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A clinical and pathological description of 320 cases of naturally acquired *Babesia rossi* infection in dogs

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ABSTRACT

Babesia rossi causes the most severe clinical disease in dogs of all the babesia parasites. We included 320 naturally-infected dogs that presented for care at the Onderstepoort Veterinary Academic Hospital between 2006 and 2016. All dogs had mono-infections confirmed by multiplex PCR. The data allowed more accurate clinical classification of the disease and identified parameters that were associated with disease severity and death. Odds ratios for dying were significant ($P < 0.05$) for increased band neutrophil count, collapse at presentation; presence of cerebral signs; hypoglycaemia; hyperlactatemia; high urea, high creatinine; hyperbilirubinaemia; hypercortisolaemia; and hypothyroxinaemia. Joint component analysis confirmed that the variables with significant odds ratios grouped together with death. Yet, multivariate logistic regression was unable to identify a group of significant independent predictors of death. Receiver Operator Characteristic curves indicated that low total thyroid hormone, high bilirubin, high serum urea and high cortisol concentrations were the variables with the highest sensitivity and specificity for death. These data provide both the clinician and researcher with a set of easily-measured laboratory and clinical assessments to classify cases into those that are uncomplicated and those that are complicated. The disease is complex and multisystemic and probably involves mechanisms more proximal in the pathogenesis than those that have been evaluated.

1. Introduction

Babesia species are considered the second most common blood parasite, after Trypanosomes, that infect mammals (Schnittger et al., 2012). They are regarded as very common pathogens of domestic dogs in areas of the world where the tick vector is present, (Collett, 2000; Matijatko et al., 2012) where they are also a significant cause of morbidity and mortality (Collett, 2000; Jacobson, 2006; Welzl et al., 2001). The most virulent canine parasite in this genus is *Babesia rossi,* which is a common and neglected cause of severe disease and death in resource-deprived countries of tropical and sub-tropical sub-Saharan Africa (Penzhorn, 2011; Schoeman, 2009).

The disease caused by babesia parasites (and *B. rossi* in particular) has been likened to human malaria caused by *Plasmodium falciparum* (Clark and Jacobson, 1998; Krause et al., 2007; Reyers et al., 1998). Although the pathology of falciparum malaria has been well described, the same cannot be said for *B. rossi* infection. Numerous, small prospective studies have evaluated specific organ systems and larger studies have been conducted retrospectively (Reyers et al., 1998). Multiple organs are affected in *B. rossi* infections and the dog disease has been loosely classified as either complicated or uncomplicated, in a similar way to falciparum malaria in humans (White et al., 2014). Complications described include anaemia (Reyers et al., 1998; Scheepers et al., 2011), haemoconcentration, haemolysis (Jacobson, 2006; Reyers et al., 1998), icterus (Jacobson and Clark, 1994), hyperlactataemia and hypoglycaemia (Keller et al., 2004; Nel et al., 2004). Acute lung injury is reported as a common cause of death by private practitioners in South Africa (Collett, 2000). The cerebral form of the *B. rossi* disease is rare (Botha, 1964a; Malherbe and Parkin, 1951b). Clinically significant (olig- or anuric) acute kidney injury is recognized in a small subset of dogs with complicated disease. ECG abnormalities, elevated cardiac troponins and myocardial haemorrhage and pericardial effusion have been described (Dvir et al., 2004; Lobetti et al., 2002). A consumptive coagulopathy and excessive pro-inflammatory response is described (Goddard et al., 2016; Goddard et al., 2013). A small study has associated *B. rossi* genotypes with specific

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disease phenotype (Matjila et al., 2009), yet the effect of parasite genotype on disease phenotype was not explored.

Despite the very common occurrence of this disease, there are no large prospective studies that provide an objective description of the disease in a cohort of molecularly confirmed, mono-infected untreated dogs. Moreover, most reviews typically examined multiple small studies that evaluated small numbers of dogs (typically less than 40 animals). Consequently, the first aim was to describe a large prospectively recruited cohort of dogs with naturally-acquired *B. rossi* infections, representing a wide variety of disease, in which a large set of parameters were measured. The data from this cohort are also strengthened by the fact that the mono-infection with *B. rossi* has been confirmed at a molecular level and all data were collected before any treatment. A secondary aim was to investigate which set of parameters/abnormalities were consistently associated with severe disease and which were most accurate at predicting an adverse outcome to facilitate better case classification.

2. Materials and methods

There were 338 cases in the cohort, but 18 of them had incomplete data sets and were excluded from all analyses, except for some of the post mortem data. The final data set included three hundred and twenty cases of *Babesia rossi* infection in dogs from 3 separate data bases that were generated between the years 2006 and 2016. Two of the cohorts have had several studies published from them (Dvir et al., 2019; Goddard et al., 2016, 2015a, b; Rees and Schoeman, 2008; Schoeman and Herrtage, 2007, 2008a; Schoeman et al., 2007b)(Koster et al., 2015). All three cohorts were collected prospectively and consisted of client-owned dogs, naturally-infected with *B. rossi* that presented for veterinary care. The research protocols for each of the three studies were approved by the Animal Ethics Committee of the University of Pretoria (Protocol no: V074-05; V055-11; V034-14). An initial diagnosis of babesiosis was made through the recognition of commensurate clinical signs and demonstration of intra-erythrocytic trophozoites and merozoites on stained thin capillary blood smears, and was later confirmed as *B. rossi* mono-infection by polymerase chain reaction (PCR) and reverse line blot (RLB) (Matjila et al., 2008). Dogs of either sex and of any breed were eligible for inclusion in the study.

