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Hypercoagulability in Dogs with Blastomycosis

M.A. McMichael, M. O'Brien, and S.A. Smith

Background: Blastomycosis is a potentially fatal fungal disease that most commonly affects humans and dogs. The organism causes systemic inflammation and has a predilection for the lungs. The inflammation might lead to a hypercoagulable state with microemboli in the pulmonary circulation which could contribute to inadequate oxygen exchange in infected dogs.

Hypothesis/Objectives: Dogs with blastomycosis will be hypercoagulable compared with healthy case-matched controls.

Animals: Client-owned dogs with a diagnosis of blastomycosis (n = 23) and healthy case-matched controls (n = 23).

Methods: Prospective case-controlled study of client-owned dogs presented to a veterinary teaching hospital with clinical signs compatible with blastomycosis. Complete blood counts, fibrinogen, PT, aPTT, thromboelastometry (TE), thrombin antithrombin complexes (TAT), and thrombin generation were evaluated.

Results: Cases had a leukocytosis compared with controls [mean (SD) 16.6 (7.6) × 10^3/μL versus 8.2 (1.8) × 10^3/μL, $P < .001$], hyperfibrinogenemia [median 784 mg/dL, range 329–1,443 versus median 178 mg/dL, range 82–257, $P < .001$], and increased TAT concentrations [mean (SD) 9.0 (5.7) μg/L versus 2.0 (2.8) μg/L, $P < .001$]. As compared to controls, cases were also hypercoagulable as evaluated by thromboelastometry and had increased in vitro thrombin generation on calibrated automated thrombography.

Conclusions and Clinical Importance: Hypercoagulability occurs in dogs with systemic blastomycosis. Additional studies are needed to explore a possible contribution of thrombogenicity to the clinical manifestations of systemic blastomycosis.

Key words: Canine; Thromboelastography; Thromboelastometry; Thrombosis.

Blastomycosis is a potentially fatal fungal disease that is contracted by inhalation of infectious spores produced by the mycelial form of the organism *Blastomyces dermatiditis*. Pulmonary blastomycosis has a broad range of clinical presentations from mild respiratory distress to acute respiratory failure. Infection rapidly progresses to acute respiratory distress syndrome and shock in 8–12% of human patients affected,[1,2] with a case fatality rate of 50–89%.[1–5] Infection results in a marked inflammatory reaction characterized by a pyogranulomatous response. An excessive inflammatory response might be contributory to the poor prognosis, and to the clinical deterioration associated with institution of antifungal treatment.[6] Excessive inflammation in infected patients might be leading to secondary hypercoagulability. Development of microemboli in the pulmonary circulation as a consequence of hypercoagulability could contribute to inadequate oxygen exchange in infected dogs.

Systemic hypercoagulability has not been previously described in association with blastomycosis, although pulmonary thromboembolism[7] and arterial thromboembolism (with atrial wall erosion by an infected lymph node)[8] occur in infected dogs and a single report describes deep venous thrombosis with pulmonary embolism in a horse with systemic blastomycosis.[9] The purpose of this study was to determine whether dogs with naturally occurring, untreated blastomycosis were hypercoagulable as compared to healthy control animals from a similar environment.

Abbreviations:

α	alpha angle
aPTT	activated partial thromboplastin time
ARDS	acute respiratory distress syndrome
CBC	complete blood count
CFT	clot formation time
CT	hematocrit, coagulation time
ETP	endogenous thrombin potential
MCF	maximum clot firmness
PT	thromboelastometry, prothrombin time
TAT	thrombin antithrombin complex

Materials and Methods

Animals

Client-owned dogs presented to the Veterinary Teaching Hospital with clinical signs consistent with blastomycosis were eligible for enrollment. All clients signed an informed consent form that was approved by the Institutional Animal Care and Use Committee at the University of Illinois. Animals were excluded if they were <5 kg body weight, had evidence of a concurrent disease known to be associated with hypercoagulability such as nephrotic syndrome, immune-mediated disease, cardiac disease, protein losing enteropathy, neoplasia, heartworm infection, or hyperadrenocorticism or had received antifungal treatment before presentation. Blastomycosis was confirmed on cytologic or histopathologic examination of tissues, aspirates, or exudates by a board certified pathologist, or by a positive blastomyces quantitative antigen enzyme immunoassay[a] test performed on urine.

