

Intraoperative Bleeding in Dogs from Grenada Seroreactive to *Anaplasma platys* and *Ehrlichia canis*

M. Lanza-Perea, U. Zieger, B.A. Quorollo, B.C. Hegarty, E.L. Pultorak, S. Kumthekar, R. Bruhl-Day, and E.B. Breitschwerdt

Background: Frequent exposure of Grenadian dogs to *Rhipicephalus sanguineus* results in *Anaplasma platys*, and *Ehrlichia canis* seroreactivity. During elective surgeries, substantial intraoperative hemorrhage occurs in some seroreactive dogs.

Objectives: To assess hemostatic parameters and bleeding tendencies as well as prevalence of PCR positivity in apparently healthy *A. platys* and *E. canis* seroreactive and seronegative free-roaming dogs from Grenada.

Animals: Forty-seven elective surgery dogs allocated to 4 groups: Seronegative control (n = 12), *A. platys* (n = 10), *E. canis* (n = 14) and *A. platys*, and *E. canis* (n = 11) seroreactive.

Methods: Preoperatively, hemostasis was assessed by platelet count, prothrombin time, activated partial thromboplastin time, and buccal mucosal bleeding time. Intra- and postoperative bleeding scores were subjectively assigned. Blood, spleen, bone marrow, and lymph node aspirates were tested by PCR.

Results: Bleeding scores in dogs coseroreactive for *A. platys* and *E. canis* were higher ($P = .015$) than those of seronegative dogs. *A. platys* DNA was amplified from 7/21 (33%) *A. platys* seroreactive dogs and from 1 *E. canis* seroreactive dog; *E. canis* DNA was amplified from 21/25 (84%) *E. canis* seroreactive dogs. *E. canis* DNA was amplified most often from blood, whereas *A. platys* DNA was amplified most often from bone marrow.

Conclusions and Clinical Importance: Apparently healthy, free-roaming dogs coseropositive for *A. platys* and *E. canis* may have increased intraoperative bleeding tendencies despite normal hemostatic parameters. Future investigations should explore the potential for vascular injury as a cause for bleeding in these dogs. Improved tick control is needed for dogs in Grenada.

Key words: Hemostasis; Infection; *Rickettsia*; Ticks.

Infection with *Anaplasma platys* and *Ehrlichia canis* is diagnosed frequently in *Rhipicephalus sanguineus*-infested dogs located on the island of Grenada, West Indies.¹ Commonly called the brown dog tick or kennel tick, *R. sanguineus* is known to transmit *E. canis* and is suspected to be the primary vector for *A. platys*. It is the only tick species known to infest Grenadian dogs.¹ After tick transmission, *A. platys* infects platelets resulting in canine infectious cyclic thrombocytopenia, and *E. canis* infects both monocytes and lymphocytes in the dog causing canine monocytic ehrlichiosis. *A. platys* and *E. canis* seroreactivity is prevalent among Grenadian dogs. In a recent island-wide

From the School of Veterinary Medicine, St. George's University, St. George's, Grenada (Lanza-Perea, Zieger, Kumthekar, Bruhl-Day); and the Intracellular Pathogens Research Laboratory (IPRL), Center for Comparative Medicine and Translational Research, College of Veterinary Medicine (CVM), North Carolina State University (NCSU), Raleigh, NC (Quorollo, Hegarty, Pultorak, Breitschwerdt).

Sample collection and blood analyses were performed at the Veterinary Surgery Laboratory, St George's University, Grenada (West Indies); DNA extraction, PCR testing and DNA sequence analyses were performed at the Intracellular Pathogens Research Laboratory (IPRL), College of Veterinary Medicine North Carolina State University (NCSU-CVM), Raleigh NC; DNA sequencing was performed by GENEWIZ, Research Triangle Park, NC.

Corresponding author: Dr E.B. Breitschwerdt, College of Veterinary Medicine, North Carolina State University, 1060 William Moore Drive, Raleigh, NC 27607; e-mail: ed_breitschwerdt@ncsu.edu.

Submitted March 10, 2014; Revised July 16, 2014; Accepted July 24, 2014.

