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Evaluation of Platelet Aggregation Using a Point-Of-Care Instrument in Retired Racing Greyhounds

C.G. Couto, A. Lara, M.C. Iazbik, and M.B. Brooks

Veterinarians involved in Greyhound rescue have anecdotally observed that 10–15% of Greyhounds bleed profusely after simple surgical procedures. In most patients, platelet counts and hemostasis profiles are normal; therefore, it is possible that these dogs have platelet dysfunction. The PFA-100[a] is a novel point-of-care platelet function analyzer that has recently been evaluated as a rapid method to assess platelet function in dogs. The objectives of this study were to characterize platelet function in a group of healthy Greyhounds by means of the PFA-100. Blood samples were collected from the jugular vein from 30 healthy Greyhounds. CBC, biochemical profile, PFA-100 assay with collagen/epinephrine (COL-EPI) and collagen/adenosindiphosphate (COL-ADP), plasma von Willebrand factor antigen concentration (vWF : Ag), and vWF collagen-binding assay (vWF : CBA) were performed. PFA-100 closure times (CTs) with COL/ADP ranged from 63 to 92 seconds (mean ± SD, 74.7 ± 7.9 seconds) and with COL/EPI from 87 to 238 seconds (138 ± 41 seconds); vWF : Ag ranged from 22 to 120% (87.52 ± 25.5%) and vWF : CBA ranged from 36 to 102% (77.4 ± 17.3%); and platelet counts ranged from 147 to 265 × 10^9/L (194.6 ± 31.64 × 10^9/L). Greyhound CTs were significantly shorter than CTs in a mixed population of 50 healthy non-Greyhound dogs, in which the COL/ADP CTs ranged from 61 to 172 seconds (mean ± SD, 87 ± 21.6 seconds), and the COL/EPI CTs ranged from 81 to 300 seconds (mean ± SD, 183 ± 67.6 seconds; $P = 0.005$ for COL/ADP CT; $P = 0.001$ for COL/EPI CT). Also, platelet counts were significantly lower ($P = 0.001$) and packed cell volume was significantly higher ($P = 0.001$) in the Greyhound than in the non-Greyhound group. The PFA-100 is a reproducible method that can be used in the clinical setting to assess platelet function in Greyhounds; however, normal CTs in healthy Greyhounds are shorter than in other breeds. The results obtained in this study will be used to screen for abnormal platelet function in Greyhounds with postoperative bleeding.

Key words: Bleeding diathesis; Dog; Platelet function; Primary hemostasis.

With the increasing popularity of retired racing Greyhounds, veterinarians are likely to examine dogs of this breed in their practices. It is therefore important to recognize the hematologic and biochemical peculiarities of this breed. For instance, mean packed cell volume (PCV), hemoglobin concentration, red blood cell count (RBC), and whole blood viscosity are higher, whereas white blood cell, neutrophil, and platelet counts are lower in Greyhounds than in other breeds.[1–3] Serum creatinine concentrations and liver transaminase activities are higher than in non-Greyhound dogs, and serum total protein, globulin, alpha-globulin, and beta-globulin concentrations are lower than in other dog breeds.[1,4–5]

In addition to known variations in physiologic variables, Greyhounds appear to be at risk for breed-specific disease syndromes. In the Greyhound community, the term ''Greyhound bleeder'' refers to dogs that bleed spontaneously after minor trauma or after a simple surgical procedure. Greyhound ''strokes'' and ''bleeding'' are also prominently mentioned in lay websites and in a survey of causes of death in Greyhounds in the UK.[6] We recently conducted a survey of 30 veterinarians involved in Greyhound rescue, inquiring about bleeding complications after surgery. Twenty veterinarians returned the survey, and the consensus opinion was that 10–15% of Greyhounds bleed profusely, frequently requiring a transfusion of blood components as a life-saving measure, 1–4 days after simple surgical procedures such as spays, neuters, dewclaw removals, or laparotomies. In those patients evaluated, the platelet count, von Willebrand factor (vWF) concentration (vWF : Ag), one-stage prothrombin time (OSPT), and activated partial thromboplastin time (APTT) were within normal limits at the time of postoperative hemorrhage. A more rigorous evaluation of hemostasis in Greyhounds is therefore required to characterize this apparent bleeding tendency.