Matched case-controls were selected from a population of 157 apparently healthy client-owned dogs living in the same local environment. All control animals were without clinical complaint and had no abnormalities identified on complete blood count or serum

From the Department of Veterinary Clinical Sciences, College of Veterinary Medicine, (McMichael, O'Brien); and the Department of Biochemistry, College of Liberal Arts and Sciences, University of Illinois, Urbana, IL (Smith).

All work was completed at the University of Illinois.

Corresponding author: M.A. McMichael, Department of Veterinary Clinical Sciences, University of Illinois at Urbana Champaign, 1008 West Hazelwood Dr., Urbana, IL 61802;
e-mail: mmcm@illinois.edu.

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biochemical panel. Cases were matched with controls by age (±1 year) and sex. Cases were also matched with controls by breed when an exact match was available from the healthy population. If no breed match was available, then a closely related breed from within the same class was selected. If no closely related breed was available, or if the case was of mixed genetic origin, then a mixed breed of similar body weight (±3 kg) was selected.

Blood Collection and Handling for Analysis

Blood was collected upon presentation via venipuncture of a jugular vein using an 18 or 20 g needle attached to a syringe, and was immediately transferred into plain, K_2EDTA, and citrate tubes, the latter at a ratio of 9 : 1 for blood: 3.2% citrate (buffered sodium citrate silicon-coated glass, 2.7 mL tube)[b] then mixed thoroughly by gentle repeated inversion. All samples were collected before initiation of antifungal treatment. Serum and EDTA blood were submitted to the clinical diagnostic laboratory. Complete blood counts were performed on a Cell-dyn 3700 hematology analyzer.[c] An aliquot of the citrated whole blood was immediately used for thromboelastometry, as described below. The remaining citrated whole blood was centrifuged at 2,500 × *g* for 10 minutes at room temperature for collection of platelet-poor plasma. Plasma was aliquoted into Eppendorf tubes and frozen at −80°C for later batch analysis.

Whole Blood Thromboelastometry

Whole blood thromboelastometry was performed on a ROTEM system[d] according to the manufacturer's instructions, by methods validated for canine whole blood.[10] Samples were evaluated at 37°C within 30 minutes of collection. Citrated whole blood samples (300 μL) were added to manufacturer supplied reagents using the supplied electronic pipette. All clotting reactions contained 20 μL of the manufacturer supplied concentrated calcium chloride reagent (startem). Reactions additionally contained either 20 μL of supplied tissue factor reagent (extem) or 20 μL of supplied ellagic acid reagent for contact activation (intem). Evaluations were performed in duplicate. Clot development was observed for 1 hour.

Variables collected from the manufacturer's modeling software included coagulation time (CT), which is lag period from initiation of the reaction until an amplitude of 2 mm is recorded; clot formation time (CFT), which is the duration necessary for amplitude to increase from 2 to 20 mm; alpha angle (α), which is the angle of change of the amplitude over the course of the CFT; and maximum clot firmness (MCF), which is the greatest amplitude achieved. Expected MCF values for extem assays were predicted from calculations based on the previously described[11] dependence of MCF on hematocrit, fibrinogen concentration, and platelet concentration.

Additional Assays of Coagulation

Prothrombin time (Neoplastine Cl+),[e] activated partial thromboplastin time (STA-aPTT, Diagnostica Stago), and fibrinogen (Fibritest-automate) [e] assays were performed on a manual fibrometer (STart4).[e] Thrombin-antithrombin complex (TAT) concentrations were measured by ELISA (Enzygnost TAT micro)[f] in duplicate, the use of which was previously validated for canine plasma.[12]

Thrombography was performed on a calibrated automated thrombograph (Thrombinoscope)[e] with the Hemker method[13] according to the manufacturer's instructions. This assay evaluates the time course of thrombin generation and inhibition in real time, interrogating the continuing generation of thrombin that occurs after formation of the fibrin clot.[14] Samples were evaluated in triplicate with coagulation triggered using manufacturer-supplied tissue factor (PPP-Low reagent) and $CaCl_2$/fluorogenic substrate (FluCa reagent) reagents. Thrombin generation was evaluated for 30 minutes. Data collected from the thrombinoscope software included lag time, time to peak thrombin activity, peak thrombin activity, and endogenous thrombin potential (ETP).