The owners were questioned regarding historical data. Dogs were evaluated clinically by the attending clinician. All samples were collected before any treatment was administered. Urine was mostly collected by cystocentesis and occasionally by free flow. Venous blood samples (EDTA and serum) were collected in Vacutainer tubes from the jugular or cephalic veins. Blood gas samples were collected anaerobically into a commercially prepared heparinized syringe (BD A-Line, arterial blood collection syringe, Becton, Dickinson and Company, UK) from the femoral artery. This sample was analysed immediately (Rapidpoint 405, Seimens). Blood glucose and lactate were determined. The EDTA samples were used for a complete blood count (ADVIA 2120, Siemens, Munich, Germany). Differential white cell counts were performed manually. EDTA anticoagulated blood was also used for DNA extraction for parasite identification. The serum sample was used for serum biochemistry determinations on an automated analyser (Cobas Integra 400 plus). Hormone analyses in the Schoeman study were performed in duplicate with kits previously validated for dogs (Cortisol (Radioimmunoassay; Cortisol, Coat-A-Count, Diagnostic Products Corp., USA); Thyrotropin (Immunoradiometric canine TSH, Coat-A-Count, Diagnostic Products Corp., USA). Hormone analysis from the Goddard and Leisewitz studies were conducted on the Immulite 1000 immunoassay system (Siemens) using canine specific reagents.

Packed cell volume and warm in-saline agglutination testing were performed on a sample from the EDTA tube. Dogs were removed from the data set retrospectively if they were subsequently proven by PCR and RLB to be infected with *B. vogeli*, *Ehrlichia canis* or *Theileria* spp. Treatment with any anti-inflammatory medication within 4 weeks prior to presentation was also a reason for exclusion. Dogs received standard care for canine babesiosis, which included antibabesial treatment with diminazene aceturate at 3.5 mg/kg and transfusion with packed red cells or whole blood and intravenous fluids as needed. In addition, any complications were treated accordingly at the discretion of the attending clinician. Outcome was recorded as short-term survival (i.e. until discharge), or death/euthanasia due to poor prognosis.

Dogs were classified as meeting the criteria for SIRS if two or more of the following parameters were met, as described by (Okano et al., 2002) and previously applied to canine babesiosis (Koster et al., 2015): a white cell count exceeding 12 000/μL or less than 4000/μL, or presence of ≥ 10% immature or band cells; a rectal temperature < 37.8 °C or > 39.7 °C; a heart rate of at least 160 beats per minute or a respiratory rate of ≥ 40 breaths per minute.

2.1. Statistics

All data were tested for normality by the Shapiro-Wilk test. Most variables were not normally distributed. The Mann-Whitney U test (2-tailed) was used to compare medians between 2 groups of non-parametric data. A Chi-square test was performed to test for associations between categorical variables and if the conditions for this test were violated, a Fisher's exact test was applied. Spearman's rank order correlation was used to assess correlations between groups of continuous data. For each continuous variable, linearity of its association with outcome (died vs. survived) was assessed by categorization into biologically meaningful categories; if the frequency of the outcome did not increase or decrease monotonically in successive categories, then the categorized variable was used. The number of SIRS criteria for which each dog was positive was compared between dogs that were able to stand and those that were recumbent, and between dogs that survived and those that died, using the two-sample Wilcoxon rank-sum (Mann-Whitney) test. Univariate logistic regression was used to estimate the odds ratio for the association of each variable with outcome. Variables associated ($P < 0.2$) with death at the univariate level were selected for inclusion in a multivariable logistic regression model which was developed by backward stepwise elimination. Further, to visually assess patterns within the data set and groupings amongst categories of variables, all variables were categorized into biologically meaningful categories and joint correspondence analysis was performed, producing scatterplots of the normalized coordinates of variables and cases in two dimensions. Commercial software packages (SPSS 24, IBM SPSS Statistics; Stata 15.1, StataCorp) were used and significance was set at $p < 0.05$.

3. Results

Unless otherwise specified, results are contained in Table S1 & Table 1.

3.1. Signalment and clinical data

Three hundred and twenty dogs of 30 different breeds (117 female; 203 male) were included from the three data bases. The median age was 20 months (range 2–156 months). The median weight was 15.6 kg (interval 1.5–65 kg). There was no significant difference between the median age or weight of dogs that lived and those that died. Median rectal temperature for the cohort was 39.6 °C (IQR 39–40.1 °C). Median rectal temperature was significantly lower in the dogs that died or were collapsed than in the dogs that survived or non-collapsed, respectively. Consequently, the odds ratio for death was 1.39 for every 1 °C drop in temperature ($p < 0.001$) (Table 1).

Cerebral babesiosis (CB) was defined as a dog showing acute cerebral signs (dramatic alteration in behaviour including coma, semi-coma, stupor and seizure activity) with a babesia-positive blood smear in the face of a normal or elevated blood glucose concentration. This

Table 1
The odds ratios for measures strongly associated with outcome in *Babesia rossi* infections in dogs.

Variable	Category	Unit of measure	Number of cases in each category	% that died in each catagory	Odds ratio	CI for odds	P value
Hct	1 (Hct < 0.15)	L/L	113	14.2	1.8	0.78-4.12	0.169
	2 (Hct0.15-0.25)		81	9.9	1.2	0.45-3.17	0.721
	3* (Hct0.25-0.55)		119	8.4	1*		
	4 (Hct > 0.55)		4	75	32.7	3.12-344.25	0.004
Band cell count	1* (0-0.49)	$\times 10^9$/L	159	5	1*	2.12-11.1	< 0.001
	2 (0.5-21.75)		132	20.45	4.9		
Rectal temperature	For every 1 °C decrease	°C	320		1.39	1.16-1.67	< 0.001
Able to stand or collapsed	Able to stand		248	14	1*	4.03-17.32	< 0.001
	Collapsed		72	24	8.36		
Cerebral signs	No cerebral signs		321	36	62.39+	8.77-infinity	< 0.001
	Cerebral signs		6	100			
SIRS	SIRS positive		161	10.6	1.3	0.61-2.71	0.42
	SIRS negative		105	13.3	1*		
Blood glucose	1 (0.1-3.2)	mmol/L	61	23	3.47	1.54-7.79	0.003
	2 (3.3-5.5)		177	7.9	1		
	3 (5.6-22.2)		79	11.4	1.5	0.62-3.62	0.37
Blood lactate	1 (0.1-2)	mmol/L	87	3.3	1		
	2 (2-23.9)		122	8.1	1.15	1.05-1.26	0.003
Urea	1 (2.7-8.9)	mmol/L	106	4.7	1*		
	2 (9.1-19.7)		40	12.5	2.89	0.79-10.57	0.109
	3 (20.4-104.3)		42	30.95	9.1	2.98-27.5	< 0.001
Creatinine	1 (18-106)	μmol/L	268	7.09	1*		
	2 (106-865)		52	36.54	7.55	3.63-15.69	< 0.001
Total bilirubin	For every 50 μmol/L increase	μmol/L	230		1.23	1.01-1.5	0.042
Cortisol	For every 50 nmol/L increase	nmol/L	241		1.15	1.08-1.22	< 0.001
Thyroid hormone (T4)	For every 5 nmol/L decrease in T4	nmol/L	242		1.63	1.2-2.194	0.002

