Thromboelastographic evaluation of haemostatic abnormalities in uncomplicated canine babesiosis

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List of Abbreviations

ARDS	Acute Respiratory Distress Syndrome
ARF	Acute renal failure
AT	Antithrombin
BSA	Bovine serum albumin
CBC	Complete blood count
DIC	Disseminated intravascular coagulation
EBV	Estimated blood volume
EDTA	Ethylenediamine tetra-acetic acid
FDP	Fibrin degradation products
G	Global clot strength
Ht	Haematocrit
IMHA	Immune-mediated haemolytic anaemia
K	Clotting time
LY 30	Percent clot lysis at 30 minutes
LY 60	Percent clot lysis at 60 minutes
MA	Maximal amplitude
MODS	Multiple organ dysfunction syndrome
OVAH	Onderstepoort Veterinary Academic Hospital
PCR	Polymerase chain reaction
PS	Phosphatidyl serine
PT	Prothrombin time

aPTT	Activated partial thromboplastin time
R	Reaction time
rHTF	Recombinant human tissue factor
RLB	Reverse line blot
SIRS	Systemic inflammatory response syndrome
SP	Split point
TEG	Thromboelastography
TF	Tissue factor
TFPI	Tissue factor plasminogen inhibitor
α	Angle

Summary

Thromboelastographic evaluation of haemostatic abnormalities in uncomplicated canine babesiosis

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Babesiosis, caused by *Babesia rossi*, is a common cause of morbidity and mortality of dogs in South Africa. Canine babesiosis can be classified either as uncomplicated or complicated based on the degree of anaemia and the severity of the presenting clinical signs.[1,2] In uncomplicated babesiosis, the clinical signs are mostly attributable to the degree of the anaemia, whereas in complicated babesiosis the disease process is characterised by additional organ involvement.[3,4] One of the most common haematological hallmarks of canine babesiosis, caused by *B. rossi*, is thrombocytopenia, which is not associated with clinical haemorrhage despite very low platelet counts that would normally cause inability to maintain normal primary haemostatic function.[5] The aim of this study was to describe the thromboelastographic findings in uncomplicated canine babesiosis and compare them with those of normal, healthy control dogs. We hypothesised that these dogs would have a normal to hypercoagulable haemostatic capacity, despite the severe thrombocytopenia, and that this could be detected with thromboelastography (TEG), which has previously been shown to correlate well with clinical signs of haemorrhage in dogs.[6]

This was a prospective, cross sectional, observational study that included 20 client-owned dogs, diagnosed with uncomplicated canine babesiosis at the

Onderstepoort Veterinary Academic Hospital (OVAH). Infection with *B. rossi* was confirmed by polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation assay. Blood samples were collected at the time of diagnosis. A group of 10 healthy control dogs were included for comparison. Antithrombin activity (AT) was measured using an automated spectrophotometric analyser (Cobas Integra 400, Roche, South Africa). D-dimer was measured using an immunometric flow-through principle (D-dimer Single test, Nycocard Reader II, Medinor A/S). Prothrombin time (PT), activated partial thromboplastin time (aPTT) and fibrinogen assays were performed on the ST art® 4 analyser (Diagnostica Stago, Roche, South Africa). TEG analysis was performed using the TEG® 5000 Thromboelastograph® Haemostasis System (Haemoscope, Pro-Gen Diagnostics (Pty) Ltd, South Africa). A complete blood count was performed on the ADVIA 2120 (Siemens, South Africa). The results of the babesiosis and control groups were compared using the Mann-Whitney U test or the Students t-test based on normality. The normality assumption for distribution of the variables in the data was evaluated using the Shapiro-Wilk test. The statistical significance was set at $p<0.01$.

The mean haematocrit (Ht) and median platelet count was significantly lower in the babesiosis group than the controls (0.29 vs. 0.50 L/L; $p<0.01$ and 22.0 vs. 374.5 x 10^9/l; $p<0.01$, respectively). There was no significant difference in any of the TEG parameters between the babesiosis group and the controls. The medians for the various TEG parameters in the babesiosis group versus the controls were; R: 5.5 vs. 4.4 min ($p=0.05$); K: 2.5 vs. 2.0 min ($p=0.08$); angle: 58.3 vs. 61.1 degrees ($p=0.35$); MA: 47.0 vs. 57.0 mm ($p=0.02$); G: 4.9 vs. 6.7 dyn/cm^2 ($p=0.02$); LY30: 0.00 vs. 0.6% ($p=0.20$); and LY60: 0.00 vs. 3.0% ($p=0.014$). The median fibrinogen concentration was

significantly higher in the babesiosis group than in the control group; 5.8 g/L (5.0 – 7.0) vs. 2.9 g/L (2.5 – 3.3); ($p<0.01$). The mean AT activity was significantly lower in the babesiosis group than in the control group; 102.6 mg/dl (89.9 – 112.8) vs. 127.8 mg/dl (110.6 – 134.8); ($p<0.01$). The median D-dimer concentration was not significantly different in the babesiosis group compared to the control group; 0.3 mg/L (0.1 – 0.4) vs. 0.1 mg/L (0.1 – 0.2); ($p=0.016$). Median PT was not significantly different in the babesiosis group compared to the control group; 6.5 sec (6.4 – 7.2) vs. 6.8 sec (6.6 – 7.5); ($p=0.14$). Median aPTT was significantly prolonged in the babesiosis group compared to the control group; 13.6 sec (12.4 – 14.5) vs. 11.5 sec (10.7 – 12.2); ($p<0.01$).

Despite the severe thrombocytopenia**,** dogs suffering from uncomplicated babesiosis did not have clinical signs of haemorrhage. The thromboelastograms of the babesiosis group were normal to hypercoagulable and thus correlated well with the clinical phenotype.

Chapter 1 Literature Review

1.1 Canine babesiosis

Babesiosis, caused by *Babesia rossi*, is a common cause of morbidity and mortality of dogs in South Africa, accounting for 12% of cases presented to the Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science of the University of Pretoria, South Africa in a survey done in 2000.[7] In an analysis of the admission records of the OVAH for the period of 2008, 331 canine cases were diagnosed with babesiosis. The intra-erythrocytic parasitaemia causes both extravascular and intravascular haemolysis.[8,9] Erythrophagocytosis of the infected erythrocytes occurs in both the spleen and liver.[10] The erythrocyte destruction and resultant anaemia is a major cause of the clinical signs of the disease.[3]

Canine babesiosis, specifically *Babesia canis*, can be classified as uncomplicated or complicated based on the degree of anaemia seen on haematology or the severity of the presenting clinical signs.[1,2] Complicated babesiosis is considered common amongst 26% of practicing veterinarians in South Africa.[7] In uncomplicated babesiosis the clinical signs are mostly attributable to the degree of the anaemia, whereas in complicated babesiosis the disease process is characterised by additional organ involvement.[3,4] Complicated babesiosis cases are described to suffer from severe systemic inflammatory response syndrome (SIRS), and multiple organ dysfunction syndrome (MODS).[1,3,4] Complications may include acute renal failure (ARF), cerebral babesiosis, disseminated intravascular coagulation (DIC), icterus and hepatopathy, immune-

mediated haemolytic anaemia (IMHA), acute respiratory distress syndrome (ARDS), pancreatitis, haemoconcentration and shock.[4,11-13]

1.2 Haemostasis

Traditionally, abnormalities in the haemostatic profile of a patient have been monitored by the changes affecting primary haemostasis (platelet count and function), secondary haemostasis (prolonged prothrombin time (PT), activated partial thromboplastin time (aPTT), decreased fibrinogen concentration) and fibrinolysis (increased FDP and D-dimer concentration).[14,15] The recent description of the cell based, tissue factor dependant model of haemostasis has increased knowledge of the complex biochemistry involved in secondary haemostasis and thus forced a review of the traditional view of the intrinsic and extrinsic pathways of coagulation.[16]

The cell based model of haemostasis incorporates the essential role of cells in *in vivo* coagulation and shows that coagulation occurs in three distinct but overlapping phases. It requires the participation of two different cell types namely cells bearing tissue factor (TF) and platelets. The model consists of the following intertwined stages namely; initiation, amplification, propagation and termination.[16]

Initiation: The intact endothelium has an antithrombotic function and prevents the initiation of coagulation during normal conditions. Cells expressing TF are generally localised within the wall of the vasculature. The endothelium and circulating monocytes can express TF when exposed to inflammatory cytokines. Initiation of coagulation occurs once endothelial injury exposes the cells bearing TF or induces TF expression. Activated factor VII (FVIIa) then rapidly binds to the exposed TF. The TF-FVIIa complex

activates FX to form the prothrombinase complex together with cofactor FV and FXI to form the tenase complex together with cofactor FVIII. The tenase complex will also activate FX. The prothrombinase complex acts as the catalyst for the conversion of pro-thrombin to thrombin. Tissue factor pathway inhibitor (TFPI) and antithrombin (AT) quickly inactivate any FXa that dissociates from the complex.

Amplification: Amplification occurs once a small amount of thrombin has been generated. Thrombin is a potent procoagulant and causes platelet activation on the surface of TF-bearing cells. The binding of the thrombin to the platelets surface causes the shuffling of membrane phospholipids to occur. This creates a procoagulant membrane surface with the release of the granule content from the platelets. The alpha granules released from the platelets contain a large amount of proteins and other substances, such as calcium, which are procoagulants. Calcium may induce a clustering of phosphatidyl serine (PS) and promote the binding of coagulation proteins to the activated membrane surface.

Propagation: Propagation is initiated with the release of the pro-coagulant alpha platelet granules, which then recruit more platelets to the site of injury. The propagation phase occurs on the surface of these platelets. The expression of ligands on the cell surface allows for cell-to-cell interaction, which leads to the aggregation of the platelets. In order for coagulation to occur effectively thrombin must be generated on the surface of the platelet in a large amount known as the burst of thrombin production. This occurs through the tenase complex activation of FX; the tenase complex is formed on the platelet surface in order to cleave FXa, since the FXa that was formed initially during the initiation phase on the TF-bearing cell surfaces is rapidly inhibited once it dissociates

from the TF. The newly generated FXa then rapidly binds to FVa and cleaves prothrombin to thrombin. This results in a burst of thrombin production in high enough quantity and speed in order to cleave fibrinogen to fibrin, resulting in the polymerization of a fibrin clot. This model adequately explains the bleeding tendencies seen with the deficiencies in FXI, FIX, and FVIII since these proteins are required for the generation of FXa and subsequent formation of thrombin on platelet membranes.

Termination: Physiological inhibitors for every aspect of haemostasis accomplish termination and control of coagulation. In primary haemostasis, the endothelial cells release ADPase and prostacyclin of which both inhibit platelet activation and aggregation. During secondary haemostasis, the TF-FVIIa-FXa complex is inhibited by TFPI. Antithrombin acts as the general inhibitor of the coagulation cascade, with special affinity for FIX, FX and FXI. Thrombomodulin expressed by the intact endothelium bind any excess/free thrombin. The thrombin–thrombomodulin complex also activates protein C and cofactor protein S, important anti-coagulants targeting FVIII and FV. The decreased activation will cause a decreased fibrin formation allowing fibrinolysis to proceed.

Hypercoagulability is considered likely in a number of inflammatory diseases that affect small animals.[4] In certain disease states, this hypercoagulability will lead to thrombus formation.[3,13] The link between inflammation and coagulation has been extensively studied and is best illustrated with the cell-based model of haemostasis.[16] Inflammation promotes coagulation and thrombin, a product of coagulation, can cause inflammation, a cycle that can progress to vascular injury.[17] A well-described consequence of babesiosis is SIRS; one can therefore assume that these proteolytic

enzymes, activating surfaces and thromboplastic substances are all present to a higher or lesser degree in these dogs.[13] Pulmonary thromboembolisms have been described at necropsy in dogs that had suffered from IMHA, cardiac disease, neoplasia, and protein losing nephropathy, hyperadrenocorticism, and sepsis.[18] One study reported that *B. canis,* using experimentally infected dogs, resulted in an inflammatory response with increased C-reactive protein (CRP), hyperfibrinoginemia, thrombocytopenia and leukopenia.[10] Another study reported raised serum levels of CRP in naturally infected *B. rossi* cases. Although this finding indicated severe inflammation, the increase in the CRP was not associated with adverse outcome.[19] DIC is a disease entity that is difficult to diagnose and treat and is potentially fatal if not recognised timeously. To date PT, aPTT, AT activity, platelet count, D-dimers, FDP and red blood cell morphology have been used to aid in the detection of DIC.[15,20] The International society on Thrombosis and Haemostasis (ISTH) developed a model for the diagnosis of DIC in people based on the approach by Bakhtiari et al. The tests used for this model includes PT, /aPTT, D-dimer, platelet count and fibrinogen concentration. Patients with DIC are identified using the following criteria; evidence of procoagulant activation, inhibitor consumption and increased fibrinolytic activity.[21,22] A similar diagnostic model, based on this approach, has been developed to diagnose DIC in dogs.[23] Thromboelastography (TEG) has been reported to provide the clinician with the ability to identify dogs in a pro-inflammatory and hypercoagulable state of DIC and affords the opportunity to differentiate these dogs from those in a consumptive but not yet in an overt stage of DIC.[24] It was reported that dogs presenting in the earlier or hypercoagulable state of DIC have an increased

chance of survival compared to dogs that present in the overt and hypocoagulable state.[6,24] TEG may thus be used for individualisation of treatment.[25]

1.3 Coagulopathy in babesiosis

The different strains of canine babesiosis are associated with varying pathogenicity. The South African strain, *B. rossi*, is highly pathogenic and associated with a higher parasitaemia, more severe anaemia and frequent complications.[2] An adequate number of normally functioning platelets are vital for primary haemostasis. One of the most common haematological hallmarks of *B. rossi* infection is thrombocytopenia, which is not associated with clinical haemorrhage despite very low platelet counts that would normally cause inability to maintain normal primary haemostatic function.[5] The author believes that the cause of this finding is multifactorial and includes the effect of haemolysis on coagulation, as well as platelet activation, due to the systemic inflammation, providing a procoagulant membrane surface upon which coagulation factors assemble.

