Received: 15 June 2022 | Accepted: 14 December 2022

DOI: 10.1111/jvim.16617

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

Carboxyhemoglobin as a diagnostic and prognostic biomarker of hemolytic anemias in dogs

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Funding information
European Veterinary Emergency and Critical Care (EVECC)

Abstract

Background: Endogenous production of carbon monoxide during hemoglobin metabolism leads to the formation of carboxyhemoglobin. Carboxyhemoglobin concentration is abnormally high in humans with hemolytic anemia (HA).

Hypothesis: Measurement of carboxyhemoglobin concentration can discriminate HA from other forms of anemia.

Animals: Twenty-seven dogs with HA (immune-mediated HA, n = 22; microangiopathic HA, n = 5), 27 dogs with non-HA (kidney disease, n = 14; immune-mediated thrombocytopenia, [n = 6]; miscellaneous, n = 7) and 24 nonanemic control dogs.

Methods: Prospective cohort study. Carboxyhemoglobin quantification, a CBC and biochemistry profile were performed upon admission, and survival to hospital discharge and at 30 days were the measured outcomes. Groups were compared by the Mann-Whitney and Kruskal-Wallis tests. Receiver-operator characteristic (ROC) analyses were used to examine the predictive utility of carboxyhemoglobin for the diagnosis of HA in anemic dogs.

Results: Carboxyhemoglobin (median [interquartile range]) differed between dogs with HA (7.7% [2.5%]) and non-HA (3.6% [1.05]; $P < .001$) and dogs with HA and nonanemic dogs (3.5% [0.65%]; $P < .001$). No difference was detected between nonHA and nonanemic dogs. The area under the ROC curve for carboxyhemoglobin as predictor of HA in anemic dogs was 0.997 (95% CI, 0.99-1.00). Three optimal cutoff points were identified, including 5.05%, 4.55% and 4.85%, with corresponding sensitivity/specificity of 92.6%/100%, 100%/92.6% and 96.3%/96.3%, respectively. Neither carboxyhemoglobin nor any of the CBC or chemistry analytes were associated with survival.

Conclusions and Clinical Importance: Carboxyhemoglobin proved an excellent predictor of HA in dogs and might constitute a useful, ancillary tool for diagnosing and monitoring hemolytic anemias.

Abbreviations: AKI, acute kidney injury; AUROCC, area under the receiver operator characteristic curve; 95% CI, 95% confidence interval; CKD, chronic kidney disease; CO, carbon monoxide; COHb, carboxyhemoglobin; HA, hemolytic anemia; HO, heme-oxygenase; ICU, intensive care unit; IMHA, immune-mediated hemolytic anemia; IQR, interquartile range; LR, likelihood ratio; MCV, mean corpuscular volume; RBC, red blood cells; ROC, receiver operator characteristic curve; SAT, saline agglutination test; WBC, white blood cells.

KEYWORDS
carbon monoxide, diagnosis, hemoglobin, immune-mediated, microangiopathic

1 | INTRODUCTION

Etiologies for hemolytic anemia (HA) in dogs are diverse, and include infections, intoxications (eg, zinc, copper, garlic/onion), Heinz body anemia, microangiopathy, hereditary diseases, transfusion reactions and splenic disease.[1] The most common etiology is immune mediated hemolytic anemia (IMHA).[1-4] Diagnosis of HA is hampered by inadequate sensitivity and specificity of existing tests. Hemoglobinemia and hemoglobinuria are specific, albeit insensitive, markers of hemolysis in 10% of cases.[5,6] Additionally, spurious hemolysis associated with blood collection is a potential cause for misinterpretation. Hyperbilirubinemia is a nonspecific marker of hemolysis which can also develop in various hepatic and cholestatic diseases.[6] Spherocytosis is highly suggestive of extravascular hemolysis in dogs, although its sensitivity for IMHA is variable among studies, ranging from 61% to 95%.[1,6,7] Differential diagnoses for spherocytosis are diverse, including IMHA, splenic diseases, microangiopathies and hereditary membrane disorders.[6] Therefore, diagnosis of HA is often based on a constellation of laboratory and imaging studies in absence of a highly sensitive and specific biomarker.

