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

2

3   **Short Title:** Diagnosis of IMHA

4

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20

21   **Abstract**

22   **Background:** A definitive diagnosis of immune-mediated hemolytic anemia (IMHA) can be difficult to

23   make. However, it is critical to differentiate IMHA from other causes of anemia due to the impact on

24   prognosis and outcome for IMHA patients. Recently published American College of Veterinary Internal

25   Medicine recommendations for the diagnosis of IMHA should be followed to concurrently confirm

26   ongoing anemia, verify *in vivo* hemolysis, and detect anti-erythrocyte antibodies. The reliability of

27   immunologic IMHA tests varies depending on which test is used and how it is performed.

28   **Objectives:** Our aims were to determine which tests are currently used in veterinary medicine to

29   diagnose IMHA and to review the utility of assays that have historically been used to diagnose IMHA.

30   **Methods:** A short survey was designed to see which diagnostic tests for IMHA were currently being

31   used by veterinary practices. The survey was distributed *via* list-serves to veterinarians and veterinary

32   technologists. A literature review was performed to report the utility of diagnostic tests for the diagnosis

33   of IMHA.

34   **Results:** Survey respondents indicated variability in test protocols used to diagnose IMHA. Most

35   respondents perform saline agglutination or Coombs' tests to detect anti-erythrocyte antibodies.

36   Additional tests that can be used to support a diagnosis of IMHA are discussed in this review.

37   **Conclusions:** A standardized diagnostic approach should be followed to differentiate IMHA from other

38   causes of anemia. Test methodology can vary from one laboratory to another, and clinicians should be

39   familiar with the procedures used by their laboratory.

40

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

42

43   **Introduction**

44   Immune-mediated hemolytic anemia (IMHA) is commonly diagnosed in canine patients with hemolytic

45   anemia. It is a less prevalent, but an equally important cause of hemolytic anemia in cats, horses, and

46   cattle.[1,2] While there are certain clinicopathologic findings supportive of an IMHA diagnosis (e.g.,

47   peripheral blood spherocytosis, RBC agglutination, demonstration of immunoglobulins attached to RBC

48   membranes), the diagnosis of IMHA is often presumptive. Misdiagnosis of IMHA is problematic because

49   of important differences in treatment decisions and prognostic consequences for patients with IMHA as

50   compared with other causes of anemia.[3] Additionally, it is critical to evaluate patients that have IMHA

51   for underlying diseases that drive the immune-mediated response. This article summarizes the results of

52   a short survey on the use of diagnostic tests for IMHA in veterinary medicine and reviews the utility of

53   diagnostic tests for IMHA.

54

55   **Diagnosis of IMHA**

56   In anemic patients, the mechanism causing the anemia (decreased RBC production, hemorrhage, or

57   hemolysis) needs to be determined. A CBC (including quantitative data and a qualitative blood film

58   review) provides fundamental information needed for the assessment of anemia. Anemia can be

59   classified as either regenerative or nonregenerative based on quantification of the reticulocytosis using

60   an absolute reticulocyte concentration or corrected reticulocyte percentage in the sample. If the anemia

61   is regenerative, blood loss or hemolytic anemia should be considered. Blood loss anemia can often be

62   differentiated from hemolytic anemia using clinical examination findings and additional

63   clinicopathologic data. Specifically, acute hemorrhage results in loss of serum proteins and,

64   consequently, decreased serum albumin and globulin concentrations. Although blood loss typically

65   results in a strongly regenerative anemia, chronic hemorrhage can cause a poorly regenerative anemia if

66   blood loss leads to iron depletion, since the resultant iron-restricted erythropoiesis impedes reticulocyte

67   production. It is important to assess patients for occult blood loss as gastrointestinal and urinary blood

68 loss can be easily missed. While the majority of IMHA patients present with a regenerative anemia, this

69 initial classification is not a certainty. IMHA can be nonregenerative or pre-regenerative if there has

70 been insufficient time for a clearly regenerative response to occur (anemia present < 5-7 days)[4] or if

71 immune-mediated destruction of erythroid precursors is occurring ("precursor-targeted immune-

72 mediated anemia").[5]

73

74 It is vital to document that true *in vivo* hemolysis is occurring in patients with IMHA. Accordingly, proper

75 collection of blood samples is critical to avoid *ex vivo* hemolysis, which can lead to false decreases in RBC

76 concentration, hematocrit (HCT), and packed cell volume (PCV); false increases in mean cell hemoglobin

77 concentration (MCHC); variable alterations in serum bilirubin concentrations (depending on the

78 methodology used); and can also interfere with several other serum chemistry values.[6] Diagnostic test

79 results that support *in vivo* hemolysis are provided in **Table 1**.

