TITLE: The utility of diagnostic tests for immune-mediated hemolytic anemia

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JOURNAL: Veterinary Clinical Pathology

PUBLISHER: Wiley

PUBLICATION DATE: 9 September 2019

DOI: 10.1111/vcp.12771

Title: The Utility of Diagnostic Tests for Immune-mediated Hemolytic Anemia

Short Title: Diagnosis of IMHA

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Abstract

Background: A definitive diagnosis of immune-mediated hemolytic anemia (IMHA) can be difficult to make. However, it is critical to differentiate IMHA from other causes of anemia due to the impact on prognosis and outcome for IMHA patients. Recently published American College of Veterinary Internal Medicine recommendations for the diagnosis of IMHA should be followed to concurrently confirm ongoing anemia, verify *in vivo* hemolysis, and detect anti-erythrocyte antibodies. The reliability of immunologic IMHA tests varies depending on which test is used and how it is performed.

Objectives: Our aims were to determine which tests are currently used in veterinary medicine to diagnose IMHA and to review the utility of assays that have historically been used to diagnose IMHA.

Methods: A short survey was designed to see which diagnostic tests for IMHA were currently being used by veterinary practices. The survey was distributed *via* list-serves to veterinarians and veterinary technologists. A literature review was performed to report the utility of diagnostic tests for the diagnosis of IMHA.

Results: Survey respondents indicated variability in test protocols used to diagnose IMHA. Most respondents perform saline agglutination or Coombs' tests to detect anti-erythrocyte antibodies. Additional tests that can be used to support a diagnosis of IMHA are discussed in this review.

Conclusions: A standardized diagnostic approach should be followed to differentiate IMHA from other causes of anemia. Test methodology can vary from one laboratory to another, and clinicians should be familiar with the procedures used by their laboratory.

Key Words: Agglutination, Anti-erythrocyte antibody, Coombs', Diagnosis, Spherocytosis

Introduction

Immune-mediated hemolytic anemia (IMHA) is commonly diagnosed in canine patients with hemolytic anemia. It is a less prevalent, but an equally important cause of hemolytic anemia in cats, horses, and cattle.[1,2] While there are certain clinicopathologic findings supportive of an IMHA diagnosis (e.g., peripheral blood spherocytosis, RBC agglutination, demonstration of immunoglobulins attached to RBC membranes), the diagnosis of IMHA is often presumptive. Misdiagnosis of IMHA is problematic because of important differences in treatment decisions and prognostic consequences for patients with IMHA as compared with other causes of anemia.[3] Additionally, it is critical to evaluate patients that have IMHA for underlying diseases that drive the immune-mediated response. This article summarizes the results of a short survey on the use of diagnostic tests for IMHA in veterinary medicine and reviews the utility of diagnostic tests for IMHA.

Diagnosis of IMHA

In anemic patients, the mechanism causing the anemia (decreased RBC production, hemorrhage, or hemolysis) needs to be determined. A CBC (including quantitative data and a qualitative blood film review) provides fundamental information needed for the assessment of anemia. Anemia can be classified as either regenerative or nonregenerative based on quantification of the reticulocytosis using an absolute reticulocyte concentration or corrected reticulocyte percentage in the sample. If the anemia is regenerative, blood loss or hemolytic anemia should be considered. Blood loss anemia can often be differentiated from hemolytic anemia using clinical examination findings and additional clinicopathologic data. Specifically, acute hemorrhage results in loss of serum proteins and, consequently, decreased serum albumin and globulin concentrations. Although blood loss typically results in a strongly regenerative anemia, chronic hemorrhage can cause a poorly regenerative anemia if blood loss leads to iron depletion, since the resultant iron-restricted erythropoiesis impedes reticulocyte production. It is important to assess patients for occult blood loss as gastrointestinal and urinary blood

loss can be easily missed. While the majority of IMHA patients present with a regenerative anemia, this initial classification is not a certainty. IMHA can be nonregenerative or pre-regenerative if there has been insufficient time for a clearly regenerative response to occur (anemia present < 5-7 days)[4] or if immune-mediated destruction of erythroid precursors is occurring ("precursor-targeted immune-mediated anemia").[5]

It is vital to document that true *in vivo* hemolysis is occurring in patients with IMHA. Accordingly, proper collection of blood samples is critical to avoid *ex vivo* hemolysis, which can lead to false decreases in RBC concentration, hematocrit (HCT), and packed cell volume (PCV); false increases in mean cell hemoglobin concentration (MCHC); variable alterations in serum bilirubin concentrations (depending on the methodology used); and can also interfere with several other serum chemistry values.[6] Diagnostic test results that support *in vivo* hemolysis are provided in **Table 1**.

To distinguish IMHA from other causes of anemia, an American College of Veterinary Internal Medicine (ACVIM) consensus statement recommends documenting all three of the following diagnostic findings as minimum criteria to diagnose IMHA in dogs:[7]

1) Decreased packed cell volume (PCV)
2) At least one of the following abnormalities supportive of hemolysis: erythrocyte ghost cells, hyperbilirubinemia, bilirubinuria, icterus, hemoglobinemia, or hemoglobinuria
3) A positive saline agglutination test that persists with washing or at least two of the following:
    a) A positive saline agglutination test without washing
    b) ≥ 5 spherocytes per 1000× microscopic field
    c) Detection of anti-erythrocyte antibodies by a Coombs' test or flow cytometry

Note that erythrocyte ghost cell numbers can markedly increase *ex vivo*, so examination of a freshly made smear (at the time of blood collection) is recommended to assess this morphologic abnormality.[6]

A short survey (Appendix A) to determine how tests are being used to diagnose IMHA was sent to an undetermined number of people in Australia, Canada, Europe, and the United States *via* list-serves and email messages. Veterinary practitioners in both general practice and specialty hospitals, veterinary clinical pathologists, and medical technologists and technicians in clinical pathology laboratories were asked to reply. Respondents were not asked to self-identify. Ninety-four people completed at least the first question of the survey.

