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Effect of dilution of canine blood samples on the specificity of saline agglutination tests for immune-mediated hemolysis

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Abstract

Background: Saline agglutination tests (SATs) are widely recommended for diagnosis of immune-mediated hemolytic anemia in dogs, but there are frequent false-positive results.

Objectives: Specificity of SATs will improve at higher saline-to-blood ratios.

Animals: One hundred fifty dogs treated at a veterinary referral hospital with hematocrits ≤30%.

Methods: Prospective diagnostic accuracy study. Immune-mediated hemolysis (IMH) was considered present if a gel direct antiglobulin test (DAT) was positive and there was clinical evidence of hemolysis (n = 9), absent if another mechanism for anemia was identified and the DAT was negative or there was no hemolysis (n = 138), and if IMH status was unclear, dogs were excluded (n = 3). Saline agglutination tests were prepared at 1 : 1, 4 : 1, 9 : 1, and 49 : 1 saline-to-blood ratios, and microscopic agglutination was considered a positive result.

Results: Specificity for IMH increased from 29% (95% confidence interval 20-38) at a 1 : 1 dilution to 97% (93-99) at a 49 : 1 dilution. Sensitivity was 88% (47-100) at 1 : 1 and 4 : 1 dilutions and 67% (30-93%) at 9 : 1 and 49 : 1 dilutions. Diagnostic accuracy increased from 33% (24–42) at 1 : 1 dilution to 95% (90-98) at 49 : 1 dilution.

Conclusions and Clinical Importance: If performed using a 49 : 1 saline-to-blood ratio, SATs achieve high specificity for IMH. Based on a gold standard of positive DAT and evidence of hemolysis, lower saline-to-blood ratio results should not be used because false-positive results are common.

KEYWORDS

accuracy, autoimmune hemolytic anemia, Coombs test, diagnosis, immune-mediated hemolytic anemia, sensitivity

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; CI, confidence interval; CKCS, Cavalier King Charles Spaniel; DAT, direct antiglobulin test; FN, false negative; FP, false positive; FUO, fever of unknown origin; GDV, gastric dilation and volvulus; GGT, gamma-glutamyl transferase; GSD, German Shepherd dog; IMH, immune-mediated hemolysis; IMHA, immune-mediated hemolytic anemia; IMPA, immune-mediated polyarthritis; ITP, immune thrombocytopenia; PBS, phosphate buffered saline; PLN, protein losing nephropathy; PSS, portosystemic shunt; RI, reference interval; SAT, saline agglutination test; TN, true negative; TP, true positive; WHWT, West Highland White Terrier.

1 | INTRODUCTION

Saline agglutination tests (SATs), also referred to as saline dispersion tests, are widely recommended for diagnosis of immune-mediated hemolytic anemia (IMHA) in dogs,[1-3] and further testing to confirm erythrocyte-bound antibody is generally considered superfluous in

the face of a positive SAT.[4] This is based on the theory that if erythrocyte aggregates remain after mixing blood with saline, they are evidence of antibody-mediated agglutination, rather than nonspecific rouleaux formation.[4] However, there is little evidence to confirm the reliability of SATs for distinguishing immune vs nonimmune-mediated erythrocyte interactions. This is concerning because a false-positive diagnosis of IMHA will delay appropriate treatment and could unnecessarily expose the patient to the risks of immunosuppressive therapy, including infection[5,6] and corticosteroid-induced gastric ulceration.[7,8]

Most studies that report SAT results also use the test as criterion for IMHA, preventing reliable assessment of sensitivity and specificity.[9-11] One exception to this reports that 4 of 20 anemic dogs with positive SATs have negative direct antiglobulin tests (DATs) and diagnoses other than IMHA.[12] However, this high false-positive rate may reflect the use of a 1 : 1 ratio of saline to blood.[12] This method is described in several sources, but several authors suggest that strong rouleaux will break apart only at higher saline-to-blood ratios.[1,3,4,13] False-positive results at low saline-to-blood ratios could also reflect difficultly in distinguishing agglutination from erythrocytes overlaid within a thick preparation. We were unable to identify any studies investigating the effect of saline-to-blood ratio on specificity, but dilutions of between 4 : 1 and 12 : 1 are suggested in textbooks and review articles,[3,13] and anecdotally, veterinary clinical pathology laboratories sometimes use much higher dilutions.

This study tested the hypothesis that the specificity of SATs for immune-mediated hemolysis (IMH) is improved at higher saline-to-blood ratios. The primary aim was to determine specificity for SATs performed at a 1 : 1, 4 : 1, 9 : 1, and 49 : 1 saline-to-blood ratio in anemic dogs presenting to a veterinary referral hospital. Secondary aims were to determine the effect of dilution on sensitivity and diagnostic accuracy of SATs.