80

81 To distinguish IMHA from other causes of anemia, an American College of Veterinary Internal Medicine

82 (ACVIM) consensus statement recommends documenting all three of the following diagnostic findings as

83 minimum criteria to diagnose IMHA in dogs:[7]

84   1) Decreased packed cell volume (PCV)

85   2) At least one of the following abnormalities supportive of hemolysis: erythrocyte ghost cells,

86   hyperbilirubinemia, bilirubinuria, icterus, hemoglobinemia, or hemoglobinuria

87   3) A positive saline agglutination test that persists with washing or at least two of the following:

88   a) A positive saline agglutination test without washing

89   b) ≥ 5 spherocytes per 1000× microscopic field

90   c) Detection of anti-erythrocyte antibodies by a Coombs' test or flow cytometry

91   Note that erythrocyte ghost cell numbers can markedly increase *ex vivo*, so examination of a freshly

92   made smear (at the time of blood collection) is recommended to assess this morphologic abnormality.[6]

93

94   A short survey (Appendix A) to determine how tests are being used to diagnose IMHA was sent to an

95   undetermined number of people in Australia, Canada, Europe, and the United States *via* list-serves and

96   email messages. Veterinary practitioners in both general practice and specialty hospitals, veterinary

97   clinical pathologists, and medical technologists and technicians in clinical pathology laboratories were

98   asked to reply. Respondents were not asked to self-identify. Ninety-four people completed at least the

99   first question of the survey.

100

101   The hematology parameters that > 75% of the respondents said were provided with a CBC are listed in

102   Figure 1. Seventy-two people (77%) indicated that, when needed, a saline agglutination test was

103   included with CBC results. An additional 13 respondents (14%) did not include a saline agglutination test

104   as part of a CB, but as an additional test. Seventy-two people (77%) recommended a test to detect anti-

105   erythrocyte antibodies (saline agglutination test, Coombs test, and/or flow cytometry). Additional tests

106   that were recommended by > 15 % of respondents included a biochemistry panel (66%), screening for

107   tick-borne diseases (60%), urinalysis (52%), and diagnostic imaging (34%).

108

109   Several tests have been used to aid in the diagnosis of IMHA, none of which are 100% sensitive and

110   specific. Differences in the protocols used to perform diagnostic tests for IMHA can dramatically affect

111   the performance of the test.[8,9] Tests for IMHA must account for physical properties associated with

112   antibody-antigen interactions, one of which is temperature-dependent antibody-antigen binding.[10]

113   Therefore, the Coombs' test is often carried out at both 37˚C and 4˚C.[11] Additionally, inclusion criteria

114   for IMHA patients vary between references, which can alter the assessment of test performance. Most

115   studies evaluating diagnostic tests for canine IMHA list auto-agglutination or spherocytosis as inclusion

116   criteria. However, Reimer *et al*. (1999) concluded that 2/70 (3%) canine patients with IMHA did not have

117   auto-agglutination or spherocytosis, nor did they have polychromasia.[12]

118

119   <u>Genetics</u>

120   Several dog breeds have a higher incidence of IMHA than the general canine population including;

121   cocker spaniels,[12–15] English springer spaniels,[12] miniature schnauzers,[13,15] and old English sheepdogs[16];

122   however, a specific gene association with IMHA has not be found. A few studies have associated the

123   presence of specific major histocompatibility complex alleles in dogs with the occurrence of immune-

124   mediated disease, but the predictive value of these associations remains uncertain.[17–19] The frequency

125   of specific dog erythrocyte antigens found during blood typing was not significantly different in 33 dogs

126   with IMHA as compared to 1,014 dogs without IMHA.[20] Therefore, expectedly, genetic testing was not

127   recommended as an additional test for IMHA by survey respondents.