The hematology parameters that > 75% of the respondents said were provided with a CBC are listed in Figure 1. Seventy-two people (77%) indicated that, when needed, a saline agglutination test was included with CBC results. An additional 13 respondents (14%) did not include a saline agglutination test as part of a CB, but as an additional test. Seventy-two people (77%) recommended a test to detect anti-erythrocyte antibodies (saline agglutination test, Coombs test, and/or flow cytometry). Additional tests that were recommended by > 15 % of respondents included a biochemistry panel (66%), screening for tick-borne diseases (60%), urinalysis (52%), and diagnostic imaging (34%).

Several tests have been used to aid in the diagnosis of IMHA, none of which are 100% sensitive and specific. Differences in the protocols used to perform diagnostic tests for IMHA can dramatically affect the performance of the test.[8,9] Tests for IMHA must account for physical properties associated with antibody-antigen interactions, one of which is temperature-dependent antibody-antigen binding.[10] Therefore, the Coombs' test is often carried out at both 37˚C and 4˚C.[11] Additionally, inclusion criteria for IMHA patients vary between references, which can alter the assessment of test performance. Most

studies evaluating diagnostic tests for canine IMHA list auto-agglutination or spherocytosis as inclusion criteria. However, Reimer *et al*. (1999) concluded that 2/70 (3%) canine patients with IMHA did not have auto-agglutination or spherocytosis, nor did they have polychromasia.[12]

Genetics

Several dog breeds have a higher incidence of IMHA than the general canine population including; cocker spaniels,[12–15] English springer spaniels,[12] miniature schnauzers,[13,15] and old English sheepdogs[16]; however, a specific gene association with IMHA has not be found. A few studies have associated the presence of specific major histocompatibility complex alleles in dogs with the occurrence of immune-mediated disease, but the predictive value of these associations remains uncertain.[17–19] The frequency of specific dog erythrocyte antigens found during blood typing was not significantly different in 33 dogs with IMHA as compared to 1,014 dogs without IMHA.[20] Therefore, expectedly, genetic testing was not recommended as an additional test for IMHA by survey respondents.

Non-specific Tests for Antibodies

Two proteins, staphylococcal protein A (SpA) and papain, have been used in several papers to document increased immunoglobulin in serum samples. SpA is an immunoglobulin binding protein produced by *Staphylococcus aureus* that has been shown to be more sensitive than indirect antiglobulin tests in dogs with IMHA.[21] Papain is an enzyme that digests antibodies into three 50 kDa segments comprised of one fragment crystallizable (Fc) and two fragment antigen-binding (Fab) regions. When using a papain test to detect anti-erythrocyte antibodies, false-positive results occur in up to 3/16 (19%) of dogs.[22] One study tested 23 papain positive anemic dogs with direct and indirect antiglobulin tests; 8/23 (35%) dogs were positive using a direct test and none were positive using an indirect test.[23] Due to the non-specific

nature of SpA and papain reactions, they are no longer recommended as diagnostic tests for IMHA and unsurprisingly were not listed as additional tests used by survey respondents.

Auto-agglutination

Red blood cell agglutination should raise suspicion for IMHA (**Fig. 2**). To determine whether agglutination is likely related to the presence of antibody or complement on the surface of the red blood cell (i.e., auto-agglutination), a saline agglutination test should be performed. It is important to note that artifactual agglutination/rouleaux is not dispersed in all patients using a 1:1 dilution. However, if agglutination persists after washing erythrocytes at least three times in isotonic or phosphate-buffered saline, it is likely that the erythrocyte clumping is true agglutination rather than rouleaux formation. The occurrence of auto-agglutination in dogs with IMHA varies from 42% to 86%.[12–16] The number of patients with a positive saline agglutination test is decreased when erythrocytes are washed extensively.[24,25] There is some evidence that the decrease in positive saline agglutination tests with washing is due to a reduction in false-positive results,[25] but increased numbers of false-negative results should also be considered.

Seventy respondents indicated whether they evaluated saline agglutination tests macroscopically or microscopically: 34 looked for microscopic agglutination, 29 looked for macroscopic and microscopic agglutination, and seven looked at the sample macroscopically only. Sixty-three people indicated how they dilute blood samples for a saline agglutination test: 31 performed a 1:4 dilution, 24 performed a 1:1 dilution, and eight performed both a 1:1 and a 1:4 dilution of the sample. Of the 28 respondents who indicated whether they wash RBCs for the saline agglutination test, 16 used unwashed RBCs, 11 used washed RBCs, and one used both.

Erythron

Spherocytosis is often used as an inclusion criterion for studies of dogs with IMHA and was included in CBC data by 90/94 (96%) of survey respondents (Fig. 1). However, IMHA also can be non-spherocytic, as the occurrence of spherocytosis in dogs with IMHA ranges from 61% to 95%.[12,13,15,16,24,26] Spherocytes are generally only recognized in dogs (owing to the pronounced central pallor of their erythrocytes). In contrast, spherocytes are rarely identified with certainty in cats, horses, and cattle since erythrocytes from these animals lack central pallor on blood smears.