Von Willebrand disease (vWD) is the most common canine hereditary hemostatic defect, and vWF-deficient Greyhounds have been identified in breed surveys.[7–8] In a recent 2-year period (July 2002 to July 2004) approximately 10% (22/216) of the Greyhounds screened at the Comparative Coagulation Section (Cornell University) had low plasma vWF : Ag, with values ≤30% (reference range >50%; M. Brooks, unpublished data). More detailed studies of primary hemostasis, including vWF and platelet function assays, are needed to clarify the clinical relevance of low vWF, in light of anecdotal reports of postsurgical and spontaneous hemorrhage in this breed. The buccal mucosa bleeding time (BMBT) has historically been used to assess platelet function and to screen for clinical expression of vWD in dogs, but this test has marked inter- and intraobserver variability, and is not highly reliable in the clinical setting.[9] Laboratory methods to assess platelet function include in vitro platelet aggregometry and flow cytometry, but these techniques are primarily limited to research institutions and are not easily applied to clinical

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studies because of the required sample preparation and sophisticated instrumentation.[10–12] Moreover, because of their high PCV and blood viscosity, and perhaps because of idiosyncrasies in platelet physiology, it has been extremely difficult for us to obtain platelet-rich plasma (PRP) for aggregometry studies in Greyhounds. Therefore, despite evaluating several techniques in the past, in vitro platelet aggregometry by means of PRP or whole blood is problematic in this breed.

The PFA-100[a] is an objective and sensitive point-of-care instrument to assess platelet function and to detect low concentration (or dysfunction) of vWF in people[13] and in dogs[14–17]; results are obtained in a short time and the instrument is easy to use. The device evaluates platelet adhesion and aggregation in citrated whole blood flowing under high shear through a capillary with an aperture coated by platelet agonists (collagen and either epinephrine or adenosine diphosphate [ADP]). The PFA-100 measures the time (closure time [CT]) required for a platelet plug to form in the capillary aperture and halt blood flow, thus simulating the in vivo conditions of endothelial damage in a capillary vessel.[18] Recently, several authors have demonstrated that the PFA-100 can detect abnormal canine platelet function associated with vWD, congenital thrombopathias, or administration of certain drugs.[14–16] This instrument has also been used to monitor response to DDAVP in dogs with type 1 vWD.[17] In addition, Tarnow et al.[b] recently demonstrated that dogs with subaortic stenosis had longer PFA-100 CTs and decreased platelet aggregation compared to control dogs. These abnormalities were attributed to decreased plasma vWF : Ag, with loss of high-molecular-weight vWF multimers, findings consistent with the classification of type 2 vWD.[b,19] Similar findings were reported in Cavalier King Charles Spaniels with myxomatous mitral valve disease.[20]

The purpose of this study was to investigate whether the PFA-100 could be used to evaluate platelet function in a group of healthy Greyhounds, and whether the results obtained were similar to those previously reported in healthy non-Greyhound dogs. Platelet count, plasma vWF : Ag, and vWF activity measured in a collagen binding assay (CBA), were correlated with the results of the PFA-100 assays.

Materials and Methods

Dogs

Thirty Greyhounds enrolled in The Ohio State University Animal Blood Bank were evaluated. There were 16 spayed females and 14 intact males; their ages ranged from 2 to 9 years (mean ± SD, 5 ± 2 years). All of the dogs were evaluated a minimum of 1 week after blood donation, and none had historical evidence of systemic disease or bleeding episodes (related or unrelated to surgical procedures) or had received any medication for 15 days before blood sampling; all dogs were serologically negative for heartworm antigen.[c] Physical examination findings were within normal limits, and blood was collected after a 12-hour fasting period for a CBC, serum biochemical profile, platelet function analysis with PFA-100, and plasma vWF : Ag and activity with CBA (vWF : CBA). Results of CBC and profile were all within the reference range for the breed.

Sample Collection and Handling

Blood samples were collected from the jugular vein through a clean and quick venipuncture in order to avoid platelet activation and sample clotting, by means of a sterile disposable 20-gauge needle attached to a 10-ml disposable syringe.[d] Blood was placed into siliconized tubes[e] without anticoagulant (2 mL), in a tube with 4 mg of ethylenediamenetetraacetic acid (EDTA; 2 mL), and in 2 tubes with 0.5 ml of 3.8% citrate (4.5 mL each).