128

129   <u>Non-specific Tests for Antibodies</u>

130   Two proteins, staphylococcal protein A (SpA) and papain, have been used in several papers to document

131   increased immunoglobulin in serum samples. SpA is an immunoglobulin binding protein produced by

132   *Staphylococcus aureus* that has been shown to be more sensitive than indirect antiglobulin tests in dogs

133   with IMHA.[21] Papain is an enzyme that digests antibodies into three 50 kDa segments comprised of one

134   fragment crystallizable (Fc) and two fragment antigen-binding (Fab) regions. When using a papain test to

135   detect anti-erythrocyte antibodies, false-positive results occur in up to 3/16 (19%) of dogs.[22] One study

136   tested 23 papain positive anemic dogs with direct and indirect antiglobulin tests; 8/23 (35%) dogs were

137   positive using a direct test and none were positive using an indirect test.[23] Due to the non-specific

138   nature of SpA and papain reactions, they are no longer recommended as diagnostic tests for IMHA and

139   unsurprisingly were not listed as additional tests used by survey respondents.

140

141   <u>Auto-agglutination</u>

142   Red blood cell agglutination should raise suspicion for IMHA (**Fig. 2**). To determine whether

143   agglutination is likely related to the presence of antibody or complement on the surface of the red blood

144   cell (i.e., auto-agglutination), a saline agglutination test should be performed. It is important to note that

145   artifactual agglutination/rouleaux is not dispersed in all patients using a 1:1 dilution. However, if

146   agglutination persists after washing erythrocytes at least three times in isotonic or phosphate-buffered

147   saline, it is likely that the erythrocyte clumping is true agglutination rather than rouleaux formation. The

148   occurrence of auto-agglutination in dogs with IMHA varies from 42% to 86%.[12–16] The number of patients

149   with a positive saline agglutination test is decreased when erythrocytes are washed extensively.[24,25]

150   There is some evidence that the decrease in positive saline agglutination tests with washing is due to a

151   reduction in false-positive results,[25] but increased numbers of false-negative results should also be

152   considered.

153

154   Seventy respondents indicated whether they evaluated saline agglutination tests macroscopically or

155   microscopically: 34 looked for microscopic agglutination, 29 looked for macroscopic and microscopic

156   agglutination, and seven looked at the sample macroscopically only. Sixty-three people indicated how

157   they dilute blood samples for a saline agglutination test: 31 performed a 1:4 dilution, 24 performed a 1:1

158   dilution, and eight performed both a 1:1 and a 1:4 dilution of the sample. Of the 28 respondents who

159   indicated whether they wash RBCs for the saline agglutination test, 16 used unwashed RBCs, 11 used

160   washed RBCs, and one used both.

161

7

162   Erythron

163   Spherocytosis is often used as an inclusion criterion for studies of dogs with IMHA and was included in

164   CBC data by 90/94 (96%) of survey respondents (Fig. 1). However, IMHA also can be non-spherocytic, as

165   the occurrence of spherocytosis in dogs with IMHA ranges from 61% to 95%.[12,13,15,16,24,26] Spherocytes are

166   generally only recognized in dogs (owing to the pronounced central pallor of their erythrocytes). In

167   contrast, spherocytes are rarely identified with certainty in cats, horses, and cattle since erythrocytes

168   from these animals lack central pallor on blood smears.

169

170   Spherocytosis and some other RBC membrane abnormalities can cause increased erythrocyte fragility.[27]

171   The osmotic fragility of erythrocytes can be tested by diluting whole blood in progressively decreasing

172   concentrations of sodium chloride (NaCl), incubating the samples for 30 minutes at room-temperature,

173   recording absorbance of the samples at 540 nm, and then creating a data curve assuming that the

174   lowest NaCl concentration causes 100% hemolysis.[27] RBC hemolysis occurs as a result of a loss of

175   osmotic regulation and volume control, which is exacerbated in a number of RBC disorders. Increased

176   RBC hemolysis during osmotic fragility testing is commonly reported in spherocytic conditions (e.g.,

177   IMHA), but can be seen in spectrin deficiency,[28] hereditary stomatocytocytosis,[29] intestinal parasite-

178   associated microcytosis, *Babesia canis* infection,[30] or non-hemolytic samples that are lipemic.