Spherocytosis and some other RBC membrane abnormalities can cause increased erythrocyte fragility.[27] The osmotic fragility of erythrocytes can be tested by diluting whole blood in progressively decreasing concentrations of sodium chloride (NaCl), incubating the samples for 30 minutes at room-temperature, recording absorbance of the samples at 540 nm, and then creating a data curve assuming that the lowest NaCl concentration causes 100% hemolysis.[27] RBC hemolysis occurs as a result of a loss of osmotic regulation and volume control, which is exacerbated in a number of RBC disorders. Increased RBC hemolysis during osmotic fragility testing is commonly reported in spherocytic conditions (e.g., IMHA), but can be seen in spectrin deficiency,[28] hereditary stomatocytocytosis,[29] intestinal parasite-associated microcytosis, *Babesia canis* infection,[30] or non-hemolytic samples that are lipemic. Erythrocyte fragility testing is not commonly available to practitioners but can provide support for ongoing hemolysis.[31] One study observed increased erythrocyte fragility in 15/15 (100%) direct antiglobuin test (DAT) positive and 4/12 (33%) DAT negative anemic dogs.[25] None (0/91) of the survey respondents indicated that they recommended osmotic fragility testing for patients suspected of having IMHA.

Most patients with IMHA have a moderate to marked regenerative anemia. A reticulocyte count was listed as a component of a CBC by 77/94 (82%) of the survey respondents (Fig. 1). The occurrence of reticulocytosis in dogs with IMHA ranges from 67% to 82%.[13,16,26] IMHA patients often have increased numbers of circulating nucleated RBCs and/or Howell-Jolly bodies. Interestingly, reticulocyte hemoglobin (HGB) content was shown to be decreased in 5/14 (36%) dogs with IMHA suggesting that iron-restricted erythropoiesis can be present in some canine IMHA patients.[32]

A diagnosis of erythroid hyperplasia in bone marrow samples is a definitive indication of erythrocyte regeneration. It is noteworthy that Weinkle *et al*. (2005) found 23/45 (51%) dogs with IMHA that underwent bone marrow analysis had erythroid hyperplasia.[13] Similarly, another study that analyzed bone marrow samples from dogs with IMHA observed erythroid hyperplasia in 6/11 (55%) samples.[15] Extramedullary hematopoiesis[33] and secondary myelodysplasia[34] also have been reported in dogs with IMHA. Bone marrow evaluation is typically recommended for suspected IMHA patients that have a nonregenerative anemia. Low numbers of survey respondents [6/91 (7%)] indicated that they recommend bone marrow aspiration or biopsy to aid in the diagnosis of IMHA. Two of these respondents specified that this recommendation was warranted in patients with persistent nonregenerative anemia.

Nonregenerative anemia has been reported in 6/23 (26%) canine IMHA patients in one study[24] and 6/20 (30%) in another.[15] In a retrospective analysis of dogs with nonregenerative anemia in which a bone marrow sample was clinically indicated, 55/82 (67%) were determined to have IMHA (based on the presence of either Coombs' positivity, auto-agglutination, or > 30% spherocytes).[35] In dogs with nonregenerative IMHA, 38/55 (69%) had erythroid hyperplasia, and 17/55 (31%) showed incomplete maturation of the erythroid line.[35] In another study of canine patients with a nonregenerative anemia

present for more than 5 days, 41/43 (95%) had erythroid hyperplasia in bone marrow samples, 23/43 (54%) had a spherocytosis, and 20/35 (57%) were positive by DAT.[36] To determine if dogs with a nonregenerative anemia > 5 days duration have precursor-targeted immune-mediated anemia, bone marrow should be evaluated for macrophage phagocytosis of erythroid precursors.[5] Weiss (2008) also evaluated 57 cats with nonregenerative anemia in which bone marrow analysis was indicated. Approximately half of the cats 28/57 (49%) were determined to have IMHA (based upon measurement of an HCT < 20% and either Coombs' positivity or auto-agglutination).[35] In cats with IMHA and nonregenerative anemia, 24/28 (86%) had erythroid hyperplasia and 4/28 (14%) showed maturation arrest of the erythroid line.[35] In another study, phagocytosis of erythroid precursors and abnormal presence of stainable iron was documented in the bone marrow of cats with both primary and secondary IMHA.[37]

In cases of IMHA with RBC agglutination in the sample, many of the measured or calculated values of the erythron are often erroneous [e.g., RBC concentration, mean cell volume (MCV), HCT, mean cell hemoglobin (MCH), MCHC, red cell distribution width (RDW)] as impedance counters will count erythrocyte clumps as single and large erythrocytes, which leads to a significant reduction in the numbers of RBCs counted and an increase in the mean size of the cells.[6] In cases of intravascular hemolysis, HGB concentration is not clinically reliable, as it represents a combination of free (plasma) and RBC HGB. If it is available, the determination of the cell hemoglobin concentration mean (CHCM) using an advanced laser cell counter could help assess RBC HGB.

Leukon

Abnormalities in leukocytes are commonly observed in patients with IMHA. Leukocytosis was reported in 43% to 99% of dogs with IMHA (WBC concentrations in these reports ranged from 5,300 cells/µL to

105,700 cells/μL).[12,15,38,39] In one study, decreased survival time of dogs with IMHA was associated with leukocytosis and lymphopenia,[40] while lymphocytosis was a positive prognostic factor in cats with IMHA.[37] Neutrophil left shifts were noted in up to 16/20 (80%) of dogs with IMHA.[15] One paper observed decreased survival rates in dogs with IMHA with band neutrophil concentrations ≥ 3000 cells/μL.[13]

IMHA patients with inflammatory leukograms typically have acute patterns such as a neutrophilia with a left shift, lymphopenia, eosinopenia, and monocytosis.[15] In severe inflammatory and erythroid regenerative conditions, a leukoerythroblastic pattern can be observed with a highly acute inflammatory leukogram and a high percentage of nucleated RBCs in different stages of maturation.[15] Rubricytosis causes a false increase in the automated WBC concentration that must be corrected mathematically after the enumeration of nucleated RBCs by blood smear review. This further emphasizes the need for blood smear examination, which can also help with the detection of neutrophil left-shifting and toxicity that can be present in IMHA cases. Sixty-five/94 (69%) survey respondents indicated that blood smear evaluation by a board-certified clinical pathologist was included in CBCs they performed or received (Fig. 1).