Copyright © 2014 by the American College of Veterinary Internal Medicine

DOI: 10.1111/jvim.12442

Abbreviations:

BCS	body condition score
CICT	canine infectious cyclic thrombocytopenia
CME	canine monocytic ehrlichiosis
ET	endothelin
GAP	<i>Anaplasma platys</i> seroreactive group
GAPec	<i>A. platys</i> and <i>E. canis</i> seroreactive group
GC	control group
GEC	<i>Ehrlichia canis</i> seroreactive group
HCT	hematocrit
IPRL	Intracellular Pathogens Research Laboratory
JSAL	junior surgery and anesthesia laboratory
NCNU-CVM	North Carolina State University College of Veterinary Medicine
RI	reference interval
rxn	reaction
SGU/SVM	St George's University School of Veterinary Surgery
TP	total protein

survey, 9% of dogs were *A. platys* seroreactive, and 34% were *E. canis* seroreactive (Lanza and Paterson, unpublished data, 2013). Presumably a portion of these seroreactive dogs are subclinically infected with 1 or both organisms.²⁻⁴ Currently, it remains unclear how often and to what extent dogs immunologically eliminate *A. platys* or *E. canis* after tick transmission, with or without developing disease.⁵ Due to the lack of long-term, serial, prospective studies, the consequences of persistent subclinical infection with these pathogens in naturally infected dogs remains incompletely understood.

Before initiation of this study, faculty surgeons at St. George's University (SGU) School of Veterinary

Medicine (SVM) had observed delayed hemostasis in approximately 30% of clinically healthy dogs seroreactive for *A. platys*, *E. canis* or both during elective surgery, resulting in excessive intraoperative bleeding (best characterized as persistent oozing of blood) into the surgical incisions. This observation is made less frequently in dogs seronegative for *A. platys*, *E. canis* or both. Therefore, the primary aims of this study were to assess hemostatic parameters and bleeding tendencies in apparently healthy *A. platys* and *E. canis* seroreactive and seronegative dogs from Grenada and to determine if seroreactive dogs were subclinically infected (ie, DNA detected in ≥ 1 tissues) with these rickettsial organisms.

Materials and Methods

Dogs

Between January 2012 and March 2013, 47 client-owned dogs admitted to SGU-SVM for elective ovariohysterectomy or castration surgery were entered into an Institutional Animal Care and Use-approved research study (IACUC-11009-R). Signed permission to obtain blood and tissue aspiration samples for research purposes was obtained from each owner. Most of these dogs originated from environments where they roam freely within the surrounding neighborhood and veterinary care is minimal or nonexistent. Before surgery, jugular blood samples were collected into ethylenediamine tetra acetic acid (EDTA) and heparin anticoagulant-coated tubes. Clinical signs and physical examination findings were recorded for each dog the day before and the day of surgery. Complete blood count (CBC) and serum biochemistry profile results were obtained preoperatively using a hematology analyzer (VetAutoread[®]) and a chemistry analyzer (VetTest[®]), respectively. To be approved for elective surgery, each dog had to be clinically healthy based upon the physical examination inclusion criteria: a minimum body condition score of 2 of 5, rectal temperature between 99.5 and 102.5°F, normal thoracic auscultation findings, bilaterally descended testicles in male dogs, and absence of any of the following: pyoderma, severe orthopedic disease, and cutaneous or intra-abdominal masses. Hematological and biochemical inclusion criteria included: hematocrit (Hct) >25%, platelet count >150 × 10³/μL, total plasma protein concentration (TP) >5.2 g/dL, alanine aminotransferase activity of 10–100 IU/L, alkaline phosphatase activity of 23–212 IU/L, blood urea nitrogen concentration 7–27 mg/dL, and serum creatinine (CREA) concentration 0.5–1.8 mg/dL. Exposure to selected vector-borne pathogens was determined using a commercial in-house enzyme-linked immunosorbent assay (ELISA)-based kit (SNAP[®] 4DX[®]). Dogs infected with *Dirofilaria immitis* were excluded from the study. Based upon ELISA results, dogs that met the inclusion criteria were allocated to 1 of 4 study groups: Control (C) dogs were *A. platys* and *E. canis* seronegative by ELISA testing, *A. platys* (Ap) dogs were seroreactive to *A. platys* only, *E. canis* (Ec) dogs were seroreactive *E. canis* only, *A. platys* and *E. canis* (ApEc) dogs were seroreactive to both *A. platys* and *E. canis*.

Anesthesia

Before surgery, all dogs were premedicated with acepromazine^b (0.05 mg/kg) and morphine^c (0.3 mg/kg), induced with propofol (Propofol, Propoflo28, 10 mg/mL propofol^d) (4 mg/kg), and maintained under isoflurane (Isoflurane IsoFlo, 250 mL⁴) anesthesia with oxygen supplementation, in accordance

with junior surgery and anesthesia laboratory (JSAL) protocols. All dogs received morphine (0.3 mg/kg) postsurgically. In addition, all female dogs and any male dog in clinically apparent pain received buprenorphine^e (0.02 mg/kg) within 5 hours after surgery.