179   Erythrocyte fragility testing is not commonly available to practitioners but can provide support for

180   ongoing hemolysis.[31] One study observed increased erythrocyte fragility in 15/15 (100%) direct

181   antiglobuin test (DAT) positive and 4/12 (33%) DAT negative anemic dogs.[25] None (0/91) of the survey

182   respondents indicated that they recommended osmotic fragility testing for patients suspected of having

183   IMHA.

184

185   Most patients with IMHA have a moderate to marked regenerative anemia. A reticulocyte count was

186   listed as a component of a CBC by 77/94 (82%) of the survey respondents (Fig. 1). The occurrence of

187   reticulocytosis in dogs with IMHA ranges from 67% to 82%.[13,16,26] IMHA patients often have increased

188   numbers of circulating nucleated RBCs and/or Howell-Jolly bodies. Interestingly, reticulocyte

189   hemoglobin (HGB) content was shown to be decreased in 5/14 (36%) dogs with IMHA suggesting that

190   iron-restricted erythropoiesis can be present in some canine IMHA patients.[32]

191

192   A diagnosis of erythroid hyperplasia in bone marrow samples is a definitive indication of erythrocyte

193   regeneration. It is noteworthy that Weinkle *et al*. (2005) found 23/45 (51%) dogs with IMHA that

194   underwent bone marrow analysis had erythroid hyperplasia.[13] Similarly, another study that analyzed

195   bone marrow samples from dogs with IMHA observed erythroid hyperplasia in 6/11 (55%) samples.[15]

196   Extramedullary hematopoiesis[33] and secondary myelodysplasia[34] also have been reported in dogs with

197   IMHA. Bone marrow evaluation is typically recommended for suspected IMHA patients that have a

198   nonregenerative anemia. Low numbers of survey respondents [6/91 (7%)] indicated that they

199   recommend bone marrow aspiration or biopsy to aid in the diagnosis of IMHA. Two of these

200   respondents specified that this recommendation was warranted in patients with persistent

201   nonregenerative anemia.

202

203   Nonregenerative anemia has been reported in 6/23 (26%) canine IMHA patients in one study[24] and 6/20

204   (30%) in another.[15] In a retrospective analysis of dogs with nonregenerative anemia in which a bone

205   marrow sample was clinically indicated, 55/82 (67%) were determined to have IMHA (based on the

206   presence of either Coombs' positivity, auto-agglutination, or > 30% spherocytes).[35] In dogs with

207   nonregenerative IMHA, 38/55 (69%) had erythroid hyperplasia, and 17/55 (31%) showed incomplete

208   maturation of the erythroid line.[35] In another study of canine patients with a nonregenerative anemia

9

209   present for more than 5 days, 41/43 (95%) had erythroid hyperplasia in bone marrow samples, 23/43

210   (54%) had a spherocytosis, and 20/35 (57%) were positive by DAT.[36] To determine if dogs with a

211   nonregenerative anemia > 5 days duration have precursor-targeted immune-mediated anemia, bone

212   marrow should be evaluated for macrophage phagocytosis of erythroid precursors.[5] Weiss (2008) also

213   evaluated 57 cats with nonregenerative anemia in which bone marrow analysis was indicated.

214   Approximately half of the cats 28/57 (49%) were determined to have IMHA (based upon measurement

215   of an HCT < 20% and either Coombs' positivity or auto-agglutination).[35] In cats with IMHA and

216   nonregenerative anemia, 24/28 (86%) had erythroid hyperplasia and 4/28 (14%) showed maturation

217   arrest of the erythroid line.[35] In another study, phagocytosis of erythroid precursors and abnormal

218   presence of stainable iron was documented in the bone marrow of cats with both primary and

219   secondary IMHA.[37]

220

221   In cases of IMHA with RBC agglutination in the sample, many of the measured or calculated values of the

222   erythron are often erroneous [e.g., RBC concentration, mean cell volume (MCV), HCT, mean cell