Serum Biochemistry and Urinalysis

Abnormalities in biochemical parameters have been associated with the clinical outcomes of IMHA patients (**Table 2**), but not all studies report the same findings. An increase in total bilirubin concentrations was observed in 60% to 100% of dogs with IMHA.[12,15,26] It is important to note that increased conjugated bilirubin can interfere with phosphorus measurements leading to pseudohypophosphatemia in patients with IMHA.[41] Although not linked to decreased survival, Klag et al. (1993) observed hemoglobinemia and/or hemoglobinuria in 4/42 (10%) dogs with IMHA.[26] There are

also a few case studies of dogs with IMHA with biochemical abnormalities consistent with distal renal tubular acidosis.[42] In cats with IMHA, hyperglobulinemia is reported to be a positive prognostic factor.[37] Biochemistry profiles were recommended as additional tests for suspected IMHA patients by 60/91 (66%) of survey respondents.

Additional serum protein parameters have been reported to be altered in dogs with IMHA. For example, cardiac troponin I was > 0.1 ng/mL in 20/27 (74%) dogs with IMHA (authors indicated that < 0.1 ng/mL is expected in healthy dogs, but a true reference interval was not provided).[43] C-reactive protein was increased in dogs with IMHA at presentation.[44–46] Alpha-1 acid glycoprotein also was increased in dogs with IMHA, while albumin can be decreased at presentation.[45] Increased serum concentrations of several cytokines have been reported in dogs with IMHA (n = 20) as compared with six healthy dogs.[46] Interleukin-15 (IL-15), IL-18, granulocyte-monocyte colony stimulating factor, and monocyte chemoattractant protein-1 concentrations were increased in animals with IMHA that died ≤ 30 days after hospital admission.[46] Similarly, IL-2, IL-6, and tumor necrosis factor-α were present at higher concentrations in dogs with primary IMHA (n = 19) when compared with dogs that had other inflammatory diseases (n = 22) or healthy dogs (n = 32).[47] In question 4, none of the survey respondents indicated that they recommended these protein assays to help diagnose patients with IMHA.

Thrombon and Coagulation

Thrombocytopenia is reported to occur in 29-70% of dogs with IMHA.[12,14,15,26,38,39] In a study of 151 dogs, a platelet concentration < 150,000 platelets/µL correlated with decreased survival rates.[13] Also, a decreased mean platelet component concentration was found in dogs with IMHA (n = 95) as compared with healthy dogs (n = 95) or sick canine patients (n = 95)[48] which could indicate increased platelet activation in IMHA patients.[49]

Considerations for severe thrombocytopenia include a consumptive process [e.g., disseminated intravascular coagulation (DIC), pulmonary thromboembolism (PTE)] or a concurrent immune-mediated thrombocytopenia (IMT). In humans, concurrent IMHA and IMT have been termed Evan's Syndrome. This disease process likely occurs in dogs; however, the presence of concurrent anti-erythrocyte and anti-platelet antibodies has rarely been documented in veterinary patients.[50,51] In 38 dogs with both anemia and thrombocytopenia, 18/38 (47%) of patients were positive by DAT for anti-erythrocyte antibodies.[52] In a similar study of 21 dogs with concurrent anemia and thrombocytopenia, auto-agglutination that persisted after washing was observed 6/21 (29%) dogs, and two of three dogs tested by DAT were positive.[53]

Several studies have assessed coagulation parameters in dogs with IMHA (**Table 3**). Importantly, increased mortality was observed in dogs with IMHA that had thrombocytopenia, prolonged prothrombin time (PT), prolonged activated partial thromboplastin time (APTT), decreased fibrinogen, or DIC.[40] Reports using thromboelastography determined that 85-100% of dogs with IMHA were hypercoagulable.[54–56] Development of DIC is observed in between 10/31 (28%)[14] and 9/20 (45%)[15] dogs with IMHA. One study reported that thromboemboli were found in 20/25 (80%) IMHA dogs at necropsy.[14] The analysis of coagulation parameters is typically recommended in IMHA patients that have clinical signs of coagulopathy. One survey respondent indicated in Question 4 that they recommended measurement of D-dimers in patients suspected of having IMHA.

<u>Indirect Antiglobulin Tests</u>

Indirect antiglobulin tests are not recommended in veterinary species due to low sensitivities and specificities. When these studies were first evaluated for utility in canine patients with IMHA, the

sensitivity and specificity of an indirect antiglobulin test were 62.5% and 96.6%, respectively.[57] The DAT performed by the same laboratory had a 83.3% sensitivity and 98.8% specificity.[57] None of the survey participants recommended indirect antiglobulin tests in Question 4.

Direct Antiglobulin Tests (DATs)

Various methods for directly detecting RBC surface-bound anti-erythrocyte immunoglobulin (Ig) and opsonizing complement protein (C3) are available. Of the 72 respondents who recommended a test to diagnose IMHA with anti-erythrocyte antibodies, 66 recommended a Coombs' test, three recommended flow cytometry and a Coombs' test, two recommended flow cytometry alone, and one recommended flow cytometry and a Coombs' test at 4°C.