2 | METHODS

2.1 | Case enrollment

Dogs treated at the Texas A&M University Veterinary Medical Teaching Hospital between May 2019 and March 2020 were eligible for enrollment if an automated hematocrit (Advia 2120, Siemans, Malvern, PA) or manual PCV for a K_2EDTA-anticoagulated blood sample was ≤30% [reference interval (RI) 35-56], and there was sufficient residual sample remaining after requested clinical testing to perform the study protocol. Ethical approval was not required for the study as no additional blood volume was collected, investigators were not involved in venipuncture and owners could refuse permission for research use of residual samples during our process of admissions. Attending clinicians were contacted to ensure no further clinical testing was required before performing the study protocol, but results of study SAT or DAT tests were not provided to the attending clinician for included cases. As described later, dogs were excluded from statistical analysis if IMH status could not be determined, and for these dogs, study results were made available to the attending clinician if requested. For dogs with multiple samples available, only the first sample meeting inclusion criteria were used.

2.2 | Criteria for IMH

Dogs were classified as affected by IMH if a gel DAT was positive and there was clinical evidence of hemolysis. Criteria for hemolysis were at least 1 of the following:

1. ≥5 spherocytes per high power field (HPF) on microscopic examination of a blood smear by a board-certified clinical pathologist.
2. Ghost cells estimated to compose >5% of erythrocytes by a board-certified veterinary clinical pathologist, for a sample collected within 1 hour of blood smear preparation.
3. Visually detectable plasma hemolysis, as assessed by a veterinary clinical pathologist, clinical pathology resident, medical technologist, or other trained staff of the veterinary clinical pathology laboratory, in samples collected across multiple venipunctures.
4. A moderate-to-large positive heme reaction on urine dipstick testing (Multistix, Siemans, Malvern, Pennsylvania) of a sample without suspected color interference, clinical findings consistent with rhabdomyolysis, or intact erythrocytes on sediment examination performed by a medical technologist or other trained staff of the veterinary clinical pathology laboratory.
5. Elevated plasma bilirubin or a moderate-to-large positive result for bilirubin urine dipstick testing without an increase in ALT, ALP, or GGT, as measured by a chemistry instrument (Vitros 4600, Ortho-Clinical Diagnostics, Raritan, New Jersey) maintained and operated by the Texas A&M Veterinary Clinical Pathology Laboratory.

Dogs were classified as unaffected by IMH in the following conditions:

1. A gel DAT was negative and there was either no clinical evidence of hemolysis or hemolysis could be explained by a nonimmune-mediated mechanism.
2. A gel DAT was positive but there was no evidence of hemolysis or hemolysis could be explained by a nonimmune-mediated mechanism.

Dogs were excluded from analysis if we did not consider IMH could be reliably diagnosed or ruled out. Criteria for exclusion were as follows:

1. Negative DAT test, lack of evidence of hemolysis, or both, but a clinical diagnosis of IMHA by the attending veterinarian.
2. Positive DAT without evidence of hemolysis, but a nonimmune-mediated cause of anemia was not identified during clinical investigations as directed by the attending veterinarian.
3. Negative DAT but clinical evidence of hemolysis, and a nonimmune-mediated mechanism for hemolysis could not be identified.
4. Complete evaluation for hemolysis could not be performed because urinalysis, biochemistry testing, or both were not

requested by the attending clinician, and a nonhemolytic cause of anemia could not be identified from the available information in the medical record.

To assist with case classification, electronic medical records from our institution and any records provided by the referring veterinarian were reviewed to determine diagnosis made by the attending veterinarian, transfusion and immunosuppressive therapies, biochemistry, hematology, and urinalysis findings relevant to determining if hemolysis was present, coagulation testing, and reticulocyte and platelet concentrations. Anemia of inflammatory disease[14] or nonhemolytic cancer-related anemia[15] were considered present if (a) anemia was nonregenerative (defined as automated reticulocytes <100 000 μL or corrected reticulocyte percentage < 1% for manual reticulocyte counts), (b) there was clinical evidence to support inflammatory disease, chronic systemic disease, or neoplasia and (c) there was no clinical evidence of blood loss. Anemia was classified as due to blood loss if there was clinical evidence of hemorrhage or recent surgery. Anemia was attributed to chemotherapy if the dog was receiving any chemotherapeutic agent other than prednisolone alone and no other cause of anemia was identified.[16-18] Hemolysis by a nonimmunological mechanism was considered likely if any of the following were present: known exposure to an oxidative toxin,[19] large numbers of Heinz bodies,[19] pkynocytes or eccentrocytes[20] on blood smear review, or documented envenomation.[21-23]

2.3 | Study testing protocol

All samples were anticoagulated using potassium EDTA. Investigators were not involved in performing venipuncture, and no attempt was made to standardize venipuncture technique. Automated hematocrit and manual PCV measurement were performed by a medical technologist or trained staff under the supervision of a medical technologist in the Texas A&M Veterinary Clinical Pathology laboratory.

Blood was refrigerated and SATs performed within 48 hours by 1 or other of the investigators. After blood had returned to room temperature, a 1 : 1 dilution was performed by transferring 5 μL of room-temperature phosphate-buffered saline (PBS, pH 7.2, Gibco, Grand Island, New York) to a glass microscope slide using a 1 to 10 μL micropipette, and then adding 5 μL of blood to the drop of saline before mixing with the pipette tip. A glass coverslip was placed over the PBS/blood mix, and immediately afterwards, the preparation was examined at ×40 magnification using a light microscope with a partially closed diaphragm. A 4 : 1 and 9 : 1 dilution was prepared identically, except 2 μL of blood was added to 8 μL of PBS for the 4 : 1 dilution and 1 μL of blood was added to 9 μL of PBS for the 9 : 1 dilution. For the 49 : 1 dilution, 1 μL of blood was added to 49 μL of PBS in a plastic 1.5 mL microcentrifuge tube and was gently mixed by manual agitation before transferring 10 μL to a glass slide and overlaying a coverslip.