223   hemoglobin (MCH), MCHC, red cell distribution width (RDW)] as impedance counters will count

224   erythrocyte clumps as single and large erythrocytes, which leads to a significant reduction in the

225   numbers of RBCs counted and an increase in the mean size of the cells.[6] In cases of intravascular

226   hemolysis, HGB concentration is not clinically reliable, as it represents a combination of free (plasma)

227   and RBC HGB. If it is available, the determination of the cell hemoglobin concentration mean (CHCM)

228   using an advanced laser cell counter could help assess RBC HGB.

229

230   Leukon

231   Abnormalities in leukocytes are commonly observed in patients with IMHA. Leukocytosis was reported

232   in 43% to 99% of dogs with IMHA (WBC concentrations in these reports ranged from 5,300 cells/μL to

233 105,700 cells/µL.[12,15,38,39] In one study, decreased survival time of dogs with IMHA was associated with

234 leukocytosis and lymphopenia,[40] while lymphocytosis was a positive prognostic factor in cats with

235 IMHA.[37] Neutrophil left shifts were noted in up to 16/20 (80%) of dogs with IMHA.[15] One paper

236 observed decreased survival rates in dogs with IMHA with band neutrophil concentrations ≥ 3000

237 cells/µL.[13]

238

239 IMHA patients with inflammatory leukograms typically have acute patterns such as a neutrophilia with a

240 left shift, lymphopenia, eosinopenia, and monocytosis.[15] In severe inflammatory and erythroid

241 regenerative conditions, a leukoerythroblastic pattern can be observed with a highly acute inflammatory

242 leukogram and a high percentage of nucleated RBCs in different stages of maturation.[15] Rubricytosis

243 causes a false increase in the automated WBC concentration that must be corrected mathematically

244 after the enumeration of nucleated RBCs by blood smear review. This further emphasizes the need for

245 blood smear examination, which can also help with the detection of neutrophil left-shifting and toxicity

246 that can be present in IMHA cases. Sixty-five/94 (69%) survey respondents indicated that blood smear

247 evaluation by a board-certified clinical pathologist was included in CBCs they performed or received (Fig.

248 1).

249

250 Serum Biochemistry and Urinalysis

251 Abnormalities in biochemical parameters have been associated with the clinical outcomes of IMHA

252 patients (**Table 2**), but not all studies report the same findings. An increase in total bilirubin

253 concentrations was observed in 60% to 100% of dogs with IMHA.[12,15,26] It is important to note that

254 increased conjugated bilirubin can interfere with phosphorus measurements leading to

255 pseudohypophosphatemia in patients with IMHA.[41] Although not linked to decreased survival, Klag et

256 al. (1993) observed hemoglobinemia and/or hemoglobinuria in 4/42 (10%) dogs with IMHA.[26] There are

11

257 also a few case studies of dogs with IMHA with biochemical abnormalities consistent with distal renal

258 tubular acidosis.[42] In cats with IMHA, hyperglobulinemia is reported to be a positive prognostic factor.[37]

259 Biochemistry profiles were recommended as additional tests for suspected IMHA patients by 60/91

260 (66%) of survey respondents.

261

262 Additional serum protein parameters have been reported to be altered in dogs with IMHA. For example,

263 cardiac troponin I was > 0.1 ng/mL in 20/27 (74%) dogs with IMHA (authors indicated that < 0.1 ng/mL is

264 expected in healthy dogs, but a true reference interval was not provided).[43] C-reactive protein was

265 increased in dogs with IMHA at presentation.[44–46] Alpha-1 acid glycoprotein also was increased in dogs

266 with IMHA, while albumin can be decreased at presentation.[45] Increased serum concentrations of

267 several cytokines have been reported in dogs with IMHA (n = 20) as compared with six healthy dogs.[46]

268 Interleukin-15 (IL-15), IL-18, granulocyte-monocyte colony stimulating factor, and monocyte

269 chemoattractant protein-1 concentrations were increased in animals with IMHA that died ≤ 30 days

270 after hospital admission.[46] Similarly, IL-2, IL-6, and tumor necrosis factor-α were present at higher

271 concentrations in dogs with primary IMHA (n = 19) when compared with dogs that had other

272 inflammatory diseases (n = 22) or healthy dogs (n = 32).[47] In question 4, none of the survey respondents

273 indicated that they recommended these protein assays to help diagnose patients with IMHA.