* Denotes the reference category and hence the OR was 1 in these categories.
+ Using exact logistic regression. Hct – hematocrit; SIRS – systemic inflammatory response syndrome.

complication was diagnosed in 6 cases and all died. The OR for death with CB was 62.39 ($p < 0.001$) (Table 1). Seventy-two of the 320 cases (22.5%) were collapsed at presentation (unable to stand unaided). Thirty-four dogs died as a result of the disease (34/320, 10.6%) and 24 of these (70.6%) were collapsed at presentation. Expectedly, collapse at presentation was significantly associated with death ($p < 0.001$) with an OR of 8.36 ($p < 0.001$) (Table 1). In the largest of the data bases (including 130 cases) 20 cases died, eight dogs received no treatment before death, because they either presented dead on arrival or died upon admission. The treated dogs that died, all died within 24 h of admission. The most common treatments provided included diminiazine aceturate, blood transfusion, crystalloid fluids, and a prokinetic drug. One dog received prednisolone.

3.2. Haematological data

Of the dogs that had haematocrit (Hct) determined at admission, 113 (35.6%) were classified as severely anaemic (Hct < 0.15%L/L) with a mortality of 14.2%, 81 (25.5%) were moderately anaemic (Hct 0.15 – 0.24 L/L) with a mortality of 8% and 70 (22.1%) cases had a mild anaemia (Hct 0.25 – 0.36 L/L) with a mortality of 11.3%. Fifty cases (15.7%) had a normal Hct with 2 deaths (4%). Four cases (1.3%) had a Hct ≥ 0.55 L/L (above the upper limit of normal) with 2 of these cases having a Hct of 0.60 L/L, whilst 3 of the 4 (75%) died. As a result, the odds ratio for death was 32.7 ($p = 0.004$) for Hct > 0.55 L/L (Table 1). The lowest Hct recorded was 0.04 L/L and that dog could stand unaided and survived. Collapsed cases had a significantly lower Hct than non-collapsed cases. There was a strong inverse correlation between Hct and absolute reticulocyte count in the 113 cases in which the absolute reticulocyte count was determined ($p < 0.01$, correlation coefficient -0.312). The median Hct of the cases that had a reticulocyte count determined was 0.235 L/L. The median absolute reticulocyte count was below 100×10^9/L in 70 of these cases (61.9%). There was a similarly significant inverse correlation between Hct and WBC ($p < 0.001$, $r_s = 0.326$, $n = 317$). There was no difference between the Hct of cases that were ISA positive (0.19 L/L) and those that were ISA negative (0.185 L/L).

Median WBC counts were significantly higher in cases that died or were collapsed than in cases that lived or were non-collapsed, respectively. Cases that died had a significantly higher median band neutrophil count than cases that survived. The odds ratio (OR) for death for a band neutrophil count $> 0.5 \times 10^9$/L (CI 2.12–11.1) was 5.9 (Table 1).

The median platelet count in the 291 cases that had a platelet count available was 20×10^9/L, which is significantly below the reference interval of 200–500 $\times 10^9$/L. Post mortem examinations were performed in 22 of the 34 cases that died and all showed macroscopically obvious haemorrhage in internal organs (Table S2).

3.3. Biochemical data

Blood lactate concentrations were determined in 209 cases which included 23 of the cases that died. One hundred and sixteen cases (55.5%) had a concentration above 2 mmol/L. The highest measure was 23.9 mmol/L. Cases that died had a significantly higher median lactate concentration (4.3 mmol/L, IQR 1.7–10.8) than cases that survived (median 2.2 mmol/L, IQR 1.4–3.4) ($p = 0.006$). Collapsed cases also had a higher median lactate than non-collapsed cases. Although the OR for adverse outcome for a lactate > 2 mmol/L was small (1.15, CI 1.05–1.26), it was nevertheless significant ($p = 0.003$). Receiver operator characteristic curve (ROC) for lactate is provided in Fig. 1. The area under the curve was 0.727 ($p = 0.035$). The cut off value for death (sensitivity 75%, specificity 75%) was 4.1 mmol/L (Tables 2 and 3) making this a useful prognostic marker.