Severe coagulopathies have been reported in *B. canis* and *B. argentina* infections.[26,27] Mechanisms for the coagulopathy postulated include direct activation by erythrocytes, altered lipid membrane structure and cytokine mediated activation. DIC has been described in naturally occurring *B. rossi* infections, using clinical, haematological, coagulation and histopathological parameters.[4] In the cases described in this small study the net effect of the clinically overt stage of DIC, the hypocoagulable state, was recognisable. DIC is the inappropriate activation of coagulation, with the net result being fibrin deposition and the formation of micro-thrombi in the vasculature.[28,29]

DIC is a complication in many diseases and the microvasculature in which DIC occurs is considered a distinct pathological organ.[29] DIC is initiated by TF in most disorders and the coagulation proceeds on the PS surfaces.[29,30] These PS surfaces are vastly increased in DIC due to the shedding of microvesicles from macrophages, monocytes, apoptotic cells and lipoproteins.[30] The most common mechanisms, which potentiate DIC, are proteolytic enzymes, activating surfaces and thromboplastic substances.[14] All of these are increased in babesiosis due to parasite destruction of the red blood cells with the release of stromal material into circulation.[8,31] A well-described consequence of babesiosis is SIRS; thus, these proteolytic enzymes, activating surfaces and thromboplastic substances are all present to a higher or lesser degree in these dogs.[13] Thrombin is pivotal in DIC since it forms the fibrin clot, activates platelets, amplifies its own production and among other things, activates other coagulation factors.[28,32] Fibrinolysis is activated simultaneously with coagulation and dissolution of the fibrin occurs.[17] In the normal animal, there is a fine balance between factors promoting coagulation (i.e. TF, inflammation, hyper-reactive platelets etc.) and those promoting anticoagulation, such as fibrinolysis and endogenous anticoagulants.[17] If this balance is disturbed the process may progress to a hypercoagulable state which will eventually lead to a hypocoagulable state and a haemorrhagic diathesis i.e. DIC, if the underlying disease is not treated appropriately.[14]

Several routine laboratory tests are traditionally utilised to diagnose and monitor abnormalities associated with primary and secondary haemostasis in animals. These tests include platelet count, platelet function, PT, aPTT, fibrin(ogen) degradation products (FDP), D-dimer concentration, AT activity and fibrinogen concentration.[33-37]

Limitations associated with the majority of these tests, however, are their insensitivity to identify the early onset of haemostatic abnormalities, as well as the fact that each test only evaluates a specific part of the haemostatic pathway.

1.4 Thromboelastography

Thromboelastography was first described in 1948 and has since been widely used in the United States and Europe especially in human medicine.[38,39] The use of TEG on human recombinant tissue factor-activated citrated whole blood has been validated for the use in dogs.[40] The potential ability of TEG to differentiate between hyper-, hypo- and normocoagulable states of haemostasis may make this assay clinically applicable for the evaluation of haemostatic disorders in dogs.

Mechanism: The TEG apparatus consists of a plastic cup and pin that is suspended by a torsion wire. The TEG analysis involves the addition of $CaCl_2$ to citrated whole blood in each TEG cup. An activator such as human recombinant tissue factor (rhTF) or kaolin can be used to reflect different pathways of activation, but also make the assay more rapid and reproducible. The pin once lowered into the cup begins an oscillation cycle. The data is then converted for display as a thromboelastogram.[25,40]

Sample: Fresh whole blood or citrated blood may be used. Fresh whole blood analysis requires a bedside machine as the sample needs to be analysed within 4 minutes, whereas citrated samples are more suitable for use in the veterinary setting where the sample is sent to a laboratory.[25] Statistical differences have been observed in citrated samples from canine blood performed at 30 and 120 minutes.[40] However, there was no overlap at either time period between patients with coagulopathies or the control

samples. It is generally agreed that blood is left to stabilise for at least 30 minutes after collection to decrease the variance of the TEG measurements since there is a tendency toward hypercoagulation at 120 minutes compared with 30 minutes.[41,42] The trend towards hypercoagulation can be explained by the fact that citrated blood does not completely inhibit thrombin formation and thus the subsequent activation of coagulation.[42] When monitoring patients with abnormal haemostasis using TEG, a fixed time point after sampling should be selected to avoid the risk of interassay variation.[40] In citrated whole blood, progressive acceleration of blood coagulation was reported to occur during the first 30 minutes after collection, which may lead to a decreased reaction time (R) and angle (α) on the thromboelastogram, as well as decreased maximal amplitude (MA). These changes can be explained by the activation of the coagulation cascade and activation of platelets during this time.[42]

TEG analyses: The TEG analyses is performed 30 minutes after blood sampling using a thromboelastograph TEG® 5000 Thromboelastograph® Haemostasis System (Haemoscope, Pro-Gen Diagnostics (Pty) Ltd, South Africa) according to a previously published method.[40] Briefly, a canine citrated whole blood sample is activated with a solution of rhTF (Innovin, Dade Behring, Marburg, Germany) in a 16:1 ratio. The rhTF solution is pre-diluted 1:2,780 in a HEPES buffer with 2% bovine serum albumin (BSA). Twenty µL (250 mM) $CaCl_2$ is added to 340 µL TF activated whole blood in the cup giving a total of 360 µL/cup and a final TF dilution of 1:50,000.

Parameters: The thromboelastography tracing, called a thromboelastogram, consists of three zones (Fig 1). The first zone, which indicates precoagulation, is represented by an initial linear segment representing the time taken from the initiation of the test to the

formation of the first fibrin strands, which causes a divergence of the line into two branches. The second zone, which represents coagulation, extends from the end of precoagulation to a maximal separation of the two branches; this represents the formation of the clot. The third zone, which represents fibrinolysis, extends from the end of coagulation until the test is ended, or until the two lines eventually converge into a single line that represents clot lysis.[24,40] Several values can be derived from the thromboelastogram, that includes R, K, α, and MA values, as well as the G value, which is derived from a calculation using the MA value. The values for clot lysis after 30 (LY30) and 60 minutes (LY60) are also derived from the tracing. The first value generated, the reaction time (R) or pre-coagulation time, represents the latency period from the time that the blood was placed in the TEG analyser until a preset fibrin formation is reached. R is related to the plasma clotting factors and inhibitor activity. The R evaluates the intrinsic pathway if kaolin is used as activator and is thus influenced by factors VIII, IX, XI and XII, or evaluates the extrinsic pathway (FVII) if TF is used as activator. The clotting time (K) represents the clot formation time and is a measurement of the rapidity of thrombin generation (clot kinetics). K is the time that corresponds to a divergence of 20 mm between the two lines and is related to clotting factors, fibrinogen and platelets. The K value is influenced by factor II, VIII, platelet count and/or function, thrombin formation, fibrin precipitation, fibrinogen concentration, and haematocrit.[25,41,43] The combined R and K values reflect coagulation time from the beginning to a predetermined clot strength. Alpha (α) is the angle between the midline and the tangent to the curve drawn from the point of 1 mm divergence, the split point (SP). Alpha represents the rapidity of fibrin build up and crosslinking. This is similar to

K, as same factors affected them both. The maximal amplitude (MA) is the maximal distance in millimetres between the two diverging lines. At this point, the clot formed entirely and thus MA represents the strength of the fibrin clot. The MA is affected by fibrin, fibrinogen concentration, platelet count and function, thrombin concentration, factor XIII and Ht. The MA is also a measure of clot stiffness and may be used to derive the global clot strength (G), a measure of the overall coagulant state. The G value is derived by the calculation: G=5000xMA/(100–MA) dyn/cm^2. Using the G value one can characterise thromboelastograms as hyper-, normo- or hypocoagulable.[6] The TEG value G<3.2 dyn/cm^2 indicates hypo-coagulability, G>7.2 dyn/cm^2 hypercoagulability and G=3.2-7.2 dyn/cm^2 normocoagulability.[43] The LY30 and LY60 values indicate fibrinolysis at 30 and 60 minutes after MA is reached.[38]

A hypocoagulable state is indicated when the R and K values are increased and the MA value is decreased and opposite changes are observed in hypercoagulable states. It has been reported that TF activated TEG is able to correctly identify dogs with clinical signs of haemorrhage, with both a higher positive predictive value (89%) and negative predictive value (98%) than the conventional coagulation profile.[6] TEG correlated more objectively to clinical signs in haemostatic dysfunction than traditionally used coagulation screens and it allows for easy and quick interpretation in patients with hyper-, hypo- or normocoagulable states.

The thromboelastogram takes into account all intravascular factors influencing coagulation in whole blood, except endothelial-dependant interactions, thus mimicking the cell based model of haemostasis. The values derived from the tracing are influenced

by multiple factors including platelet count, platelet function, Ht and fibrinogen concentration, which is all systematically abnormal in *B. rossi* infected dogs.

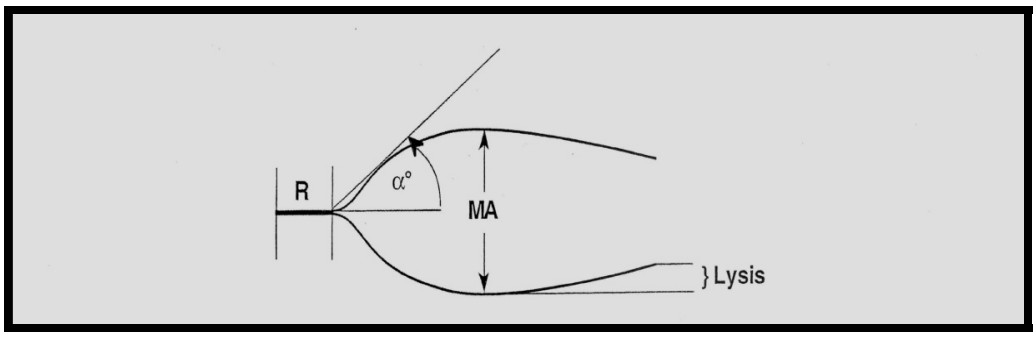

Figure 1. A schematic thromboelastographic (TEG® 5000 Thromboelastograph® Haemostasis System (Haemoscope Corp, Stokie, IL) trace is composed of four principal parameters. Reaction time (R), clotting initiation time recorded in minutes; angle α, fibrin polymerization rate, the tangent to the TEG® curve from the R point; and maximal amplitude (MA) and overall clot strength are recorded in millimetres. Lysis 30 is the percentile decrement in the TEG® amplitude 30 minutes after acquisition of the MA, a measure of clot stability.

1.5 The influence of platelets and haematocrit on thromboelastography

Thromboelastography closely resembles coagulation as it occurs *in vivo,* incorporating all the intravascular components of the haemostatic system except the endothelium. TEG evaluates all the steps in haemostasis and is influenced by factors such as the Ht, platelet count and platelet function.[6,40] Platelet concentration affects the overall clot strength and several studies have shown that a decreased platelet count leads to hypocoagulable thromboelastograms.[41,44,45] One of the most common haematological hallmarks for canine babesiosis, caused by *B. rossi*, is marked thrombocytopenia. A retrospective study reported that 62% of dogs suffering from canine babesiosis, presented to the OVAH, had platelet counts below 25.0 x 10^9/L.[5]

In uncomplicated babesiosis, the clinical signs are mostly attributable to the degree of the anaemia, despite the severe thrombocytopenia.[3] The anaemia must be considered when evaluating the results, since various studies show that alteration of the Ht can lead to hypo-, and hypercoagulable tracings.[46-55] Studies have thus far concentrated on the effects of progressive whole blood loss, serial haemodilution, decreased red cell mass as well as the induction of *in vitro* haemolysis. However, it is important to understand that anaemia seen with babesiosis is due to haemolysis, rather than whole blood loss, leading to a loss in only the red blood cell mass of the patient.

1.6 The utilisation of thromboelastography in veterinary medicine

TEG has been validated for use in dogs and several studies have been conducted using TEG in animal models for human research.[6,25,40,41,56,57] TEG has been successfully used to identify hypercoagulability in dogs with parvoviral enteritis, protein losing nephropathy, neoplasia and DIC.[6,24,56-58] TEG has also identified early haemostatic abnormalities in dogs that are in a pro-inflammatory and hypercoagulable state of DIC.[24] TEG may thus be used for individualization of treatment.[25]

Chapter 2 Study Objectives

2.1 Hypothesis and statement of the problem

Canine babesiosis caused by *B. rossi* is associated with severe thrombocytopenia; yet infected dogs rarely show clinical signs of haemorrhage. Several routine laboratory tests are traditionally utilised to diagnose and monitor abnormalities associated with primary and secondary haemostasis in animals. The traditional assays have limitations associated with their insensitivity to identify early haemostatic abnormalities.

The purpose of this study was to describe the thromboelastographic evaluation of coagulation in dogs with uncomplicated babesiosis. This study, to our knowledge, is the only study that has evaluated the haemostatic capacity *in vivo*, using TEG, in dogs with uncomplicated babesiosis.

The hypotheses were as follows:

1. Thromboelastography will detect that dogs with uncomplicated babesiosis have normal to hypercoagulable haemostatic capacity despite severe thrombocytopenia.
2. Severe thrombocytopenia, as seen with *B. rossi,* will not have a significant influence on the TEG values and will show that TEG can be a valuable assay for detection of haemostatic abnormalities in dogs with uncomplicated babesiosis.

3. The TEG results will correlate with the results from traditional assays including PT, aPTT, fibrinogen, AT activity and D-dimers.

2.2 Objectives of this study

1. To identify significant changes in the TEG values seen in dogs suffering from uncomplicated canine babesiosis caused by *B. rossi* compared to healthy control dogs.
2. To assess the effect that severe thrombocytopenia will have on the TEG values.
3. To identify whether TEG will indicate haemostatic abnormalities compared to traditional assays.

2.3 Benefits arising from the project

1. To investigate the influence of severe thrombocytopenia on TEG values in dogs suffering from uncomplicated canine babesiosis caused by *B. rossi*; which may optimise treatment and consequently outcome.

2. To serve as a baseline for future studies evaluating coagulopathies in dogs suffering from complicated babesiosis.

3. The research conducted serves as a partial fulfilment of the principal investigator's MSc (Vet Sci) degree.

Chapter 3 Material and methods

3.1 Model system

A prospective, cross-sectional, observational study.