Coombs' Tests

Coombs' tests are often performed using a microtiter plate format. Additional methods include gel-based microcolumn, immunochromatographic strip, and capillary DAT assays. Good agreement has been reported between results of the Coombs' test and these methods.[25] False-positive DAT results have been reported in anemic dogs. In theory, false-positive results could be due to technical difficulties (e.g., nonspecific absorption of the antibody, incomplete washing, contamination, assignment of an inappropriate cut off) but patient factors are critical to consider. Dogs that recently received a transfusion can have a positive DAT.[8,58] Also, dogs with an autoimmune disease that are positive for antinuclear antibodies have been reported to be DAT positive without conclusive evidence of IMHA.[59] Additionally, horses with equine infectious anemia can have a positive Coombs' test.[60]

The Coombs' test uses species-specific antibodies to detect Ig and/or C3 bound to erythrocytes in a patient blood sample. A positive test results in RBC agglutination. Sixty-five of the people surveyed

specified if they recommended a Coombs' test when auto-agglutination was observed, 50 people recommended a Coombs' test if no auto-agglutination was seen (7 specified use of a microtiter plate, and 2 recommended an immunochromatographic strip DAT assay). Fifteen people recommended a Coombs' test with or without auto-agglutination (1 specified the use of a microtiter plate, and another recommended the use of a strip DAT assay). The authors agree with the recent ACVIM consensus statement,[7] which indicates that a Coombs' test is unnecessary if true auto-agglutination that persists after washing is present.

When a Coombs' test is warranted, polyvalent and monovalent test antibodies are available. These antibodies are pre-adsorbed onto RBCs from healthy dogs before use in the test. False-negative results can occur with either type of test antibody; therefore, including both polyvalent and monovalent antibodies in a Coombs' test can be beneficial.[11] Including both polyvalent and monovalent antibodies increased test performance in a study that reported a sensitivity of 82% and a specificity of 95% when antibodies were combined.[8]

The antibody binding reaction is temperature-dependent, so it is recommended that testing is performed at both 37°C and 4°C.[11] Thirty people specified that they ran a Coombs' test at 37°C, 11 respondents performed a Coombs' test at 37°C and 4°C, 1 respondent performed the test at 37°C and room temperature, and (as mentioned above)one person performed flow cytometry plus a Coombs' test at 4°C.

Also, prozone effects are commonly reported when agglutination is not observed at low serum dilutions (i.e., high antibody concentrations) but is observed at higher serum dilutions (i.e., low antibody concentrations). This is due to the presence of excess immunoglobulins that interfere with agglutination

induced by the interactions of the test antibodies with Ig and C3 on the RBCs.[61] This improper test antibody to anti-RBC Ig ratio leads to false-negative results if there are not enough serial serum dilutions tested. A prozone effect was observed in 17/126 (13%) samples tested by Piek *et al*. (2012).[62]

The reported performance of Coombs' tests vary, likely due to different samples, protocols, and test reagents that are used at different laboratories. Important positive control samples for the Coombs' test include Ig-coated and complement-coated canine RBCs, but these reagents are not readily available.[9] It has been reported that the use of whole blood in EDTA or acid citrate dextrose (ACD) yields similar results; however, ACD anticoagulants were preferred in one study because of increased sample hemolysis in EDTA.[9] False-negative results that reduce the sensitivity, and negative predictive value (NPV) of the Coombs' test can be caused by physical properties of the test antibodies (e.g., low antibody affinity, an inappropriate antibody ratio, steric hindrance), poor technique (e.g., excessive washing, delayed processing, assignment of an inappropriate cut-off), or patient factors (drug-dependent reactions, blood transfusions, steroid administration).[8]

In dogs with IMHA, one study reported that a low percentage, 17/46 (37%), of patients had positive Coombs' test results,[12] but other studies indicated that 77% of dogs with IMHA were positive.[13,14] In a small study of 12 dogs, the sensitivity, specificity, positive predictive value (PPV), and NPV of the Coombs' test was 58%, 100%, 100%, and 62%, respectively.[63] Similarly, Quigley *et al*. (2001) calculated a PPV of 100% and an NPV of 68%.[64] In a study of cats with IMHA, 2/89 (2%) healthy cats were reported to have a strongly positive Coombs' test at 37˚C.[65] Another study reported that 0/14 (0%) nonanemic cats and 18/55 (33%) anemic cats were Coombs' positive.[66] Of the 18 Coombs' positive cats, 15 were diagnosed with primary IMHA, two were feline leukemia virus positive (FeLV), and one had cholangiohepatitis.[66] An older manuscript indicated a weak positive Coombs' test at 4˚C in 9/20 (45%)