Blood glucose (BG) measurements were available in 317 cases. Sixty-one cases (19.2%) were hypoglycaemic (blood glucose < 3.3 mmol/L). Seventy-nine cases (24.9%) were hyperglycaemic (> 5.5 mmol/L). There was no difference in BG concentrations between cases that were collapsed and non-collapsed and cases that lived and died, yet the odds ratio for dying if hypoglycaemia was present was

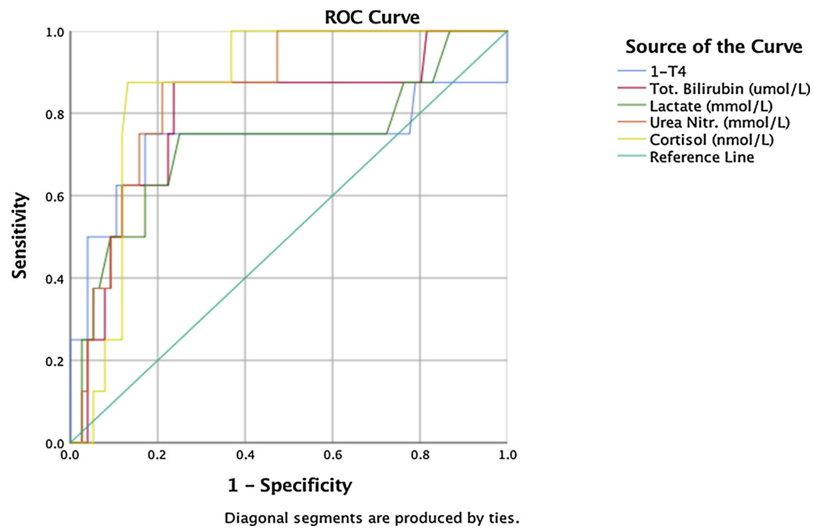

Fig. 1. Receiver operator characteristic curve for 1-T4, total bilirubin, lactate, urea and cortisol concentrations in serum that had a P < 0.05 and an AUC > 0.7 comparing cases that died versus cases that survived.

Table 2
Area under the receiver operating characteristic curves for 1-T4, total bilirubin, lactate, urea and cortisol concentrations in serum that had a P < 0.05 and an AUC > 0.7. AUC: area under the curve; 95% CI: 95% confidence interval.

Analyte For survivors versus non-survivors	AUC	95% CI	P-value
Cortisol	0.863	0.772-0.953	0.001
Urea	0.854	0.743-0.964	0.001
Total bilirubin	0.795	0.62-0.97	0.006
1-T4	0.733	0.475-0.99	0.031
Lactate	0.727	0.506-0.948	0.035

Table 3
Cut-off values and sensitivities and specificities for the analytes with an AUC > 0.7 and P < 0.05 discriminating between survivors and non-survivors. For cortisol, urea, lactate and total bilirubin, values above the cut off are associated with death whilst fro T4 values below the cut off are associated with death. T4: total thyroid hormone.

Analyte	Normal range	Cut-off	Sensitivity	Specificity
Cortisol	10-160 nmol/L	388	87.5	82.9
Urea	2.3-8.9 mmol/L	14	87.5	78.9
Lactate	< 2 mmol/L	4.1	75	75
Total bilirubin	1-6.8 μmol/L	14.7	87.5	76.3
T4	13-45 nmol/L	4.8	75	81.6

3.47 (CI: 1.54–7.79; p = 0.003) (Table 1). Glucose and lactate were significantly negatively correlated (p < 0.001, correlation coefficient -0.28).

Serum bilirubin concentration was measured in 230 cases. The serum bilirubin concentration was above the reference interval in 130 cases (56.5%). Collapsed dogs and dogs that died had a significantly higher bilirubin than non-collapsed cases and cases that survived, respectively. For every 50 μmol/L increase in bilirubin the OR for death increased by 1.23 (CI 1.01–1.5, p = 0.042) (Table 1). The ROC curve for bilirubin (Fig. 1) and area under the curve (AUC) data (Tables 2 and 3) demonstrate that a bilirubin > 14.7 μmol/L had a sensitivity of 75% and specificity of 81.6% for predicting death, making this a useful prognostic marker. Serum ALP and ALT changes were largely insignificant. Albumin was measured in 294 cases. The median albumin concentration was 23.3 g/L (IQR 20.3–27.9 g/L). Two-hundred and fourteen cases were hypoalbuminaemic (72.8%) whereas 13 cases (4,4%) had an albumin below 15 g/L. Albumin concentration was significantly lower in collapsed dogs than non-collapsed. Globulin concentrations were unremarkable in all groups.

Creatinine was measured in 320 cases. The median serum creatinine concentration was 66.5 μmol/L (IQR 50–92). One-hundred and twenty-one cases (37.8%) were below the reference interval and 52 cases (16.3%) measured above the reference interval. Collapsed cases and cases that died had significantly higher concentrations than non-collapsed cases and survivors, respectively. Creatinine was significantly correlated with urea concentration (p < 0.001, r_s 0.385) and with serum inorganic phosphate (SIP) (p < 0.001, r_s = 0.278). The OR for death with a creatinine > 106 μmol/L was 7.55. Urea was measured in 188 cases. The median serum urea concentration was 8.05 mmol/L (IQR 5.6–18.28). No cases measured below the reference interval whilst 82 (43.6%) were above the reference interval. The OR for death with a urea > 20 mmol/L was 30.95. Creatinine was 1.5× increased above the top of the normal interval (safely azotaemic) in 22/320 cases (6.8%). Ten of these 22 cases died (45.5%). Eleven of these 22 cases also had urea determined and all 11 had concentrations in excess of 1.5x top normal. Collapsed cases and non-survivors had significantly higher urea than non-collapsed cases and survivors, respectively. Over 65% of the 170 cases in this series had a urine specific gravity (SG) above 1.030 indicating adequate concentrating ability. It is however important to view this measure in the context of the majority of the samples having had macroscopic and/or dipstick evidence of haemoglobinuria.