3.2 Experimental design

The study was approved by the Animal Use and Care Committee and the Research Committee of the University of Pretoria (protocol number V51/09). The research project and data collection was conducted at the Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa, between February and September 2010. The study included 30 dogs in total. The babesiosis group comprised twenty client-owned dogs, with uncomplicated babesiosis. The dogs were examined at the outpatient's clinic of the Onderstepoort Veterinary Academic Hospital (OVAH). Eligible dogs were of any breed and of either sex, provided they were older than 12 weeks of age, weighed more than 6 kg and were naturally infected by *B. rossi* parasites. The designated controls comprised ten healthy, client-owned dogs, admitted to the OVAH for routine ovariohysterectomies or castration, or for blood donation. The control dog population was selected to match (age and sex) that of the babesiosis dogs. Owner consent was necessary for inclusion of all the cases in the study (Appendix A). Diagnosis of canine babesiosis was made on the basis of the morphological demonstration of the intra-erythrocytic babesia trophozite on a thin capillary blood film stained with Romanowsky

stain (Kyro-quick, Kyron Laboratories PTY Ltd, South Africa). Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB) hybridisation assay confirmed infection with *B. rossi* and ruled out co-infection with *Ehrlichia canis*. The data collection sheet (Appendix B) was utilised to record all the results of the basic clinical parameters obtained during the thorough clinical examination of all the dogs.

Exclusion criteria:

- Dogs presenting with *B. vogeli,* concurrent *E. canis* or *Theileria* infections;
- that had clinical or laboratory evidence of complications associated with canine babesiosis that included ARF, neurological signs, ARDS, haemoconcentration, secondary IMHA and icterus;
- or any concurrent inflammatory disease conditions, any known cardiac disease, any known neoplastic disease, any obvious infections or wounds, or any signs of trauma (e.g. fractures, contusions and wounds indicating a motor vehicle accident or fighting);
- or have been exposed to any medication known to interfere with normal haemostasis including prednisolone, aspirin, non-steroidal anti-inflammatory drugs, or heparin products either at presentation or at 4 weeks prior to presentation.

3.3 Experimental procedures

At presentation, prior to any treatment, a serum sample, sodium citrate sample and an EDTA sample was collected, in this specific order, from the jugular vein of each dog

using a 21-gauge venoject needle in a 3 ml vacutainer tube (BD Vacutainer tube, S.A. Scientific, Bryanston, South Africa) 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 EDTA sample was used to determine the platelet count and Ht. The TEG analysis was performed on the sodium citrate sample 30 minutes after collection. The remaining citrated sample was centrifuged at 2100 g for 8 minutes, after which the plasma was harvested and stored at -80ºC (Forma Scientific -86°C freezer). The coagulation profile analyses were performed as a batch within 4 months of collection and included PT, aPTT, D-dimer, AT and fibrinogen. All assays performed adhered strictly to the manufacturer's instructions.

Thromboelastography

The citrated whole blood collected for the TEG tracings was allowed to stand for 30 minutes to allow for the settling of the platelets activity levels. The TEG tracing was performed at 30 minutes from the time of collection. The analysis performed on the TEG® 5000 Thromboelastograph® Haemostasis System (Haemoscope, Pro-Gen Diagnostics (Pty) Ltd, South Africa) according to a previously published method used tissue factor as the activator.[40]

Complete blood count (CBC)

An EDTA blood sample was utilised to perform a CBC, on an automated cell counter (Advia 2120, Siemens, South Africa), to determine the Ht and platelet count of the dogs.

PT and aPTT assays

Citrated plasma was used to perform both the PT and aPTT assays and were performed on the ST art® 4 analyser (Diagnostica Stago, Roche, South Africa) using the Neoplastine® CI Plus (Diagnostica Stago, Roche, South Africa) reagent kit for PT, and the C.K. Prest® (Diagnostica Stago, Roche, South Africa) reagent kit for aPTT.

Fibrinogen assay

Citrated plasma was used to perform the assay. The fibrinogen assay was performed on the ST art® 4 analyser (Diagnostica Stago, Roche, South Africa) using the Sta-Fib 2 (Diagnostica Stago, Roche, South Africa) reagent kit on citrated plasma.

D-dimer assay

Citrated plasma was used to perform the assay. The assay was performed, according to the manufacturer's instructions, using an immunometric flow-through principle (D-dimer single test, NYCOCARD Reader, ILEX South Africa (Pty) Ltd.

Antithrombin assay

Antithrombin activity in the citrated plasma was determined utilizing a thrombin dependent chromogenic substrate assay (Cobas Integra 400 plus, AT III cassette reagent, Diagnostica Stago, Roche, South Africa) on an automated analyser.

DNA extraction and PCR

DNA was extracted from the EDTA whole blood sample using the QIAmp® blood and tissue extraction kit (Qiagen, Hilden, Germany. Molecular diagnosis of *B. rossi* and exclusion of other *Babesia* species, *Ehrlichia* and *Anaplasma* species was performed using PCR and RLB assay as previously described [59,60]

3.4 Observations

The data collection sheet (Appendix B) recorded details pertaining to clinical findings, CBC results, urinalysis, faecal flotation and routine coagulation assays.

Data captured from the TEG tracing, which included the four parameters (R, K, MA and α) and G was transferred onto a spreadsheet in Microsoft Excel and then made available for statistical analysis.

3.5 Statistical considerations

The normality assumption for distribution of the variables in the data was evaluated using the Shapiro-Wilk test. Those variables, which were normally distributed, were tested using the Students t-test and the Levene test was used to check for the equality of variance of the babesiosis and control groups. Variables, which were not normally distributed, were tested using the non-parametric Mann Whitney-U test. Data analysis revealed that Ht and AT were normally distributed variables whereas all other

parameters were not normally distributed. Significance was set at $p<0.01$ to adjust for multiple comparisons and reduce the chance of a type I error.

Box plots (representing the interquartile range) of the platelets, TEG variables, PT, aPTT, fibrinogen and D-dimer were created to indicate the distribution of the various values of the babesiosis group as compared to the control group.

Chapter 4 Results

4.1 Distribution of Breed, Age and Sex

The median age for the babesiosis group was 6 years (Range: 7 months – 11 years). The group consisted of eleven females and nine males. Breeds included four rottweilers, three cross breed dogs, three Boerboels, two Jack Russell terriers, two dachshunds and one of each of the following, fox terrier, toy pomeranian, Labrador retriever, Staffordshire terrier, Chow-chow and maltese. The control group was matched for age and sex. The median age for the control group was 3 years (Range: 6 months – 6 years). The group consisted of six females and four males. Breeds included three beagles, two Boerboels, two German Shepherds one Bouvier de Flanders, one cross breed and one Rhodesian ridgeback.

4.2 Comparison of the thromboelastographic values between the babesiosis dogs and control groups

There were no significant differences found in the median values of any of the TEG parameters between the babesiosis and the control groups. The medians (Interquartile range) for the various TEG parameters in the babesiosis group versus the control group were as follows: R time was 5.5 min. (4.9 – 7.6) vs. 4.4 min. (3.8 – 5.5); (p=0.05). The K time was 2.5 min. (1.7 – 3.9) vs. 2.0 min. (1.7 – 2.1); (p=0.08). The α was 58.3 degrees (45.3 – 66.0) vs. 61.1 degrees (59.0 – 65.4); (p=0.35). The MA value was 47.0 mm (40.5 – 56.6) vs. 57.0 mm (53.7 – 60.3); (p=0.02). The G value was 4.9 dyn/cm^2 (3.4 –

6.5) vs. 6.7 dyn/cm^2 (5.9 – 7.6); (p=0.02). The LY30 value was 0.00% (0.0 – 0.03) vs. 0.6% (0.0 – 2.7); (p=0.20). The LY60 value was 0.00% (0.0 – 0.9) vs. 3.0% (0.1 – 6.9); (p=0.014). (Fig. 2 – 8)

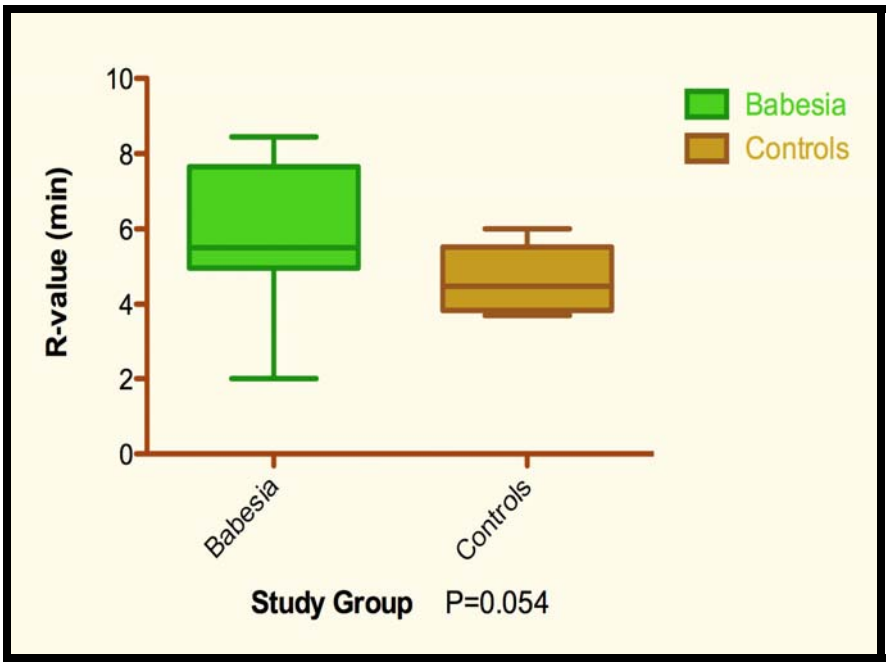

Figure 2. Box plot (representing the interquartile range) of the R-value in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the R-value. (Ref range: 3-9 min)

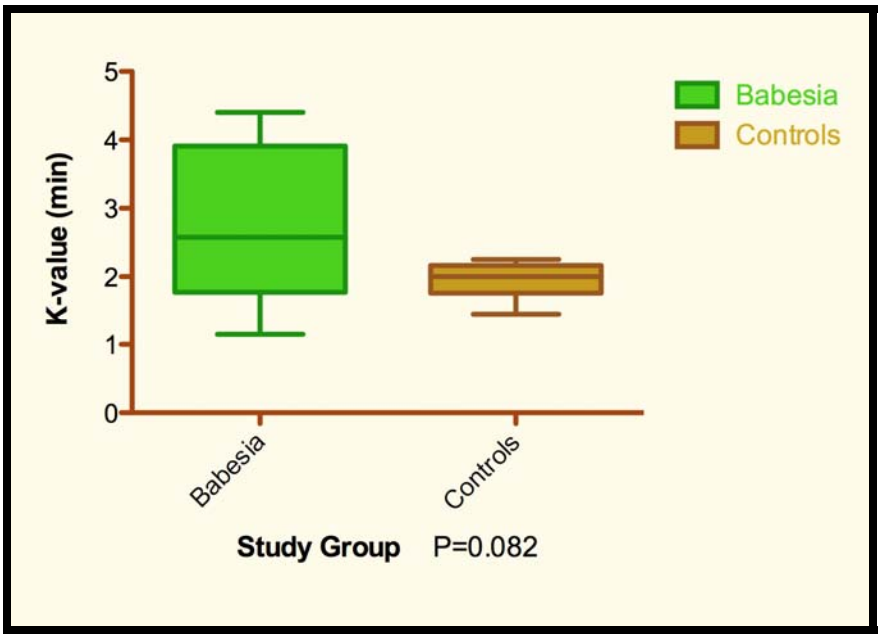

Figure 3. Box plot (representing the interquartile range) of the K-value in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the K-value. (Ref range: 2-8 min)

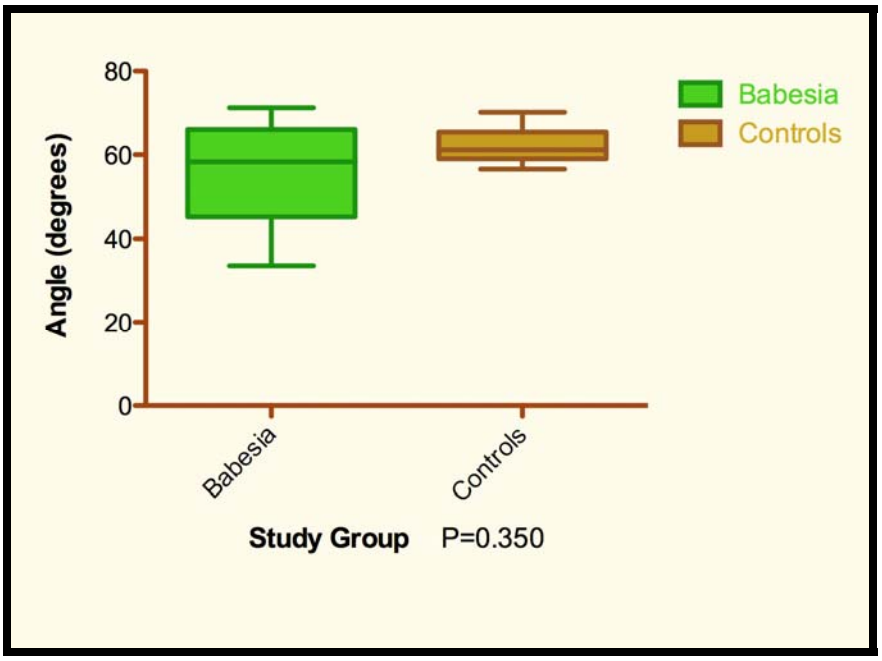

Figure 4. Box plot (representing the interquartile range) of the angle in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the angle. (Ref range: 27-59 degrees)