377 healthy cats and a positive Coombs' test at 4˚C and 37˚C in 16/20 (80%) anemic cats (12 of the Coombs'
378 positive anemic cats were FeLV positive).[67]
379
380 It is expected that transfusion reactions can cause positive DAT results.[58,68,69] Honeckman *et al*.
381 indicated that transfusions given 3 to 21 days before a Coombs' test could be particularly problematic.[58]
382 However, only one study was found that reported DAT test results in seven dogs that had been given a
383 recent transfusion; results of two DAT test kits were reported.[8] Samples were interpreted as truly
384 positive for five dogs (by at least one Coomb's test kit), falsely positive for one dog, and falsely negative
385 for one dog. This study did not specify the length of time between when the transfusion was given and
386 when the diagnostic testing was done. Interestingly, in humans, blood typing is recommended either
387 prior to transfusion or a minimum of 3 months after transfusion to avoid erroneous results.[70]
388
389 Ninety-one of the 94 survey respondents indicated if they would interpret any diagnostic results with
390 caution following a transfusion. The timeframe of concern for people who interpreted results with more
391 caution varied (Fig. 3). It is evident from this small survey that there is uncertainty if recent transfusion
392 would cause false-positive results in CBC, agglutination, or DAT assays.
393
394 *Flow Cytometry Methods*
395 Flow cytometry can also be used to detect immunoglobulins bound to RBCs. One of the first evaluations
396 of flow cytometry as a diagnostic test for IMHA compared data from 12 dogs and three horses with
397 IMHA to 12 healthy animals from each respective species.[63] They reported low specificity of a goat anti-
398 equine IgG but 100% specificity of goat anti-equine IgG F(ab')2 fragment in their assay.[63] In dogs, by
399 pooling anti-canine IgG, IgM, IgA, and C3 antibodies, the sensitivity of the test was 100%, specificity was
400 87.5%, PPV was 92%, and NPV was 100%.[63] There was no prozone effect with this assay.[63] Quigley et al.

(2001) reported a PPV of 100%, and an NPV of 93% when they used flow cytometry to evaluate 13 dogs with IMHA and 13 healthy dogs.[64] In 2008, Morley *et al*. published an assessment of the utility of flow cytometry to detect anti-erythrocyte antibodies in dogs.[71] They found 26/147 (18%) anemic patients had detectable anti-erythrocyte antibodies. This included 17/22 (77%) IMHA patients, 5/14 (36%) IMT patients, and 3/71 (4%) cancer patients. However, 12/145 (8%) nonanemic dogs also were positive for anti-erythrocyte antibodies, which included 3/5 (60%) patients with infectious disease and 5/81 (6%) cancer patients, and the test had a PPV of 70% and NPV of 95%.

Direct Enzyme-Linked Antiglobulin Tests (DELATs)

Immunoglobulins and C3 bound to RBCs are detected by comparing the absorbance of patient samples to healthy control animals in the DELAT. Early evaluation of a DELAT indicated that 1 mg/mL ρ-nitrophenyl phosphate in carbonate buffer is the preferred substrate for the reaction, however false-positive results were observed in 31/60 (52%) dogs tested.[72] Another evaluation of DELAT performance yielded comparable results with a Coombs' test; 12/23 (52%) samples were Coombs' positive, while 13/23 (57%) were DELAT positive.[73] To the authors' knowledge, this test for IMHA is no longer readily available.

Guidelines for Performing Diagnostic Tests for IMHA

As mentioned previously, technical difficulties and test protocols can profoundly affect the performance of diagnostic tests for IMHA. Tests performed at 4°C, room temperature, and 37°C can provide different results. This document includes two example protocols for saline agglutination testing (Appendix B) and an example of Coombs' testing (Appendix C). In different diagnostic laboratories, it is expected that these protocols will be performed differently but that proper procedures will yield adequate results.

Conclusions

Making a definitive diagnosis of IMHA can be difficult due to the variability in patient presentation and diagnostic test performance. Recommended tests for diagnosing IMHA in anemic patients include: 1) a CBC with verified reticulocyte count, manual PCV with assessment of plasma color, and microscopic examination of a (preferably fresh) blood smear, 2) serum chemistry profile, 3) urinalysis, 4) saline agglutination test (preferably with washing), 5) Coombs' test or flow cytometric analysis if the saline agglutination test is negative, 6) coagulation testing for thrombocytopenic patients and patients with clinical signs of coagulopathy, and 7) tests to determine if any underlying disease is present (e.g., drug or toxin exposure, infection, inflammation, neoplasia, other autoimmune diseases). Our survey proved that clinicians choose different tests to diagnose IMHA and that laboratories perform tests differently. Therefore, it is recommended that veterinarians contact a clinical pathologist or technicians or technologist at the diagnostic laboratory they use to obtain details about the reliability of specific tests being performed to diagnose IMHA.

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638 **Table 1.** Clinical pathology findings that support the diagnosis of hemolytic anemia.[6]

CBC/Blood smear exam	Serum Biochemistry	Urinalysis
Increased polychromasia	Hyperbilirubinemia	Hyperbilirubinuria
Reticulocytosis ± rubricytosis ± Howell-Jolly bodies	Hemoglobinemia [i]	Hemoglobinuria [i]
Macrocytosis & anisocytosis	Decreased haptoglobin concentration [i]	Increased urobilinogen
Decreased MCHC [e] or increased MCHC [i]	Decreased hemopexin concentration [i]	
Spherocytes [e]; ghost cells [I] – immune-mediated damage or other cause		
Heinz bodies [e]; eccentrocytes[I] – oxidative damage		
Hemoparasites – direct physical &/or immune-mediated damage		
Schistocytes [I]; acanthocytes [I]; keratocytes [I] – direct physical damage		

639 e = specifically associated with extravascular hemolysis

640 i = specifically associated with intravascular hemolysis

641 MCHC = mean cell hemoglobin concentration

642

643 **Table 2.** Serum biochemistry results associated with decreased survival in IMHA patients.

Abnormality	Species	References
Hyperbilirubinemia	Dog	[12–14,40]
	Cat	[37]
Hyperlactatemia	Dog	[74]
Increased alanine aminotransferase	Dog	[40]
Increased aspartate aminotransferase	Dog	[40]
Increased urea nitrogen	Dog	[40]
Increased alkaline phosphatase	Dog	[12]
Hypoalbuminemia (< 3.0 mg/dL)	Dog	[13]
Hypophosphatemia (< 3.5 mEq/L)	Dog	[13]
Increased creatine kinase (> 250 U/L)	Dog	[13]

644

Table 3. Evidence of coagulation abnormalities in dogs with IMHA.