Arterial blood gases were measured before any supplemental oxygen therapy and corrected for body temperature in 98 cases. The median pO_2 was 91.4 mmHg (IQR 81–101.2 mmHg). Eighteen cases had a pO_2 < 60 mmHg (18.1%) and only 3 of these cases died (16.6%). There was no difference between survivors and non-survivors or between collapsed and non-collapsed dogs. The median arterial-alveolar oxygen difference ($AaDO_2$) was 20.5 mmHg (IQR 10.9–38.4 mmHg); (N < 15 mmHg;). The median arterial pCO_2 was 25.4 mmHg (IQR 21.8–29.5). One-hundred and six cases measured < 31 mmHg and only one case was > 43 mmHg (normal 31–43 mmHg). There was no difference between cases that survived and cases that died. In contrast, collapsed cases had a significantly lower pCO_2 than non-collapsed cases. The median pH for the dogs in which it was determined was 7.429 (IQR 7.378–7.467) which is within the normal interval (7.35–7.46). Collapsed cases and non-survivors had significantly lower pH than non-collapsed cases and survivors, respectively. Forty cases (31.7%) had a pH < 7.398 and the same number had a pH > 7.45. The median HCO_3 was lower in the collapsed than the non-collapsed

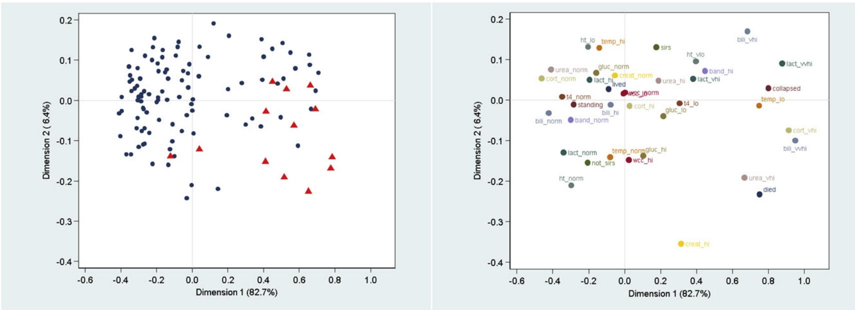

Fig. 2. a and b.Joint Components Analysis for cases that died. 1(a): Dots represent dogs that survived the infection; triangles represent cases that died. 1(b) Each point represents a clinical, hematological or biochemical measure (norm: normal; hi: high; vhi: very high; vvhi: very very high; lo: low; ht: hematocrit; temp: rectal temperature; gluc: glucose; lact: lactate; cort: cortisol; t4: total thyroid hormone; bili: bilirubin; band: band cell count; wcc: white cell count; sirs: systemic inflammatory syndrome).

group, but there was no significant difference between survivors and non-survivors. Seventy-nine cases (62.9%) had a HCO_3 less than 18 and no case had a value above 26 mEq/L. Forty-eight cases (37.2%) had an AG $>$ 16 mmol/L and 5 cases (3.9%) had an AG $<$ 8 mmol/L. There was a significantly higher median AG in the collapsed compared to the non-collapsed cases. Of the 252 cases in which the data were generated, 98 cases (76%) showed a reduction in pCO_2 in excess of 2 mmHg of the calculated drop that should have been experienced considering the drop in HCO_3 (0.7 mmHg decrease in pCO_2 for every 1 mEq decrease in HCO_3). This demonstrates that a mixed acid base disorder (metabolic acidosis with respiratory alkalosis) is very common in this disease. In the cases that died, the HCO_3 was below normal in 9 of the 11 cases in which it was determined. The actual drop in pCO_2 fell within 2 mmHg of the calculated change in only 1 case and exceeds this in 10 cases, thus identifying a mixed disorder (concurrent metabolic acidosis and respiratory alkalosis).

The median serum concentrations of sodium, potassium, chloride, calcium and phosphate ions were determined. There were no significant differences between any of the groups for sodium or potassium. Chloride was however lower in the cases that died compared to the survivors. In addition, ionized calcium concentrations were significantly lower in the collapsed and died cases than in non-collapsed or survived cases, respectively. In contrast, serum phosphate concentrations were significantly higher in collapsed and died cases than in non-collapsed and survived cases, respectively.

The presence or absence of SIRS was determined in 266 cases (See Tables S4 A and S4 B, Supplementary data). One hundred and sixty-one cases were diagnosed with SIRS (60.5%). Nine of these (3%) met 4 diagnostic criteria, whereas 51 (19%) met 3 criteria and 101 (38%) met 2 criteria for the diagnosis of SIRS. Collapsed dogs (n = 72 of the 320) were significantly more likely to have a diagnosis of SIRS than non-collapsed dogs (p = 0.021). Yet, SIRS positive cases were not more likely to die than SIRS negative cases. Dogs that were collapsed were positive for more SIRS criteria than those that were able to stand (p = 0.005). There was no significant difference in the number of SIRS criteria for which dogs were positive between those that survived and those that died (p = 0.919).

Basal serum cortisol was measured in 244 cases. One hundred and twenty-two cases (50%) had a basal serum cortisol > 160 nmol/L (normal = 10–160 nmol/L). There was a significantly higher median cortisol concentration in cases that died or were collapsed than in cases that survived or were non-collapsed, respectively. The OR for death increased 1.15 times for every 50 nmol/L increase in cortisol (CI 1.08–1.22, p $<$ 0.001). (Table 1). Total thyroid hormone (TT4) was determined in 245 cases One hundred and nine cases (44.5%) had TT4 $<$ 13 nmol/L (N = 13–45 nmol/L). TT4 was significantly lower in dogs that died or collapsed compared to dogs that survived or non-collapsed, respectively. The OR for death increased 1.63 times for every 5 nmol/L decrease in TT4 concentration (CI 1.2–2.2, p = 0.002).