Carboxyhemoglobin (COHb) is a stable complex of carbon monoxide (CO) and hemoglobin and serves as a useful biomarker of blood CO concentration.[8,9] Fires and environmental contamination are leading causes of severe CO intoxication. However, endogenous CO production also occurs under physiological and pathological conditions because of metabolism of heme-containing proteins, most notably of Hb.[8-10] This process is mediated by heme-oxygenase (HO), which catalyzes the degradation of heme into CO, Fe^{++} and biliverdin. Thus, hemoglobin serves both as the principal source and the target of endogenous CO.[9] Basal COHb concentration rarely exceeds 1% in healthy, nonsmoking humans, but increases in states of accelerated hemoglobin metabolism.[8,9,11-13] Furthermore, measurement of COHb is not affected by hemolysis, lipemia or hyperbilirubinemia,[14] rendering it a potentially useful predictor of hemolysis.

The deleterious effects of CO on arterial oxygen content are 2-fold: First, it decreases the oxygen-carrying capacity of hemoglobin owing to its greater affinity for heme groups, as compared to oxygen. Second, binding of CO to hemoglobin at 1 of the 4 heme sites increases the affinity of oxygen at the remaining sites, and consequently reduces oxygen release to tissues. Combined, these effects can impair oxygen delivery to organs and exacerbate tissue hypoxia.[15,16]

Our main hypotheses were that COHb would be higher in dogs with HA, compared to anemic dogs without hemolysis, and in nonsurvivors compared to survivors. Therefore, we sought to investigate the discriminatory utility of COHb for the diagnosis of hemolysis in anemic dogs, and explore associations between COHb and survival and between COHb and laboratory analytes.

2 | MATERIALS AND METHODS

2.1 | Dogs and definitions

The study was conducted at a private referral center, between 2021 and 2022 and was approved by the national council for experiments on animal subjects. Previous RBC transfusions constituted an exclusion criterion.

2.2 | Collection of samples and laboratory methods

A CBC, biochemistry, and venous blood gas analysis, including COHb measurement, were performed in all dogs upon admission. Samples for CBC were collected in potassium-EDTA tubes and analyzed within 60 minutes from collection (IDEXX ProCyte Dx, IDEXX Laboratories, Westbrook, ME). For the purpose of saline agglutination test (SAT), 1 drop of EDTA-anticoagulated blood with 4 drops of saline, at room temperature, were thoroughly mixed prior to microscopic examination. DiffQuick-stained smears were evaluated by a board-certified internal medicine specialist (R. Nivy) for the presence of polychromasia, nucleated RBC, ghost cells, spherocytes, schistocytes and white blood cell (WBC) morphology.

Routine biochemistry was performed on heparinized blood (Catalyst One, IDEXX Laboratories, Westbrook, Maine). Measurement of COHb concentration (presented as % of total hemoglobin) was carried out in heparinized blood with the use of GEM 4000 premier system with iQM (Barcelona, Spain), This system is routinely used for measurement of electrolytes in all dogs admitted to the referral center wherein the study was conducted. Therefore, no additional blood was drawn for the purpose of this study. Urinalysis was performed in all dogs with pigmenturia, azotemia, or suspected urinary tract infection.

2.3 | Definitions

Anemic dogs (red blood cell [RBC] count $<5.65 \times 10^6/\mu L$; hematocrit <37.3%) were prospectively enrolled and allocated to either the HA or non-HA group. In accordance with the ACVIM consensus statement,[17] dogs were included in the IMHA group only if both a positive SAT without washing and spherocytosis in the blood smear were confirmed by a board-certified internist. Subsequently, dogs in the IMHA group were evaluated for the presence of hemolysis. Criteria for hemolysis included hyperbilirubinemia in absence of functional hepatic disease/cholestasis/sepsis, hemoglobinemia, hemoglobinuria, the identification of ghost cells in the blood smear, or a combination of

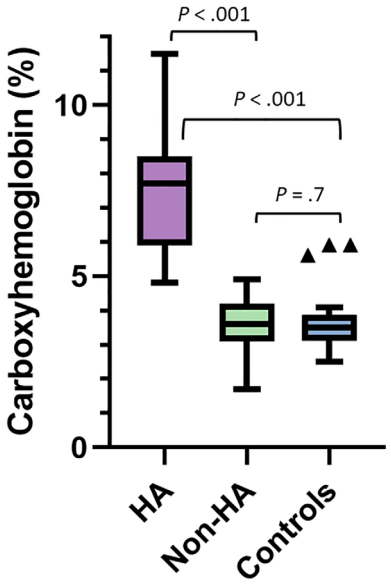

FIGURE 1 Box and whiskers plots for carboxyhemoglobin concentrations, presented as % of total hemoglobin, in 27 dogs with hemolytic anemia (HA), 27 dogs with non-HA, and 24, nonanemic control dogs (including blood donors and dogs presenting for elective orthopedic procedures). The box extends from the 25th to the 75th percentiles, while the whiskers show the minimum and maximum values. Carboxyhemoglobin concentrations (median [interquartile range]) significantly differed between the HA (7.7% [2.5%]) and either the non-HA group (3.6% [1.05]; $P < .001$) or control group (3.5% [0.65%]; $P < .001$) but not between the last 2 groups ($P = .7$)

