THROMBOELASTOGRAPHIC PLATELET MAPPING IN DOGS WITH COMPLICATED BABESIA ROSSI INFECTION

PLATELET MAPPING IN CANINE BABESIOSIS

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ABSTRACT

Background: Dogs with Babesia rossi infection display a normocoagulable thromboelastogram, despite being markedly thrombocytopenic. This is purportedly due to large-scale platelet activation. Thromboelastographic platelet mapping (TEG-PM) evaluates individual contributions of thrombin, fibrinogen and platelets to clot formation, and may elucidate some of the pathomechanisms of thrombocytopenia-associated hemostatic alterations.

Objective: This study investigated potential differences in TEG-PM variables in dogs with complicated B. rossi infection compared to healthy controls, and whether these variables correlated with indices of platelet activation.

Methods: The maximum amplitude (MA) following thrombin generation (MA\textsubscript{Thrombin}) was determined using kaolin-activated TEG. TEG-PM variables included MA following addition of platelet agonists arachidonic acid (MA\textsubscript{AA}) and adenosine diphosphate (MA\textsubscript{ADP}), and MA due to fibrin alone (MA\textsubscript{Fibrin}). In addition, platelet indices and fibrinogen concentration were determined.

Results: Thirteen dogs with complicated B. rossi infection and five healthy controls were included. The median MA\textsubscript{Fibrin} and fibrinogen concentration were significantly higher ($P < .01$ for both) and platelet count was significantly lower ($P < .01$) in the babesiosis group versus controls. No significant differences were found for MA\textsubscript{Thrombin} and MA\textsubscript{AA/ADP}. MA\textsubscript{Fibrin} was positively correlated with fibrinogen concentration ($r = 0.735$), mean platelet volume ($r = 0.517$) and mean platelet mass ($r = 0.498$), and negatively correlated with hematocrit ($r = -0.685$), platelet count ($r = -0.476$) and plateletcrit ($r = -0.479$) ($P < .05$ for all).
**Conclusions:** This study suggests that the presence of hyperfibrinogenemia offsets the severe thrombocytopenia associated with *B. rossi* to result in normal thromboelastograms and lack of overt clinical bleeding.

**Keywords**

Dogs; Hyperfibrinogenemia; MA_{Fibrin}; Thrombocytopenia
INTRODUCTION

Canine babesiosis is an intra-erythrocytic tick-borne protozoal disease, of which *Babesia rossi* is the most virulent form.¹ The pathogenesis involves a marked pro-inflammatory host response which is likely responsible for the development of complicated disease.² ³ Severe thrombocytopenia is common in canine babesiosis, yet such cases seldom display overt signs of hemorrhage⁴ ⁵, and have been reported to be normocoagulable.⁵ While evidence exists that platelet activation secondary to the inflammatory process may offset the thrombocytopenia⁵–⁷, the receptor-mediated mechanisms by which this occurs are unknown.

Platelet activation occurs when an agonist binds to a receptor, leading to activation of downstream signaling pathways to result in degranulation, translocation of negatively charged membrane phospholipids to the outer leaflet, and activation of the glycoprotein (GP)_{IIbIIIa} receptor responsible for binding fibrinogen. Agonists include thrombin, collagen, adenosine diphosphate (ADP), and thromboxane A₂ (TxA₂) generated from arachidonic acid (AA).⁸ Activated platelets provide a procoagulant surface for large-scale thrombin generation.⁸ ⁹