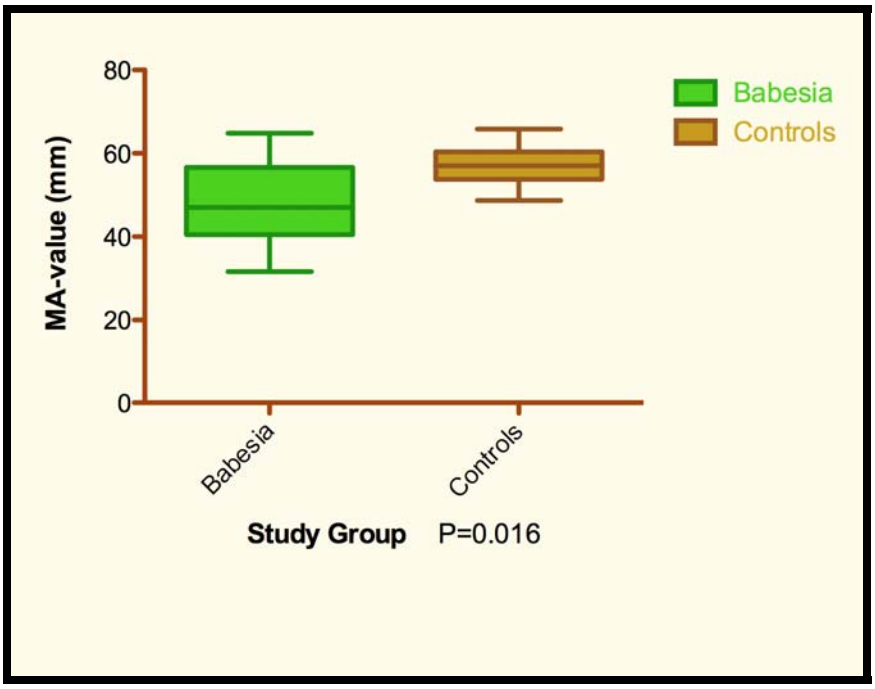

Figure 5. Box plot (representing the interquartile range) of the MA-value in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the MA-value. (Ref range: 39-59 mm)

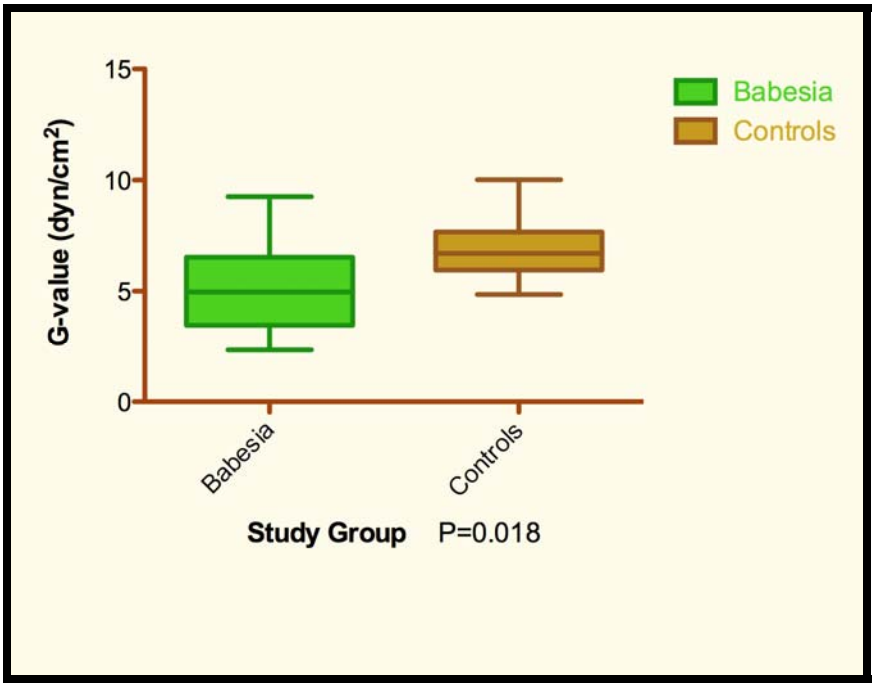

Figure 6. Box plot (representing the interquartile range) of the G-value in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the G-value. (Ref range: 3.2-7.2 dyn/cm^2)

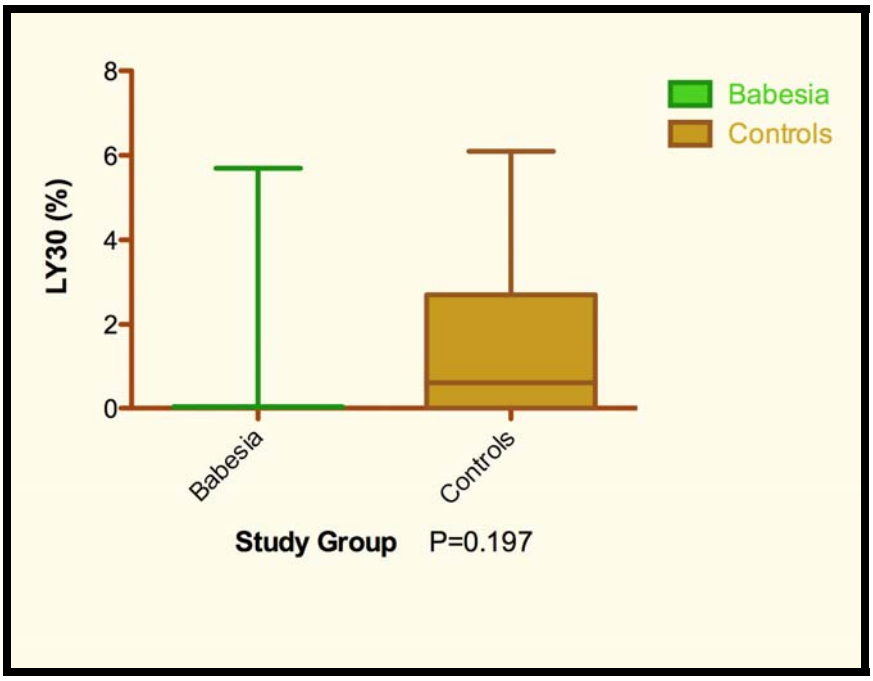

Figure 7. Box plot (representing the interquartile range) of the LY30 value in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the LY30 value. (Ref range: 0-2%)

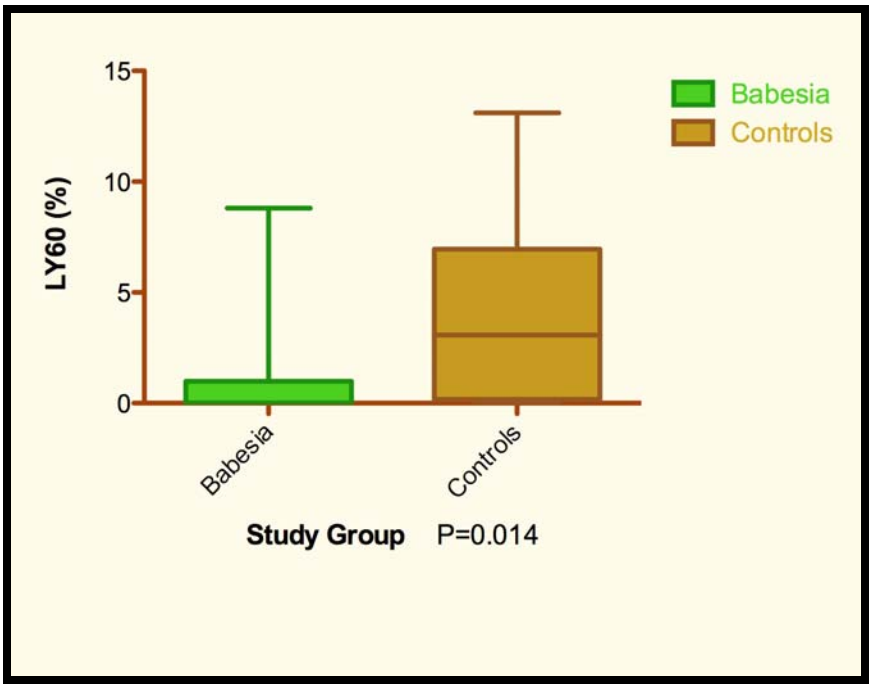

Figure 8. Box plot (representing the interquartile range) of the LY60 value in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the LY60 value. (Ref range: 0-8%)

4.3 Assay Results: Platelet, Haematocrit and Coagulation

The median platelet count was significantly lower in the babesiosis group than in the control group; 22.0 x 10^9/L (14.25 – 44.25) vs. 374.5 x 10^9/L (267.0 – 415.0); ($p<0.01$). (Fig. 9) No significant platelet aggregation, which could have contributed to the low platelet count, was reported in the samples. The mean Ht was significantly lower in the babesiosis group than in the control group; 0.29 L/L (0.20 – 0.35) vs. 0.50 L/L (0.42 – 0.55); ($p<0.01$). (Fig. 10) The median fibrinogen concentration was significantly higher in the babesiosis group than in the control group; 5.8 g/L (5.0 – 7.0) vs. 2.9 g/L (2.5 – 3.3); ($p<0.01$). (Fig. 11) The mean AT activity was significantly lower in the babesiosis group than in the control group; 102.6% (89.9 – 112.8) vs. 127.8% (110.6 – 134.8); ($p<0.01$). (Fig. 12) The median D-dimer concentration was not significantly different in the babesiosis group compared to the control group; 0.3 mg/L (0.1 – 0.4) vs. 0.1 mg/L (0.1 – 0.2); ($p=0.016$). (Fig. 13) Median PT was not significantly different in the babesiosis group compared to the control group; 6.5 sec (6.4 – 7.2) vs. 6.8 sec (6.6 – 7.5); ($p=0.14$). (Fig. 14) Median aPTT was significantly prolonged in the babesiosis group compared to the control group; 13.6 sec (12.4 – 14.5) vs. 11.5 sec (10.7 – 12.2); ($p<0.01$). (Fig. 15)

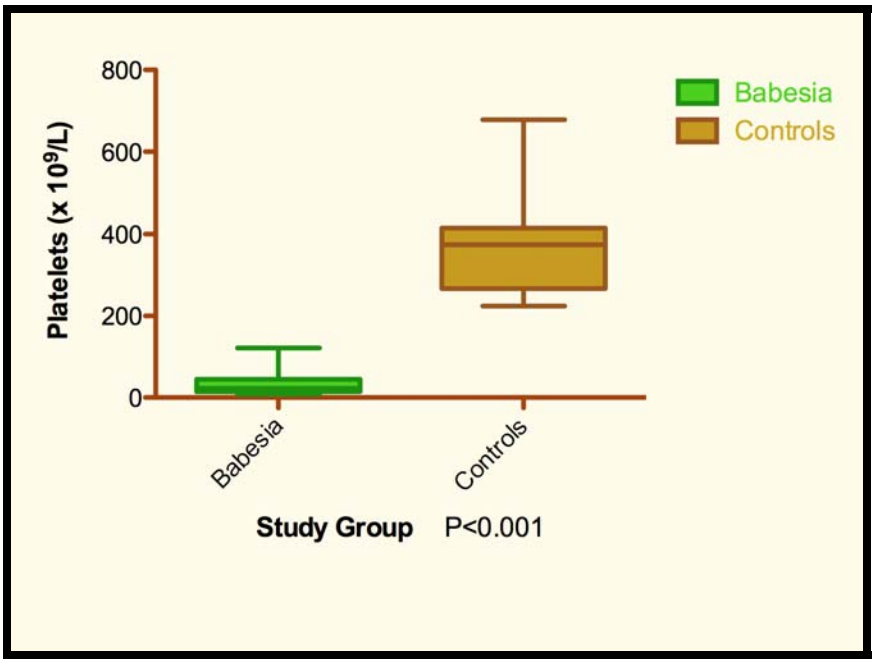

Figure 9. Box plot (representing the interquartile range) of the platelet count in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the platelet count. (Ref range: 200-500 x10^9/L)

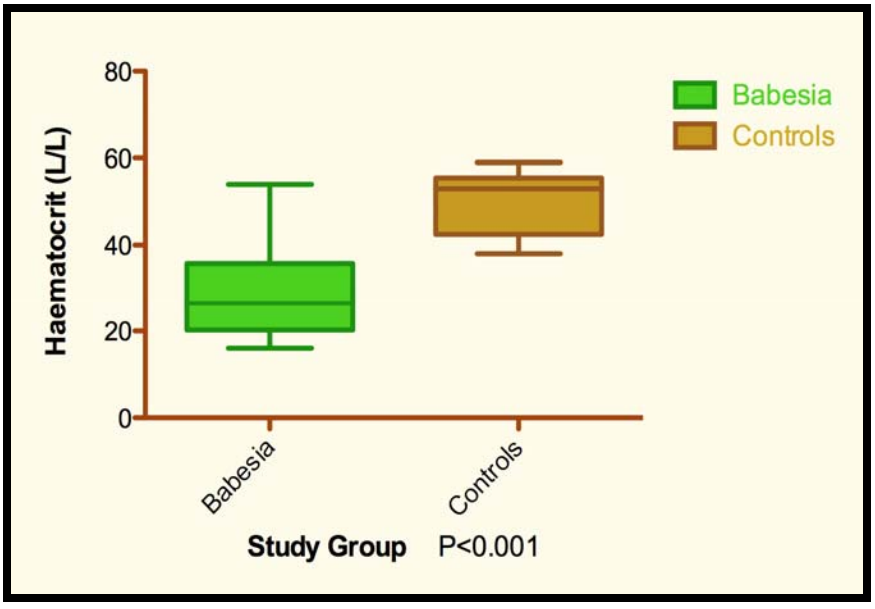

Figure 10. Box plot (representing the interquartile range) of the Ht in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the Ht. (Ref range: 0.37-0.55 L/L)

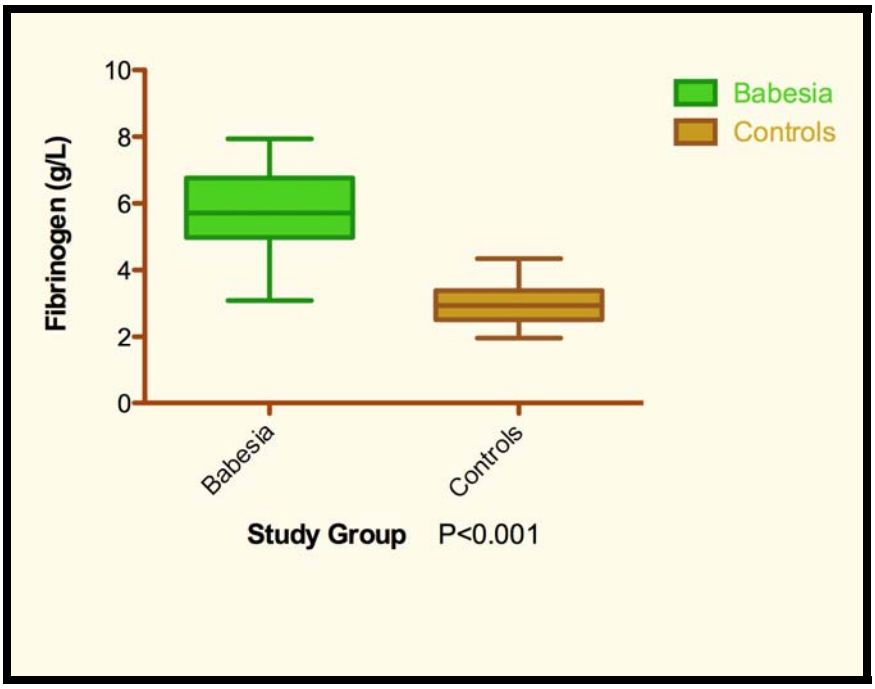

Figure 11. Box plot (representing the interquartile range) of the fibrinogen concentration in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the fibrinogen concentration. (Ref range: 2-4 g/L)