For each dilution, 5 × 40 objective fields were selected at random for microscopic investigation. Random selection was achieved by the investigator looking away from the microscope while moving the microscope stage. The SAT was considered positive if at least 1 field had ≥1 group of 4 or more erythrocytes, or at least 2 groups of 3 erythrocytes, which did not break apart if the coverslip was gently tapped with the tip of a pen (Figure 1). If the field was composed of dense layers of erythrocytes, it was reported as too thick to read. If all 5 fields were too thick to read, the results at that dilution were excluded from statistical analysis.

A gel DAT (Canine Gel Test DAT, Alvedia, Limonest, France) was performed according to the manufacturer's instructions. EDTA blood samples were centrifuged at 1000*g* for 2 minutes and plasma was discarded. Ten microliters of the remaining erythrocytes were transferred to 20 drops (approximately 1 mL) of the manufacturer-supplied dilutant in a 5 mL plastic tube. After mixing by gentle swirling, 30 μL of blood was added to a gel tube, containing anti-dog IgG, IgM, or C3 antibodies. After enrolling the first 22 cases, the manufacturer provided an antibody-free autocontrol tube. Gel tubes were centrifuged for 10 minutes at 200*g* in a swing bucket centrifuge. The gel tube was considered positive if there was a line of erythrocytes at the top of the gel; weak positive if there were erythrocytes suspended within the gel; and negative if all erythrocytes were at the base of the gel tube. Tests were considered to have failed if both the test and autocontrol tube were positive or weak positive.

2.4 | Statistical analysis and sample size calculation

Sensitivity, specificity, diagnostic accuracy, and their 95% confidence intervals were calculated for each SAT dilution. Equations were as follows, where TP indicates true positive, TN true negative, FP false positive, and FN false negative:

$$\text{Sensitivity}\,(\%) = [\text{TP}/(\text{TP}+\text{FN})] \times 100,$$

$$\text{Specificity}\,(\%) = [\text{TN}/(\text{TN}+\text{FP})] \times 100,$$

$$\text{Diagnostic accuracy}\,(\%) = \left[\frac{\text{TP}+\text{TN}}{(\text{TP}+\text{TN}+\text{FP}+\text{FN})} \times 100\right].$$

The proportion of samples classified as false positive was compared between dilutions using a McNemar test for paired data. As 6 comparisons were performed, a Bonferroni correction was calculated, resulting in $P < .008$ being considered significant. Globulin concentration was compared between dogs with false-positive and true-negative results at each dilution by independent *t* test, after confirming normal data distribution using the D'Agostino-Pearson test. As 4 comparisons were performed, a Bonferroni correction was calculated, resulting in $P < .013$ being considered significant.

One hundred eleven true-negative samples were needed for the McNemar test to detect a 15% shift in samples from false positive to true negative between 2 dilutions and 0% shift from true negative to false positive with 80% power and a type I error of 0.001. Magnitude of change was selected based on the specificity reported for a 1 : 1

