthy status was defined as an unremarkable history including no chronic disease or any disease in the preceding 14 days, an unremarkable physical examination, normal urinalysis and no clinically significant haematology or biochemistry abnormalities.

Sampling timeline

All papers followed the same basic sampling timeline as outlined in Figure 2. Envenomated dogs were examined, and blood (serum and citrated plasma) and free-catch urine samples taken, at five timepoints after bite: T1: presentation (0.5-7.5 hours, h), T2: 12 (±2) h, T3: 36 (±2) h, T4: 36 (±2) h and T5: 14 days, with ranges of 10-21 days (paper I) and 10-23 days (papers II and III). In paper IV, T2 and T4 had a greater time range than in the other papers (±4 hours) and T5 ranged from 10-32 days. Control dogs (papers II-IV) were sampled at a single timepoint.

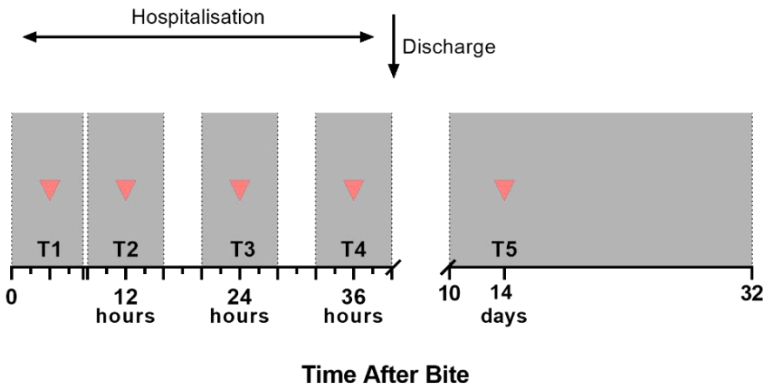

Figure 2. Sampling timeline for envenomated dogs in papers I-IV. T1 (presentation), T2 (12 ± 2 hours), T3 (24 ± 2 hours) and T4 (36 ± 2 hours) and T5 (10-32 days). In paper IV, T2 and T4 corresponded to 12 ± 4 hours, and 36 ± 4 hours.

The reader is referred to the individual papers for a comprehensive overview of the methodologies employed. Specific methods worthy of extra attention are described below.

Snakebite severity scoring (SSS)

In papers I-III, scoring systems were used to record severity of clinical signs. In paper I a simple three-point scoring system was adapted from a grading system for *V. berus* and V. aspis envenomation in humans (Audebert et al., 1992) and previously adapted for a canine study (Palviainen et al., 2013). The original grading system was shown to correlate with serum venom concentrations in humans (Audebert et al., 1994a) and thus thought to be good indicator of venom dose and thereby, severity. The scoring is based on the degree of swelling and systemic signs of envenomation with grade one being local swelling only and grade three being extensive swelling with marked systemic signs of envenomation (see paper I for full details).

Since there is no consensus for severity scoring after *V. berus* envenomation in dogs and the original scoring system by Audebert et al (1992) is based on somewhat subjective criteria, papers II and III made use of an adapted version of a more comprehensive grading system, previously validated for crotalid snakebites in humans (Dart et al., 1996). This grading system includes more specific assessment of the local wound as well as respiratory, cardiovascular, gastrointestinal, haemostatic and central nervous systems. Given the differences in expected venom effects between *V. berus* and crotalids and the need for a system not reliant on laboratory parameters of coagulation, central nervous system parameters were changed to general demeanour parameters, and haematological parameters were removed. Thus, adapting the scoring from a 20-point to a 16-point grading system (see papers II and III for full details).

Blood pressure measurement

Papers I-III included indirect blood pressure measurements using an oscillometric technique (Cardell®). The blood pressure measurement procedure was standardised using a cuff size of approximately 40% limb circumference, placed on the distal radius or metatarsus with the dog in lateral recumbency, 12 measurements were made and systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressures recorded. The first two measurements and any obvious outliers were excluded, and the mean of the remaining values used in analyses.

Ethics

All studies included in this thesis complied with the national animal welfare rules and regulations for the use of animals in research. Written owner consent was obtained for all participating dogs, prior to enrolment.

Results

An overview of research questions, materials and methods, and a summary of the results for each of the four papers included in this thesis, are provided in Tables 1-4.

Discussion

This PhD work has identified cardiac, renal and coagulopathic effects in dogs envenomated by *V. berus*, that may be occult and do not necessarily correlate with severity of clinical signs. These are important features for clinicians to be aware of, both in terms of patient monitoring and communicating the importance of monitoring dogs bitten by *V. berus* despite relatively mild clinical signs, to dog owners.