these findings.[17] Accordingly, IMHA cases were classified as "diagnostic for IMHA" if at least 1 criterion for hemolysis had been identified, or as "supportive of IMHA," if only a positive SAT and spherocytosis, but no indicators of hemolysis, had been identified. Microangiopathy was defined by the presence of schistocytosis, a negative SAT and identification of an underlying etiology such as splenic torsion or mass. Dogs in the non-HA group were included if they had presented with a nonregenerative anemia of any cause or a regenerative anemia secondary to blood loss, in absence of the above-mentioned criteria for immune-mediated destruction and hemolysis. Chronic kidney disease (CKD) and acute kidney injury (AKI) were diagnosed in accordance with the International Renal Interest Society guidelines. Immune-mediated thrombocytopenia was diagnosed in dogs with severe thrombocytopenia (<50 $\times$ 10^3/μL), in which an underlying infectious, neoplastic, or splenic disease had not been identified in abdominal ultrasound, thoracic radiographs, and PCR tests. Iron deficiency was suspected based on the presence of marked microcytosis that was not breed-related (mean corpuscular volume, <55 fL; reference interval, 61.6-73.5 fL) in dogs with evidence of chronic blood loss (eg, severe infestation with ectoparasites or gastric ulcers). Dogs presenting for elective orthopedic surgery or blood donation, with no medical history or laboratory abnormalities, were chosen as the third, control group by convenience sampling. Survival to discharge and 1-month, postdischarge survival were used as outcome measures.

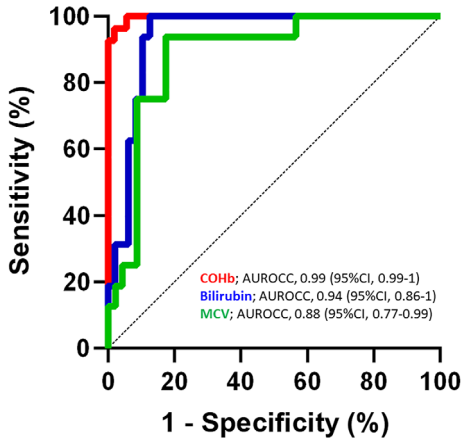

FIGURE 2 ROC curves of carboxyhemoglobin (%), mean corpuscular volume of erythrocytes (fL) and blood bilirubin concentration (mg/dL) at presentation as predictors of hemolytic anemia among a general group of anemic dogs of different etiologies. The reference line is colored black

2.4 | Statistical analyses

Statistical analyses were performed using a statistical software package (SPSS 25.0 for Windows, IBM, Armonk, New York), while Figures 1 and 2 were obtained using GraphPad Prism (version 9.3.1 for Windows, San Diego, California). Owing to data distribution, continuous variables were described as medians and interquartile ranges (IQR) and the nonparametric Mann-Whitney *U*-test and Kruskal-Wallis tests were used to compare continuous variables between 2 and 3 outcome groups, respectively. The Fisher's exact test was applied for testing associations between categorical variables. Spearman rank correlation coefficients were calculated for assessment of the strength of associations between continuous variables. The receiver operator characteristic (ROC) analysis was used to evaluate several laboratory analytes, including COHb, as predictors of hemolysis or survival. The maximal point of the Youden's index (Sen-[1-Spec]) was used as the optimal cut-off value for ROC analyses. Likelihood ratios (LR) were computed to assess the contribution of COHb measurement on the probability of being HA. All tests were 2-tailed, and $P < .05$ was considered significant except in the case of the pairwise comparisons in which the Bonferroni corrected *P*-value was used. Bonferroni corrections were applied to correct for multiple comparisons.