274

275 <u>Thrombon and Coagulation</u>

276 Thrombocytopenia is reported to occur in 29-70% of dogs with IMHA.[12,14,15,26,38,39] In a study of 151 dogs,

277 a platelet concentration < 150,000 platelets/μL correlated with decreased survival rates.[13] Also, a

278 decreased mean platelet component concentration was found in dogs with IMHA (n = 95) as compared

279 with healthy dogs (n = 95) or sick canine patients (n = 95)[48] which could indicate increased platelet

280 activation in IMHA patients.[49]

281

282 Considerations for severe thrombocytopenia include a consumptive process [e.g., disseminated

283 intravascular coagulation (DIC), pulmonary thromboembolism (PTE)] or a concurrent immune-mediated

284 thrombocytopenia (IMT). In humans, concurrent IMHA and IMT have been termed Evan's Syndrome.

285 This disease process likely occurs in dogs; however, the presence of concurrent anti-erythrocyte and

286 anti-platelet antibodies has rarely been documented in veterinary patients.[50,51] In 38 dogs with both

287 anemia and thrombocytopenia, 18/38 (47%) of patients were positive by DAT for anti-erythrocyte

288 antibodies.[52] In a similar study of 21 dogs with concurrent anemia and thrombocytopenia, auto-

289 agglutination that persisted after washing was observed 6/21 (29%) dogs, and two of three dogs tested

290 by DAT were positive.[53]

291

292 Several studies have assessed coagulation parameters in dogs with IMHA (**Table 3**). Importantly,

293 increased mortality was observed in dogs with IMHA that had thrombocytopenia, prolonged

294 prothrombin time (PT), prolonged activated partial thromboplastin time (APTT), decreased fibrinogen,

295 or DIC.[40] Reports using thromboelastography determined that 85-100% of dogs with IMHA were

296 hypercoagulable.[54–56] Development of DIC is observed in between 10/31 (28%)[14] and 9/20 (45%)[15] dogs

297 with IMHA. One study reported that thromboemboli were found in 20/25 (80%) IMHA dogs at

298 necropsy.[14] The analysis of coagulation parameters is typically recommended in IMHA patients that

299 have clinical signs of coagulopathy. One survey respondent indicated in Question 4 that they

300 recommended measurement of D-dimers in patients suspected of having IMHA.

301

302 <u>Indirect Antiglobulin Tests</u>

303 Indirect antiglobulin tests are not recommended in veterinary species due to low sensitivities and

304 specificities. When these studies were first evaluated for utility in canine patients with IMHA, the

305   sensitivity and specificity of an indirect antiglobulin test were 62.5% and 96.6%, respectively.[57] The DAT

306   performed by the same laboratory had a 83.3% sensitivity and 98.8% specificity.[57] None of the survey

307   participants recommended indirect antiglobulin tests in Question 4.

308

309   Direct Antiglobulin Tests (DATs)

310   Various methods for directly detecting RBC surface-bound anti-erythrocyte immunoglobulin (Ig) and

311   opsonizing complement protein (C3) are available. Of the 72 respondents who recommended a test to

312   diagnose IMHA with anti-erythrocyte antibodies, 66 recommended a Coombs' test, three recommended

313   flow cytometry and a Coombs' test, two recommended flow cytometry alone, and one recommended

314   flow cytometry and a Coombs' test at 4°C.