Statistical Analysis

Shapiro-Wilk tests were used to evaluate normality of both case and control datasets. Parametric datasets were compared using *t*-tests, whereas nonparametric sets were compared using Mann-Whitney Rank Sum tests. Statistical comparisons were performed using commercial software.[g] Significance was defined at a $P < .05$.

Results

Whole blood thromboelastometry was performed at presentation on 28 dogs with clinical signs consistent with blastomycosis. Five dogs were later excluded from further analysis either because of identification of concomitant disease, or lack of confirmation of infection with *Blastomyces*. For the 23 cases with confirmed blastomycosis, the median age was 4.1 years (range 1.5–11.8 years) and sex distribution was 14 males (3 intact) and 9 females (2 intact). Each case was matched with 1 control, resulting in a total of 23 controls.

Ocular (n = 11; 48%), respiratory (n = 8; 35%), and skin (n = 6; 26%) lesions predominated. Thoracic radiographs obtained at the time of diagnosis were abnormal in 19 (90%) of those in which radiographs were available (n = 21). Abnormal Roentgen signs included a diffuse military pattern (n = 7, 37%), nodular interstitial pattern (n = 3, 16%), thoracic lymphadenopathy (n = 4, 21%), lung mass (n = 5, 26%), and consolidated lung/lobar sign (n = 3, 16%).

The diagnosis of blastomycosis was obtained from a single test method in 17 animals (74%). Four (17%) dogs were diagnosed solely through lymph node cytologic examination, 4 (17%) dogs were diagnosed solely by impression smear cytology from a skin lesion, 4 dogs were diagnosed solely from ocular aspiration or histopathology, 3 (13%) dogs were diagnosed solely by positive cytologic examination of lung aspirates, and 2 (9%) dogs were diagnosed solely from a positive urine antigen result. Other means of diagnosis included fine needle aspiration of bone, and necropsy. The remainder (n = 6, 26%) tested positive for blastomycosis on more than 1 test method.

Complete blood counts indicated that dogs with blastomycosis had slightly lower indicators of circulating red cell mass than did apparently healthy dogs, although the erythrocyte concentrations (Fig 1A) and hematocrits (Supplemental Figure) for cases were all within reference interval. Cases had a significant leukocytosis characterized by a mature neutrophilia and monocytosis. Mean (SD) leukocyte concentrations were significantly higher for cases [16.6 (7.6) × 10^3/μL] as compared to controls [8.2 (1.8) × 10^3/μL; $P < .001$] (Fig 1B). Similarly, mean (SD) segmented neutrophil concentrations were significantly higher for cases [13.2 (6.8) × 10^3/μL] as compared to controls [5.1

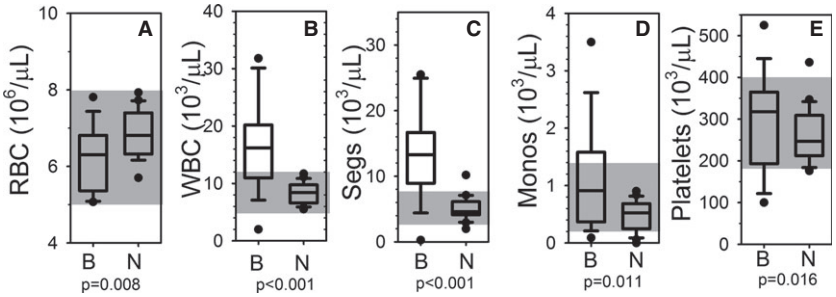

Fig 1. Blood cells. Box and whisker plots describing results of complete blood counts for the blastomycosis cohort ("B") and the normal control cohort ("N"). Boxes represent the 25–75th percentile, central lines represent median values, whiskers indicate the 5–95th percentile, and outliers are described by filled circles. The gray region indicates reference interval. **(A)** Red blood cell concentration (RBC), **(B)** total white blood cell concentration (WBC), **(C)** segmented neutrophil concentration (Segs), **(D)** monocyte concentration (Monos), and **(E)** platelet concentration (platelets).

$(1.7) \times 10^3/\mu L$; $P < .001$] (Fig 1C). Mean (SD) monocyte concentrations were also significantly higher for cases [1.1 $(0.9) \times 10^3/\mu L$] versus controls [0.5 $(0.3) \times 10^3/\mu L$; $P = .011$] (Fig 1D). Platelet concentrations were more variable in the case cohort than in the control cohort, but the median was significantly higher for cases [$3.18 \times 10^5/\mu L$] as compared to controls [$2.47 \times 10^5/\mu L$; $P = .016$] (Fig 1E).