Coagulation Testing

A buccal mucosal bleeding time (BMBT) test was performed the day before surgery using a bleeding time device^f and filter paper. Time until cessation of bleeding was recorded. A previously published reference range of 1.7–4.2 minutes was considered a normal bleeding time.⁶ Because of their fractious nature, bleeding time assessment was performed on the day of surgery for 5 dogs (5/47; 10.6%) after sedation with acepromazine and morphine. Sedation with these drugs does not interfere with clot formation.⁷ Prothrombin time (PT) and activated partial thromboplastin time (aPTT) were determined using citrated whole blood samples and measured with a coagulation analyzer.⁸ A bleeding index based on a previous evaluation of bleeding times in greyhound dogs,⁸ originally adapted and modified from a study evaluating children with idiopathic thrombocytopenic purpura,⁹ was used to measure the peri- and intraoperative bleeding tendencies for each dog based on the following scale: 0-no oozing; 1-mild oozing; 2-oozing requiring constant blotting; 3-moderate to severe oozing, postoperative seroma formation, or prolonged bleeding from venipuncture sites without a measurable decline in postoperative Hct; 4-severe oozing with a decrease of >6% in postoperative Hct. Using the bleeding index, 4 bleeding assessments were scored by the attending faculty surgeon during the surgical procedure at the following times: (1) during the initial surgical incision and after initially blotting the superficial skin incision; (2) intraoperatively during the first testicular or ovarian pedicle ligation; (3) after closing the median raphe (males) or linea alba (females) and (4) 3 hours postoperatively. The total bleeding score was calculated by adding the 4 subjective assessments for each dog.

Tissue Aspiration

Tissue aspirates were aseptically collected to test for the presence of *A. platys* and *E. canis* DNA by PCR. Whereas in dorsal recumbency and under general anesthesia, fine needle aspirates (FNA) of the spleen were collected aseptically using a 23-gauge, 1.5 in. needle attached to a 5 mL syringe. Spleen samples from female dogs were collected after visualizing the spleen, whereas spleen samples from male dogs were collected using ultrasound guidance. Bone marrow aspirates were obtained from the iliac crest. After a local injection of bupivacaine (10 mg/dog), a small skin incision was made and a Jamshidi needle advanced into the iliac marrow space. Bone marrow was aspirated into EDTA-coated 20 mL syringes. Popliteal lymph node FNAs were collected using a 23-gauge needle attached to a 5 mL syringe. All samples were stored in 0.25–0.5 mL of sterile 0.9% NaCl at –20°C for DNA extraction.

DNA Extraction

Samples for DNA extraction consisted of EDTA-anticoagulated whole blood as well as splenic, bone marrow and lymph node aspirates. Samples were shipped on dry ice to NCSU for DNA extraction and PCR testing. Two-hundred microliters of whole blood or cell suspensions from tissue aspirates were used for DNA extraction, performed on the QIA-symphony.^h Extracted DNA was quantified by spectrophotometry using NanoDrop ND-1000.ⁱ Ratios of A260/A280 nm and A260/A230 nm were used to assess DNA purity.

Polymerase Chain Reaction

All PCR sample preparations were performed in a biocontainment hood with UV light decontamination capabilities. All PCRs were performed in an Eppendorf thermocycler.³ Oligonucleotides and PCR reaction conditions designed to amplify a 420 bp fragment of the *16S* rRNA gene and a 620 bp fragment of the *GroEL* gene have been described previously.^{10,11} Positive plasmid (*Anaplasma* or *Ehrlichia 16S* rRNA DNA and *Anaplasma GroEL* DNA, respectively) and negative (RNase-free, molecular-grade water and uninfected canine genomic DNA) controls were included in all of the assays. The *A. platys p44* gene was amplified using Apl_p44F3 (5'-GCT AAG TGG AGC GGT GGC GAT GAC AG) and Apl_p44R3 (5'-CGA TCT CCG CCG CTT TCG TAT TCT TC) as forward and reverse primers to amplify a 510 bp fragment. The reaction was performed in a 25 μ L final volume reaction containing 12.5 μ L of MyTaq,^k 0.3 μ L of 50 μ M of each primer,¹ 7 or 2 μ L of filter-sterilized, molecular-grade water and 5 or 10 μ L of extracted DNA template. PCR was performed under the following conditions: a single hot start cycle at 94°C for 3 minutes, followed by 55 cycles of denaturation at 94°C for 15 seconds, annealing at 70°C for 10 seconds, and extension at 72°C for 30 seconds, followed by a single cycle at 72°C for 1 minute. Oligonucleotides designed to amplify a 1,623 bp fragment of the *E. canis p140* gene were Ec_VNTR108_F (5'-CAA CCT GC TGT GAA TGA CAA) and Ec_VNTR108_R (5'-AAC AGG ATT ACC ATC TGC AAC A) as forward and reverse primers, respectively. The reaction was performed in a 25 μ L final volume reaction containing 12.5 μ L of MyTaq,^k 0.3 μ L of 50 μ M of each primer,¹ 7 or 2 μ L of filter-sterilized, molecular-grade water and 5 or 10 μ L of extracted DNA template. Polymerase chain reaction was performed under the following conditions: a single hot start cycle at 94°C for 3 minutes, followed by 55 cycles of denaturation at 94°C for 20 seconds, annealing at 65°C for 30 seconds, and extension at 72°C for 1 minute, followed by a single cycle at 72°C for 3 minutes. Positive controls (*A. platys* or *E. canis* DNA, respectively) and negative (RNase-free, molecular-grade water and uninfected canine genomic DNA) controls were included in both of the