The sample in EDTA was used for a CBC and platelet count; the nonanticoagulated blood sample and one of the tubes with citrate were centrifuged at 3,000 rpm for 10 minutes within an hour of blood collection, and the serum and plasma were separated; the serum was used for a biochemical profile, and the plasma was transferred to polypropylene transfer tubes and immediately frozen at −20°C for determinations of vWF : Ag and vWF : CBA. For the latter, samples were analyzed within 30 days of collection at the Comparative Coagulation Section (Animal Health Diagnostic Laboratory, Cornell University, Ithaca, NY). The second tube of citrated blood was stored at 24°C and processed in the PFA-100 between 30 minutes and 3 hours after collection.

Procedures

Complete blood counts were performed in a standard fashion by means of a Cell Dyne 3500R[f]; serum biochemical profiles were done on a Hitachi 911 Automatic Analyzer.[g]

Platelet function was evaluated with the PFA-100 by using citrated blood according to the manufacturer's instructions. This device has been described elsewhere in detail.[18,21] A daily self-test was always done before performing any assays. The reagents were stored at 4°C and prewarmed at room temperature no less than 15 minutes before use. After gentle mixing of the blood sample by means of a manual pipette, 800 µl was placed in each of the disposable cartridges in the device.

CTs were obtained using collagen/epinephrine (COL/EPI) and collagen/ADP (COL/ADP) cartridges. For each cartridge type, results were based on the mean of duplicate tests; the maximum time measured by the instrument is 300 seconds.[18,21] The device automatically calculates the mean of the 2 assays and the coefficient of variation (CV) as a measure of reproducibility. If results of duplicate tests deviated more than 20% from the mean, a third test was performed, provided that the sample volume allowed it.

Von Willebrand Factor Assays

Plasma vWF : Ag was measured in 23 of the Greyhounds by enzyme-linked immunosorbent assay configured with monoclonal anti-canine vWF antibodies.[22] This assay is a quantitative measure of total plasma vWF and is insensitive to abnormalities of vWF multimer size or function. The biologic activity of vWF was evaluated in a subgroup of 15 Greyhounds with the vWF : CBA, which assesses the ability of vWF to bind to collagen coated to a solid-phase support, primarily through its high-molecular weight multimers.[22–24] For these analyses, a previously described canine vWF : CBA[22] was modified by use of a commercially available mixed type I/II bovine collagen[h] to coat the microtiter plates. The procedures, reagents, and buffers for sample dilution and detection of bound vWF were the same as described for the vWF : Ag assay.[25]

Statistical Analysis

Graph Pad Prism software[i] was used for statistical analysis. Statistical descriptive parameters were calculated for all variables. The Kolmorogov-Smirnov test was used to assess normal distribution of the variables analyzed before using parametric

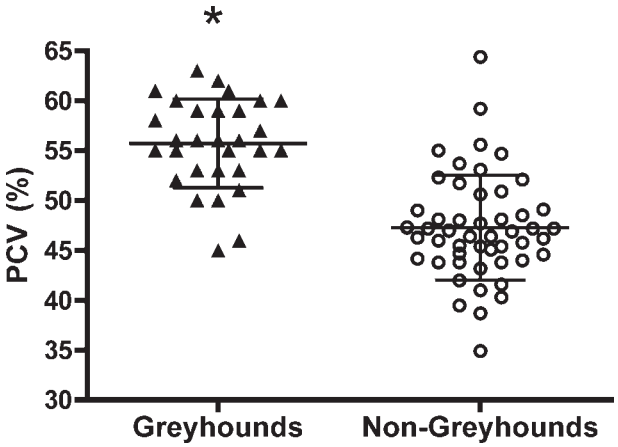

Fig 1. Scatter plot distribution of PCVs in 30 healthy Greyhounds and 50 healthy non-Greyhound dogs. Horizontal bars indicate mean ± SD for each group. Asterisk denotes significant difference between groups.

methods. Closure time data from Greyhounds and a historical group of normal non-Greyhound dogs evaluated by us by means of the same sample handling and testing methods[26] were compared by means of ANOVA for repeated measures; potential differences related to sex were also analyzed with this method. Mean CT in both groups was compared with other continuous variables by means of linear regression and Pearson's correlation coefficient. Student's *t*-test for independent variables was used to compare variables other than closure time between both groups of dogs. Differences were considered statistically significant at $P < 0.05$. For calculation purposes, CTs of >300 seconds were considered to be 300 seconds.