Post mortem examinations were conducted on 25 of the 34 cases that died across the 3 studies. The most common macroscopic lesions are tabulated, from most- to least common, in Table S2. Splenomegaly was observed in all fatal cases. Visceral haemorrhages were typically ecchymoses and suggilations and were most commonly observed in the epicardium and myocardium of the heart as well as the diaphragm and occasionally the thoracic pleurae. Anaemia was also commonly observed. Acute interstitial pneumonia was identified in the majority of cases, based on the observation of diffusely increased consistency of the lungs, due to severe oedema and congestion with multifocal haemorrhages. In approximately a quarter of the cases, segmental haemorrhage was evident in the gastrointestinal tract, while in roughly half the cases serosanguinous effusions were observed in body cavities. Only 4 of 6 cases that showed neurological signs before death were submitted for post mortem examination. In all these cases, petechiae and/or larger haemorrhages and foci of malacia could be identified macroscopically and/or in histological sections through the grey matter of the brain. Nephrosis was observed in 4 cases. In all these cases serum urea and creatinine were also significantly elevated.

3.4. *Findings clustering with a poor outcome*

Identifying the most common abnormalities and determining which of these abnormalities occur together as overlapping syndromes, provides a helpful overview of the disease. Table S3 lists the abnormalities seen and ranks them from most to least common. Table 1 lists the abnormalities most strongly associated with outcome; listing those factors that demonstrated a significant odds ratio. Fig. 1 provides the receiver operating characteristic curves (ROC) for those measures that are most predictive of death. Table 2 provides the area under the curve (AUC) data for these measures and Table 3 provides the cut-offs with their sensitivities and specificities for these measures. Fig. 2 shows scatterplots of the normalized coordinates from a joint correspondence analysis, both for individual cases and for variable categories. It is clear that cases that died clustered together (Fig. 2a), and that these cases also clustered together with laboratory and clinical features associated with death, such as collapsed state, elevated urea, bilirubin and cortisol, and low body temperature and low thyroxine concentrations (Fig. 2b). Due to missing data, many cases, including all the cerebral cases, were not included in the joint correspondence analysis. Despite the fact that many variables were strongly associated with death at the univariate level, a multiple logistic regression model was unable to identify a group of significant independent predictors of death. Therefore, only univariate results are reported.

4. Discussion

The data presented here represent the largest cohort of molecularly confirmed, untreated, naturally-acquired mono-infections with *B. rossi* in the literature. Reflection on the data highlighted the complexity of the disease and on why evaluation of the disease at a clinical level is unable to shed significant new light on the disease pathomechanisms. Finding novel pathogenic pathways that lend themselves to therapeutic intervention is unlikely to progress using the current tools. Investigations will need to be directed at events more proximal in the disease process if we wish to understand this disease better (and other diseases like it) and make an impact on its high morbidity and mortality.

Anaemia was a very common finding in this cohort of dogs, with 83.3% of all the cases having a Hct below normal. This was in line with what has been shown previously (Reyers et al., 1998). Mortality was highest in the very small group (n = 4) that were haemoconcentrated, where 3 of 4 cases died, whilst the next highest mortality was in the severely anaemic group where it was 14.2%. Whilst anaemia is not a reliable predictor of death, haemoconcentration is. The pathogenesis of the haemoconcentration is not understood, but it is not related to significant dehydration. It is always accompanied by multiple organ failure and hence probably represents a relative haemoconcentration due to plasma loss through an excessively permeable endothelium (Ince et al., 2016). Understandably, the collapsed group of dogs had a lower median Hct than the non-collapsed group. A rather simplistic view of the disease pathogenesis centres around anaemia-associated hypoxia and lactacidosis (Button, 1979). The mechanisms inducing anaemia have been poorly investigated. The high incidence of haemoglobinuria is testimony to the very important role haemolysis plays in the anaemia. A suggested mechanism is decreased red cell production, which most likely occurs through inflammation-induced erythroid hypoplasia, dyserythropoeisis and concomitant infections and infestations (Chang and Stevenson, 2004; Menendez et al., 2000). Coomb's positive anaemia has described in canine babesiosis (Reyers et al., (1998). He reported that 88% of cases were positive, but that the majority of these cases did not show significant spherocytosis or agglutination and were thus not classified clinically as having immune mediated haemolytic anaemia (IMHA) (Reyers et al., 1998). Secondary warm-in-saline-agglutinating IMHA was present in 10.3% of the dogs in this study and positivity did not affect outcome nor the degree of anaemia at admission. The absolute reticulocyte count was $< 100 \times 10^9$/L in close to 70% of the cases in which reticulocyte counts were measured. This is consistent with a very slight regenerative response to the anaemia, which could be due to dyserythropoeisis. Similarly, poorly regenerative bone marrow responses have been reported before (Scheepers et al., 2011). In one study, erythropoietin levels in babesia-infected dogs were found to be appropriate for the level of anaemia and the same has been found to be true for malaria (Chang and Stevenson, 2004). The bone marrow's putative inability to respond to erythropoeitin plays a role in the inadequate response to the anaemia in this infection (McDevitt et al., 2004).

The median WBC count was within the reference interval for all groups, except the non-collapsed group where it was slightly below the reference interval. This has been described previously (Rautenbach et al., 2017; Scheepers et al., 2011; Weltan et al., 2008; Welzl et al., 2001) and similar findings have been reported for human malaria infections (Philipose and Umashankar, 2016). The relatively normal total WBC count, in the face of an elevated band neutrophil count (which was significantly higher in the cases that died), underline the inflammatory nature of this disease and the association between disease severity and the severity of systemic inflammation. Similar findings are true in sepsis, where the role of activated neutrophils is undisputed (Lehr et al., 2000). The interaction between leukocytes (specifically neutrophils) and the endothelium in septic states is important and well documented (Lehr et al., 2000) and we posit that similar mechanisms are likely at play in *B. rossi*-induced pathology.