3 | RESULTS

3.1 | Study group demographics

The study group comprised 27 dogs with HA (11 males [neutered, 9]; 16 females [neutered, 13]; median [IQR] age, 8 [4.1] years), 27 dogs with non-HA (14 males [neutered, 8]; 13 females [neutered, 9]; age, 11 [4.8] years) and 24 control dogs (9 males [neutered, 7]; 15 females [neutered, 8]; age, 2.5 [5.6] years). Age significantly differed between

TABLE 1 Selected CBC, chemistry and blood gas analytes in anemic dogs with or without hemolysis

Analytes[a]	Hemolytic anemia	Nonhemolytic anemia	Reference interval	*P* value
Leukocytes ($\times 10^3/\mu L$)	17.38 (19.01)	13.13 (12.98)	5.05-16.76	.18
Red blood cells ($\times 10^6/\mu L$)	1.98 (1.42)	4.05 (1.14)	5.65-8.87	<.001
Hemoglobin (g/dL)	5.2 (2.85)	9.20 (3.60)	13.1-20.5	.003
Hematocrit (%)	15.7 (10)	25.20 (9.92)	37.3-61.7	<.001
MCV (fL)	76 (14)	62.3 (4.7)	61.6-73.5	<.001
MCHC (g/dL)	32.7 (6.75)	36.3 (1.85)	32.0-37.9	.18
RDW (%)	22.2 (7.37)	18 (6.5)	13.6-21.7	.14
Platelets ($10^3/\mu L$)	127 (84)	115 (399)	148-484	.9
Albumin (g/dL)	3.1 (0.55)	2.8 (0.825)	2.3-4.0	.07
Bilirubin (mg/dL)	1.7 (10.3)	0.2 (0.22)	0.0-0.9	<.001
Lactate (mmol/L)	2.45 (4.3)	1.3 (1.1)	<2.5	<.001
Carboxyhemoglobin (%)	7.7 (2.5)	3.6 (1.05)	N/A	<.001
Methemoglobin (%)	1.75 (2.2)	0.85 (1.1)	N/A	.008[b]

Abbreviations: MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; RDW, red blood cell distribution width.
[a]Results are presented as median (interquartile range); The Mann-Whitney *U*-test was used to compare the 2 groups.
[b]Significance was lost after correction for multiple comparisons.

groups, both between the HA and non-HA groups, as well as between the controls and either of the other 2 groups ($P < .001$ for all comparisons).

Dogs in the HA group comprised 21 dogs with IMHA, 5 dogs with microangiopathic HA, and 1 dog with Evans syndrome. Eighteen of the dogs had findings diagnostic for IMHA, with a positive SAT, spherocytosis, and hyperbilirubinemia. In 3 dogs with signs of immune-mediated RBC destruction (a positive SAT and spherocytosis), the evidence for IMHA was deemed supportive in absence of markers of hemolysis. The 1 dog with Evans syndrome had findings diagnostic for IMHA and a platelet count of $5 \times 10^3/\mu L$, with no evidence of active bleeding to any organ or body cavity. Dogs with microangiopathic HA had splenic hemangiosarcoma (n = 3), or splenic torsion (n = 2) and presented with marked schistocytosis, hemoglobinemia and hemoglobinuria.

Diagnoses in the non-HA group included CKD (n = 11), immune-mediated thrombocytopenia (n = 6), AKI (n = 3), iron deficiency (n = 2), anemia of chronic disease (n = 2; 1 with megaesophagus and aspiration pneumonia and 1 with a nonbleeding, *Spirocerca lupi*-associated esophageal mass). Hypoadrenocorticism, Sertoli cell tumor, and bone marrow lymphoma were diagnosed in 1 dog each. None of the dogs in any of the 3 groups had received blood transfusions or medications that could have increased carboxyhemoglobin concentrations before enrollment.

3.2 | Clinicopathological findings

Anemia was significantly more severe, and the mean corpuscular volume was higher in dogs with HA, compared to dogs with non-HA (Table 1). Additional differences included higher lactate and bilirubin concentrations in dogs with HA (Table 1). No other hepatic functional variables beyond total bilirubin were altered in dogs from the HA group.