assays. PCR products were visualized on a 2.0% agarose gel in 1 \times Tris-acetate-EDTA buffer with ethidium bromide staining and sized by comparison with a DNA ladder as a molecular marker. Sensitivities for each target are as follows: *16S* rRNA 10 copies/reaction (rxn), *GroEL* 10 copies/rxn, *A. platys p44* 3 copies/rxn; sensitivity for *E. canis p140* copy has not been determined. Amplified DNA was sequenced directly by GENEWIZ^m and alignments were compared with GenBank sequences.ⁿ

Statistical Analysis

Statistical analysis was performed using statistical software^o to assess associations among altered coagulation parameters, demographic, hematologic, and biochemical parameters and *A. platys* and or *E. canis* seroreactivity using a one-way ANOVA, and or Mann-Whitney *U*-test, depending on the distribution of the data. Normality was determined using Shapiro-Wilks test. Multiple comparisons were performed using Dunnett's method or a Bonferroni correction. The chi-squared test was used to evaluate comparisons between demographic variables and seroreactive status. Fisher's exact test was used when the observed frequencies were <5. Differences in the proportion of PCR positives by whole blood or tissue type were determined using McNemar's test on paired proportions.

Results

Forty-seven dogs (C, n = 12; Ap, n = 10; Ec, n = 14; ApEc, n = 11) satisfying the inclusion criteria were admitted to the study.

Demographic, hematological, biochemical parameters for each of the 4 groups of Grenadian dogs are summarized in Table 1. Most dogs were mixed breeds, referred to as "Pothounds," Grenada's indigenous mongrel breed. The remaining 3 dogs were Pomeranian-Pekingese crosses, commonly referred to as "Pompek" in Grenada, but this mixed breed is not a

Table 1. Demographic, hematological, biochemical parameters in the four groups of Grenadian dogs.

Parameters	Median (Range)			
	C	Ap	Ec	ApEc
Males ^a	3 (25%)	8 (80%)	11 (80%)	5 (45.5%)
Females	9 (75%)	2 (20%)	3 (20%)	6 (54.5%)
Age in months: median (range)	6 (5–24)	11 (4.5–36)	24 (4–60)	36 (7–72)
Pothound ^a	12 (100)	9 (90.0)	13 (92.9)	10 (90.9)
Pompek	0 (0.0)	1 (10.0)	1 (7.1)	1 (9.1)
Weight (kg)	9.5 (5–15.2)	11.1 (4–15)	10.1 (5.8–14.8)	14 (5.3–17.5)
Body condition score	4.8 (3–5)	4.2 (3–5)	4 (2.5–5)	5 (4–6)
Hematocrit (%)	42.3 (27.9–48.6)	40.5 (33.9–46.6)	38.4 (29–44.8)	44.6 (27–51.5)
Platelet count ($\times 10^3/\mu$ L)	240 (165–500)	195 (150–270)	195 (150–390)	195 (165–300)
PT	7.3 (7.1–12.3)	7.1 (5.7–9.3)	7.5 (5.8–13.7)	8.15 (6.6–12.3)
aPTT	14.1 (5.9–21.3)	13.3 (12.3–17.1)	14.3 (6.9–17.3)	13.05 (6.5–14.3)
BMT	112.5 (92–200)	110 (40–237)	130 (61–174)	112.5 (40–200)
Total bleeding score	2 (1.0–7.0)	2.5 (1.5–5.0)	3.5 (1.5–6.0)	4.5 (2.0–7.5)
TP (g/dL)	7.1 (6–8.4)	6.7 (6–9)	7.6 (6.6–9.5)	8.3 (6.7–9.5)
Albumin (g/dL) ^b	3.05 (2.4–3.7)	2.8 (2.8–3.1)	2.7 (2.3–3.1)	3.1 (2.7–3.4)
Globulins (g/dL) ^b	3.95 (2.4–5.1)	3.75 (3.2–6.1)	5 (3.9–6.4)	5.5 (4.9–6.3)

C, seronegative controls; Ap, Ap seroreactive; Ec, Ec seroreactive; Ap/Ec, seroreactive to both organisms.