Thrombocytopenia is a very consistent finding in *B. rossi* infections (Goddard et al., 2015b; Kettner et al., 2003) and indeed in babesia infections generally (human and animal) (Solano-Gallego et al., 2016; Vannier et al., 2015) as well as in human malaria (Horstmann et al., 1981). Despite the median platelet count in dogs in this series being $< 50 \times 10^9$/L, clinically obvious bleeding was not seen in any case, yet macroscopically obvious haemorrhage was a very common finding on post mortem. The lack of clinical bleeding has been attributed to the presence of significantly larger, more active platelets in the presence of high concentrations of fibrinogen, despite the severe thrombocytopenia (Goddard et al., 2015b). The mechanisms responsible for this consistent finding have been attributed to disseminated intravascular coagulation (Goddard et al., 2013). Immune mediated destruction has also been speculated (Paim et al., 2012). Reduced production is very unlikely, because the large sized platelets present in circulation are evidence of a strong bone marrow response (Goddard et al., 2015b).

Hyperlactatemia has consistently been shown to be related to the severity of *B. rossi* infections and the work presented in this study is consistent with this (Jacobson and Lobetti, 2005; Leisewitz et al., 2001; Nel et al., 2004). It is also clear that a spot lactate measured at admission is not the strongest predictor of poor outcome, but rather the failure of lactate concentration to reduce over the first 8 h after the initiation of treatment (Nel et al., 2004). Perturbations in blood glucose measurements were present in over 40% of the cases in this study with around half of these being hypoglycaemic and the other half being hyperglycaemic. In this series, hypoglycaemia was more common in the collapsed group, but there was no significant difference between the medians of the group that died and the survivors, nor between the non-collapsed and collapsed groups. A previous study has shown an association between poor outcome and admission hypoglycaemia (Nel et al., 2004). In paediatric malaria, hypoglycaemia is seen despite adequate levels of gluconeogenic substrates and in the face of appropriately low insulin levels as has been shown in babesia-infected dogs (Rees and Schoeman, 2008). This argues against a substrate deficiency or excess insulin as the cause. Other causes of hypoglycaemia, such as increased glucose consumption, depletion of hepatic glycogen stores, and hepatic dysfunction with impaired gluconeogenesis, are speculated to play more important roles in the pathophysiology of hypoglycaemia in canine babesiosis (Rees and Schoeman, 2008). The hyperglycemia may in part be due to the activation of counter-regulatory hormone and cytokine responses (McCowen et al., 2001). The cytokine responses in babesia are comparable to those seen in sepsis and malaria (Goddard et al., 2016; Leisewitz et al., 2019).

Hyperbilirubinemia and icterus were common in this cohort and, interestingly, high concentrations were associated with poor outcome. Just over 16% of cases were clinically icteric. This is around half of the proportion of cases reported elsewhere (Jacobson and Clark, 1994). As reported before, there was a negative correlation between bilirubin and Hct, lending credence to a pre-hepatic mechanism to this finding. Icterus is however likely due to a combination of hepatic damage (Gilles et al., 1953) and haemolysis (thus being both pre-heptic and hepatic). Mild hypoalbuminemia was common in this cohort, and has been reported before for babesiosis (Sudhakara Reddy et al., 2016) but is unlikely to be due to hepatic synthetic failure. The acute pansystemic sepsis-like state of the disease, more than likely, results in a reduction in albumin as a result of a negative acute phase response and a leaky endothelium (Cray et al., 2009; Ince et al., 2016). The liver is clearly an organ that is caught up and stressed (indeed overwhelmed) by the complex extrahepatic pathology of the disease, but it is unlikely to be an organ of primary importance and it is also not an organ likely to fail or become so dysfunctional as to be a direct cause of death.

Renal function has been assessed in several previous studies and the general consensus is that although there is evidence of renal damage, renal failure is a rare event (Lobetti and Jacobson, 2001). Acute subclinical renal injury is however common and mostly limited to the renal tubule (Defauw et al., 2018, 2017). The traditional markers of renal function (urea, creatinine and urine specific gravity) are not helpful in detecting the early and milder glomerular and tubular malfunction caused by babesiosis. High creatinine was however a poor prognostic indicator in this series and, as such, acute renal failure is an important life threatening, albeit rare, complication. Creatinine is a good correlate of glomerular filtration rate. Urea is affected by extra-renal factors, because its increase is commonly disproportionate to that of creatinine (without obvious dehydration). This finding has been reported previously (de Scally et al., 2006). The source of the urea is likely to be largely pre-renal. One possible cause could be hyperureagenesis due to erythrocyte components being released during haemolysis and blood from enteric bleeding which is occasionally seen with babesiosis. The amount of haemoglobin that must be metabolized in severe haemolysis is in excess of 10 fold the normal daily load (Maegraith et al., 1957). Urea was unaffected in a study that evaluated the effect of free haemoglobin on the canine kidney in which haemoglobin was infused in concentrations comparable to what is seen with natural babesia infection (Lobetti et al., 1996). Babesiosis causes obvious coagulopathy and hence this proposed mechanism may also be at play here (Goddard et al., 2013). Other possible causes include hypotension (Schetters et al., 2009), dehydration (Schetters et al., 1998) or rhabdomyolsis (Jacobson and Lobetti, 1996; Welzl et al., 2001). For the first time, this study demonstrated an association between high urea and poor outcome - hence whatever the cause of the elevated urea, it is probably reflective of pathology in a crucial process or processes. An inability to raise the urine SG above 1.030 in the dog is regarded as possibly reflective of tubular malfunction. It is however important to view this measure in the context of the majority of the samples having a haemoglobinuria. Urine haemoglobin has been shown to artefactually raise urine SG in a significant proportion of babesia cases and, as such, urine osmolality is needed to give a true reflection of tubular function (Defauw et al., 2018). So, despite the fact that the SG in our series was > 1.030 in over 65% of cases, this is clearly not a true reflection of normal renal tubular function.