3.3 | Carboxyhemoglobin concentration and outcome

Carboxyhemoglobin concentrations (median [IQR]) differed between dogs with HA (7.7% [2.5%]) and non-HA (3.6% [1.05]; $P < .001$) and dogs with HA and nonanemic dogs (3.5% [0.65%]; $P < .001$). No difference was found between non-HA and nonanemic dogs ($P = .7$; Figure 1). The area under the ROC curve (AUROCC) of COHb as predictor of HA among all anemic dogs (ie, the HA and the non-HA groups combined) was 0.997 (95% confidence interval [CI], 0.99-1.00; Figure 2). Three cut-off points with the least misclassifications were identified, including 5.05%, 4.55% and 4.85%, with corresponding sensitivity/specificity of 92.6%/100%, 100%/92.6% and 96.3%/96.3%, respectively. The LR of a positive test using the cut-off value of 4.85% was 26, while that of a negative test was 0.04.

The AUROCC of serum bilirubin concentration or mean corpuscular volume (MCV) of erythrocytes, as predictors of HA among anemic dogs, was 0.94 (95% CI, 0.86-1.00) and 0.884 (95% CI, 0.77-0.99), respectively (Figure 2).

Weak to moderate correlations were identified between the COHb concentration and hematocrit ($\rho = -584$; $P < .001$) and bilirubin concentration ($\rho = 611$; $P < .001$) in the entire group of anemic dogs. Statistical significance was lost for each of these correlations when they were investigated only in dogs with HA. Twenty-one (78%) dogs with non-HA survived to discharge. Among dogs with HA in general, or IMHA in particular, survival to discharge and 1-month survival were 55% and 41%, or 50% and 36%, respectively. Follow-up was incomplete in dogs with non-HA, and therefore 1-month survival could not be documented.

Carboxyhemoglobin concentration did not discriminate survivors from nonsurvivors among dogs with HA ($P = .72$), IMHA ($P = .58$) or dogs with non-HA ($P = .39$). Correspondingly, the AUROCC of COHb

as predictor of 1-month survival was 0.542 (95% CI, 0.305-0.781) and 0.619 (95% CI, 0.366-0.871) for dogs with HA or non-HA, respectively.

Statistically significant associations between outcome and the hematocrit, potassium/albumin/bilirubin/lactate concentration, WBC count or the platelet count were not found.

4 | DISCUSSION

In the present study, COHb demonstrated excellent discriminatory ability for the diagnosis of hemolysis among anemic dogs. It exhibited superior sensitivity and specificity, when compared to other markers of hemolysis (bilirubin) and to reported sensitivities of the saline-agglutination/direct antiglobulin tests. Conversely, it was not associated with severity of hemolysis, and failed to discriminate survivors from nonsurvivors, when measured upon admission.

Blood COHb concentrations increase during hemolysis because of increased hemoglobin catabolism.[8,11-13] Additionally, IMHA (the leading etiology of HA in dogs) is a proinflammatory disease, characterized by increased cytokine concentrations.[3,18-20] Some of these, including interleukin-1,-6,-10, and TNF-α induce HO-1 overexpression,[9] thereby further increasing CO production and COHb formation. In humans, a plethora of studies demonstrate increased COHb concentrations during hemolysis, including in patients with auto-immune HA, thrombotic microangiopathy, sickle cell disease, extracorporeal membrane oxygenation-related hemolysis, alloimmunization in newborns and hereditary enzyme deficiencies.[8,12,13,21-23] In dogs there is a dearth of information regarding COHb in the context of HA, barring 2 studies which demonstrate high COHb concentrations in 5, *Babesia canis*-infected dogs,[24] and in dogs that are experimentally subjected to extracorporeal hemolysis.[25] Two large, retrospective studies in humans demonstrate the diagnostic utility of COHb for HA among 185 newborn babies with hyperbilirubinemia[12] and 187 intensive care unit (ICU) patients.[11] The performance of COHb was comparable to the present findings, when judged by ROC analysis, with AUROCCs in the range of 0.92-0.93 in these 2 studies. COHb measurements in those patients, and the proposed optimal cut-off points, were lower by more than half the proposed cut-off points herein (2%-2.2%, compared to 4.55%-5.05%), even though the same blood gas analyzer was used in 1 of the studies.[11] Furthermore, COHb concentrations in control dogs in the present study exceeded the proposed cut-off points in humans. These discrepancies might therefore reflect interspecies differences in endogenous COHb production.

The LR of a positive test in our study (defined as COHb >4.85% for the purpose of analysis) demonstrates the usefulness of COHb in the diagnosis of HA. If the pretest probability of HA in a group of anemic dogs is 2.5%,[26] a positive COHb test will result in posttest probability of 40%. Alternatively, a pretest probability of 6.5% for HA among a group of dogs with regenerative anemia[26] will yield a posttest probability of 64%, when COHb concentrations exceed 4.85%. Thus, COHb proves to be a highly useful ancillary diagnostic tool for HA in dogs, and future studies are warranted to investigate its added value when combined with other measures of hemolysis.