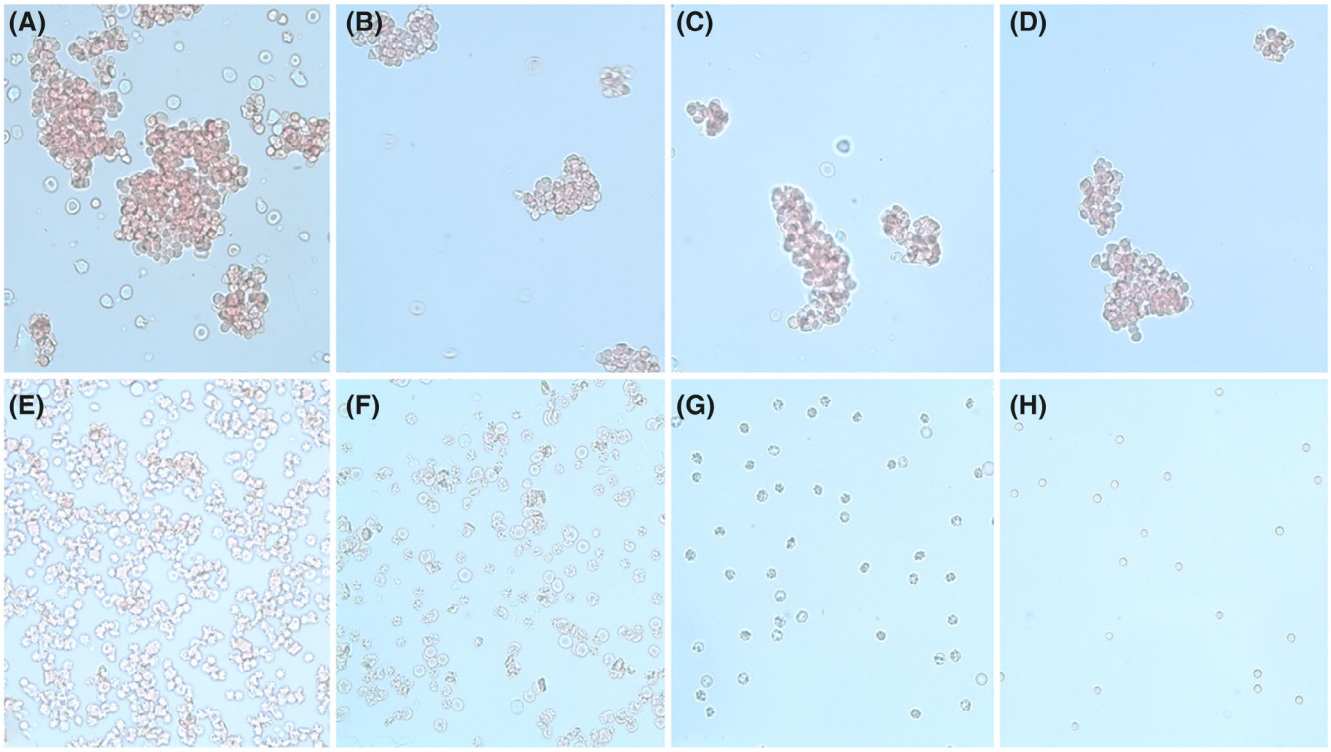

FIGURE 1 Examples of saline agglutination tests performed at increasing saline-to-blood ratios (×40 objective, unstained). Saline and blood from a dog with immune-mediated hemolytic anemia (IMHA) (A-D) and a dog with anemia of inflammatory disease (E-H) was mixed in 1 : 1 (A,E), 4 : 1 (B,F), 9 : 1 (C,G), and 49 : 1 (D,H) ratios. The IMHA case demonstrates persistent agglutination at all saline-to-blood ratios. For the anemia of inflammatory disease case, the 1 : 1 and 4 : 1 dilutions were considered positive because there were groups of 4 or more red cells. In some areas, these groups or erythrocytes have an obvious "stacks of coins" arrangement (ie, rouleaux), but there are also overlapping rouleaux mimicking agglutination. At 9 : 1 and 49 : 1 saline-to-blood ratios, rouleaux have fully dispersed

saline dilution test and 4 : 1 saline dilution test in published studies.[9,12] To ensure at least 111 true-negative cases were included in statistical analysis, sample size was increased to 150. Sample size calculation and statistical analysis were performed using the MedCalc statistical software (MedCalc, Ostend, Belgium).

3 | RESULTS

3.1 | Signalment and excluded cases

Signalment and diagnosis are summarized (Table 1). One hundred fifty anemic dogs were enrolled but 3 of 150 (1 DAT negative, 2 DAT positive) were subsequently excluded because IMHA status was unclear. Details of the excluded cases are included in the supplementary material.

3.2 | IMHA status and DAT test results

Of the 147 included dogs, 138 did not meet our criteria for IMHA. For 2/138 non-IMH cases, the DAT failed (ie, positive autocontrol). Neither case met criteria for hemolysis, and both had evidence of a nonhemolytic cause of anemia (Table 1). For the 27 of 138 non-IMH cases, the DAT was positive, but our criteria for hemolysis were not met. For 20 of 27 non-IMH cases, the DAT was rated as weak positive. For the 109 of 138 remaining non-IMH cases, the DAT was negative. None of the non-IMH cases were diagnosed with IMHA by their attending clinician. For the 9 dogs meeting our criteria for IMH, 8 of 9 were diagnosed with IMHA by their attending clinician and 1 of 9 had a history of multiple blood transfusions for bleeding due to hemophilia A, including a noncross-matched transfusion the day before presentation, and had clinical signs consistent with an immunological hemolytic transfusion reaction. For the dog with a transfusion reaction, the gel DAT was weakly positive, and for the 8 dogs with IMHA, the gel DAT was strongly positive. Clinical details and relevant laboratory testing for included dogs are summarized in supplementary material.

3.3 | Saline agglutination results

Increasing dilution increased specificity and diagnostic accuracy, but reduced sensitivity for IMH (Table 2). Proportion of false-positive results was significantly greater when a 1 : 1 dilution was compared

to 4 : 1, 9 : 1, or 49 : 1 dilutions, when a 4 : 1 dilution was compared to 9 : 1 or 49 : 1, and when 9 : 1 was compared to 49 : 1 dilution ($P < .0001$ for all comparisons). Diagnoses in dogs with false-positive and false-negative results at each dilution are summarized in Supplementary Table 2. For the 4 of 9 dogs with IMH that had received at least 1 dose of an immunosuppressive agent before sample collection, 3 of 4 had positive SATs at all dilutions, including a dog that had received at least 2 months of immunosuppression. The remaining dog had a negative SAT at 9 : 1 and 49 : 1 dilutions, and tests were too thick to be assessed at 1 : 1 and 4 : 1 dilutions. At a 1 : 1 dilution, total