^aExpressed as total number (n) and percentage within the group (%).

^bAlbumin and Globulin values from 10/12 dogs in C, 8/10 in Ap, 13/14 in Ec, and 8/11 in ApEc (not analyzed statistically because of missing values).

first generation cross. Median age was 2 years (range, 5 months to 6 years) and the median weight was 11.5 kg (range, 5–18 kg). Dogs in ApEc ($P < .001$) and Ec ($P = .004$) were significantly older than the dogs in the C group. As would be expected from the defined inclusion criteria, there were no presurgical statistical differences in Hct, platelet count, or TP among the 4 study groups. Eosinophilia, with a median of $2.71 \times 10^3/\mu\text{L}$ (range, $1\text{--}6.86 \times 10^3/\mu\text{L}$), was common among all 4 study groups. Albumin and globulin concentrations were not available for 8 (17.0%) dogs. Therefore, no statistical analysis was performed for these parameters.

Presurgical coagulation parameters (PT, aPTT) and BMBT were within reference intervals (RI) for all dogs and did not vary by study group (Table 1). Based upon the 4 subjective assessments of intra- and postoperative bleeding by staff surgeons, the bleeding scores significantly differed among groups ($P = .02$), with the following total median scores per group (out of a possible 16): C = 2.0, Ap = 2.5, Ec = 3.25, and ApEc = 4.5. Bleeding scores in ApEc were significantly different from C by both Dunnett's method ($P = .015$) and Bonferroni correction ($P = .03$). Ap and Ec bleeding scores did not significantly differ from C or ApEc.

Of the Ap seroreactive dogs (including coseroreactives), 7/21 (33%) were *A. platys* PCR+ and of the Ec seroreactive (including coseroreactives), 21/25 (84%) were *E. canis* PCR+. One dog in the Ec group was *A. platys* PCR+. Of the ApEc coseroreactive dogs, *E. canis* DNA was amplified from 9/11 (81.8%) and *A. platys* DNA was amplified from 4/11 (36.4%) with 3 dogs being coinfecting. Presurgical coagulation parameters and subjective bleeding scores did not vary by PCR positive status. Pathogen DNA was not amplified from blood, spleen, bone marrow, or lymph node tissues from any C (*A. platys*/*E. canis* seronegative) dog. Only *A. platys* DNA was amplified from Ap, whereas 1 Ec dog was concurrently infected with *E. canis* and *A. platys*.

Of the *E. canis* PCR+ dogs, pathogen DNA was most often amplified from blood (19/21, 90.4%), followed by bone marrow (17/21, 81.0%), lymph node (10/21, 47.6%), and spleen (4/21, 19.0%) with significant differences seen between whole blood versus lymph node ($P = .02$) or spleen ($P < .01$), and bone marrow versus spleen ($P < .01$), whereas blood versus bone marrow, lymph node versus bone marrow, and lymph node versus spleen did not differ. In contrast to the *E. canis* PCR+ dogs, of the *A. platys* PCR+ dogs, pathogen DNA was amplified more often from bone marrow (7/8, 87.5%), followed by blood (6/8, 75.0%) and spleen (5/8, 62.5%), but not from lymph nodes. There were no significant differences in the proportion of *A. platys* DNA amplified from the different tissue sources. Of the 4 *A. platys* and *E. canis* coinfecting dogs (PCR+ for both organisms in seropositive groups Ec, $n = 1$ and ApEc, $n = 3$), *E. canis* DNA was amplified from blood (3/4, 75%), bone marrow (3/4, 75%), and lymph node (1/4, 25%) but not from spleen.

Amplified PCR products were sequenced directly and alignments were made with NCBI GenBank sequences.¹¹ Sequence identities for the partial *16S* rRNA, *GroEL*, *A. platys p44*, and *E. canis p140* genes were as follows (all with 100% sequence coverage): 100% similar to *16S* rRNA (361 bp) *E. canis* (NR_074283) or (355 bp) *A. platys* (KF360841); 100% similar to *GroEL* (549 bp) *E. canis* (U96731) or 99% similar to (567 bp) *A. platys* (EF201806); 99% similar to *p44* (503 bp) *A. platys p44* expression locus, *p44* clones 1–3 (GU357491-3); and 100% similar to *p140* (985 bp) *E. canis* locus tag Ecag_0017 (CP000107.1) and 99% similar to *E. canis p140* (EU118964.1).