Thromboelastography (TEG) is a global assessment of hemostasis¹⁰, and relies on thrombin generation to cleave fibrinogen and activate platelets.¹¹ The maximum amplitude (MA) is a function of the dynamic interaction between platelets and fibrinogen.¹⁰ This modality provides information regarding the patient’s maximum hemostatic potential, but is non-specific for platelet activity.¹⁰ Thromboelastographic platelet mapping (TEG-PM) evaluates the individual contributions of thrombin, fibrin and platelet activity to clot formation.¹² Heparinized blood, in combination with an activator, bypasses thrombin activity, allowing for assessment of the contributions of fibrin and milder platelet agonists such as ADP and AA.¹¹ ¹²
In people, TEG-PM is frequently employed to monitor the efficacy of antiplatelet therapy. TEG-PM has been reported to be useful for identifying alterations in platelet receptor function during cardiopulmonary bypass, traumatic brain injury and multisystem trauma.16 There is a paucity of literature regarding TEG-PM in veterinary medicine. To date, pilot studies have been conducted in dogs and cats, and a small number of studies have used TEG-PM to assess the effects of various medications on platelet function.18–20 Only one study has focused on TEG-PM findings in dogs with a clinical disease associated with hypercoagulability, namely hyperadrenocorticism (HAC).21

The objectives of this study were to investigate possible differences in TEG-PM variables in dogs with complicated B. rossi infection compared to healthy controls, and determine whether TEG-PM variables correlated with any hematological variables.

**MATERIALS AND METHODS**

This prospective study was conducted on client-owned dogs presenting to the Onderstepoort Veterinary Academic Hospital (OVAH) of the University of Pretoria, South Africa. The babesiosis group consisted of dogs naturally infected with B. rossi with clinical or laboratory evidence of complicated disease. The initial diagnosis of babesiosis was made by demonstrating the presence of intra-erythrocytic trophozoites on a stained thin blood film. Infection with B. rossi was confirmed by means of PCR and reverse line blot (RLB).22,23 Healthy dogs presenting for routine sterilization or blood donation were included as controls. This study was approved by the University of Pretoria’s Animal Ethics Committee (Protocol number V098-15; 30 October 2015). Signed owner consent was required to enroll an animal in the study.
Animals

Dogs of any breed and either sex with demonstrable parasitemia were considered for inclusion into the babesiosis group, provided they were older than six months and weighed more than 3.5 kg. The infected dogs had to demonstrate one or more of the following manifestations of complicated babesiosis: secondary immune-mediated hemolytic anemia (IMHA; marked spherocytosis, positive warm in-saline agglutination or Coombs test result), acute kidney injury (AKI; oliguria/anuria and persistent azotemia unresponsive to appropriate fluid therapy), hypoglycemia (blood glucose < 3.3 mmol/L), cerebral babesiosis (neurological signs not attributable to any other cause), hepatopathy with cholestasis (icterus, marked bilirubinuria, hyperbilirubinemia, raised liver enzyme activities), acute respiratory distress syndrome (ARDS; dyspnea, adventitious lung sounds, frothy blood-tinged nasal discharge, blood-gas evidence of ventilation-perfusion mismatch, radiological evidence of edema and lung consolidation), hemoconcentration (congested mucous membranes, high-normal or raised HCT with evidence of concurrent intravascular hemolysis), and pancreatitis (vomiting, cranial abdominal pain, melena and icterus; raised serum lipase activity; or ultrasonographic evidence of acute pancreatitis).1,24,25

The *Babesia*-infected dogs were treated according to the standard protocol in use at the OVAH. This included treatment with diminazene aceturate (Berenil RTU 0.07 g/mL, Intervet South Africa, Spartan, Kempton Park, South Africa), transfusion with blood products, or intravenous fluid therapy as deemed necessary. Complications were treated as needed. Dogs in the control group were deemed clinically healthy based on a physical examination, CBC and blood smear evaluation, serum biochemical profile, and urine analysis, and if they were free of any parasitemia as determined by PCR and RLB. The same requirements regarding age and weight, as mentioned for the babesiosis group, applied.
For both groups, dogs were excluded if they had clinical evidence of any concurrent inflammatory or neoplastic condition, or if any evidence of trauma or wounds was present. Dogs treated with any medications known to interfere with platelet function within four weeks prior to presentation were excluded. Dogs were also excluded if they were found to be co-infected with other tick-borne agents, such as *Ehrlichia canis*, *B. vogeli*, *Theileria* and *Anaplasma* species, as determined by PCR and RLB.