The cardiac arrhythmias detected in paper I appeared to be well-tolerated and thus their clinical relevance is unclear. However, given the previously discussed concerns regarding VT, findings in this thesis work suggest that prolonged (48 hour) ECG monitoring for the development of VT in particular, is a sensible recommendation in dogs bitten by *V. berus.* This which would allow rapid initiation of antiarrhythmic treatment where necessary. Rapid VT occurs after *V. berus* bite and its presence does not appear to correlate with severity of clinical signs of envenomation. Arrhythmias may also last into day two after bite. A general recommendation after *V. berus* bite has been for the dog to avoid exertion in the time between hospital discharge (typically 36-48 hours after bite) and a re-examination 14 days later. Bearing in mind the incidence of myocardial injury and that a number of dogs in paper I were still arrhythmic at discharge, this recommendation also appears to be justified in want of studies specifically examining arrhythmias and myocardial injury in the period between discharge and re-examination.

The absence of azotaemia does not rule out the presence of AKI in envenomated dogs. These studies suggest a likely mild transient AKI. Whilst all dogs in papers II and III received IVFT and knowledge regarding the severity of AKI in the absence of IVFT, is therefore not known, supportive, targeted IVFT would appear to be a sensible recommendation (Prowle et al., 2014, Brienza et al., 2009).

Dogs are persistently hypercoagulable after *V. berus* envenomation. Although the significance of the procoagulant state is unclear and diagnosis of thromboembolic disease is difficult in dogs, it would seem wise to be aware of the potential for thromboembolic disease and monitor for associated acute neurological and respiratory signs.

Based on this work, recommendations for the treatment and management of *V. berus* envenomated dogs include: 36-48-hour hospitalisation including IVFT and monitoring with a focus on arrhythmias and clinical signs of thromboembolic disease.

In many ways these recommendations were already implemented in the clinics involved in these studies, prior to initiation of the work included in this thesis. This work contributes to the general evidence base for the treatment and management of *V. berus* envenomation in dogs. More specifically, an added focus on arrhythmia monitoring and an awareness of AKI and hypercoagulability, have been achieved.

Paper	Research questions	Materials and Methods	Statistics	Main Limitations	Key findings
I	***1. What is the incidence, nature and duration of myocardial injury in dogs bitten by V. berus?*** ***2. Can SSS at presentation indicate the development of myocardial injury?*** ***3. Can cTnI concentrations measured early after bite be used as an indicator for the development of arrhythmias?***	21 dogs bitten by *V. berus* in 2018. 3-point SSS at presentation. Holter AECG from presentation to up to 48 hours after bite, and a 5 min ECG at T5. Arrhythmias were graded 0-3 based on frequency and severity, where 0 was considered normal. Serial cTnI measurements T1-T5. Indirect blood pressure measurements T1-T5.	Comparison of cTnI concentrations between arrhythmia grades 0 vs 1-3, at each timepoint: *Wilcoxon exact test.* Comparison of cTnI concentration between SS scores at presentation: *Steel Dwass for multiple comparisons*. Comparisons between SSS and arrhythmia grade: *contingency table analysis: Fishers exact test.* Assessment of cTnI concentrations in relation to arrhythmia grade: *Receiver operating characteristics (ROC) curve analysis.*	Small sample size. Confounding effect of antivenom treatment.	**1**. Arrhythmias were detected in 12/21 dogs (57%). All arrhythmias were ventricular in origin. VT was observed in 6 dogs (29%). No arrhythmias were detected 14 days after bite. Increased cTnI concentrations were found at one timepoint or more, in 17 dogs (81%). cTnI was normalised in all but one dog, 14 days after bite. **2**. SSS at presentation was not a useful indicator of cardiac effects. **3**. cTnI concentrations ≥ 1.89, 12 hours after bite may indicate the development of VT.

Table 1. Summary of paper I.