315

316   *Coombs' Tests*

317   Coombs' tests are often performed using a microtiter plate format. Additional methods include gel-

318   based microcolumn, immunochromatographic strip, and capillary DAT assays. Good agreement has been

319   reported between results of the Coombs' test and these methods.[25] False-positive DAT results have

320   been reported in anemic dogs. In theory, false-positive results could be due to technical difficulties (e.g.,

321   nonspecific absorption of the antibody, incomplete washing, contamination, assignment of an

322   inappropriate cut off) but patient factors are critical to consider. Dogs that recently received a

323   transfusion can have a positive DAT.[8,58] Also, dogs with an autoimmune disease that are positive for

324   antinuclear antibodies have been reported to be DAT positive without conclusive evidence of IMHA.[59]

325   Additionally, horses with equine infectious anemia can have a positive Coombs' test.[60]

326

327   The Coombs' test uses species-specific antibodies to detect Ig and/or C3 bound to erythrocytes in a

328   patient blood sample. A positive test results in RBC agglutination. Sixty-five of the people surveyed

14

329   specified if they recommended a Coombs' test when auto-agglutination was observed, 50 people

330   recommended a Coombs' test if no auto-agglutination was seen (7 specified use of a microtiter plate,

331   and 2 recommended an immunochromatographic strip DAT assay). Fifteen people recommended a

332   Coombs' test with or without auto-agglutination (1 specified the use of a microtiter plate, and another

333   recommended the use of a strip DAT assay). The authors agree with the recent ACVIM consensus

334   statement,[7] which indicates that a Coombs' test is unnecessary if true auto-agglutination that persists

335   after washing is present.

336

337   When a Coombs' test is warranted, polyvalent and monovalent test antibodies are available. These

338   antibodies are pre-adsorbed onto RBCs from healthy dogs before use in the test. False-negative results

339   can occur with either type of test antibody; therefore, including both polyvalent and monovalent

340   antibodies in a Coombs' test can be beneficial.[11] Including both polyvalent and monovalent antibodies

341   increased test performance in a study that reported a sensitivity of 82% and a specificity of 95% when

342   antibodies were combined.[8]

343

344   The antibody binding reaction is temperature-dependent, so it is recommended that testing is

345   performed at both 37°C and 4°C.[11] Thirty people specified that they ran a Coombs' test at 37°C, 11

346   respondents performed a Coombs' test at 37°C and 4°C, 1 respondent performed the test at 37°C and

347   room temperature, and (as mentioned above)one person performed flow cytometry plus a Coombs' test

348   at 4°C.

349

350   Also, prozone effects are commonly reported when agglutination is not observed at low serum dilutions

351   (i.e., high antibody concentrations) but is observed at higher serum dilutions (i.e., low antibody

352   concentrations). This is due to the presence of excess immunoglobulins that interfere with agglutination

353 induced by the interactions of the test antibodies with Ig and C3 on the RBCs.[61] This improper test

354 antibody to anti-RBC Ig ratio leads to false-negative results if there are not enough serial serum dilutions

355 tested. A prozone effect was observed in 17/126 (13%) samples tested by Piek *et al*. (2012).[62]

356

357 The reported performance of Coombs' tests vary, likely due to different samples, protocols, and test

358 reagents that are used at different laboratories. Important positive control samples for the Coombs' test

359 include Ig-coated and complement-coated canine RBCs, but these reagents are not readily available.[9] It

360 has been reported that the use of whole blood in EDTA or acid citrate dextrose (ACD) yields similar

361 results; however, ACD anticoagulants were preferred in one study because of increased sample

362 hemolysis in EDTA.[9] False-negative results that reduce the sensitivity, and negative predictive value

363 (NPV) of the Coombs' test can be caused by physical properties of the test antibodies (e.g., low antibody

364 affinity, an inappropriate antibody ratio, steric hindrance), poor technique (e.g., excessive washing,

365 delayed processing, assignment of an inappropriate cut-off), or patient factors (drug-dependent

366 reactions, blood transfusions, steroid administration).[8]

367

368 In dogs with IMHA, one study reported that a low percentage, 17/46 (37%), of patients had positive

369 Coombs' test results,[12] but other studies indicated that 77% of dogs with IMHA were positive.[13,14] In a

370 small study of 12 dogs, the sensitivity, specificity, positive predictive value (PPV), and NPV of the

371 Coombs' test was 58%, 100%, 100%, and 62%, respectively.[63] Similarly, Quigley *et al*. (2001) calculated a

372 PPV of 100% and an NPV of 68%.[64] In a study of cats with IMHA, 2/89 (2%) healthy cats were reported

373 to have a strongly positive Coombs' test at 37˚C.[65] Another study reported that 0/14 (0%) nonanemic

374 cats and 18/55 (33%) anemic cats were Coombs' positive.[66] Of the 18 Coombs' positive cats, 15 were

375 diagnosed with primary IMHA, two were feline leukemia virus positive (FeLV), and one had

376 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.