**Sample collection**

At presentation and prior to any treatment (including blood products or intravenous fluids), blood was collected from the jugular vein from each dog using a 21-gauge needle by careful venipuncture with minimum stasis. Blood was collected into serum, sodium citrate, lithium heparin and EDTA vacutainer tubes (BD Biosciences, Franklin Lakes, NJ, USA), in this order. All tubes were filled, using vacuum assistance, to ensure the correct ratio of anticoagulant to blood. The EDTA sample was used to perform the CBC and the PCR/RLB analysis. Standard kaolin-activated TEG was performed on the sodium citrate sample, after which the remainder was centrifuged within one hour at 2100 g for 8 minutes. The plasma was stored at –80 °C for determination of fibrinogen concentration and antithrombin activity at a later stage. The lithium heparin sample was used to perform the TEG-PM analysis.

**Thromboelastographic platelet mapping analysis**

Two thromboelastograph hemostasis analyzers (TEG 5000 Thrombelastograph Hemostasis System, Haemonetics Corporation, Braintree, MA, USA) were used. The sodium citrate and lithium heparin samples were left to equilibrate at room temperature for 30 minutes post-
collection while four TEG cups were pre-warmed to 37 °C. Following equilibration, 1 mL of citrated blood was added to a kaolin-coated vial, which was gently inverted five times. Following addition of 20 µL CaCl₂ to the cup in channel 1, 340 µL of citrated kaolin blood was transferred to this cup. Standard TEG analysis was performed to determine MA due to the action of thrombin (MA<sub>Thrombin</sub>). To determine the contribution of cross-linked fibrin to clot formation, 10 µL of the activator substance (Activator F, Haemonetics Corporation, Braintree, MA, USA), a reagent containing reptilase (an enzyme with thrombin-like activity capable of cleaving fibrinogen to fibrin) and Factor XIII (required for fibrin cross-linking) were transferred to the cup in channel 2, followed by 360 µL of heparinized blood. The mixture in the cup was gently mixed by partially aspirating the contents three times, after which MA<sub>Fibrin</sub> was measured. To evaluate the contribution of platelet agonist receptors to clot formation, 10 µL of Activator F and 360 µL of heparinized blood were added to channels 3 and 4. Then, 10 µL of ADP were added to channel 3 to yield a final ADP concentration of 2 µmol/L. Similarly, 10 µL of AA were added to channel 4 to result in a final AA concentration of 1 mmol/L. Both samples were gently mixed, after which the MA<sub>ADP</sub> and MA<sub>AA</sub> were determined.

**Hematology variables**

A CBC was performed on an optical-based automated hematology system (ADVIA 2120, Siemens, Munich, Germany) and variables of interest included HCT, platelet count (PLT), plateletcrit (PCT), MPV, mean platelet mass (MPM), and mean platelet component concentration (MPC). Analysis was performed within 30 minutes of sample collection.
Plasma fibrinogen concentration and antithrombin activity

Plasma fibrinogen concentration was determined by the Clauss method using an automated coagulometric analyzer (ACL Elite, Instrumentation Laboratory, Bedford, MA, USA). Plasma antithrombin (AT) activity was assessed using a thrombin-dependent chromogenic substrate assay (Precimat Chromogen, Roche, Basel, Switzerland) on an automated analyzer (Cobas Integra 400 Plus, Roche, Basel, Switzerland). A normal pooled control sample, with a presumed activity of 100%, was run with each batch of tests, and the patient AT activity was normalized against the pooled control sample.

Statistical analysis

Statistical analyses were performed using a commercial software package (SPSS Statistics 24.0 Software; SPSS Inc., Armonk, NY, USA). Data were inspected for normality using the Shapiro-Wilk test and found not to display a normal distribution. Differences between groups were assessed using the Mann-Whitney U test. Correlations were determined using Spearman’s rank correlation coefficient. Gender proportions between groups were compared using the Fisher’s exact test. Data are presented as median and interquartile range (IQR). In addition, interindividual CV for patient and control groups were calculated for the TEG-PM variables by dividing the group standard deviation by the group mean and multiplying this by 100. A P value of < .05 was considered statistically significant.