The results of routine screening plasma coagulation assays (PT and aPTT) were unremarkable, with results for cases falling primarily within reference interval, and not statistically different from controls (Fig 2A,B). Hyperfibrinogenemia (Fig 2C) was identified in 100% of cases, and plasma fibrinogen concentration was significantly different between cases (median 784 mg/dL, range 329–1,443) and controls (median 178 mg/dL, range 82–257; $P < .001$). Mean (SD) TAT concentration was also significantly higher in cases [9.0 (5.7) μg/L] than in controls [2.0 (2.8) μg/L; $P < .001$].

Whole blood samples from cases appeared hypercoagulable on thromboelastometry regardless of whether the trigger was tissue factor (extem, Fig 3) or ellagic acid (intem, Fig 4). Although no cases had an abnormal extem CT, and only 5 cases (22%) had an abnormal intem CT, there was a statistically significant difference between cases and controls. Variables describing the kinetics of clot formation (CFT and α) were hypercoagulable on either extem or intem for 100% of cases. MCF values were above reference interval for 22 samples, with the remaining sample having an MCF at the upper limit of reference interval on 1 of the 2 assays. When datasets for each of the 4 variables were compared between cases and controls, all comparisons indicated statistically significant differences between the 2 populations. Bland-Altman analysis comparing predicted MCF to the actual measured MCF indicated that effects of the slightly low hematocrit, high fibrinogen, and generally higher platelet concentrations in the samples from cases did not account for the abnormally high MCF values (Fig 4E).

Calibrated automated thrombography indicated that thrombin generation was excessive in cases as compared to controls, with all 4 measured variables [lag time

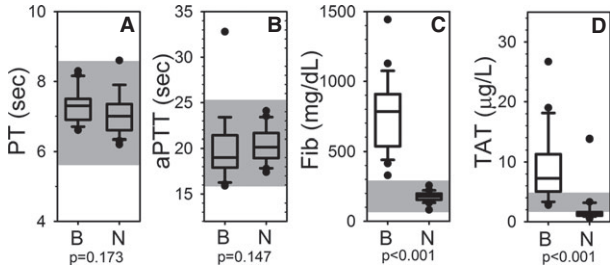

Fig 2. Coagulation assays. Box and whisker plots describing results of routine plasma coagulation assays for the blastomycosis cohort ("B") and the normal control cohort ("N"). Boxes represent the 25–75th percentile, central lines represent median values, whiskers indicate the 5–95th percentile, and outliers are described by filled circles. The gray region indicates reference interval. **(A)** Prothrombin time (PT), **(B)** activated partial thromboplastin time (aPTT), **(C)** fibrinogen concentration (Fib), and **(D)** thrombin-antithrombin complex concentration (TAT).

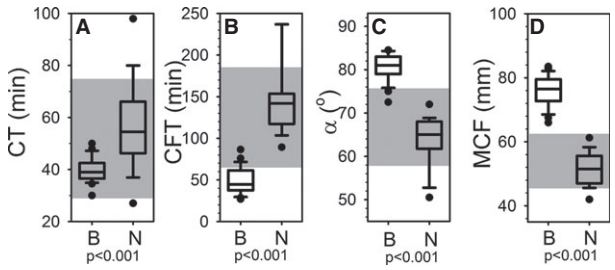

Fig 3. Extem thromboelastometry. Box and whisker plots describing results of extem thromboelastometry assays for the blastomycosis cohort ("B") and the normal control cohort ("N"). Boxes represent the 25–75th percentile, central lines represent median values, whiskers indicate the 5–95th percentile, and outliers are described by filled circles. The gray region indicates reference interval. **(A)** Coagulation time (CT), **(B)** clot formation time (CFT), **(C)** alpha angle (α), and **(D)** maximum clot firmness (MCF). **(E)** Bland-Altman plot comparing predicted maximum clot firmness (MCF) to measured MCF. Predicted MCF was calculated using the described[11] dependence of MCF on hematocrit, fibrinogen concentration, and platelet concentration. MCF values for the control cohort are denoted by □. MCF values for the blastomycosis case cohort are denoted by ▼.