Discussion

Based on this study, the majority (21/25; 84%) of clinically healthy, *E. canis* seroreactive dogs in Grenada are subclinically infected, whereas one-third (7/21; 33%) of *A. platys* seroreactive dogs are subclinically infected. Because this study did not investigate preoperative antibiotic treatment in this population of dogs, it is unknown if treatment would prevent or decrease intraoperative bleeding. Future studies with preoperative antibiotic treatment are warranted. These findings do, however, highlight the prevalence of tick-borne infections in dogs residing in Grenada and support the need for improved tick prevention and tick-borne disease-related education among owners and shelters in Grenada.

Excessive bleeding during elective surgeries in a subset of clinically healthy dogs with *A. platys* and *E. canis* exposure motivated this investigation. Prolonged bleeding increases surgical time, obscures visualization of the surgical field and can predispose the dog to intraoperative and postoperative complications. Therefore, preoperative identification of dogs prone to bleeding could facilitate corrective or preventive interventions before scheduling elective surgery. In this study, subjectively determined bleeding scores made during elective surgery were significantly higher in dogs seroreactive to both *E. canis* and *A. platys* (8/11; 72%, had total bleeding scores >4). Dogs seroreactive to only *E. canis* or only *A. platys* did not have an increased bleeding tendency. Although 6/14 (43%) dogs seroreactive only to *E. canis* had subjective bleeding scores >4 compared to only 1/12 (8.3%) seronegative control dogs, there was no significant difference in bleeding tendencies between either the *E. canis* or *A. platys* seroreactive dogs and the seronegative controls. Future studies with a larger population of dogs should be considered to evaluate the independent effect of *E. canis* seroreactivity as a predictor of increased intraoperative hemorrhage.

None of the standard clinicopathological hemostatic tests were predictive of increased intraoperative hemorrhage. All dogs with prolonged bleeding had normal platelet counts and normal coagulation parameters (BMBT, PT, aPTT), which did not vary among groups. Therefore, these tests did not identify dogs with potential bleeding tendencies, suggesting that the

increased intraoperative bleeding in our study was not attributed to platelet numbers, platelet function, or intrinsic and extrinsic coagulation factors.

As described above, the median bleeding score was significantly higher in dogs seroreactive to *A. platys* and *E. canis* than in seronegative controls. Based on PCR results, nearly all *E. canis* seroreactive dogs were subclinically infected. These data may signify the importance of *E. canis* antibody-antigen complexes contributing to the higher bleeding tendency seen in the coseroreactive dogs, and potentially underscore a synergistic effect in dogs seroreactive to or infected with both pathogens. Vasculitis, which was not detectable by the buccal mucosal bleeding time in our study, is 1 possible cause of the extravasation of blood. An experimental infection study¹² suggested that *E. canis* immune-mediated vasculitis may result in perivascular cuffs from deposited antigens and immune cells in blood vessel walls. Thrombocytopenia because of decreased platelet function also has been reported in association with canine ehrlichiosis,¹³ but normal bleeding times did not support thrombocytopenia in these dogs. Although this study was not designed to evaluate the potential for vascular injury, the possibility that subclinical infection with *A. platys* and *E. canis* may result in vascular injury could be further explored with histopathological examination of blood vessels and by measuring molecular markers of vascular damage, such as endothelin, vascular endothelial growth factor, circulating endothelial cells, plasmatic thrombomodulin, von Willebrand factor¹⁴ and intercellular and vascular cell adhesion molecules.¹⁵

Based on PCR results, *A. platys* and *E. canis* seroreactive, clinically healthy Grenadian dogs frequently are infected with 1 or both organisms, similar to other Caribbean islands.¹⁶ The molecular prevalence of *A. platys* was substantially lower than *E. canis* in these dogs. Several potential factors may contribute to this difference, including cyclic rickettsemia induced by *A. platys*,¹⁷ earlier immunological clearance of *A. platys* versus persistent *E. canis* ehrlichemia,^{18,19} or potentially a more recent infection with *E. canis* as compared to *A. platys*. Experimentally, dogs coinfecting with *A. platys* and *E. canis* remain *A. platys*-infected for a longer period than dogs infected with *A. platys* only.¹⁸ Failure to amplify *A. platys* DNA from seroreactive dogs may be because of low pathogen levels or poor specimen type or quality. The latter seems unlikely as the same tissue extractions were used for *E. canis* PCR.