RESULTS

Animals

Thirteen dogs were included in the babesiosis group, and five dogs in the control group. There were no significant differences in age between the groups, with the median patient age 24
months (12–52) and the median control age 48 months (19–82). The ratio of male: female for the Babesia-infected dogs was 7: 6 and the controls 3: 2, with no significant difference between the groups. The following complications were observed either singly or in combination: IMHA ($n = 4$), ARDS ($n = 2$), hepatopathy with cholestasis ($n = 3$), AKI ($n = 2$), neurological signs ($n = 4$), hypoglycemia ($n = 1$) and hemoconcentration ($n = 3$).

**Thromboelastographic platelet mapping analysis**

The TEG-PM variables are shown in Table 1. Median $MA_{\text{Thrombin}}$ did not differ significantly between the groups (Figure 1A); however, $MA_{\text{Fibrin}}$ was significantly higher in the babesiosis group compared to the control group (Figure 1B). No significant differences were observed between groups for median $MA_{\text{ADP}}$ (Figure 1C) and $MA_{\text{AA}}$ (Figure 1D). Inter-individual CV for the babesiosis and control groups were 21.1% and 5.9%, respectively for $MA_{\text{Thrombin}}$; 38.9% and 41.9%, respectively for $MA_{\text{Fibrin}}$; 48.3% and 92.9%, respectively for $MA_{\text{ADP}}$; and 38.7% and 41.6%, respectively for $MA_{\text{AA}}$. Representative TEG-PM tracings from a dog with babesiosis and a control dog are also shown to illustrate these findings (Figure 2).

**Hematological variables**

Results are shown in Table 1. The median HCT, PLT and PCT were significantly decreased, with the MPV and MPM significantly increased in the babesiosis group compared to the control group. No significant difference was noted for MPC.
<table>
<thead>
<tr>
<th>Variable (Unit)</th>
<th>Control dogs (n = 5)</th>
<th>Babesia-infected dogs (n = 13)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median (IQR)</td>
<td>Range</td>
<td>Median (IQR)</td>
</tr>
<tr>
<td>MA\textsubscript{Thrombin} (mm)</td>
<td>56.3 (55.9–61.0)</td>
<td>55.8–63.9</td>
<td>51.0 (43.1–62.2)</td>
</tr>
<tr>
<td>MA\textsubscript{Fibrin} (mm)</td>
<td>5.6 (3.4–7.8)</td>
<td>2.5–8.4</td>
<td>28.3 (23.5–36.6)</td>
</tr>
<tr>
<td>MA\textsubscript{ADP} (mm)</td>
<td>15.9 (7.8–60.5)</td>
<td>7.6–69.4</td>
<td>29.5 (24.2–48.1)</td>
</tr>
<tr>
<td>MA\textsubscript{AA} (mm)</td>
<td>44.8 (28.6–61.4)</td>
<td>16.6–65.7</td>
<td>44.9 (29.4–58.9)</td>
</tr>
<tr>
<td>Hematocrit (L/L)</td>
<td>0.49 (0.47–0.56)</td>
<td>0.47–0.58</td>
<td>0.18 (0.14–0.34)</td>
</tr>
<tr>
<td>Platelet count (×10(^9)/L)</td>
<td>225 (155–347)</td>
<td>151–467</td>
<td>29 (22–50)</td>
</tr>
<tr>
<td>Plateletcrit (%)</td>
<td>0.22 (0.18–0.43)</td>
<td>0.18–0.55</td>
<td>0.06 (0.04–0.11)</td>
</tr>
<tr>
<td>Mean platelet volume (fL)</td>
<td>11.8 (10.6–12.8)</td>
<td>9.6–13.6</td>
<td>21.1 (16.6–23.2)</td>
</tr>
<tr>
<td>Mean platelet mass (pg)</td>
<td>2.24 (1.74–2.42)</td>
<td>1.60–2.53</td>
<td>3.09 (2.68–3.48)</td>
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<tr>
<td>Mean platelet component concentration (g/dL)</td>
<td>21.5 (18.6–23.0)</td>
<td>16.9–24.0</td>
<td>19.7 (19.0–22.0)</td>
</tr>
<tr>
<td>Fibrinogen (g/L)</td>
<td>2.6 (1.9–3.0)</td>
<td>1.7–3.0</td>
<td>6.9 (6.3–7.0)</td>
</tr>
<tr>
<td>Antithrombin activity (%)</td>
<td>106 (99–109)</td>
<td>94–110</td>
<td>78 (71–96)</td>
</tr>
</tbody>
</table>