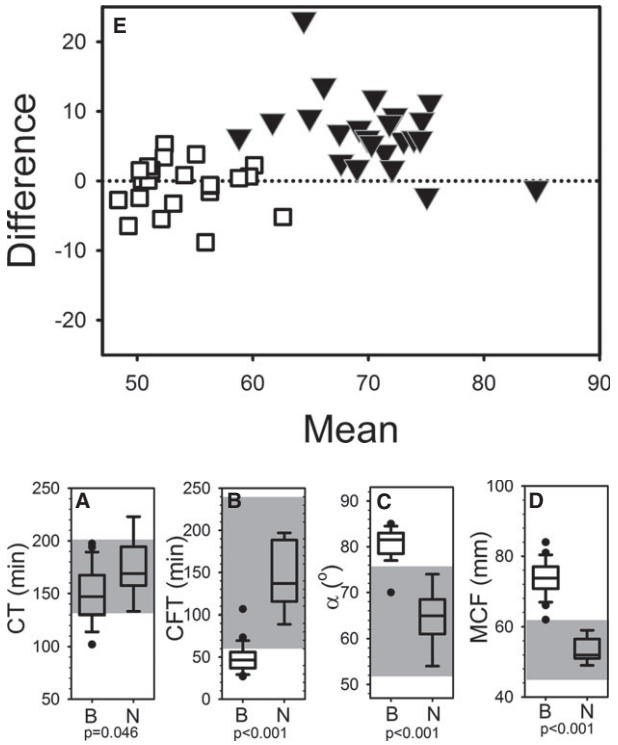

Fig 4. Intem thromboelastometry. Box and whisker plots **(A–D)** describing results of intem thromboelastometry assays for the blastomycosis cohort ("B") and the normal control cohort ("N"). Boxes represent the 25–75th percentile, central lines represent median values, whiskers indicate the 5–95th percentile, and outliers are described by filled circles. The gray region indicates reference interval. (A) Coagulation time (CT), (B) clot formation time (CFT), (C) alpha angle (α), and (D) maximum clot firmness (MCF).

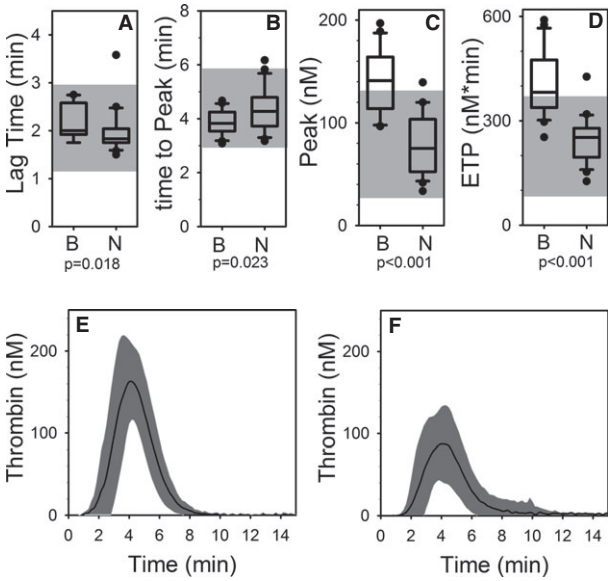

Fig 5. Calibrated automated thrombography. Box and whisker plots **(A–D)** of describing results of calibrated automated thrombography for the blastomycosis cohort ("B") and the normal control cohort ("N"). Boxes represent the 25–75th percentile, central lines represent median values, whiskers indicate the 5–95th percentile, and outliers are described by filled circles. The gray region indicates reference interval. (A) Lag time (CT), (B) time to peak thrombin (time to peak), (C) peak thrombin (peak), (D) endogenous thrombin potential (ETP), **(E)** Mean (solid line) ±1 standard deviation (gray area) curves describing the time course of thrombin generation for cases. **(F)** Mean (solid line) ±1 standard deviation (gray area) curves describing the time course of thrombin generation for controls.

(minutes), $P = .018$; time to peak (minutes), $P = .023$; peak (nM), $P < .001$; endogenous thrombin potential (nM), $P < .001$] statistically different between cases and controls (Fig 5).

Discussion

This study documents significant hypercoagulability in systemic blastomycosis in dogs including elevations in plasma fibrinogen concentration, hypercoagulable thromboelastometry tracings, and excessive in vitro and in vivo thrombin generation.