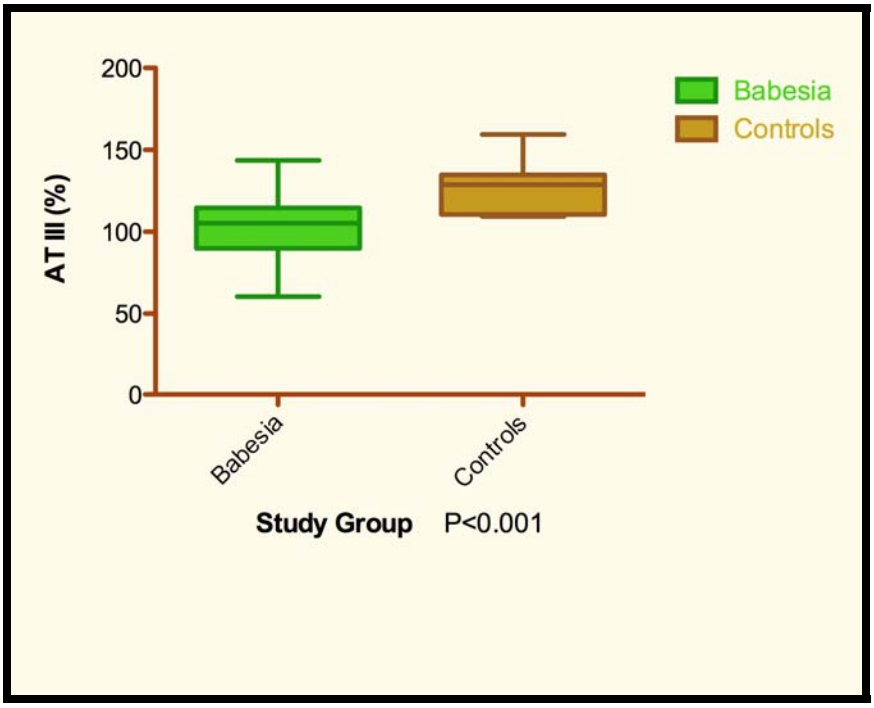

Figure 12. Box plot (representing the interquartile range) of the AT activity in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the AT activity. (Ref range: 80-135%)

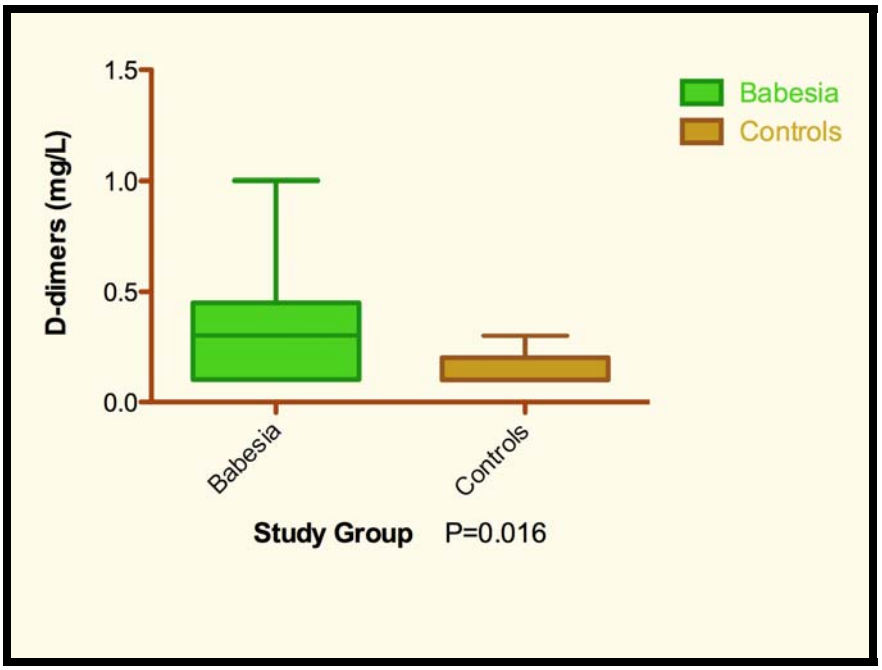

Figure 13. Box plot (representing the interquartile range) of the D-dimer concentration in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of the D-dimer concentration. (Ref range: <0.5 mg/dL)

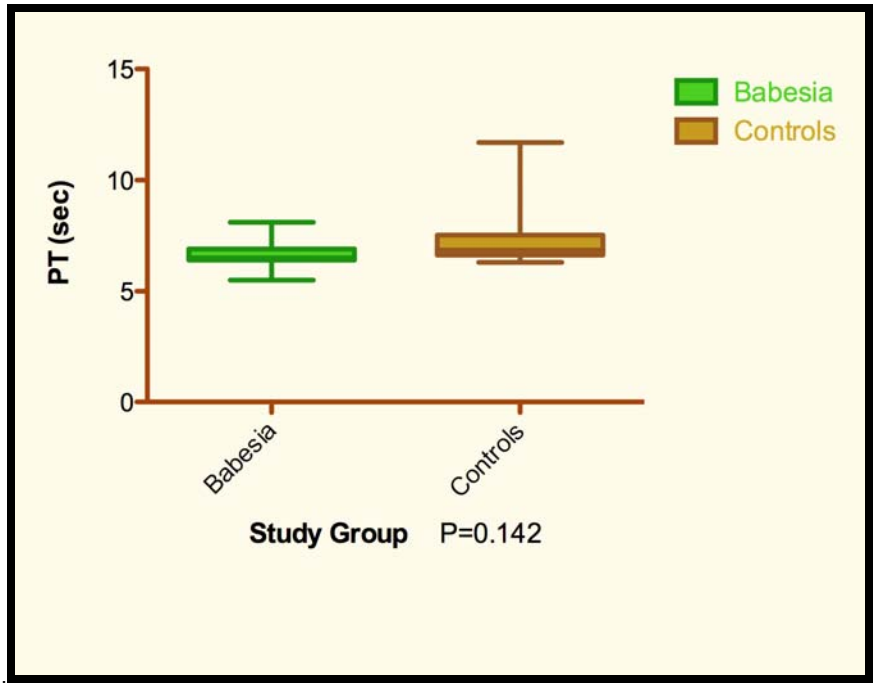

Figure 14. Box plot (representing the interquartile range) of PT in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of PT. (Ref range: <6.9 sec)

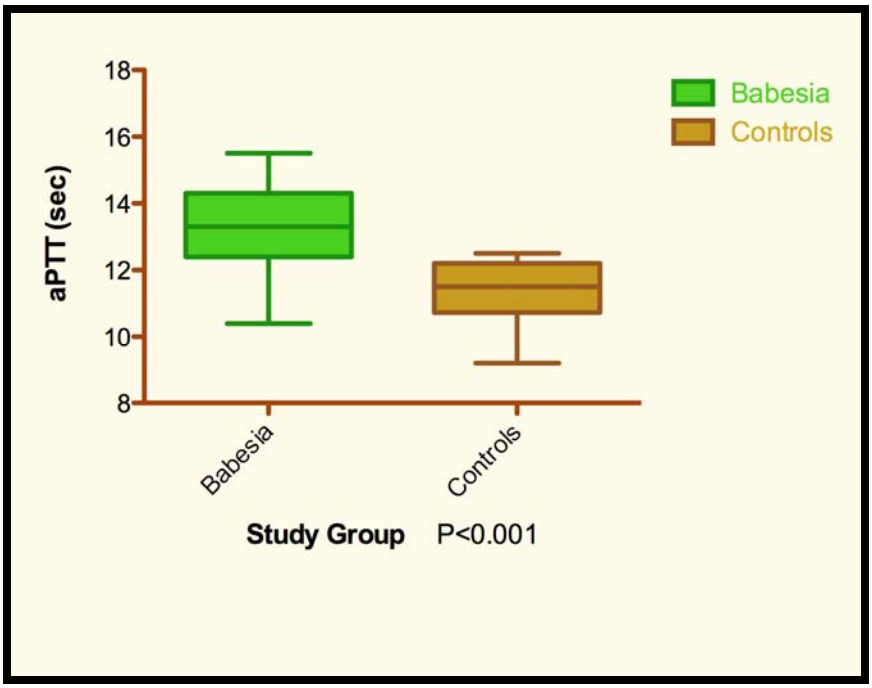

Figure 15. Box plot (representing the interquartile range) of aPTT in the babesiosis- and control groups. The graph displays as horizontal lines the 10th, 25th, 50th, 75th and 90th percentiles of aPTT. (Ref range: <12.9 sec)

Table 1. Median (IQR range) of all the coagulation analyses for the babesiosis and control groups with respective *p*-values. Mean (range) included for haematocrit and antithrombin activity.

Parameter (unit) (Reference range)	**Babesiosis group** Mean/median (IQR range)	**Control group** Mean/median (IQR range)	***P*-value**
Plt (10^9 /L) (200 – 500)	22.0 (14.25 – 44.25)	374.5 (267.0 – 415.0)	<0.001 *
Ht (L/L) (0.37 – 0.55)	0.29 (0.20 – 0.35)	0.50 (0.42 – 0.55)	<0.001 *
R – time (minutes) (3 – 9)	5.5 (4.9 – 7.6)	4.4 (3.8 – 5.5)	0.054
K – time (minutes) (2 – 8)	2.5 (1.7 – 3.9)	2.0 (1.7 – 2.1)	0.082
Angle (degrees) (27 – 59)	58.3 (45.3 – 66.0)	61.1 (59.0 – 65.4)	0.350
MA (mm) (39 – 59)	47.0 (40.5 – 56.6)	57.0 (53.7 – 60.3)	0.016
G (dyn/cm^2) (3.2 – 7.2)	4.9 (3.4 – 6.5)	6.7 (5.9 – 7.6)	0.018
LY30 (%) (0 – 2)	0.0 (0.0 – 0.03)	0.6 (0.0 – 2.7)	0.197
LY60 (%) (0 – 8)	0.0 (0.0 – 0.9)	3.0 (0.1 – 6.9)	0.014
PT (seconds) (< 6.9)	6.5 (6.4 – 7.2)	6.8 (6.6 – 7.5)	0.142
aPTT(seconds)(< 12.9)	13.6 (12.4 – 14.5)	11.5 (10.7 – 12.2)	<0.001 *
AT (%) (80 – 135)	102.6 (89.9 – 112.8)	127.8 (110.6 – 134.8)	<0.001 *
Fibrinogen (g/L) (2 – 4)	5.8 (5.0 – 7.0)	2.9 (2.5 – 3.3)	<0.001 *
D-dimers (mg/L) (<0.5)	0.3 (0.1 – 0.4)	0.1 (0.1 – 0.2)	0.016

The * denote statistically significant differences between groups.

Platelet count (Plt); Haematocrit (Ht); Reaction time (R); Clotting time (K); Alpha (α);Maximal amplitude (MA); Global clot strength (G); Percent lysis at 30 minutes (LY30); Percent lysis at 60 minutes (LY60); activated partial thromboplastin time (aPTT); Prothrombin time (PT); Antithrombin (AT); Number of dogs (n).

Chapter 5 Discussion

This study demonstrated that there was no significant difference in any of the TEG parameters between the babesiosis group with a median platelet count of 22.0 x 10^9/L and a control group consisting of healthy dogs with normal platelet counts. One of the haematological hallmarks for canine babesiosis, caused by *B. rossi*, is thrombocytopenia, which is not associated with clinical haemorrhage despite very low platelet counts that would normally cause an inability to maintain normal primary haemostatic function.[5] TEG thus demonstrated that dogs with severe thrombocytopenia secondary to *B. rossi* infection have normal overall haemostatic function, which correlates with the clinical presentation.

An adequate number of normally functioning platelets are vital for primary haemostasis.[41,61,62] A study on the influence of platelet count and activity on TEG parameters reported that the MA is the parameter that best represents the effect of platelet count on TEG.[41] Platelet counts below 66 x 10^9/L have been shown to affect the TEG values K, MA, and G in studies conducted in people; with the results being a smaller MA and prolonged K time [45] Platelet counts have also been shown to affect the TEG values K, MA and G in studies conducted in dogs.[24,63] Both studies found similar results with regards to the platelet count's influence on the TEG tracing, and confirmed that a low MA (<40mm), despite adequate clotting factor replacement and a normal R time, should raise the possibility for the need of a platelet transfusion. However, due to the logarithmic relationship between platelet count and MA, TEG cannot provide a sensitive reflection of platelet function or number for routine clinical practice.[41,45]

Personal communication by the co-workers on this study, Wiinberg et al., indicated that deviations in the TEG tracing with regards to the platelet count in dogs is set closer to values of 40 × 10^9/L or less. However, despite a median platelet count of 22 x 10^9/L, the thromboelastograms of the babesiosis group in this study did not have a decreased median MA and some of these dogs with severe thrombocytopenia even had an increased MA. (Fig. 16 &17) The authors believe that the cause of this finding is multifactorial and includes the effect of haemolysis on coagulation, as well as platelet activation, due to the systemic inflammation, providing a procoagulant membrane surface upon which coagulation factors assemble.