*Denotes statistical significance between groups.

Data are represented as medians (IQR), followed by the minimum-maximum range. MA indicates maximum amplitude; IQR, interquartile range.
Figure 1. Dot plots of the thromboelastographic platelet mapping variables (A) $\text{MA}_{\text{Thrombin}}$, (B) $\text{MA}_{\text{Fibrin}}$, (C) $\text{MA}_{\text{ADP}}$, (D) $\text{MA}_{\text{AA}}$ of the Babesia-infected group compared with the control group. Each dot represents an individual result, and the group medians are indicated by the horizontal lines. Maximum amplitude = MA; ADP = adenosine diphosphate; AA = arachidonic acid
Figure 2. Thromboelastographic platelet mapping tracings from (A) a healthy control dog and (B) a dog with complicated *Babesia rossi* infection. The white line indicates the maximum amplitude due to thrombin generation (MA_{Thrombin}); the green and pink lines denote the maximum amplitude due to fibrin (MA_{Fibrin}) and the maximum amplitude due to adenosine diphosphate (MA_{ADP}), respectively.
Plasma fibrinogen concentration and antithrombin activity

Results are shown in Table 1. The median fibrinogen concentration was significantly increased and the AT activity was significantly decreased in the babesiosis group compared to the control group.

Correlation analysis

The $\text{MA}_{\text{Thrombin}}$ had significant strong positive correlations with PLT ($r = 0.709; P = 0.001$) and PCT ($r = 0.707; P = 0.001$). The $\text{MA}_{\text{Fibrin}}$ had significant moderate to strong positive correlations with MPV ($r = 0.523; P = 0.026$), MPM ($r = 0.509; P = 0.031$) and fibrinogen concentration ($r = 0.742; P < 0.001$), and significant moderate to strong negative correlations with HCT ($r = -0.690; P = 0.002$), PLT ($r = -0.478; P = 0.045$), and PCT ($r = -0.481; P = 0.044$).

DISCUSSION

This is the first report to show the effect of hyperfibrinogenemia, using TEG-PM, on the hemostatic function of dogs with complicated babesiosis. The findings suggest that the presence of marked hyperfibrinogenemia and the resultant contribution of fibrin to clot strength offsets the severe thrombocytopenia associated with babesiosis, to result in a lack of clinical bleeding.

The study showed no significant difference for $\text{MA}_{\text{Thrombin}}$ between the $\text{Babesia}$-infected and healthy control dogs. $\text{MA}_{\text{Thrombin}}$ is the maximum amplitude determined using standard kaolin-activated TEG. Our results are similar to a previous report on uncomplicated babesiosis, using tissue factor-activated TEG. The authors in that study suggested two possible reasons for their findings. Since TEG is influenced by HCT, PLT, platelet function, fibrinogen concentration and AT activity, the normocoagulable thromboelastograms could have been the result of the opposing forces of these variables on the overall tracing. The second explanation was that
Babesia-infected dogs remained normocoagulable, despite severe thrombocytopenia, due to marked platelet activation associated with inflammation. Subsequently, studies confirmed the presence of activated platelets in dogs with babesiosis based on the presence of known and surrogate markers of platelet activation, namely increased circulating platelet-monocyte aggregates and increased MPV and MPM.\textsuperscript{6,7} Although MA\textsubscript{Thrombin} may be affected by PLT, platelet function and plasma fibrinogen concentration\textsuperscript{10}, our study showed a significant correlation with PLT only, but not with any of the markers for platelet activity. HCT is also an important variable to consider, because it has been shown that decreased HCT results in more hypercoagulable viscoelastic tracings.\textsuperscript{26,27} However, similar to a previous report, no correlation was seen with HCT in our study cohort.\textsuperscript{5}