The present study tested only the usefulness of COHb as a marker of hemolysis. While immune-mediated anemia is a leading cause of hemolysis, it is not invariably hemolytic. Cases of precursor-targeted immune-mediated anemia and pure-red cell aplasia might lack criteria of hemolysis and have low COHb cocentrations.[27,28] Conversely, cases of internal bleeding might result in increased COHb concentrations secondary to hematoma formation and hemoglobin degradation.[29] These caveats should be considered when interpreting COHb concentrations in anemic dogs.

In the present study, COHb was not associated with the RBC count or hemoglobin concentration in dogs with HA. This surprising finding might have several explanations. First, a moderate, inverse correlation was found between hematocrit and COHb in the general group of anemic dogs (both the HA and non-HA groups, combined). However, statistical significance was lost when this was tested only in dogs with HA, possibly because of the limited range and sample size. Second, COHb is a measure of increased hemoglobin catabolism, while RBC indices reflect the balance between the rate of erythropoiesis versus that of RBC clearance. In humans, a low inverse correlation ($r = -.442$; $P < .001$) was found between COHb and hemoglobin in ICU patients,[11] but not in newborns with hyperbilirubinemia.[12] Furthermore, glucose-6-phosphate dehydrogenase deficiency in humans can cause severe hemolysis, including hyperbilirubinemia and marked increases in COHb concentrations, in absence of anemia.[23] Thus, under certain disease conditions, discordant hemoglobin and COHb results might exist.

Carboxyhemoglobin failed to discriminate survivors from nonsurvivors in any of the study groups herein. Lack of association between the magnitude of hemolysis and the magnitude of COHb is a possible explanation. Failure to reach reported toxic concentrations (ie, >10%-15%), above which clinical signs appear, is another possible explanation. Lastly, in 2 large retrospective studies of 868 critically-ill, human patients[30] and 466 dogs with respiratory diseases,[31] COHb was not associated with death.

This study has several limitations. First, COHb measurement is unavailable in most private veterinary practices. However, it is highly stable in heparinized blood for up to 4 weeks,[32] and can be measured in many emergency and referral centers. Second, differential diagnoses for elevated COHb other than hemolysis and CO intoxication are myriad, and include internal bleeding, exposure to drugs, sepsis, hepatic and respiratory diseases, and various additional inflammatory diseases.[8] However, except for internal bleeding,[8,29] COHb is only mildly elevated under these conditions and is far below the proposed cutoffs for the diagnosis of HA. Third, COHb was measured in transfusion-naïve dogs, and therefore its discriminatory utility might not apply to dogs that have recently been transfused. Fourth, hyperbilirubinemia was used to classify most IMHA cases as "diagnostic for IMHA," based on the ACVIM consensus statement on the diagnosis of IMHA.[17] While all other markers of liver function, other than

bilirubin concentration, were within their respective reference intervals, one cannot exclude a functional liver disease or posthepatic cholestasis as possible etiologies for hyperbilirubinemia. Under those circumstances, those cases would have been classified as "supportive of IMHA."[17] Lastly, autoimmunity was demonstrated by the presence of a positive SAT vis-à-vis spherocytosis, rather than a positive direct antiglobulin test or flow cytometry. However, the SAT and direct antiglobulin test have comparable sensitivities and specificities,[6,17] and are both acceptable as diagnostic criteria for IMHA.[17]

In conclusion, COHb proved an excellent biomarker for HA, and might prove a useful ancillary test in questionable cases. Notwithstanding its diagnostic utility, it was not associated with magnitude of hemolysis, and cannot be recommended as a marker of disease severity or outcome based on the findings herein.

ACKNOWLEDGMENT

Funding provided by the European Veterinary Emergency and Critical Care (EVECC) research grant 2021. Preliminary results were presented as an oral abstract at the 19th EVECC congress, June 2022.

CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the national council for experiments on animal subjects (February 28, 2021).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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How to cite this article: Nivy R, Sutton G, Bruchim Y. Carboxyhemoglobin as a diagnostic and prognostic biomarker of hemolytic anemias in dogs. *J Vet Intern Med*. 2023;37(1): 110-116. doi:10.1111/jvim.16617