TABLE 1 Signalment and mechanisms of anemia

Age, years (median, range)	Breeds	Sex	Principal mechanism of anemia
DAT positive, evidence of hemolysis (n = 9)			
4 (2–8)	Beagle (1), Border Collie (1), Dachshund (1), Doberman (1), English Bulldog (1), Jack Russell Terrier (3), Shetland Sheepdog (1)	FI (2); FS (4), MN (3)	*Immune-mediated hemolysis n = 9* [primary IMHA (6); secondary IMHA (2) (cephalosporins 1, pregnancy, 1); immunological hemolytic transfusion reaction (1)]
DAT positive, no evidence of hemolysis (n = 27)			
6 (0.6-13)	Beagle (1), Bernese Mountain Dog (1), Black Mouth Cur (1), Border Collie (2), Chihuahua (1), CKCS (1), Cocker Spaniel (1), Dogo Argentino (1), English Bulldog (1), GSD (1), Jack Russell Terrier (1), Labrador (1), Maltese (2), Miniature Schnauzer (1), Mix (3), Poodle (1), Pug (1), Rottweiler (1), Siberian Husky (2), Silky Terrier (1), Yorkshire Terrier (2)	FI (4), FN (9), MI (6), MN (8)	*AID/non-hemolytic paraneoplastic anemia n = 10* [congestive heart failure (1); congenital (1) (PSS 1); infectious (4) (distemper 1, pythiosis 1, septic abdomen 1, wound infection 1); inflammatory (1) (pancreatitis 1); neoplastic (3) (lymphoma 3)] *Blood loss n = 12* [bleeding associated with nongastrointestinal neoplasm (2) (hemangiosarcoma 1, splenic stromal sarcoma 1); gastrointestinal lesion (4); ITP (2); surgical blood loss (1); trauma (2); suspected hemophilia (1)] *Chemotherapy n = 3* [lymphoma/lymphoid leukemia (3)] *Other n = 2* [phenobarbital toxicity (1), splenic torsion (1)]
DAT negative (109)			
10 (0.3-16)	American Bulldog (1), Australian Cattle Dog (1), Basset Hound (1), Beagle (3), Bichon Frise (2), Black Mouth Cur (1), Blue Heeler (1), Boerboel (1), Border Collie (1), Boston Terrier (1), Boxer (2), Bull Terrier (2), Catahoula Hog Dog (1), Chihuahua (5), CKCS (1), Cocker Spaniel (4), Dachshund (6), Doberman (1), English Shepherd (1), Foxhound (1), GSD (1), Giant Schnauzer (1), Golden Retriever (5), Great Pyrenees (1), Greyhound (1), Jack Russel Terrier (2), Labrador (6), Maltese (2), Mastiff (1), Miniature Schnauzer (4), Mix (16), Pekinese (1), Pembroke Welsh Corgi (1), Pitbull (5), Pomeranian (1), Poodle (7), Pug (2), Rottweiler (1), Samoyed (1), Shetland Sheepdog (2), Shiba Inu (1), Shih Tzu (4), Siberian Husky (2), WHWT (2), Yorkshire Terrier (2)	FI (14); FS (42); MI (13), MN (40)	*AID/nonhemolytic paraneoplastic anemia n = 40* [congenital (1) (PSS 1); endocrine (2) (diabetes mellitus 1, hypoadrenocorticism 1); immune-mediated (4) (myositis 1, IMPA 1, polyneuritis 1, PLN 1); infectious (10) (abdominal abscess 1, bacterial hepatitis 1, *Dirofilaria immitis* 1, endocarditis 1, osteomyelitis 1, otitis media 1, *Spirocerca lupi* 1, wound infection 3); inflammatory (5) (aspiration pneumonia 2, bile peritonitis 1, pancreatitis 2); multiple chronic systemic disorders (2); neoplasia (10) (disseminated adenocarcinoma 3, lymphoma 3, meningioma 1, osteosarcoma 1, soft-tissue sarcoma 1, suspected but not histologically confirmed 1); open/other (6) (acute retinal detachment 1, FUO 1, chronic diarrhea 1, pneumopericardium after GDV surgery 1, pulmonary hypertension 1, cerebrovascular event 1)]

TABLE 1 (Continued)

Age, years (median, range)	Breeds	Sex	Principal mechanism of anemia
			Blood loss n = 45 [coagulopathy secondary to hepatic histoplasmosis (1); gastrointestinal lesions (9); hypoadrenocorticism (1); idiopathic hematuria (1), ITP (12), bleeding associated with nongastrointestinal neoplasm (5) (disseminated neuroendocrine carcinoma 1, hemangiosarcoma 4); surgical blood loss (9), hemorrhage secondary to thrombocytopenia associated with a large splenic sarcoma (1), trauma (6)] *Chemotherapy n = 12* [lymphoma/lymphoid leukemia (6); mast cell neoplasia (4); multiple myeloma (1); heart-base tumor (1)] *Renal failure n = 7* [acute on chronic 2; chronic 5] *Other n = 5* [hemodilution due to large volume resuscitation after anesthesia adverse event 1; ehrlichiosis-associated pancytopenia 1; parvo infection 1; PIMA, 1; venom-induced hemolysis, 1]
DAT failed, no clinical evidence of hemolysis (2)			
0.3-11	Mastiff (1), Mix (1)	FN (1); MI (1)	*Anemia of inflammatory disease n = 1* [sepsis associated with osteomyelitis (1)] *Blood loss n = 1* [gastrointestinal blood loss associated with chemotherapy for lymphoma (1)]
Excluded (n = 3)			
8 (7-10)	Brussels Griffon (1), Dachshund (1), Mix (1)	FN (3)	*Unknown n = 3*