In this study, DNA was extracted from 4 different tissues from each dog. *E. canis* DNA was amplified more often from whole blood and bone marrow than from spleen and lymph nodes. These results differ from previous studies, where splenic aspiration specimens used for PCR testing were considered more sensitive when compared to whole blood or bone marrow specimens.^{19–21} Differences among studies may be related to regional differences among *E. canis* strains, strain-specific tissue predilections, variable sample collection techniques, or variability among DNA tissue extractions.^{19,22,23}

This study has several limitations. Our inclusion criteria selected only dogs that were deemed acceptable surgical candidates for elective castration or ovariectomy. As such, the population consisted predominantly of young dogs and, based upon our inclusion criteria, differences between CBC and serum biochemical test results could not be evaluated. Dogs, however, were significantly older in groups seroreactive to ApEc (7–72 months; $P < .001$) and Ec (4–60 months; $P = .004$) than in the control group (5–24 months). Thus, it is possible the increased bleeding scores seen in the ApEc group were age related. The surgeons assigning bleeding scores were masked for 70% of the cases, but became unmasked during the final stages of the study when attempting to select dogs that satisfied the defined group entry criteria. Also, DNA amplification by PCR is rarely 100% sensitive or specific, thus false negative and false positive results are possible. All DNA was measured to ensure adequate sample template per reaction and multiple genes were targeted, thus improving the chances of detection. We conclude that a substantial proportion of clinically healthy, Grenadian dogs seroreactive for *A. platys*, *E. canis* or both are subclinically infected with one or both of these tick-borne pathogens, supporting the need for acaricide administration in dogs from Grenada. Dogs with combined *E. canis* and *A. platys* seroreactivity had the highest intraoperative bleeding tendencies, an observation that warrants further studies to assess if *A. platys*/*E. canis* coseroreactivity is a useful predictor for increased bleeding tendencies during surgery. The study did not identify a coagulation parameter that would be predictive of excessive bleeding during elective surgery and failed to identify a cause for the intraoperative bleeding tendency. Because platelet number, platelet function, and defects in intrinsic and extrinsic coagulation parameters do not appear to be responsible for the bleeding tendency documented in these dogs, future studies should investigate whether vasculitis or alterations in endothelial integrity in connection with *E. canis* and *A. platys* exposure contribute to excessive bleeding.

Footnotes

^a IDEXX, Westbrook, ME

^b Acepromazine Acepromazine inj 10 mg/mL – 50 mL, Med-Pharmex, Inc., Pomona, CA

^c Morphine; Morphine sulfate 15 mg/mL-1 mL vial, Martindale, Essex, UK

^d Abbott laboratories, Abbott Park, IL

^e Buprenorphine Temgesic injection 0.3 mg/mL, Reckitt Benckiser, Berkshire, UK

^f Surgicutt bleeding device, ITC, Edison, NJ

^g ThromboScreen 400c machine; Pacific Hemostasis, A Fisher Scientific Company, Waltham, MA

^h QIA Symphony DNA Mini Kit (192) Qiagen, cat. no. 931236, Valencia, CA

ⁱ NanoDrop is a trademark of Thermo Fisher Scientific, Wilmington, DE

^j Mastercycler EPgradient, Westbury, NY

- ^k HS Mix (2X) Bioline cat: BIO-25046, Taunton, MA
^l Sigma-Aldrich, St. Louis, MO
^m GENEWIZ, Inc. Research Triangle Park, NC
ⁿ AlignX software, Vector NTI Suite 6.0, InforMax, Inc, Bethesda, MD
^o SAS/STAT 9.2; SAS Institute, Cary, NC

Acknowledgments

The authors thank Mrs Tonya Lee for editorial assistance.

This study was supported by a SGU Small Research Grant Initiative (GSP\SRGI\12002) and in part by the NCSU-CVM Vector Borne Diseases Research Foundation Account and the State of North Carolina. Dr Barbara Qurollo, a postdoctoral research fellow in the IPRL, was supported through a grant from IDEXX Laboratories, Westbrook, ME and Elizabeth Pultorak, a Ph. D. candidate in infectious disease epidemiology, received stipend support from Bayer Animal Health.

Conflict of Interest Declaration: The authors disclose no conflict of interest.