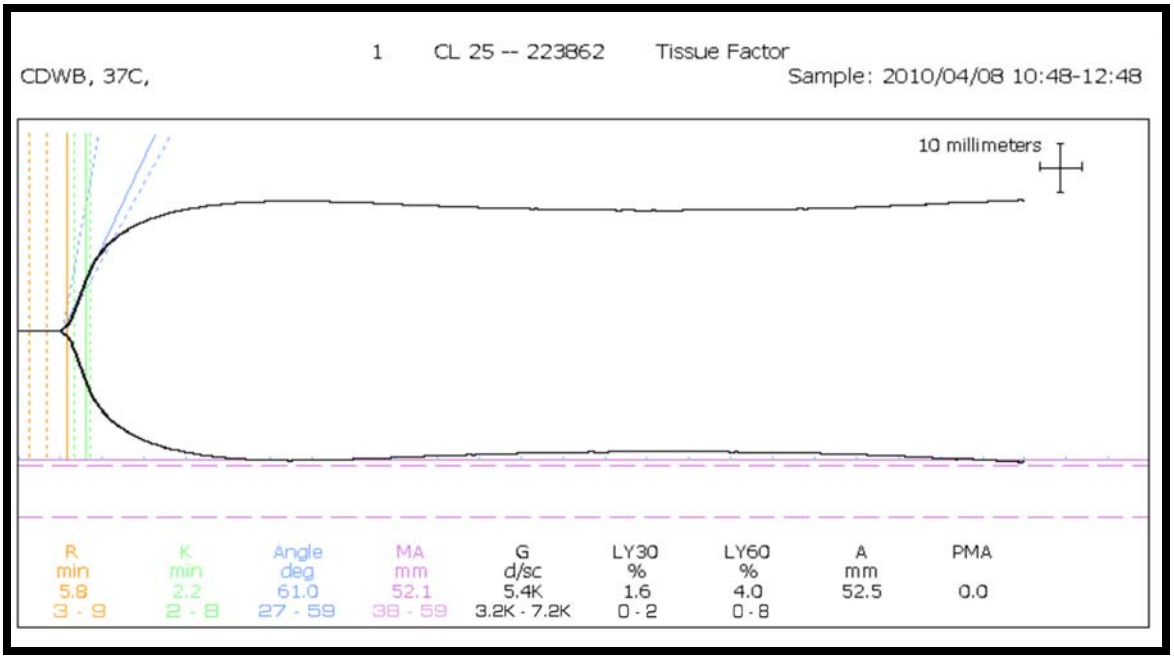

Figure 16. Example of a normocoagulable thromboelastogram of a dog in the babesiosis group. The platelet count was equal to 13 x 10^9/L and Ht was 0.30 L/L. The fibrinogen concentration was 5.84 g/L.

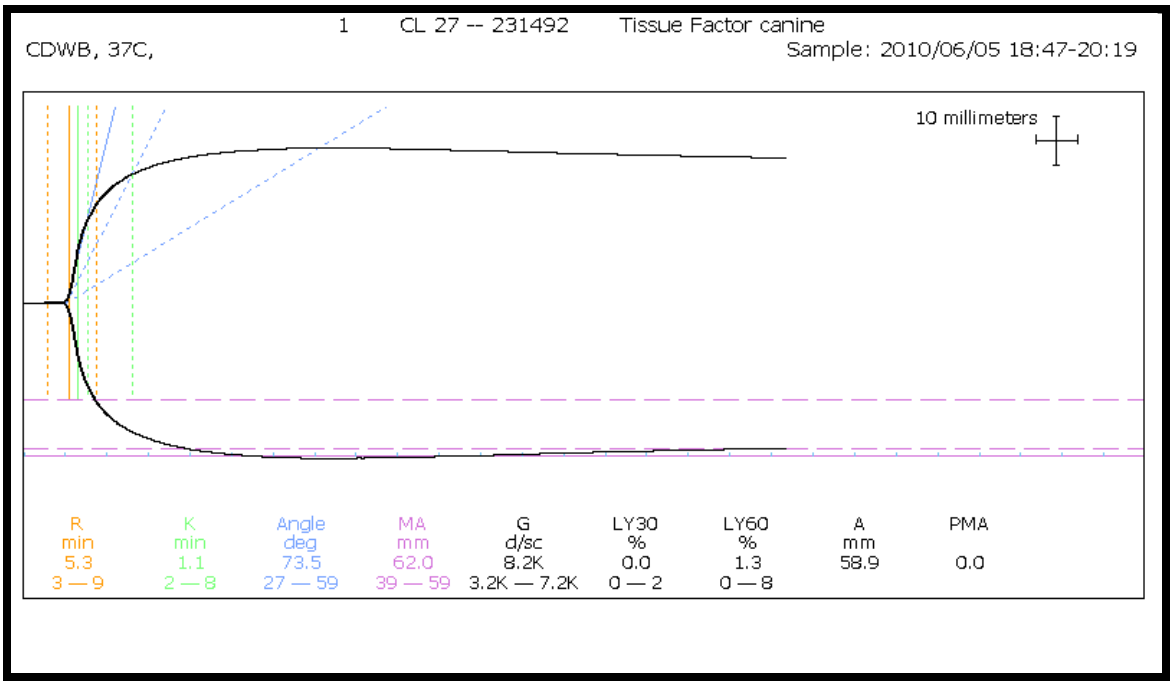

Figure 17. Example of a hypercoagulable thromboelastogram of a dog in the babesiosis group. The platelet count was equal to 120 x 10^9/L and Ht was 0.19 L/ L. The fibrinogen concentration was 3.09 g/L.

The babesiosis group in this study had a mean Ht of 0.29 L/L. In uncomplicated babesiosis, the clinical signs are mostly attributable to the degree of the anaemia.[3] Since the thromboelastogram is influenced by all components of coagulation, the anaemia in dogs suffering from babesiosis has to be considered when evaluating the results. The effects of progressive whole blood loss were studied in 87 adult humans, of which none were receiving anticoagulant therapy or had any alterations in coagulation or liver function. TEG analysis showed a trend towards increasing coagulability with progressive blood loss. Two of four patients with an 80% loss of estimated blood volume maintained normal to enhanced coagulation status whilst the other two patients developed clinical and TEG evidence of hypercoagulopathy.[46] Studies conducted on the effects of a decreased red cell mass have reported that this can lead to hypercoagulable tracings.[47-49] However, it is still unclear whether this hypercoagulability

due to decreased red cell mass is a true reflection of *in vivo* hypercoagulability.[47-50] Moreover, studies conducted on *in vitro* and *in vivo* haemodilution, using various diluents, or on the effects of decreased red cell mass, have had varied results, with thromboelastograms ranging from hyper- to hypocoagulable.[47-55,64] The effect of serial haemodilution on whole blood coagulation was investigated using hydroxyethyl starch, albumin and 0.9% saline. Of the haemodiluted samples up to 75% demonstrated a dose dependant increase in R and a decrease in both α and MA. The largest decrease in haemostatic function was noted when 5% albumin was used as the diluent.[55] The mechanism for the reported albumin mediated impairment of haemostasis after a 75% haemodilution was secondary due to the severe reduction in whole blood ionised calcium.[55] This study further found that after approximately 75% haemodilution, R values were decreased, K values were unchanged, α values were increased and MA values decreased. *In vivo* haemodilution accelerated clot formation but the clot had decreased viscoelastic strength.[53] In contrast to experimental haemodilution, the anaemia present in canine babesiosis is due to haemolysis, caused by the intra-erythrocytic parasite.[65] The local disruption of the red blood cells leads to the exposure of intracellular stromal material, especially phospholipids, which trigger the coagulation system.[8,9] Yet, ironically, the induction of *in vitro* haemolysis (either mechanically induced or by freeze-thawing), conducted in two separate studies on canine and equine citrated whole blood, revealed decreased MA and G values consistent with hypocoagulability.[51,52] The study conducted on canine citrated whole blood concluded that the hypocoagulable tracings were due to the platelets becoming refractory after activation during haemolysis.[52] The activation during haemolysis was due to either

degranulation or fluid uptake.[52] The study conducted on equine citrated whole blood concluded that the hypocoagulable tracings were due to the reduction of red blood cell mass.[51] It is the authors' contention that the release of red blood cell stromal content during parasite-induced haemolysis may contribute to a state of hypercoagulability in babesiosis, because it provides a readily available platform for the initiation of coagulation by tissue factor activation.[9]

Hypercoagulability is considered likely in a number of inflammatory diseases of small animals and may result in clot formation. Studies have reported pulmonary thromboembolism at necropsy in dogs, which had suffered from IMHA, cardiac disease, neoplasia, protein losing nephropathy, hyperadrenocorticism, and sepsis.[18] Both *B. canis* and *B. rossi* result in a systemic inflammatory response with increased C-reactive protein, hyperfibrinoginemia, thrombocytopenia and leukopenia.[10,13,19] Additionally, inflammation promotes coagulation and thrombin can in turn cause inflammation, creating a cycle, which can progress to vascular injury.[17] Although platelets are activated by many stimuli, thrombin is the most potent platelet activator.[66] Platelet activation and coagulation collectively determine the haemostatic activity of plasma - both are intertwined and thrombin is instrumental in each process.[66] Thus the inter-relationship between inflammation and coagulation has been well described and their molecular links are unquestioned.[16] In the light of the above, the significantly higher median fibrinogen level in the babesiosis group as compared to the controls is consistent with an acute phase response. Yet, hyperfibrinoginemia could also have occurred due to the preferred binding of thrombin to thrombomodulin on endothelial surfaces, instead of fibrinogen, during the advanced stages of coagulation.[32] Further

evidence for the concomitant activation of coagulation was supported by the mean AT activity, which was significantly lower in the babesiosis group as compared to controls. During severe inflammation AT activity is decreased due to impaired synthesis as a result of a negative acute phase response, degradation by granulocytic elastase, consumption as a consequence of ongoing thrombin formation and impairment due to the reduced availability of glycosaminoglycans.[28,67] Despite activation of coagulation, the median D-dimer value was not significantly different in the babesiosis group as compared to the controls and the D-dimer levels of the babesiosis group mostly fell within the normal range of <0.5 mg/l. This either indicates that increased thrombosis was unlikely in the babesiosis group or that there was complete suppression of the fibrinolytic system as has been reported in sepsis in people.[29] The TEG parameters for fibrinolysis (LY30 and LY60) further support this finding, since these values were not significantly different and the babesiosis group fell within the normal ranges, indicating that there was no measurable activation of the fibrinolytic system.

The median PT was not significantly prolonged between the babesiosis and control groups; however the median aPTT was significantly prolonged in the babesiosis group as compared to the control group. This study did not further investigate the finding of a prolonged aPTT in the presence of a normocoagulable thromboelastogram; however the findings are in accordance with other recent studies. A prolonged aPTT has been reported in a study on IMHA in dogs, in conjunction with hypercoagulable TEG values.[68] Prolonged aPTT has also been reported in Bernese Mountain dogs with normal TEG tracings and it was speculated that this could be due to anti-phospholipid antibodies.[69] Thus, an isolated prolonged aPTT may be due to the anti- phospholipid

syndrome. The influence of antiphospholipid antibodies on the aPTT values in our study is unknown, however it is likely that they play a role, since the anaemia seen with canine babesiosis is due to haemolysis, and the local disruption of the red blood cells leads to the exposure of intracellular stromal material, especially phospholipids, which could trigger an immune response.[8,9]

Limitations to this study included the small sample size which cannot preclude a type II error from having occurred. Secondly, the Ht values of the babesiosis dogs ranged from 0.16 L/L – 0.54 L/L. This broad range makes it difficult to draw inferences on the effect of Ht on the TEG tracings in this study. Consequently, future studies should be conducted in which larger numbers of babesiosis cases are categorised into several Ht ranges, so that Ht can be incorporated as an ordinal variable in multivariate analysis to examine the effect of a parasite-induced reduction in Ht on TEG tracings.

Chapter 6 Conclusion

In conclusion, this prospective, cross-sectional observational study has shown that dogs suffering from uncomplicated babesiosis, caused by *B. rossi*, have normo- to hypercoagulable thromboelastograms in the face of severe thrombocytopenia; which may be the result of the marked inflammatory process present in babesiosis.

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APPENDIX A

A. Owner Consent Form - Babesiosis Group

Your dog has been diagnosed with Babesia rossi commonly known as babesiosis/tick fever/biliary. At present, we are conducting a study to evaluate the blood clotting function in dogs with babesiosis. This study will require the collection of three blood samples taken at presentation. The collection of these samples will in no way be detrimental to your pet and at no time will the study change the treatment your pet would normally receive.

No additional costs will be charged to you for the collection of the samples or the blood tests required for this study. The treatment of your dog will be at the discretion of the attending clinician and will not be changed for the purpose of this trial.

Thank you for allowing your pet to be entered into this trial. It is our hope that the information gained will improve our understanding and future treatment of babesiosis. Should you have any further enquiries about the trial please contact:

Dr. C.E. Liebenberg
Department of Small Animal Medicine
Onderstepoort Veterinary Academic Hospital
Tel: 012 529-8140/8096

I, _____, hereby give permission that my dog _____, a _____ may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore, I understand that no additional costs will be incurred to me in respect of this trial for the collection of blood samples or the blood test required.

Signed at Onderstepoort on the _____ day of _____2009

Signature Owner/Agent_____

Home Tel: _____

Work Tel: _____

Cell No: _____

B. Owner Consent Form - Sterilization/Castration Control Group

Your dog has been admitted to the Department of Surgery, Onderstepoort Veterinary Academic Hospital for a routine sterilization (ovariohysterectomy/castration). Whilst your pet is awaiting surgery, we would like to collect three blood samples. This is to aid us in a clinical study on the blood clotting abnormalities in dogs suffering from babesiosis (tick bite fever/biliary). Your pet will serve as the healthy and normal result for the blood test.

The volume of blood we will collect will in no way harm your pet or change the procedure for which your pet was admitted for.

Thank you for your willingness to participate in this clinical trial. Should you have any further enquiries about the trial please contact:

Dr. C.E. Liebenberg

Department of Small Animal Medicine

Onderstepoort Veterinary Academic Hospital

Tel: 012 529-8140/8096

I, _____, hereby give permission that my dog _____, a _____ may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore, I understand that no additional costs will be incurred to me in respect of this trial for the collection of blood samples or the blood test required over and above the normal ovariohysterectomy or castration costs.