Note: Of the 150 dogs enrolled in the study, 9 met our criteria for immune-mediated hemolysis, 138 (27 DAT positive, 109 DAT negative, and 2 with failed DAT tests) were anemic due to mechanisms other than immune-mediated hemolysis and 3 were excluded because immune-mediated hemolysis could not be definitively diagnosed or ruled out.

Abbreviations: AID, anemia of inflammatory disease; CKCS, Cavalier King Charles spaniel; DAT, direct antiglobulin test; FUO, fever of unknown origin; GDV, gastric dilation and volvulus; GSD, German Shepherd dog; IMHA, immune-mediated hemolytic anemia; IMPA, immune-mediated polyarthritis; ITP, immune thrombocytopenia; PIMA, precursor-targeting immune-mediated anemia; PLN, protein-losing nephropathy; WHWT, West Highland White Terrier.

protein was significantly higher in dogs with false-positive results (median 6.5 g/dL; range, 3.2-9.1) than those with true-negative results (median 5.9; range, 4.0-8.1; $P = .009$).

On dried blood smear examination, autoagglutination was present in 6 of 9 cases with IMH, all of which had a positive SAT at the 49 : 1 dilution. Macroscopic agglutination was observed in 4 of 9 IMH cases, all of which were positive at the 49 : 1 dilution. One non-IMH case had apparent macroscopic agglutination and autoagglutination on blood smear examination. Agglutination persisted after warming blood to 39°C but dispersed after washing red cells repeatedly, suggestive of strong rouleaux formation.

Mean globulin concentrations were higher for dogs with false-positive results than dogs with true-negative results at all concentrations, but our requirement for statistical significance of $P < .013$ was not met at any dilution [1 : 1 mean for true negatives 3.0 g/dL, SD 0.6, mean for false positives 3.4 g/dL, SD 0.7, $P = .035$; 4 : 1 mean for true negatives 3.1 g/dL, SD 0.6, mean for false positives 3.4 g/dL, SD 0.8, $P = .014$; 9 : 1 mean for true negatives 3.2 g/dL, SD 0.6, mean for false positives 3.3 g/dL, SD 0.8, $P = .041$; 49 : 1 true negatives 3.2 g/dL, SD 0.7, false positives 3.6 g/dL, SD 0.8, $P = .45$].

4 | DISCUSSION

Performing SATs at a 49 : 1 saline-to-blood ratio resulted in high specificity for IMH, while the lower dilutions resulted in frequent false-positive results. This high specificity supports the continuing use of SATs as an inexpensive, patient-side screening tests for IMHA,

TABLE 2 Diagnostic performance of SATs performed at saline to blood ratios of 1 : 1, 4 : 1, 9 : 1, and 49 : 1 for diagnosis of immune-mediated hemolysis

		Saline-to-blood ratio			
	SAT result	1 : 1	4 : 1	9 : 1	49 : 1
IMH (n = 9)	Too thick to read	1/9	1/9	0/9	0/9
	Positive	7/9	7/9	6/9	6/9
	Negative	1/9	1/9	3/9	3/9
Non-IMH/DAT positive (n = 27)	Too thick to read	10/27	0/27	0/27	0/27
	Positive	11/27	15/27	10/27	1/27
	Negative	6/27	12/27	17/27	26/27
Non-IMH/DAT negative (n = 109)	Too thick to read	23/109	1/109	0/109	0/109
	Positive	62/109	55/109	31/109	3/109
	Negative	24/109	53/109	78/109	106/109
Non-IMH/DAT failed (n = 2)	Too thick to read	0/2	0/2	0/2	0/2
	Positive	2/2	0/2	0/2	0/2
	Negative	0/2	2/2	2/2	2/2
Sensitivity, % (95% CI)		88 (47–100)	88 (47–100)	67 (30–93)	67 (30–93)
Specificity, % (95% CI)		29 (20-38)	49 (40-58)	70 (62-78)	97 (93-99)
Diagnostic accuracy, % (95% CI)		33 (24-42)	51 (43-59)	70 (62-77)	95 (90-98)

Abbreviations: CI, confidence interval; DAT, direct antiglobulin test; IMH, immune-mediated hemolysis; SAT, saline agglutination test.

provided dilution is adequate. Persistent erythrocyte aggregates at a 49 : 1 dilution are supportive of IMH, but a small number of false-positive results did occur at a 49 : 1 dilution and there is the possibility of clinically insignificant cold agglutinins.[24] Therefore, a positive SAT should continue to be correlated with other clinical findings, with a particular emphasis on confirming hemolysis, before considering the diagnosis of IMHA definitive.[1] As sensitivity is low at the 49 : 1 dilution, a negative SAT result does not rule out IMHA.