Results

Packed Cell Volume and Platelet Count

PCVs ranged from 45 to 63% (mean ± SD, 55.7 ± 4.5%; reference range 36–54%) and platelet counts ranged from 147 to 265 × 10^9/L (mean ± SD, 194.6 ± 31.6 × 10^9/L; reference range 106–424 × 10^9/L) (Figs 1, 2). In the historical non-Greyhound group, the PCV ranged from 34.9 to 64.4% (mean ± SD, 47.3 ± 5.2%; reference range 36–54%) and the platelet count ranged from 150 to 405 × 10^9/L (mean ± SD, 261.3 ± 68.2 × 10^9/L; reference range 106–424 × 10^9/L) (Figs 1, 2). The PCV was significantly higher ($P < 0.001$) and the platelet count significantly lower ($P < 0.001$) in the Greyhound group than in the historical controls.

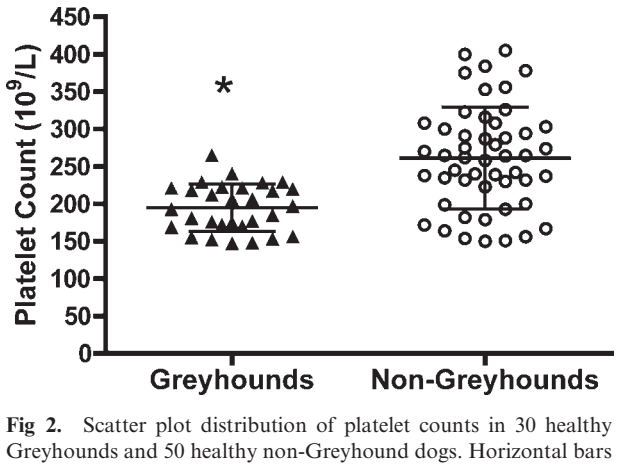

Fig 2. Scatter plot distribution of platelet counts in 30 healthy Greyhounds and 50 healthy non-Greyhound dogs. Horizontal bars indicate mean ± SD for each group. Asterisk denotes significant difference between groups.

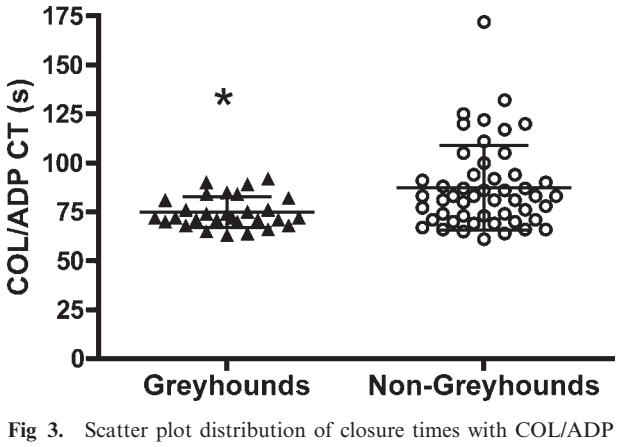

Fig 3. Scatter plot distribution of closure times with COL/ADP in 30 healthy Greyhounds and 50 healthy non-Greyhound dogs. Symbols represent means of duplicate readings for each dog. Horizontal bars indicate mean ± SD for each group. Asterisk denotes significant difference between groups.

Platelet Function

The mean CTs obtained by means of COL/ADP in the Greyhound group ranged from 63 to 92 seconds (mean ± SD, 74.7 ± 7.9 seconds; Fig 3); the mean CTs obtained by means of COL/EPI ranged from 87 to 238 seconds (mean ± SD, 138 ± 4 seconds; Fig 4). There was no significant correlation between CTs with COL/ADP and COL/EPI ($P = 0.382$, r = 0.168). None of the Greyhounds had a mean CT of 300 seconds.

The mean CV for COL/ADP CT was 5 ± 3.8% (range 0–13%), and for COL/EPI the mean CV was 11.2 ± 8.2% (range 0–29%); 4 dogs had a COL/EPI CV higher than 20%. There was no significant correlation between

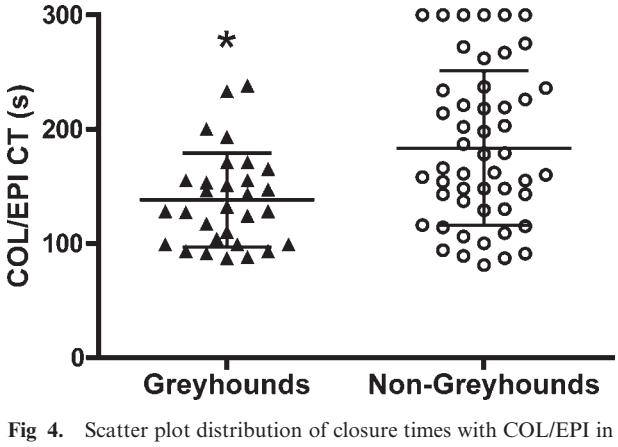

Fig 4. Scatter plot distribution of closure times with COL/EPI in 30 healthy Greyhounds and 50 healthy non-Greyhound dogs. Symbols represent means of duplicate readings for each dog. Horizontal bars indicate mean ± SD for each group. Asterisk denotes significant difference between groups.

sex or age and CT with either agonist, nor between CT and platelet count.