Signed at Onderstepoort on the _____ day of _____2009

Signature Owner/Agent_____

Home Tel: _____

Work Tel: _____

Cell No: _____

C. Owner Consent Form - Blood Donor Control Group

Your dog whilst donating blood can aid in a clinical trial by allowing us to collect three blood samples. This clinical study is on the blood clotting abnormalities in dogs suffering from babesiosis (tick bite fever/biliary). Your pet will serve as the healthy and normal result for the blood test.

The volume of blood we will collect will in no way harm your pet or change the procedure for normal blood collection.

Thank you for your willingness to participate in this clinical trial. Should you have any further enquiries about the trial please contact:

Dr. C.E. Liebenberg

Department of Small Animal Medicine

Onderstepoort Veterinary Academic Hospital

Tel: 012 529-8140/8096

I, _____, hereby give permission that my dog _____, a _____ may participate in this clinical study conducted at the Onderstepoort Veterinary Academic Hospital.

I understand that this study will in no way harm my dog. Furthermore, I understand that no additional costs will be incurred to me in respect of this trial for the collection of blood samples or the blood test required.

Signed at Onderstepoort on the _____ day of _____2009

Signature Owner/Agent_____

Home Tel: _____

Work Tel: _____

Cell No: _____

Appendix B: Data Collection Sheet

Date of admission:

Patient Sticker

Trial Number:

Sample	Collection procedure	Tests and Results			
1.Serum	1x 3ml vacutainer Store remaining sample at -80 ºC	TSP		Alp	
		Alb		Alt	
		Glob		Na+	
		Urea		K+	
		Creat		Ca+	
2.Citrate	2x 3ml vacutainer TEG tracing performed at 30 min All coagulation tests-Samples to be batched Citrated plasma stored at -80 ºC	TEG tracing			
		PT/PTT			
		D-Dimers			
		Fibrinogen			
		Antithrombin III			
4.EDTA	1x 3ml vacutainer PCR & RLB – samples to be batched .Plasma stored at -80 ºC Plasma stored	**CBC**			
		Ht		Retic%	
		Nmat		Platelets	
		Nimm			
		Mono			
		Lymph			
		E.canis	PCR		
			RLB		
5.Urine	5ml sample via cystocentesis Store remaining sample at -80 ºC	**Urinalysis**			
		Ph		Colour	
		S.G.		Clear?	
		Proteins		Smell	
		Bilirubin			
		Urobilino			
		Haem/bld			
		Urine	UPC		
			Culture		
			Antibiogr.		
6. In saline agglut.	Collected at admission	Insa			
7.Faecal float	Collected at admission	Float			

APPENDIX C: Summary of sample collection and handling

Sample	Sample handling	Procedure	Storage
1. Serum	• Collection at presentation	• Storage	• Centrifuge for 10 minutes at 5000rpm • Stored at -80ºC
2. 3.8% Na-Citrate	• Collection at presentation • 9:1 ratio • Allow to stand for 30min • Evaluate at 30min • 3 ml sample	• TEG tracing (duplicate) • PT/ aPTT • D-dimers • Fibrinogen • Antithrombin	• Room temp • Room temp/frozen • Room temp • Room temp/frozen • Room temp/frozen
3. EDTA	• Collection prior to treatment • Blood smear within 30min • Evaluate sample within 4 • Hours for full blood count • PCR sample to be batched and run within 1 month of collection. • PCR E.canis	• Full blood count (Automated cell count) • Blood film evaluation • PCR babesia • PCR E. canis	• Room temperature • EDTA to be centrifuged at 5000 rpm for 10 minutes, serum to be stored in 0.5ml aliquot at - 20ºC
4. in Saline	• Collection prior to treatment	• In saline aggluti-nation	
5. Faeces	• Collect at least 0.5 gram faeces per rectum	• Faecal flotation	
6. Urine	• Collect 5ml via urinary catheter or free flow if no catheter is placed	• Urinalysis, dipstick and sediment	

Appendix D: Raw Data

Table 2. Babesiosis Group: Raw data of Age, Breed, Sex, Weight, Clinical Examination, History and Faecal Floatation.

Case Number	Breed	Age	Gender	Body Weight	Spayed/Castrated/Intact	Rx admit/prior	Habitus	Temp.°C	Pulse	Resp	M.Mem.	CRT	Complaint/Clinical/History/Outcome	Comments	Faecal
CL 01	Spaniel	2 years	Female	16kg	Not recorded	Asprin	4+	39.9	124	36	Pale pink	1 sec	Asprin previous day	CL 01 - CL 10 TEG tracing	neg
CL 02	Jack Russel	1 Years	Female	12kg	Not recorded	None	Depressed	40.5	120	30	Light pink	1 sec	Full mammary glands	not used in TEG trial, TEG	neg
CL 03	Boerboel	1 Years	Male	52kg	Intact	None	Depressed	40.4	154	32	Pink	1 sec	Developed uveitis	data lost due to technical	2+ancy
CL 04	Jack Russel	4 Years	Male	4.4kg	Not recorded	None	Slight depression	39.7	132	48	Pale pink	1.5 sec	Recovered	error.	neg
CL 05	Cross	3 Years	Male	22kg	Not recorded	None	Depressed	38.9	114	24	Pale pink	<1 sec	Reduced appetite,diarrhoea with malena		2+ancy+tox
CL 06	Cross	1 Years	Female	32kg	Spayed	None	1+	40.2	140	40	Pink	1 sec	Recovered		2+ancy
CL 07	Dach.	9 Years	Male	8kg	Castrated	None	2+	40	128	32	Pink	1 sec	"Cushinoid"appearance		neg
CL 08	Pek.	2 Years	Female	6kg	Spayed	None	Depressed	39.2	112	30	Pale pink	1 sec	Low parasitaemia		2+ancy
CL 09	G.Dane	3.5 Years	Female	60kg	Spayed	Metronidazole	Depressed	40.5	90	28	Pale pink	1 sec	Diarrhoea prior to babesia		neg
CL 10	Dach.	9 Years	Female	7.8kg	Spayed	None	Depressed	39.4	122	20	Pink	1 sec	Anorexia of 2 days duration		neg
CL 11	Rottweiler	3 Years	Female	28kg	Not recorded	None	Depressed	40.2	100	28	Pale pink	1 sec	Lethargy of 3 days duration		neg
CL 12	Boerboel	11 Years	Male	40kg	Spayed	None	Depressed	40.2	80	28	Pale pink	1 sec	Anorexia of 1 days duration		neg
CL 13	Staffie	3 Years	Female	17kg	Intact	None	Depressed	42	160	30	Pale	1.5 sec	Anorexia,severe parasitaemia,died		2+ancy
CL 14	Cross	1 Year	Female	12.80kg	Intact	None	4+	38.8	86	36	Pink	1 sec	Consult for enucleation,incidental babesia		neg
CL 15	Dach.	6 Years	Female	6.5kg	Spayed	None	Slight depression	39.8	180	28	Pale pink	1 sec	Anorexia of 1 days duration		neg
CL 16	Toy.Pom	6 Years	Male	3.2kg	Castrated	None	Depressed	40.1	140	36	Pale	1 sec	Anorexia of 1 weeks duration		neg
CL 17	Chow	6 Years	Female	22kg	Spayed	Fluids	Depressed	40.1	120	30	Pale	1.5 sec	Lethargy of 1 weeks duration		neg
CL 18	Fox Terrier	9 Years	Female	7.60kg	Spayed	None	4+	40	140	26	Pale	1 sec	Owner noticed orange coloured stool		neg
CL 19	Rottweiler	3 Years	Female	28.6kg	Spayed	None	Slight depression	40.2	80	34	Pink	1 sec	On heat,mated, Flea allergy with hot spots		neg
CL 20	Boerboel	5 Months	Male	12kg	Intact	None	Depressed	40.1	180	40	Pale	1 sec	Anorexia of 1 days duration		2+ancy
CL 21	Maltese	10 Years	Female	6kg	Not recorded	None	Depressed	40	120	30	Pale	1.5 sec	Lethargic		neg
CL 22	Jack Russel	13 Years	Male	7kg	Intact	Doramectin	Depressed	40.1	100	36	Pale	1 sec	Previous Dx. And Rx. for S.Lupi		neg
CL 23	Jack Russel	7 Months	Male	5.8kg	Intact	None	Depressed	40.2	160	Panting	Pale pink	1 sec	Scrotal oedema		4+ancy
CL 24	Rottweiler	7 Years	Female	39.20kg	Intact	None	1+	40.2	120	Panting	Pale	1.5 sec	Died -Thrombis in mesenteric artery 1week later		neg
CL 25	Boerboel	11 Months	Female	41kg	Intact	None	Depressed	40.5	100	50	Pale	1.5 sec	Recovered		neg
CL 26	Dach.	1 Year	Male	5kg	Intact	None	Depressed	37	104	40	Pale	1 sec	Developed icterus and renal failure next day		4+ancy
CL 27	Labrador	2 years	Male	25.8kg	Intact	Asprin 200mg	Depressed	40.1	80	26	Pale	1.5 sec	Owner admin. 200mg asprin on day of consult		neg
CL 28	Cross	3 Years	Female	27kg	Intact	None	Depressed	40	100	30	Pink	1 sec	Mild decrease in appetite		neg
CL 29	Rottweiler	7 Months	Male	25.4kg	Intact	None	Slight depression	39.9	100	28	Pale pink	1 sec	Anorexia of 2 days duration		neg
CL 30	Cross	3 Years	Male	17kg	Intact	None	Slight depression	40	100	30	Pale	1 sec	Anorexia of 5 days duration, 3/5 B.C.S		neg
CL 31	Bull terrier	1,9 Years	Male	23kg	Not recorded	None	Slight depression	40	110	Panting	Pale	1 sec	Lethargic		2+ancy

Table 3. Control Group: Raw data of Age, Breed, Sex, Weight, Clinical Examination, History and Faecal Floatation.

Case Number	Breed	Age	Gender	Body Weight	**S**payed/**C**astrated/**I**ntact	Rx admit/prior	Habitus	Temp.°C	Pulse	Resp	M.Mem.	CRT	Clinical & History	Faecal
CL Con 1	Bouvier	2 Years	Female	40kg	Intact	None	4+	38.8	70	Panting	Pink	1 sec	Healthy breeding bitch	neg
CL Con 2	Beagle	1 Year	Female	6kg	Intact	None	4+	38.8	120	30	Pink	1 sec	Not used - early pregnant	neg
CL Con 3	Bouvier	3 Years	Female	38kg	Intact	None	4+	38.6	80	28	Pink	1 sec	Not used for TEG trial - thrombocytopaenia	neg
CL Con 4	Labrador	5 Years	Female	36kg	Spayed	None	4+	38.9	100	28	Pink	1 sec	Not used for TEG trial -Lipaemic serum sample	neg
CL Con 5	Pyr.mount.dog	7 Years	Female	40kg	Spayed	None	4+	Not taken	80	26	Pink	1 sec	Not used for TEG trial - thrombocytopaenia	neg
CL Con 6	Irish wolfhound	4 Years	Male	68kg	Intact	None	4+	38.8	80	30	Pink	1 sec	Not used for TEG trial - mild thrombocytopaenia	neg
CL Con 7	Rhod.ridgeback	5 Years	Male	38kg	Intact	None	4+	38.7	90	26	Pink	1 sec	Healthy blood donor	neg
CL Con 8	Cross	3Years	Female	12kg	Intact	Maxitrol	4+	39.8	120	Panting	Pink	1 sec	Unilateral traumatic cataract -rx:maxitrol	neg
Cl Con 9	G.S.D	3 Years	Male	38kg	Intact	None	4+	Not taken	90	Panting	Pink	1 sec	Healthy blood donor	neg
CL Con 10	G.S.D	8 Years	Female	30kg	Intact	None	4+	38.9	80	Panting	Pink	1 sec	Not used for TEG trial -Lipaemic serum sample	neg
CL Con 11	Cross	4 Years	Female	15kg	Spayed	None	4+	38.9	100	26	Pink	1 sec	Not used for TEG trial - polycythaemia	neg
CL Con 12	Boerboel	3 Years	Female	35kg	Spayed	None	4+	38.8	80	20	Pink	1 sec	Healthy breeding bitch	neg
CL Con 13	Boerboel	6 Months	Male	15kg	Intact	None	4+	38.6	100	30	Pink	1 sec	Healthy, fully vaccinated	neg
CL Con 14	G.S.D	6 Years	Female	40kg	Intact	Glugosaminoglycans	4+	38.7	88	24	Pink	1 sec	Urinary tract infection	neg
CL Con 15	Beagle	6 Years	Male	12kg	Castrated	None	4+	38.8	90	34	Pink	1 sec	Healthy,fully vaccinated	neg
CL Con 16	Beagle	2 Years	Male	8kg	Castrated	None	4+	39.1	94	22	Pink	1 sec	Not used for TEG trial -prolonged PT/PTT	neg
CL Con 17	Beagle	1 Year	Female	6kg	Spayed	None	4+	38.3	120	30	Pink	1 sec	Healthy, fully vaccinated	neg
CL Con 18	Beagle	3 Years	Female	7kg	Spayed	None	4+	38.3	102	36	Pink	1 sec	Healthy,fully vaccinated	neg

Table 4. Babesiosis Group: Raw data of Platelet, Ht, TEG values and traditional coagulation assays.