Abnormality	Dogs with IMHA affected	References
Increased prothrombin time	10-28%	[14,15]
Increased activated partial thromboplastic time	45-47%	[14,15]
Increased fibrin degradation products	57-60%	[14,15]
Increased fibrinogen	17/20 (85%)	[15]
Increased D-dimers	16/20 (80%)	[15]
Decreased anti-thrombin III	10/20 (50%)	[15]
Increased Russell viper venom time	7/20 (35%)	[15]
Increased von Willebrand factor associated antigen	9/20 (45%)	[15]
Increased Kaolin clotting time	3/20 (15%)	[15]
Increased P-selectin*	15/20 (75%)	[75]
Hypercoagulability using thromboelastography	85-100%	[54–56]
Disseminated intravascular coagulation	28-45%	[14,15]
Thromboemboli found at necropsy	20/25 (80%)	[14]

* A second paper saw no increase in P-selectin.[13]

Figure Legends:

Figure 1. Proportions (above 75%) of hematologic parameters reported or received on the CBC results of patients suspected of having IMHA by survey respondents (n=94).

Figure 2. Microscopic evidence of agglutination in a wet-mount saline agglutination test. Peripheral blood in EDTA (0.15%) was diluted 1:4 in isotonic saline (0.9% NaCl). A drop of the mixture was placed on a glass slide, and a coverslip was placed over the drop. Grape-like aggregates of erythrocytes can be observed (unstained, 200× magnification).

Figure 3. Survey responses indicating the post-transfusion time-frame in days (d) during which respondents would cautiously interpret hematologic test results that support a diagnosis of IMHA (n=94).

Survey of Tests Used to Diagnose Immune Mediated Hemolytic Anemia (IMHA) in Veterinary Medicine

Designed by the Diagnostic Task Force of the Veterinary and Comparative Clinical Immunology Society

Data from this survey will be presented at the ACVP/ASVCP Meeting in November 2018. Thank you for taking the time to answer these questions about the diagnosis of IMHA.

Question Title

1. Which parameters do you report or receive as part of a complete blood count when an animal is suspected of having IMHA? Please check all that apply.

- [ ] Packed cell volume (PCV)
- [ ] Plasma Total Protein (TP)
- [ ] Fibrinogen using heat precipitation
- [ ] Fibrinogen using a turbidimetric assay
- [ ] Hemolysis
- [ ] Icterus
- [ ] Lipemia
- [ ] Red blood cell (RBC) count
- [ ] Hematocrit
- [ ] Hemoglobin concentration
- [ ] Mean corpuscular volume (MCV)
- [ ] Mean corpuscular hemoglobin (MCH)
- [ ] Mean corpuscular hemoglobin content (MCHC)
- [ ] Red cell distribution width (RDW)
- [ ] Spherocytes
- [ ] Ghost cells
- [ ] Acanthocytes
- [ ] Shistocytes
- [ ] Keratocytes
- [ ] Hemoparasites
- [ ] Relative numbers of nucleated RBCs (nRBCs/100 WBCs)
- [ ] Absolute nRBC count
- [ ] Manual reticulocyte count using New Methyene Blue
- [ ] Manual reticulocyte count using Brilliant Cresyl Blue
- [ ] Automated hematology instrument reticulocyte count
- [ ] Average mean reticulocyte volume
- [ ] Average reticulocyte hemoglobin concentration
- [ ] Average hemoglobin content of reticulocytes
- [ ] White blood cell count

- [ ] Automated hematology instrument differential count
- [ ] Manual 200-cell differential count
- [ ] Manual 100-cell differential count
- [ ] Blood smear review by a board-certified clinical pathologist
- [ ] Saline agglutination test at a 1:1 dilution
- [ ] Saline agglutination test at a 1:4 dilution
- [ ] Saline agglutination test without extensive washing
- [ ] Saline agglutination test with extensive washing
- [ ] Saline agglutination test by macroscopic evaluation
- [ ] Saline agglutination test by microscopic evaluation

Question Title

2. If not already included in the CBC, what additional testing do you recommend for IMHA? Please check all that apply.

- [ ] Saline agglutination test at a 1:1 dilution
- [ ] Saline agglutination test at a 1:4 dilution
- [ ] Saline agglutination test without extensive washing
- [ ] Saline agglutination test with extensive washing
- [ ] Saline agglutination test by macroscopic evaluation
- [ ] Saline agglutination test by microscopic evaluation
- [ ] Direct antigen testing using Coombs' test only if there is no auto-agglutination
- [ ] Direct antigen testing using Coombs' test with or without auto-agglutination
- [ ] Direct antigen testing using Coombs' test at 37 degrees C
- [ ] Direct antigen testing using Coombs' test at 4 degrees C
- [ ] Direct antigen testing using a Coombs' test microtiter assay
- [ ] Direct antigen testing using a Coombs' test gel microcolumn assay
- [ ] Direct antigen testing using a Coombs' test immunochromatographic strip assay
- [ ] Direct antigen testing using a Coombs' test capillary assay
- [ ] Flow cytometry to detect anti-RBC antibodies
- [ ] Biochemistry panel
- [ ] Urinalysis
- [ ] Bone marrow aspiration needle biopsy
- [ ] Bone marrow core biopsy
- [ ] Infectious disease screening for tick-borne diseases
- [ ] Infectious disease screening for viral diseases
- [ ] Infectious disease screening for a large panel of different organisms

- [ ] Anti-nuclear antigen testing
- [ ] Osmotic fragility testing
- [ ] Diagnostic imaging

Question Title

3. If the animal was transfused recently, do you interpret test results more cautiously? Please check all that apply.

- [ ] Yes, if the transfusion occurred within the last 24 hours
- [ ] Yes, if the transfusion occurred 1-6 days ago
- [ ] Yes, if the transfusion occurred 7-14 days ago
- [ ] No

Question Title

4. Please indicate if you use a test method or report out a parameter that you feel supports a diagnosis of IMHA, but was not listed in Question 1 or Question 2.