References

1. Yabsley MJ, McKibben J, Macpherson CN, et al. Prevalence of *Ehrlichia canis*, *Anaplasma platys*, *Babesia canis vogeli*, *Hepatozoon canis*, *Bartonella vinsonii berkhoffii*, and *Rickettsia* spp. in dogs from Grenada. *Vet Parasitol* 2008;151:279–285.
2. Baneth G, Waner T, Koplak A, et al. Survey of *Ehrlichia canis* antibodies among dogs in Israel. *Vet Rec* 1996;138:257–259.
3. Botros BA, Elmolla MS, Salib AW, et al. Canine ehrlichiosis in Egypt: Sero-epidemiological survey. *Onderstepoort J Vet Res* 1995;62:41–43.
4. Kelly PJ, Xu C, Lucas H, et al. Ehrlichiosis, babesiosis, anaplasmosis and hepatozoonosis in dogs from St. Kitts, West Indies. *PLoS One* 2013;8:e53450.
5. Neer TM, Breitschwerdt EB, Greene RT, et al. Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the ACVIM. *American College of Veterinary Internal Medicine. J Vet Intern Med* 2002;16:309–315.
6. Bromel K. Buccal mucosal bleeding time. In: Ettinger SJ, Feldman EC, eds. *Textbook of Veterinary Internal Medicine*, 7th ed. Guelph, ON: Saunders; 2010:364–365.
7. Mylonakis ME, Kazakos GM, Pardali D, et al. Bleeding time in healthy dogs sedated with morphine and medetomidine. *Vet Rec* 2011;169:470.
8. Lara-Garcia A, Couto CG, Iazbik MC, et al. Postoperative bleeding in retired racing greyhounds. *J Vet Intern Med* 2008;22:525–533.
9. Buchanan GR, Adix L. Grading for hemorrhage in children with idiopathic thrombocytopenic purpura. *J Pediatr* 2002;141:683–688.
10. Barber R, Li Q, Diniz P, et al. Evaluation of brain tissue or cerebrospinal fluid with broadly reactive polymerase chain reaction for *Ehrlichia*, *Anaplasma*, spotted fever group *Rickettsia*, *Bartonella*, and *Borrelia* Species in canine neurological diseases (109 Cases). *J Vet Intern Med* 2010;24:372–378.
11. Eddlestone SM, Diniz PP, Neer TM, et al. Doxycycline clearance of experimentally induced chronic *Ehrlichia canis* infection in dogs. *J Vet Intern Med* 2007;21:1237–1242.
12. De Castro MB, Machado RZ, de Aquino LP, et al. Experimental acute canine monocytic ehrlichiosis: Clinicopathological and immunopathological findings. *Vet Parasitol* 2004;119:73–86.
13. Harrus S, Waner T, Eldor A, et al. Platelet dysfunction associated with experimental acute canine ehrlichiosis. *Vet Rec* 1996;139:290–293.
14. George F, Brouqui P, Boffa M, et al. Demonstration of *Rickettsia conorii*-induced endothelial injury *in vivo* by measuring circulating endothelial cells, thrombomodulin, and von Willebrand factor in patients with Mediterranean spotted fever. *Blood* 1993;82:2109–2116.
15. Van Gorp E, Suharti C, Ten Cate H, et al. Review: Infectious diseases and coagulation disorders. *J Infect Dis* 1999;180:176–186.
16. Loftis AD, Kelly PJ, Freeman MD, et al. Tick-borne pathogens and disease in dogs on St. Kitts, West Indies. *Vet Parasitol* 2013;196:44–49.
17. Varela A. Tick-borne Ehrlichiae and Rickettsiae of dogs. In: Bowman DD, ed. *Companion and Exotic Animal Parasitology*. Ithaca, NY: International Veterinary Information Service [IVIS]; 2003. Document No.A0315.0503. Available at: http://www.ivis.org/advances/parasit_Bowman/varela/chapter. Accessed August 20, 2014.
18. Gaunt S, Beall M, Stillman B, et al. Experimental infection and co-infection of dogs with *Anaplasma platys* and *Ehrlichia canis*: Hematologic, serologic and molecular findings. *Parasit Vectors* 2010;3:33.
19. Harrus S, Waner T. Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): An overview. *Vet J* 2011;187:292–296.
20. Harrus S, Waner T, Aizenberg I, et al. Amplification of ehrlichial DNA from dogs 34 months after infection with *Ehrlichia canis*. *J Clin Microbiol* 1998;36:73–76.
21. Harrus S, Waner T, Mahan S, Bark H. *Animal Rickettsial Pathogens—Family Anaplasmataceae. Pathogenesis of Bacterial Infections in Animals*, 3rd ed. Hoboken, NJ: Blackwell Publishing; 2004:426.
22. Asgarali Z, Pargass I, Adam J, et al. Haematological parameters in stray dogs seropositive and seronegative to *Ehrlichia canis* in North Trinidad. *Ticks Tick Borne Dis* 2012;3:207–211.
23. Vieira RFdC, Biondo AW, Guimarães AMS, et al. Ehrlichiosis in Brazil. *Rev Bras Parasitol Vet* 2011;20:01–12.