In the non-Greyhound historical control group, CTs with COL/ADP ranged from 61 to 172 seconds (mean ± SD, 87 ± 21.6 seconds); CTs with COL/EPI ranged from 81 to 300 seconds (mean ± SD, 183 ± 67.6 seconds). The mean CV for COL/ADP CT was 6.3 ± 4.7% (range 0–21%), and only 1 of the dogs had a CV higher than 20%. The mean CV for COL/EPI CT was 6.7 ± 6.9% (range 0–28.5%) and all but 2 dogs had CV less than 20%.[26]

The CTs obtained by means of both COL/ADP ($P = 0.005$) and COL/EPI ($P = 0.001$) were significantly shorter in the Greyhounds than in the non-Greyhound historical control dogs.

vWF Determinations

Plasma vWF : Ag (n = 23) ranged from 22 to 120% (mean ± SD, 87.5 ± 25.2%; reference range >50%). Three dogs had vWF : Ag below 2 SD from the group mean, with values of 49%, 34%, and 22%, respectively. The dog with 49% vWF : Ag also had the longest COL/EPI CT; however, this dog's COL/ADP CT was 85 seconds. The results of vWF : CBA (n = 15) ranged from 36 to 102% (mean ± SD, 77.4 ± 17.3%; reference range >50%), with all but 1 dog having values within the reference range. This dog (vWF : CBA = 36%) had vWF : Ag of 49%. The ratio of vWF : Ag to vWF : CBA ranged from 1.04 to 1.48 (mean ± SD, 1.23 ± 0.13; reference range <2), indicating proportionate values for protein concentration and function. The vWF : Ag and vWF : CBA results were strongly correlated ($P < 0.01$, $r = 0.862$). No significant correlation was found between vWF : Ag or vWF : CBA and COL/ADP CT or COL/EPI CT. Interestingly, vWF : CBA was significantly correlated with PCV ($P < 0.05$, $r = 0.531$).

Discussion

Spontaneous bleeding or bleeding after minimally invasive surgical procedures, such as ovariohysterectomy, orchiectomy, or dewclaw removal, appears to be relatively common in Greyhounds. We have also observed severe postoperative bleeding in Greyhounds 1–4 days after limb amputation for osteosarcoma or trauma, to the point that the majority of those dogs received blood component therapy (ie, fresh-frozen plasma or packed red blood cells). The affected dogs have no consistent abnormalities of OSPT, APTT, platelet count, BMBT, or vWF : Ag. These findings indicate that common canine bleeding disorders, such as thrombocytopenia and coagulation factor and vWF deficiencies, are unlikely to be the cause of abnormal bleeding in Greyhounds. These screening tests, however, do not evaluate all aspects of hemostasis and cannot rule out primary vascular defects, platelet or vWF dysfunction, or defects of fibrin clot stabilization and lysis.

Therefore, we undertook validation of the PFA-100 to determine the suitability of CT as a screening test of platelet function in Greyhounds, for future studies of spontaneous or postoperative bleeding in this breed.

Using the PFA-100, the mean CTs in the Greyhound group were significantly shorter than in the non-Greyhound historical control group with both COL/ADP and COL/EPI. However, both the COL/ADP and COL/EPI CT ranges were similar to those previously published in dogs. Callan and Giger[15] reported COL/ADP CTs of 52 to 86 seconds and COL/EPI CTs of 97–225 seconds, whereas Mischke and Keidel reported COL/ADP and COL/EPI CTs of 53–98 seconds and 92–300 seconds, respectively.[16] COL/ADP CTs in Greyhounds in our study ranged from 63 to 92 seconds, and CTs with COL/EPI ranged from 87 to 238 seconds. Interestingly, the lower platelet count in the breed was not associated with a prolongation of CTs with either agonist. In fact, Greyhounds had shorter CTs, likely because of the higher PCV in the breed.[1,3] Anemia results in marked prolongation of CTs with both COL/ADP and COL/EPI, to the point that the instruction manual of the PFA-100 recommends that this test should not be performed in anemic human patients (PCV < 35%).[j] Mischke and Keidel[16] demonstrated that decreasing the hematocrit of normal dogs from 40% to 30% by dilution with PRP and platelet-poor plasma resulted in a significant prolongation of CTs ($P = 0.0049$). Callan and Giger[15] also revealed that serial dilution of canine blood with PRP results in progressive prolongation of the COL/ADP CT; all dogs with PCVs between 18 and 23% had COL/ADP CTs of >300 seconds.