Appendix B

Saline agglutination:

The following is a very basic protocol for testing for agglutination.[1] This protocol is likely to give some false positive results.[2] Reagents needed include isotonic saline, a dropper, a clean glass microscope slide, a wooden applicator stick, and a microscope. Results using fresh EDTA blood from the patient should be compared to fresh EDTA blood from a normal animal of the same species.

- Place one drop (50 µL) of saline onto a glass slide
- Place an applicator stick in a tube of well-mixed whole blood and stir
- Tap the blood-filled applicator stick into the drop of saline and stir
    - This should produce a 1:4 to 1:10 dilution of blood[3]
    - If needed, a more accurate dilution of blood can be tested
        - Add 5-10 µL of whole blood to the drop of saline and stir
- Evaluate the slide for agglutination macroscopically and microscopically at 100× magnification
    - Agglutination appears as grape-like clusters of RBCs (Fig. 2) and must be distinguished from rouleaux where erythrocytes are arranged in linear stacks.
    - If agglutination is present, the result is positive and supports a diagnosis of IMHA

A more stringent protocol for testing for agglutination is listed below.[1] This protocol can give some false negative results. Reagents needed include isotonic saline, a clean glass microscope slide, micropipettes, a centrifuge, and a microscope. Results using fresh EDTA blood from the patient should be compared to fresh EDTA blood from a normal animal of the same species.

- Wash RBCs
    - Dilute 100 µL of whole blood in 900 µL of isotonic saline to wash the RBCs
    - Centrifuge the sample for 5 minutes at 1500 × g to pellet the RBCs

    - Remove the supernatant
    - Re-suspend the RBC pellet in 900 µL of isotonic saline
- Wash the sample in saline (as outlined above) at least 2 additional times
- Place 50 µL of the re-suspended sample onto a glass slide
- Evaluate the slide for agglutination macroscopically and microscopically at 100× magnification
    - Agglutination appears as grape-like clusters of RBCs (Fig. 2) and must be distinguished from rouleaux where erythrocytes are arranged in linear stacks.
    - If agglutination is present, the result is positive and supports a diagnosis of IMHA

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Appendix C

Coombs' test:

Test kits for Coombs' tests are available for dogs, cats, and horses.[1–3] Instructions provided in the kit should be followed. Reagents typically include rabbit antiserum that has been adsorbed with normal species-specific RBCs so that the reagents are unlikely to react with erythrocytes from healthy animals. Blood collected in EDTA, ACD, or heparin can be used. Ideally, results using fresh whole blood from the patient should be compared to fresh whole blood from a normal animal of the same species. However, samples can be stored at 4˚C for up to 7 days before testing.[4]

- Wash RBCs
    - Centrifuge blood for 5 minutes at 1500 × g
    - Remove 100 µL of packed RBCs
    - Add 4.9 mL phosphate buffered saline (PBS) and mix
    - Centrifuge the sample for 5 minutes at 1500 × g
    - Remove the supernatant
    - Re-suspend the RBC pellet in 4.9 mL PBS
- Wash the sample in PBS (as outlined above) at least 2 additional times
- Dilute the Coombs' test reagent
    - Label 2 sets of 10 test tubes: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, auto, neg
        - Note that the use of microwell titer plates in place of test tubes can dramatically reduce the amount of serum needed.
        - Higher dilutions are recommended based upon the findings of Piek *et al*..[5]
    - Add 100 µL PBS to each test tube
    - Add 100 µL species-specific Coombs' reagent to the test tube labeled 1:2
    - Transfer 100 µL solution from tube 1:2 to tube 1:4

- Transfer 100 µL solution from tube 1:4 to tube 1:8
- Transfer 100 µL solution from tube 1:8 to tube 1:16
- Transfer 100 µL solution from tube 1:16 to tube 1:32
- Transfer 100 µL solution from tube 1:32 to tube 1:64
- Transfer 100 µL solution from tube 1:64 to tube 1:128
- Transfer 100 µL solution from tube 1:128 to tube 1:256

- Perform the Coombs' test
  - Add 100 µL of washed RBCs from the patient to test tubes 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and auto
  - Add 100 µL of washed RBCs from the normal animal to test tube neg
  - Incubate one set of 10 test tubes for 30 minutes at 37˚C
  - Incubate the other set of 10 test tubes for 30 minutes at 4˚C
  - Centrifuge for 1 minute at 1500 × g
  - Hold each tube up to the light at a 45˚ angle and mix by tapping
  - If RBCs stay in a pellet or break off in clumps, the sample is positive for macro-agglutination
- Evaluate the slides for agglutination macroscopically and microscopically at 100× magnification
  - Place 50 µL each sample onto glass slides
    - Agglutination appears as grape-like clusters of RBCs and must be distinguished from rouleaux where erythrocytes are arranged in linear stacks (Fig. 2).
    - If agglutination is present, the result is positive and supports a diagnosis of IMHA

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