Hypercoagulability has not been recognized in this population, because abnormalities are not detected on routine plasma coagulation assays in humans and dogs with blastomycosis. Results of PT and aPTT were not abnormal, although these routine coagulation assays are designed for evaluating plasma for evidence of *hypo*coagulability rather than *hyper*coagulability, and are insensitive to changes that increase the rate of thrombin generation.

Plasma fibrinogen concentration was markedly increased for cases as compared to controls; not unsurprising as systemic fungal infection is associated with inflammation. The neutrophilic leukocytosis in these infected dogs supports an ongoing systemic inflammatory response.[15,16] Concomitant hyperfibrinogenemia occurs in disease states associated with thrombosis in dogs, including neoplasia,[17] protein losing nephropathy,[18] and babesiosis.[19] Hyperfibrinogenemia in humans is associated with increased risk of cardiovascular disease and arterial and venous thrombosis, with higher risk of death or thrombosis in subjects with the highest plasma fibrinogen concentration.[20–28] There is a direct causal relationship between hyperfibrinogenemia and thrombosis in mice, where increased fibrinogen concentration increases thrombus fibrin content, promotes faster fibrin formation, and increases fibrin network density, strength, and stability.[29]

Results of thromboelastometry assays also indicated evidence of marked hypercoagulability in the blood from these dogs. Use of thromboelastometry has become increasingly popular to evaluate for hypercoagulability, owing to the insensitivity of standard plasma assays. This approach is subject to some limitations. Depending on the methodology used, results might be influenced by *ex vivo* changes. We avoided this issue by using an approach documented to be insensitive to variability in sample handling.[10] Whole blood thromboelastometry is sensitive to increased plasma fibrinogen concentration,[30,31] so we would expect that the hyperfi-

brinogenemia contributed to the evidence of abnormal clot formation on thromboelastometry, particularly the abnormal MCF values.

Mild decreases in hematocrit can artifactually impact the results obtained with whole blood thromboelastometry, causing apparent hypercoagulability.[11,32] Although the animals in our case cohort had hematocrits mostly with reference interval, there was a statistically significant difference between cases and controls. Even such minor differences in red cell mass might contribute to apparent but artifactual hypercoagulability on thromboelastometry.[11,32]

To determine whether or not the lower hematocrit could have been primarily responsible for the apparent hypercoagulability we observed on thromboelastometry, we used the previously described mathematical relationship between hematocrit, fibrinogen concentration, platelet concentration, and thromboelastometry to predict MCF values[11] for these samples. These calculations indicated that the slightly low hematocrit and high fibrinogen could not have accounted for all of the apparent hypercoagulability indicated via the thromboelastometry results, suggesting another component was contributory.

We further evaluated plasma from the case cohort using thrombography, which is not influenced by either fibrinogen concentration or hematocrit as it evaluates real time thrombin generation in vitro in the plasma sample.[13] Thrombin generation was abnormal in the case cohort, with evidence of both more rapid thrombin generation (shortened lag time and time to peak) and greater amount of thrombin generated (higher peak, higher area under the curve or ETP).

Lastly, the finding of elevations in plasma TAT concentration provides strong support for the presence of in vivo hypercoagulability in dogs with blastomycosis, as formation of TAT complexes is a specific marker of ongoing in vivo thrombin generation.

The hypercoagulability documented in cases in this report indicates that these dogs are prothrombotic, and could develop thrombi that adversely impact the perfusion component of gaseous exchange. Additional studies are needed to further explore a possible contribution of thrombogenicity to the clinical manifestations of systemic blastomycosis. Thromboprophylaxis might potentially have a role in improving outcome in this population.

Footnotes

[a] Mira Vista Laboratory, Indianapolis, IN
[b] BD, Franklin Lakes, NJ
[c] Abbott Diagnostics, Abbott Park, IL
[d] Tem International GmbH, Munich, Germany
[e] Diagnostica Stago, Assiernes, France
[f] Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany
[g] Sigma Stat 2.03, SPSS Inc, Chicago, IL

Acknowledgments

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Conflict of Interest Declaration: Stephanie Smith is an associate editor for the Journal of Veterinary Internal Medicine.

Off-label Antimicrobial Declaration: The authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Fig S1. Hematocrit (HCT). Box and whisker plots describing results of HCT for blastomycosis cohort ("B") and the normal control cohort ("N"). Boxes represent the 25-75th percentile, central lines represent median values, whiskers indicate the 5-95th percentile. The gray region indicates reference interval.