A low PCV decreases blood viscosity, and hence increases blood flow; moreover, anemia results in alterations of blood cell distribution in the vessels, and probably also in the capillary tube of the instrument, so that platelets are flowing more centrally and have less contact with the surface.[16,27] In humans, increases in the numbers of circulating red blood cells result in increases in the radial platelet movement; as a consequence, the interaction of red blood cells with the endothelium also increases.[27,28] This likely explains why the bleeding time in human uremic patients shortens after red blood cell transfusions.[28,29]

Greyhounds have significantly higher PCVs and whole blood viscosity than non-Greyhound dogs[30]; therefore, the opposite mechanism to that in anemic patients may be in operation (ie, platelet distribution is peripheral, thus increasing interaction with the surface). In humans, the CT shortens after artificial increases of hematocrit by the addition of autologous red blood cells, independently of the platelet count.[29]

Greyhounds also have significantly higher arterial blood pressure than non-Greyhound dogs.[31,32] The observed shorter CTs may be an adaptive platelet response to accommodate higher shear in the breed. In conclusion, the significantly shorter CTs in the Greyhound likely represent yet another manifestation of the distinct hemorrheology of the breed.

Several authors have already pointed out that, because of the variability of the COL/EPI results in dogs, these cartridges are not suited for platelet function evaluation in this species.[15,16] We obtained similar results in Greyhounds; there was no correlation between COL/ADP and COL/EPI CTs ($P = 0.382$), and the CV of

COL/EPI CTs was higher than with COL/ADP. Studies performed with turbidimetric aggregometry may corroborate this theory, because canine platelets exhibit a variable sensitivity to epinephrine-induced platelet aggregation, and that variability not only is interindividual, but also may be influenced by the breed.[33,34]

Three of the study dogs were vWF-deficient (vWF : Ag range 22–49%) and had a proportionate reduction in vWF activity (vWF : Ag to vWF : CBA ratio <2). These findings meet the criteria for a classification of Type 1 vWD.[19,22] Their COL/ADP CTs (82 to 85 seconds) were within the observed range for the remaining Greyhounds and our historical controls,[26] compatible with the reported variable sensitivity of PFA-100 CT to detect mild to moderate Type 1 vWD in human patients.[13,35] A previous study failed to demonstrate correlation of COL/ADP CTs with residual vWF : Ag in a population of 11 vWF-deficient dogs (vWF : Ag range 0–33%); however, all deficient dogs in that study had long CTs.[15] Further evaluation of the PFA 100 is needed in order to determine its ability to detect mild vWF deficiency and whether CT is related to clinical expression of a bleeding tendency in vWF-deficient dogs.

In conclusion, the PFA-100 is an easy-to-use, clinician-friendly method to assess platelet function in dogs. Our observed values of healthy Greyhound CTs are similar to reported values for other breeds,[15,16,20] indicating that the idiosyncrasies of Greyhound blood do not preclude the use of PFA-100 screening in this breed. Because of the variability of the BMBT and the technical difficulties related to obtaining PRP for aggregometry in this breed, the PFA-100 seems especially suited as a point-of-care instrument to evaluate primary hemostasis in Greyhounds with signs of a bleeding diathesis.

Footnotes

[a] PFA-100, Dade Behring, West Sacramento, CA
[b] Tarnow I, Falk T, Kristensen AM, et al. Decreased platelet function in dogs with subaortic stenosis. J Vet Intern Med 2004;18:449 (abstract)
[c] Dirochek, Synbiotics, San Diego, CA
[d] Monoject, Sherwood, St. Louis, MO
[e] Vacutainer tubes, Monoject, Sherwood, St. Louis, MO
[f] Abbott Diagnostics, Abbott Park, IL
[g] Roche Laboratories, Indianapolis, IN
[h] Vitrogen 100, Cohesion, Palo Alto, CA
[i] Graph Pad Software, San Diego, CA
[j] Dade PFA-100 Reagents, November 1998 edition, Dade Behring Inc, Newark, DE

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