MA\textsubscript{Fibrin} was significantly increased in the Babesia-infected dogs compared to the controls. Rotational thromboelastometry (ROTEM) studies have demonstrated that adequate fibrinogen concentrations are critical for clot formation.\textsuperscript{28–30} Even in the face of severe thrombocytopenia, the maximum clot elasticity (MCE) determined by ROTEM increased exponentially when increasing concentrations of fibrinogen were added.\textsuperscript{29} Moreover, the use of ROTEM, modified by the addition of cytochalasin-D (FIBTEM) to isolate the contribution of fibrinogen to clot strength, showed that MCE\textsubscript{Fibtem} displayed a similar exponential increase as fibrinogen concentrations increased.\textsuperscript{29} Canine babesiosis is characterized by an acute phase response with increases in acute phase proteins, including fibrinogen\textsuperscript{5,31–33}, and all but one of the Babesia-infected dogs in our study were hyperfibrinogenemic. The strong positive correlation between MA\textsubscript{Fibrin} and plasma fibrinogen concentration in our study supports previous reports on the effect of hyperfibrinogenemia in coagulation, especially in the presence of thrombocytopenia. Similar findings have been reported in dogs with pituitary-dependent HAC,
in which affected dogs had hyperfibrinogenemia and higher median $M_{nf}^{\text{Fibrin}}$ values than healthy controls at all assessed time points.\textsuperscript{21} The HAC study also reported a weak positive correlation between $M_{nf}^{\text{Fibrin}}$ and plasma fibrinogen concentration.

Interestingly, the contribution of platelets to clot formation increases in a similar fibrinogen concentration-dependent pattern. Each platelet bears numerous GP\textsubscript{IIbIIIa} receptors on its surface, and when activated by thrombin, platelets are able to bind high numbers of fibrinogen molecules.\textsuperscript{34} Thus, each individual platelet may become increasingly engaged with fibrinogen via its GP\textsubscript{IIbIIIa} receptors as the overall platelet count decreases and platelet volume increases.\textsuperscript{29} Given the inflammatory nature of the disease which could potentially result in both hyperfibrinogenemia and platelet activation, it is plausible that this mechanism is also at play in dogs with babesiosis. This may explain the significant and positive correlation of $M_{nf}^{\text{Fibrin}}$ with MPV and MPM. Moreover, enhanced thrombopoiesis and platelet regeneration in response to the inflammatory process and thrombocytopenia may have also resulted in increased MPV and MPM. Younger platelets tend to be larger and more hemostatically active, and both platelet size and immaturity are independent determinants of platelet function.\textsuperscript{35,36} The negative correlation between $M_{nf}^{\text{Fibrin}}$ and PLT is also noteworthy and supports the theory of platelets becoming increasingly engaged with fibrinogen as their numbers decrease, as previously stated.\textsuperscript{29} A possible explanation for the negative correlation with HCT is that a defined volume of anticoagulated blood is used for the TEG assay and erythrocytes act as a functional diluent for plasma, limiting the volume of plasma and plasma coagulation proteins, included in the assay. A whole blood sample with a low HCT will consequently allow for more plasma coagulation proteins, specifically fibrinogen, to be included in the assay.\textsuperscript{26,27}
Besides hyperfibrinogenemia, other reasons for high $\text{MA}_{\text{Fibrin}}$ include prior platelet sensitization and AT deficiency.\textsuperscript{11} Although prior platelet sensitization during blood collection cannot be excluded\textsuperscript{11,17}, a standardized sampling method was employed for our study, which makes this an unlikely reason for the significant difference between the groups. Since heparin requires AT to inhibit thrombin, it is conceivable that the lower median AT activities observed in dogs with babesiosis could have contributed to the increased $\text{MA}_{\text{Fibrin}}$ values. However, no significant correlation was observed between $\text{MA}_{\text{Fibrin}}$ and AT activity in our study. In addition, although the study on dogs with HAC reported persistently elevated $\text{MA}_{\text{Fibrin}}$ compared to controls, the median AT activity was within the reference interval, which further mitigates against low AT activity as a possible cause.