	Plt	Ht	R	K	Angle	MA	G	LY30	LY60	A	PT	PTT	AT III	Fib	D-dimers
Normals	200-500	37-55	3-9	2-8	27-59	39-59	3.2-7.2	0-2	0-8		6.8	12.6	80-135	2-4	<0.5
BABESIA															
CL10.22793	57	37	7.10	2.50	55.90	64.85	9.25	0.00	0.00	64.20	6.60	14.60	98.70	7.18	0.10
CL11.227968	18	30	6.50	3.25	46.25	39.55	3.25	0.00	0.00	41.90	5.50	14.00	115.20	6.34	0.30
CL12.178303	20	31	8.30	4.10	45.00	38.30	3.10	0.00	0.00	43.25	6.50	14.10	105.80	5.58	0.30
CL14.228041	36	32	5.65	2.65	54.85	47.90	4.60	0.20	2.05	44.55	6.90	14.30	88.30	3.83	0.30
CL15.228084	28	42	8.05	3.25	48.65	55.20	6.15	0.00	0.00	57.60	6.40	15.50	124.40	7.94	0.70
CL16.181807	45	20	4.75	1.45	68.80	60.45	7.65	5.70	8.80	53.65	6.40	12.40	95.00	5.93	0.20
CL17.228128	51	19	3.10	1.55	67.55	55.50	6.25	2.45	5.45	52.10	6.40	12.40	87.40	3.88	0.30
CL18.132219	8	45	2.00	4.25	65.70	31.60	2.35	0.00	0.00	34.65	6.10	10.40	143.50	5.71	0.90
CL19.116236	24	26	4.95	1.75	64.75	56.95	6.65	0.00	0.00	57.85	6.70	11.80	112.70	5.07	0.30
CL20.228265	17	16	5.30	2.25	58.45	46.20	4.30	0.00	0.00	48.15	7.40	12.80	110.00	4.14	0.50
CL21.158851	14	20	3.90	2.85	66.15	37.45	3.00	0.00	0.00	42.55	7.60	15.00	83.80	4.98	0.30
CL22.220885	15	27	7.90	4.35	44.65	34.15	2.60	0.00	0.00	38.40	6.50	13.30	108.70	6.08	1.00
CL23.229442	54	26	5.35	1.85	65.25	45.60	4.15	0.05	0.20	51.05	6.50	13.30	104.60	6.76	0.30
CL24.229465	33	23	5.00	1.95	63.30	55.65	6.30	0.00	0.65	52.75	6.20	14.10	105.90	7.26	0.50
CL25.223862	13	30	5.65	2.20	58.15	52.20	5.45	1.60	4.35	54.05	6.10	15.40	100.27	5.84	0.10
CL27.231492	120	19	5.25	1.15	71.25	62.95	8.50	0.00	1.00	60.25	7.50	13.90	70.61	3.09	0.10
CL28.229547	13	43	7.65	4.40	39.95	45.20	4.10	0.00	0.50	44.40	6.40	10.80	119.62	5.21	0.10
CL29.227828	15	21	8.45	3.65	36.80	45.75	4.20	0.00	0.00	44.40	6.40	10.80	119.62	5.21	0.10
CL30.207879	42	21	5.25	1.35	70.30	56.95	6.60	0.00	0.90	56.45	>60	>120	60.40	>70	0.20
CL31.223909	12	54	7.65	4.00	33.60	43.45	5.35	0.00	0.00	48.25	8.10	13.30	104.61	7.67	0.10

Table 5. Control Group: Raw data of Platelet, Ht, TEG values and traditional coagulation assays.

	Plt	Ht	R	K	Angle	MA	G	LY30	LY60	A	PT	PTT	AT III	Fib	D-dimers
Normals	200-500	37-55	3-9	2-8	27-59	39-59	3.2-7.2	0-2	0-8		6.8	12.6	80-135	2-4	<0.5
CONTROLS															
CLCon1.213798	237	41	3.87	2.25	56.60	57.85	6.95	1.45	6.20	45.25	7.40	12.10	126.80	1.96	0.30
CLCon7.181101	277	54	5.45	2.15	60.20	59.35	7.30	0.00	0.00	58.95	6.80	11.70	130.40	2.69	0.20
CLCon8.119129	357	59	3.70	1.80	65.40	50.80	5.20	1.20	5.40	39.20	7.00	9.20	159.40	2.93	0.10
CLCon9.101400	224	52	3.75	2.20	59.85	54.75	6.20	0.00	1.35	48.20	6.80	11.00	130.20	3.07	0.10
CLCon12.148631	291	48	4.05	1.80	65.40	48.70	4.85	5.85	13.10	30.75	7.90	11.80	127.40	2.54	0.10
CLCon13.227271	401	38	6.00	1.45	70.15	65.85	10.00	6.10	9.25	50.10	6.90	12.50	110.50	4.35	0.20
CLCon14.186072	457	54	5.05	2.05	58.85	56.05	6.35	1.65	4.8	49.1	6.7	11.3	132.8	3.34	0.1
CLCon15.OTAU	400	55	4.90	2.00	59.10	59.10	7.20	0.00	0.10	56.05	6.30	9.90	110.67	2.44	0.10
CLCon17.OTAU	392	57	3.95	2.00	63.80	56.25	6.45	0.00	1.25	52.40	6.40	11.00	109.40	2.94	0.10
CLCon18.OTAU	678	43	5.75	1.65	62.15	63.45	8.70	0.00	0.20	61.95	11.70	12.50	140.87	3.52	0.10

Table 6. Babesiosis Group: Raw data of CBC.

Case Number	Hb g/l	RCC X10^12/L	Ht L/L	MCV fl	MHC g/dl	MCHC g/dl	RDW %	WCC X10^9/l	AbN (tot)X10^9/l	AbNmatx10^9/l	AbNimmX10^9/l	AbLymphoX10^9/l	AbMonoX10^9/l	AbEos X10^9/l	AbBaso X10^9/l	Plt C X10^9/l	MPV fl	Aniso	Normo %	M act	Retic %
CL 01	116	4.94	0.34	68.9	23.5	34.1	13.4	3.06	2.33	2.14	0.18	0.55	0.18	0	0	16	16	2+	6	3+	/
CL 02	77	3.31	0.23	69.3	23.2	33.5	13.8	7.19	4.89	4.46	0.43	2.01	0.29	0	0	51	15.7	2+	6	1+	/
CL 03	100	4.301	0.3	69.4	23.3	33.6	14.6	5.96	3.81	3.28	0.54	1.19	0.95	0	0	22	16.7	3+	9	2+	/
CL 04	38	1.81	0.13	73.2	21.2	28.9	18.8	8.53	5.72	4.95	0.77	2.13	0.51	0.17	0	24	14.5	3+	7	1+	8.27
CL 05	37	1.63	0.12	72	22.8	31.7	17.9	5.12	3.48	3.48	0	1.13	0.36	0.15	0	64	12.7	3+	2	2+	0.3
CL 06	89	3.83	0.27	69.4	23.1	33.3	15.2	13.02	10.42	9.9	0.52	1.69	0.91	0	0	47	17.5	3+	3	2+	1.83
CL 07	83	3.66	0.26	71.3	22.7	31.8	15.6	13.74	12.23	10.72	1.51	0.41	1.1	0	0	60	16.1	4+	10	2+	2.76
CL 08	105	4.69	0.31	66.2	22.5	33.9	13.8	3.23	1.68	1.36	0.32	1.29	0.19	0.06	0	40	10.8	1+	/	1+	/
CL 09	88	3.73	0.27	73.2	23.6	32.3	18.5	13.45	9.68	7.8	1.88	2.42	1.35	0	0	57	17	5+	10	2+	5.47
CL 10	127	5.58	0.37	67	22.8	34	12.9	5.14	3.24	2.88	0.36	1.18	0.62	0.1	0	57	21.2	2+	/	2+	/
CL 11	103	4.36	0.301	69.2	23.6	34.1	14.9	14.24	11.53	7.26	4.27	1.57	1	0.14	0	18	17.6	4+	3	3+	/
CL 12	109	4.52	0.31	67.6	24.2	35.7	15.4	3.11	2.11	1.93	0.19	0.81	0.19	0	0	20	13.2	1+	10	4+	/
CL 13	74	3.05	0.21	69.5	24.4	35.1	15.9	8.1	4.37	2.75	1.62	2.75	0.97	0	0	21	12.8	3+	38	2+	2.51
CL 14	102	4.56	0.32	70.6	22.4	31.7	17.3	6.66	4.28	4.14	0.13	1.74	0.53	0.09	0.03	36	14.2	3+	4	2+	/
CL 15	140	5.87	0.42	71.8	22.8	33.1	13	3.64	2.91	2.62	0.29	0.66	0.07	0	0	28	19.9	1+	/	1+	/
CL 16	62	2.66	0.201	75	23.4	31.2	18.1	4.4	4.05	3.78	0.26	0.18	0.18	0	0	45	14.7	5+	14	1+	6.6
CL 17	53	2.61	0.19	74.1	20.4	27.5	18	3.73	2.61	2.46	0.15	0.67	0.37	0.07	0	51	12.8	4+	/	1+	1.49
CL 18	145	6.28	0.45	71.6	23.1	32.3	12.2	2.34	1.73	1.54	0.19	0.56	0	0.05	0	8	20.5	1+	/	1+	/
CL 19	86	3.79	0.26	65.8	22.1	33.5	14.4	5.89	3.48	3.42	0.06	2.12	0.24	0.06	0	24	16.6	2+	2	1+	/
CL 20	52	2.4	0.16	66.6	21.7	32.5	17.8	8.5	7.14	7.14	0	1.11	0.26	0	0	17	17.4	4+	/	3+	5.48
CL 21	67	2.84	0.2	71.3	23.4	32.9	14.3	2.38	1.62	0.71	0.9	0.33	0.38	0.05	0	14	14	3+	50	3+	/
CL 22	87	3.8	0.27	71.8	22.8	31.8	15.2	6.03	3.74	3.44	0.3	1.75	0.48	0.06	0	15	19.8	2+	7	1+	0.37
CL 23	74	3.36	0.26	76.4	22	28.8	14.3	5.92	3.5	3.27	0.23	1.43	0.77	0.2	0.02	54	14.2	3+	12	1+	2.05
CL 24	78	3.36	0.23	68.1	23.2	34.1	14.3	8.91	7.48	6.77	0.71	0.62	0.71	0.09	0	33	15.6	1+	2	/	1
CL 25	97	4.13	0.3	71.5	23.5	32.8	13	5.88	/	3.47	0	2.23	0.18	0	0	13	19.9	2+	1	1+	/
CL 26	128	5.63	0.35	61.8	22.8	36.8	16.5	16.2	/	8.75	3.89	1.3	2.27	0	0	30	13.2	3+	8	2+	/
CL 27	61	2.78	0.19	68.5	21.3	32.2	15.8	9.98	/	6.59	0.6	2.1	0.5	0.2	0	120	13.1	5+	4	1+	0.71
CL 28	146	5.88	0.43	72.6	24.8	34.2	13.2	4.14	/	3.24	0.5	0.17	0.17	0.08	0	13	17.6	2+	/	1+	/
CL 29	67	2.95	0.21	71.2	21.7	31.7	13.6	5	/	3.9	0	0.8	0.25	0.05	0	15	15.1	2+	/	1+	0.96
CL 30	67	2.9	0.21	74	23.2	31.3	18.1	10.41	/	8.74	0.21	1.25	0.1	0.1	0	42	13.7	5+	6	1+	3.75
CL 31	178	7.95	0.54	67.4	22.4	33.2	16.7	8.57	/	5.91	0.86	1.63	0.17	0	0	12	131	/	/	1+	/

Table 7. Control Group: Raw data of CBC.

Case Number	Hb g/l	RCC x10^12/L	Ht L/L	MCV fl	MCH g/dl	MCHC g/dl	RDW %	WCC x10^9/l	AbN (tot)x10^9/l	AbNmatx10^9/l	AbNimmX10^9/l	AbLymphox10^9/l	AbMonox10^9/l	AbEos x10^9/l	AbBaso X10^9/l	Plt C X10^9/l	MPV fl	Aniso
CL Con 1	136	6.27	0.41	65.6	21.8	33.2	13	6.58	3.16	3.03	0.13	1.84	0.39	0.92	0.26	237	11.6	1+
CL Con 2	181	7.74	0.54	69.4	23.3	33.6	13.3	15.9	10.49	10.49	0	3.82	0.32	1.27	0	312	8.1	1+
CL Con 3	151	6.85	0.46	67.4	22	32.7	12.6	8.03	4.66	4.66	0	2.25	0.48	0.48	0.16	32	14.8	1+
CL Con 4	182	7.81	0.56	71.2	23.3	32.7	13.6	9.08	5.99	5.99	0	1.82	0.18	1.09	0	460	10.3	1+
CL Con 5	151	6.33	0.45	70.8	23.8	33.7	15.3	10.06	7.44	7.44	0	1.81	0.2	0.6	0	52	13.7	3+
CL Con 6	174	7.49	0.53	71.2	23.2	32.6	13.6	6.69	4.42	4.42	0	1.07	0.13	0.94	0.13	197	10.2	1+
CL Con 7	180	7.75	0.54	69.3	23.2	33.5	14.1	10.26	6.57	6.57	0	3.28	0.41	0	0	277	7.5	0+
CL Con 8	194	8.31	0.59	71.3	23.4	32.8	12.7	7.44	3.57	3.42	0.15	1.04	0.6	2.23	0	357	7.5	1+
CL Con 9	172	7.09	0.52	73.1	24.2	33.2	13.8	10.28	4.32	4.32	0	1.64	1.03	3.29	0	224	8.9	1+
CL Con 10	186	7.95	0.54	68	23.5	34.5	13.4	7.35	3.53	3.53	0	1.91	0.15	1.76	0	212	7.5	1+
CL Con 11	198	8.22	0.601	72.5	24.1	33.2	13.7	9.58	5.72	5.72	0	2.85	0.35	0.62	0.04	320	7.8	1+
CL Con 12	160	6.73	0.48	71.5	23.8	33.3	13.6	15.88	10.66	10.66	0	3.11	1.4	0.68	0.03	291	8.7	2+
CL Con 13	125	5.31	0.38	71.1	23.6	33.2	14.7	19.87	9.14	8.74	0.4	7.95	1.19	1.59	0	401	8.5	2+
CL Con 14	181	7.68	0.54	70.3	23.6	33.5	13	10.54	7.73	7.73	0	1.8	0.26	0.73	0.02	457	8.5	1+
CL Con 15	184	7.5	0.55	73.3	24.5	33.4	13.2	8.47	4.91	4.91	0.34	2.46	0.08	0.51	0.17	400	7.8	2+
CL Con 16	174	7.44	0.52	69.3	23.4	33.8	14.3	9.56	3.35	3.35	0.48	4.97	0.19	0.19	0.38	377	8.9	2+
CL Con 17	182	7.9	0.57	71.6	23.1	32.2	14.1	12.74	5.1	5.1	0	4.33	0.51	2.8	0	392	8.3	1+
CL Con 18	142	6.25	0.43	68.6	22.7	33.1	14.1	9.34	4.67	4.67	0	2.9	0.19	1.59	0	678	9.5	1+