To ensure preparation was comparable throughout the study, we used micropipettes to prepare the 49 : 1 dilution. This degree of accuracy is probably unnecessary for clinical point-of-care testing and the necessary equipment may not be consistently available. A less precise approach would likely be acceptable, providing the preparation results in erythrocytes or erythrocyte aggregates being separated by considerable clear space (Figure 1). For example, a similar dilution is routinely prepared at our institution by briefly dipping a wooden applicator stick into EDTA blood before submerging it in 2 mL of saline, mixing gently, and using a transfer pipette to add a drop to a glass slide. However, further studies are needed to confirm the same specificity can be achieved without precise measurement.

Sensitivity for IMH did reduce with increasing saline dilution, but this should be interpreted cautiously. Firstly, the number of dogs meeting our criteria for IMH was low, resulting in a wide confidence interval for our estimate of sensitivity. Secondly, as reported in previous studies, not all IMHA cases display spontaneous agglutination.[1] Given the high rate of false-positive results at lower dilutions, it is likely that for at least some of our IMH cases, positive results at lower dilutions were the result of persistent rouleaux or erythrocytes overlaid in thick preparations, rather than antibody-mediated agglutination. For IMHA, a false-positive diagnosis carries significant risk to the patient[5] and there are assays other than SATs that can provide evidence of erythrocyte-bound antibody, including DATs, flow cytometry or microscopic confirmation of large numbers of spherocytes.[1] Therefore, adopting a method for SATs that increases specificity at the expense of sensitivity is clinically appropriate.

It is surprising that there have not been more extensive previous efforts to standardize the SAT technique. SATs are not used as a criterion for diagnosis of autoimmune hemolytic anemia in humans,[25] and we struggled to definitively identify the origins of the SAT in veterinary medicine. It seems likely that the test was adopted from procedures to identify incompatible donor-recipient pairs before blood transfusion.[26] Regardless of the test origins, it seems unlikely that a true 1 : 1 dilution was ever intended to be assessed microscopically for diagnosis of IMHA, as such preparations are frequently too thick to examine. As SATs have been adopted without extensive published validation, confusion may have developed between techniques for assessing macroscopic agglutination (ie, visible to the eye) and microscopic agglutination,[3] and between techniques designed to allow the user to distinguish the "stack-of-coins" appearance of rouleaux from the "bunch-of-grapes" like appearance of agglutination vs techniques that break apart rouleaux.[13] It is also possible that instructions to add 1 drop of blood to 1 drop of saline were not intended to imply that the 2 drops should be of equal volume.

There is also no published consensus on what constitutes a positive SAT result. We considered even small groups of erythrocytes evidence of agglutination. This was based on our clinical experience, in which such findings are typically reported as weak positive or "plus/minus" results. In the current study, all dogs with IMHA and a positive SAT at a 49 : 1 saline-to-blood ratio had large aggregates involving many >4 cells, which were obvious using a ×10 objective. In contrast,

3 of 4 false-positive results at the 49 : 1 ratio resulted from small aggregates of 3 or 4 erythrocytes. Investigation of a larger number of IMHA cases is needed before concluding that small aggregates of erythrocytes are not evidence of agglutination, but our results suggest that until such studies becomes available, weak positive SATs should be confirmed by DAT. This should not be extrapolated to interpretation of pretransfusion slide-based crossmatches,[27,28] as in this setting, failure to recognize incompatibility risks an immunological transfusion reaction,[29] whereas false-positive results are of little clinical relevance if other donors are available.

An additional challenge was reliably determining if a patient had an immune-mediated component to their anemia. We could not directly follow the recent ACVIM consensus statement on diagnosis of IMHA because this includes SATs as a diagnostic criterion.[1] Instead, we required a positive DAT and clinical evidence of hemolysis. We acknowledge that DAT-negative IMHA can occur,[9,30,31] and that some clinical signs of hemolysis, such as icterus, may not be present early in the course of hemolysis or after initiation of immunosuppression.[32] Similarly, hemolysis can arise through nonimmune-mediated mechanisms,[21,33,34] and some signs consistent with hemolysis can be artifacts (eg, hemolyzed plasma due to traumatic venipuncture or lysis of erythrocytes in urine leading to confusion between intravascular hemolysis and hemorrhage into the urinary tract)[35] or the result of nonhemolytic disease (eg, hyperbilirubinemia due to hepatic[36] or post-hepatic disease).[37] Large numbers of spherocytes are strongly, but not exclusively, associated with IMHA.[1] Of the DAT negative dogs in the current study, only 1 had ≥5 spherocytes per HPF and this dog had been attacked by large numbers of bees. Although immunosuppression has been suggested as a treatment for dogs with bee venom-induced hemolysis,[21] bee venom constituents can directly cause erythrocyte membrane loss[38] and we considered it likely that spherocytes in this case were an example of nonantibody-mediated spherocytosis. There is also the concern that some of the DAT-positive cases classified as anemic because of a nonimmune-mediated process could have been affected by secondary IMHA, triggered either by an underlying disease, such as neoplasia, or by a drug treatment.[1] Additionally, we did not exclude dogs that had been previously transfused[25,39,40] or received immunosuppression,[39] which may have influenced DAT results.[1] Therefore, it is possible that some of our case classifications are incorrect, but there was agreement between our assessment of IMH and the attending clinician's diagnosis for all dogs included in assessment of test performance. As we did not release DAT or SAT results to clinicians unless dogs were excluded from the study, this suggests that our classifications were consistent with current clinical practice.