Paper	Research question	Materials and Methods	Statistics	Main Limitations	Key findings
II	***1. Do dogs bitten by V. berus sustain an acute kidney injury?*** ***2. How long does the injury last last?*** ***3. Does the severity of clinical signs give an indication of AKI?***	35 dogs bitten by *V. berus* in 2018 and 35 healthy control dogs. Urine samples at T2-T5. Serum samples at T1-T5. Control dogs were sampled at a single timepoint. <u>Urinary biomarkers normalised to creatinine</u>: - **albumin** - **OPN** - **KIM-1** - **MCP-1** - **IL-8** - **cystatin C** - **NGAL** - **ALP** - **GGT** <u>Serum biomarkers</u>: **SDMA** and **creatinine** <u>Other parameters</u>: 16-point **SSS** at T1-T5 and indirect **blood pressure** measurements at T1-T5.	1. Comparisons of biomarker/Cr ratios between cases and controls: *Wilcoxon rank sum test or t-test with Bonferroni correction.* 2. Repeated measurements of biomarker/Cr ratios in envenomated dogs: *Mixed model analysis* *Fixed effects*: time, age, weight, sex *Random effect*: dog. 3. Correlations between SSS and peak biomarker ratios: *Spearman's rank correlation*.	High number of samples with values below the limit of quantification (LOQ) for the assay, for ALP, IL-8, and albumin. This resulted in unusable ALP results. Uncertain specificity of OPN, NGAL, MCP-1 and Il-8, for AKI. Confounding effect of antivenom treatment.	**1**. There was evidence of non-azotemic kidney tubular injury during the first 36 hours after *V. berus* bites in dogs. **2.** Urinary biomarker/creatinine ratios were normalised 14 days after bite. **3.** SSS was not a useful indicator of increased urinary AKI biomarker/creatinine ratios.

Table 2. Summary of paper II.

Paper	Research question	Materials and Methods	Statistics	Main Limitations	Key findings
III	***1. Do dogs bitten by V. berus sustain an acute kidney injury*** ***2.How long does the injury last?*** ***3. Does the severity of clinical signs give an indication of AKI?***	36 dogs bitten by *V. berus* in 2018 and 34 healthy control dogs. Urine samples and serum samples at T1-T5. Control dogs sampled at a single timepoint. <u>Urine biomarkers</u>: - **clusterin** (with and without normalisation to urine creatinine). - **cystatin B** (with and without normalisation to urine creatinine). - **UPC** <u>Serum biomarkers</u>: **Creatinine** and **SDMA.** <u>Other parameters</u>: 16-point **SSS** at T1-T5 and indirect **blood pressure** measurements T1-T5.	1. Comparison of biomarkers concentrations between cases and controls: *Wilcoxon rank sum test.* 2. Proportions of samples with biomarker concentrations over the reference interval compared to controls, at each timepoint: *Fishers exact test with Bonferroni correction*. 3. Correlations between SSS and biomarker concentrations: *Spearman's rank correlation.*	High number of samples with clusterin and cystatin B below the LOQ for the assay. Uncertain specificity of cystatin B for AKI. Confounding effect of antivenom treatment.	**1**. Absolut urine cystatin B concentrations and values normalised to urinary creatinine were higher in envenomated dogs than in controls at T1-T4. When clusterin was normalised to urinary creatinine, levels were significantly higher at T3 and T4 in envenomated dogs compared to controls. **2**. Urinary cystatin B values were not significantly different to controls at T5. **3**. SSS correlated with absolute cystatin B concentrations at T1.

Table 3. Summary of paper III.

.Paper	Research question	Materials and Methods	Statistics	Main Limitations	Key findings
IV	***1. What is the global coagulation status of dogs envenomated by V. berus?*** ***2. Is there a difference in coagulation status between dogs treated with and without antivenom?***	28 dogs bitten by *V. berus* in 2017 and 28 healthy control dogs. Citrated plasma samples taken at T1-T5 in envenomated dogs and a single timepoint in controls. Thrombin generation measured by **CAT** (lag time, peak and ETP). **TAT** complexes. **PS equivalents**.	1. Comparisons of CAT parameters, TAT and PS equivalents between cases and controls: *Steels test for multiple comparisons.* 2. Comparisons between dogs treated with and without antivenom at each timepoint: *Wilcoxon rank sum test.* 3. Analysis of proportions of dogs with peak, ETP and LT values above or below the control group at presentation: *Fishers exact test.* 4. Repeated measurements of CAT parameters, TAT and PS equivalents between envenomated dogs: *Mixed model analysis* *Fixed effects:* timepoint and antivenom treatment. *Random effect*: dog.	Large number of samples excluded due to haemolysis.	**1**. Envenomated dogs were hypercoagulable compared to controls, already at presentation and still at 15 days after bite. **2.** Dogs treated with antivenom may be less hypercoagulable than non-antivenom treated dogs. Lag time might serve as a diagnostic test for envenomation.

Table 4. Summary of paper IV.

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