Previous studies in dogs\textsuperscript{17}, cats\textsuperscript{11} and horses\textsuperscript{18} showed that the $\text{MA}_{\text{Fibrin}}$ is prone to a high degree of intra-and inter-individual variability, with extremely high CVs being obtained in the dog study ($>100\%$). Similarly, the HAC study found significant overlap between $\text{MA}_{\text{Fibrin}}$ in affected dogs and controls.\textsuperscript{21} In our study, the inter-individual CVs for both the babesiosis and control groups were significantly lower (38.9\% and 41.6\%, respectively) than previously reported values; and although the sample sizes were small, no overlap was seen between the groups. This, together with a plausible underlying pathomechanism to explain the increased $\text{MA}_{\text{Fibrin}}$, suggests that the difference between the groups is due to disease and not biological or analytical variation. Nevertheless, assay analytical precision, as well as biological variation, should be investigated further.

The lack of significant difference between groups for $\text{MA}_{\text{ADP}}$ and $\text{MA}_{\text{AA}}$ was unexpected, given that platelet activation has been reported to occur in dogs with canine babesiosis. However, the inter-individual variation for both variables was significant, which is similar to previous
studies in dogs\textsuperscript{17,37} and cats.\textsuperscript{11} There are several potential explanations for the lack of significance, such as the use of suboptimal agonist concentrations.\textsuperscript{17} The TEG-PM kit contains 2 \textmu{}mol/L ADP and 1 mmol/L AA, compared to 5–20 \textmu{}mol/L of ADP and 1 mmol/L AA typically required in aggregometry.\textsuperscript{20} An additional reason could be that the functions of the receptors for ADP and TXA\textsubscript{2} may not be altered appreciably in all dogs during \textit{B. rossi} infection, especially considering the clinical and clinicopathologic heterogeneity seen in infected dogs.\textsuperscript{24} However, the study cohort included dogs with complicated disease, which is associated with an excessive pro-inflammatory response.\textsuperscript{3} Since ADP is released by platelet dense granules during platelet activation to augment the activation response initiated by other agonists\textsuperscript{8}, and AA is an important inflammatory mediator, it would seem unlikely that the function of these receptors was unaltered in this population of dogs. It is also possible that the variation in the results is due to biologic variability, as a great deal of heterogeneity exists regarding the response of platelets to various agonists in dogs.\textsuperscript{37–39} The lower HCT could also have affected both the MA\textsubscript{AA} and MA\textsubscript{ADP} of the \textit{Babesia}-infected group; however, no significant correlation with HCT was observed.

Our study had some limitations. Sample sizes were small due to financial constraints, which may have resulted in failure to detect significant changes in the MA\textsubscript{ADP} and MA\textsubscript{AA}. Due to the nature of sample collection from clinical cases, sampling at a standardized time point in the disease course was not possible. This may also have introduced heterogeneity into the study population. More reliable assays for determination of platelet activation, such as flow cytometry or aggregometry, were not performed, thus it was not possible to show conclusively, using surrogate markers of platelet activation such as raised MPM and MPV, that platelet activation occurred in the dogs of this study. In addition, the TEG-PM assay has not been validated for use
in dogs, but several published studies have used this methodology.\textsuperscript{11,17–21} Lastly, the analytical variation of the variables is presently unknown.

In conclusion, the findings of this study suggest that hyperfibrinogenemia in the presence of large platelets, secondary to either concurrent activation or regeneration, overrides the severe thrombocytopenia associated with canine babesiosis to result in normal thromboelastograms and absence of clinical bleeding.

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