There is considerable variability in reported sensitivity and specificity for both traditional test-tube or microtiter plate DATs and flow cytometry for identification of erythrocyte-bound antibody.[9,12,31,41-43] In some cases, this likely reflects variable case and control selection between studies, but variations in test protocols and reagents are also likely a contributing factor.[1,31] Variation in reported test performance creates difficulty when comparing between studies that use DAT as an inclusion criteria for IMH. We therefore opted to use a commercial gel DAT, rather than an in-house DAT protocol for assessment of erythrocyte-bound antibody, as it can be readily replicated by other authors. The gel format of this test has been only recently become commercially available, and a sensitivity and specificity study has yet to be published. In the current study, all cases with a clinical diagnosis of IMHA were DAT positive, and 27 of 136 dogs classified as affected by nonimmune-mediated anemia had a positive DAT and negative autocontrol tube, equivalent to a specificity of 80%.

The gel DAT was strongly positive in all our IMHA cases, and for the dog with a transfusion reaction, the gel was rated as weak positive because approximately half the erythrocytes were at the top of the gel and the other half at the base of the tube. Potentially, this could be consistent with 2 populations of erythrocytes, antibody-positive transfused donor cells and antibody-negative recipient cells, but this was not confirmed through further testing. Most of the positive DAT results in dogs without evidence of IMH were weak, with most erythrocytes reaching the base of the tube but a small number caught within the gel. These positive results in dogs without hemolysis may be genuine evidence of erythrocyte-associated antibodies, as can occur due to passive antibody absorption in patients with increased globulins.[44] However, discussion with the manufacturer identified the possibility that false positives may have occurred because we used our own centrifuge, rather than a manufacturer-supplied centrifuge. If gel tubes shifted during centrifugation, or the centrifugal force exerted was not optimal, it is possible that this may have led to erythrocytes becoming trapped against the sides of the tube or within the gel, creating weak false-positive results. It should also be noted that during the initial stages of the study, the gel DAT kit did not include an autocontrol tube. Of the 22 dogs tested during this period, there were 2 dogs classified as affected by IMH and 6 as DAT-positive, nonimmune-mediated anemias. It is possible that had an autocontrol tube been prepared, some of these positive DAT tests might instead have been classified as test failures. As we required both a positive DAT and evidence of hemolysis to reach a diagnosis of IMHA and our classifications agreed with clinical diagnoses, we consider it unlikely that these limitations had a major impact on our diagnostic classifications.

When discussing limitations, it is also important to note that there is a wide variation in the method for SATs used by different veterinarians and clinical pathology laboratories, and our findings cannot be assumed to be directly transferable to all the protocols in current use. Notably, we sought to use a simple method that replicates the approach we have seen used in multiple clinical settings, and did not investigate if more complex protocols, such as washing erythrocytes, improve performance. We also did not investigate the causes of false-positive results, but it is possible that if we had for example attempted to use lower volumes of the blood and saline, we might have improved performance at the lower dilutions. Similarly, because we were attempting to mimic routine practice in our institution, we did not routinely prewarm blood and saline to body temperature to prevent false-positive results due to cold agglutinins. However, we would suggest that this is a sensible follow-up step in clinical cases with

positive SATs but with clinical signs that are not consistent with warm agglutinin IMHA. Our inclusion criteria may also have influenced our findings. We included all dogs with a PCV of 30% or below, as a recent study reported a high proportion of dogs with IMHA had mild to moderate anemia. Inclusion of dogs with relatively mild anemia likely explains the relatively high proportion of dogs with SATs that were too thick to read at low saline-to-blood ratios. Our assessment of test performance is also likely influenced by our inclusion criteria, specifically it is likely that had we performed SATs only in dogs with a high clinical suspicion of IMHA or agglutination visible to the naked eye, we would have had fewer false-positive results. These findings are therefore most analogous to a clinical setting where SATs are performed as a reflex test in anemic patients rather than targeted at those with a high likelihood of IMHA.

In conclusion, SATs can achieve high specificity for diagnosis of IMH when a 49 : 1 saline-to-blood ratio is used. As false-positive results for dogs with nonimmune-mediated anemias are uncommon at a 49 : 1 ratio, a positive SAT is supportive of IMHA, provided that other clinical signs are consistent with the diagnosis. As sensitivity is limited, a negative result in a dog with clinical evidence of hemolysis does not rule out IMHA and should be followed by further testing for antierythrocyte antibodies.

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CONFLICT OF INTEREST DECLARATION

Alvedia donated the gel antiglobulin kits and provided guidance on their use, but did not have input into study design or manuscript preparation.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Not required because the study used only remnant blood remaining after diagnostic testing, and owners had the option to deny permission for use of remnant material during the admissions process.

HUMAN ETHICS APPROVAL DECLARATION

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

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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