Acute respiratory distress syndrome (ARDS) is undoubtedly a feature of severe babesiosis (Collett, 2000; Daste et al., 2013) and it is perceived as being uniformly fatal. Hypoxemia is most likely caused by pulmonary oedema in babesiosis and was present in 18/98 (18%) of cases in this study, but with only 3 of these dogs dying. The numbers were too small to allow for a meaningful statistical analysis. It is quite possible that fulminant lung oedema is an agonal event and that arterial blood gas analysis collected at admission is not reflective of these terminal moments. Acute lung injury (ALI) is a common finding (seen in just under two thirds of cases on post mortem). Inappropriate hypocapnia is common in babesiosis and probably represents the sepsis-like condition of the disease (Leisewitz et al., 2001) and the interstitial pneumonitis seen as a component of the ALI. Lung injury is a feature of severe infectious multisystemic diseases like sepsis, human babesiosis and malaria (Happel et al., 2004; Horowitz et al., 1994; Taylor et al., 2012) and, as such, it should come as no surprise that babesiosis is capable of causing severe lung injury. Acidaemia was a common finding in the collapsed dogs and in those that died. In addition, this was typically accompanied by an anion gap metabolic acidosis in these groups. The frequency of inappropriate hypocapnia also signifies how commonly mixed imbalances are present (metabolic acidosis and respiratory alkalosis). The complex multisystemic nature of the disease and the common findings of severe haemolytic anaemia, hyperlactatemia and ALI are obvious contributors to these findings.

Systemic inflammatory response syndrome is a concept used to describe a complex pathophysiologic response to a range of insults including infection, trauma, burns and pancreatitis (Balk, 2014). It has been used in dogs and has shown itself useful as a measure of illness severity and outcome (Balk, 2014; Brady and Otto, 2001; Kilpatrick et al., 2016; Pashmakova et al., 2014). Previous work has demonstrated that the cluster of findings used to diagnose SIRS are useful in predicting outcome in dogs with sepsis (Rau et al., 2007) and primary hepatitis (Kilpatrick et al., 2016). The syndrome has also previously been specifically examined in *B. rossi* infections in dogs (Welzl et al., 2001). Despite SIRS being significantly more common in the complicated cases, it was not predictive of death.

Cerebral babesiosis (CB) is rare and devastating in dogs. There are several old reports of the disease (Basson and Pienaar, 1965; Botha, 1964b; Malherbe and Parkin, 1951a; Piercy, 1947; Purchase and Kabete, 1947). The incidence of this complication was low in our study (16% of all deaths and 1.3% of all cases) as has been confirmed in others (Jacobson, 2006) and the mortality was extremely high as described before (Welzl et al., 2001). It is clear that from a pathological perspective, cerebral haemorrhage is the hallmark sign. The clinical definition of this complication in the living animal is more problematic than the pathological one in the dead animal. The pathology of this complication has never been well-described and the pathogenesis is unknown.

Cortisol and thyroid hormone levels have previously been evaluated in 95 cases of canine babesiosis and found to be strongly predictive of disease severity and outcome (Schoeman et al., 2007b). In our study of over 240 cases in which basal serum cortisol and thyroxine concentrations were obtained, these findings were again confirmed. These increased cortisol concentrations have been attributed to CRH-mediated ACTH secretion (Turrin and Rivest, 2004), caused mainly by cytokines such as IL6, IL1ß and TNF (Turnbull and Rivier, 1999), non-ACTH factors that directly stimulate the adrenal gland (Andreis et al., 1991; Ehrhart-Bornstein et al., 1998) and recently, also to decreased cortisol breakdown, resulting in significantly prolonged half-life of endogenous as well as exogenous cortisol (Boonen et al., 2013). Other canine studies have also documented marked reductions in T3, T4 and free T4, especially in dogs with sepsis and demonstrated that free T4 is less affected by illness than total T4 (Kantrowitz et al., 2001; Mooney et al., 2008; Schoeman et al., 2007a; Schoeman and Herrtage, 2008b).

This is the first published case series describing the macro-pathology of this disease. Post mortem findings are consistent with the severe multisystemic nature of the disease and are consistent with the anti-mortal findings. One remarkable feature of the post mortem examinations was the presence of macroscopic visceral haemorrhages despite the lack of clinical haemorrhage in the living animal. The disease has however been reported to result in a significant coagulopathy (Goddard et al., 2013; Liebenberg et al., 2013). All animals that died, did so within 24 h of admission. The greatest majority of deaths have repeatedly been reported to occur within 24 h of hospital admission (Keller et al., 2004; Nel et al., 2004; Schoeman et al., 2007b).

It is not surprising that logistic regression failed to identify a cluster of factors, independently associated with death. Babesiosis is a complex multisystemic disease. The alterations seen in the measured variables are all distal in the disease process, reflecting dysfunction or failure in organ systems and likely no more than indirect indicators of a much more proximal organ/tissue failure (such as the endothelium). For example, cortisol and thyroid hormone, although being strongly associated with the death, are global measures reflective of a whole body crisis and as such are influenced by a myriad of events more proximal in the disease process. Thus despite several of the variables we measured being strongly associated with poor outcome at the univariate level, a multivariable regression model was unable to estimate the independent "effect" of any individual variable. This reflects the fact that we have not yet identified variable(s) that are really proximal in the disease pathogenesis; the search for those key events must continue if we are to understand the pathophysiology of this disease and in so doing, be able to make an impact on outcome.

Defining how objectively measurable analytes cluster together with specific disease phenotypes provides objective ways of classifying the disease as uncomplicated or complicated. Such classification is useful to both the clinician and the researcher. To this end this data base was large enough to facilitate the calculation of ORs which confirmed that a high band neutrophil cell count (reflective of the very inflammatory nature of the disease), collapse at presentation, the presence of CNS signs, hypoglycaemia, hyperlactatemia, increase serum urea concentration, elevated creatinine, hyperbilirubinemia, hypercortisolaemia and low total thyroid hormone concentration provide a significantly increased odds of death. In addition to the ORs we have been able to generate ROC curves that highlight that total TT4, total bilirubin, serum urea and cortisol are the 4 measurables that are most indicative of death at admission. Cut-off values for these measures provide useful handles for clinicians and researchers alike and allow for an objective case classification system.

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Appendix A. Supplementary data

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