401 (2001) reported a PPV of 100%, and an NPV of 93% when they used flow cytometry to evaluate 13 dogs

402 with IMHA and 13 healthy dogs.[64] In 2008, Morley *et al*. published an assessment of the utility of flow

403 cytometry to detect anti-erythrocyte antibodies in dogs.[71] They found 26/147 (18%) anemic patients

404 had detectable anti-erythrocyte antibodies. This included 17/22 (77%) IMHA patients, 5/14 (36%) IMT

405 patients, and 3/71 (4%) cancer patients. However, 12/145 (8%) nonanemic dogs also were positive for

406 anti-erythrocyte antibodies, which included 3/5 (60%) patients with infectious disease and 5/81 (6%)

407 cancer patients, and the test had a PPV of 70% and NPV of 95%.

408

409 *Direct Enzyme-Linked Antiglobulin Tests (DELATs)*

410 Immunoglobulins and C3 bound to RBCs are detected by comparing the absorbance of patient samples

411 to healthy control animals in the DELAT. Early evaluation of a DELAT indicated that 1 mg/mL ρ-

412 nitrophenyl phosphate in carbonate buffer is the preferred substrate for the reaction, however false-

413 positive results were observed in 31/60 (52%) dogs tested.[72] Another evaluation of DELAT performance

414 yielded comparable results with a Coombs' test; 12/23 (52%) samples were Coombs' positive, while

415 13/23 (57%) were DELAT positive.[73] To the authors' knowledge, this test for IMHA is no longer readily

416 available.

417

418 **Guidelines for Performing Diagnostic Tests for IMHA**

419 As mentioned previously, technical difficulties and test protocols can profoundly affect the performance

420 of diagnostic tests for IMHA. Tests performed at 4°C, room temperature, and 37°C can provide different

421 results. This document includes two example protocols for saline agglutination testing (Appendix B) and

422 an example of Coombs' testing (Appendix C). In different diagnostic laboratories, it is expected that

423 these protocols will be performed differently but that proper procedures will yield adequate results.

424

425 **Conclusions**

426 Making a definitive diagnosis of IMHA can be difficult due to the variability in patient presentation and

427 diagnostic test performance. Recommended tests for diagnosing IMHA in anemic patients include: 1) a

428 CBC with verified reticulocyte count, manual PCV with assessment of plasma color, and microscopic

429 examination of a (preferably fresh) blood smear, 2) serum chemistry profile, 3) urinalysis, 4) saline

430 agglutination test (preferably with washing), 5) Coombs' test or flow cytometric analysis if the saline

431 agglutination test is negative, 6) coagulation testing for thrombocytopenic patients and patients with

432 clinical signs of coagulopathy, and 7) tests to determine if any underlying disease is present (e.g., drug or

433 toxin exposure, infection, inflammation, neoplasia, other autoimmune diseases). Our survey proved that

434 clinicians choose different tests to diagnose IMHA and that laboratories perform tests differently.

435 Therefore, it is recommended that veterinarians contact a clinical pathologist or technicians or

436 technologist at the diagnostic laboratory they use to obtain details about the reliability of specific tests

437 being performed to diagnose IMHA.

438

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637

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	Increased urobilinogen
Decreased MCHC [e] or increased MCHC [i]	concentration [i]	
Spherocytes [e]; ghost cells [I] – immune-mediated damage or other cause	Decreased hemopexin concentration [i]	
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

645 **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]

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

31

647 **Figure Legends:**

648 **Figure 1.** Proportions (above 75%) of hematologic parameters reported or received on the CBC results of

649 patients suspected of having IMHA by survey respondents (n=94).

650

651 **Figure 2.** Microscopic evidence of agglutination in a wet-mount saline agglutination test. Peripheral

652 blood in EDTA (0.15%) was diluted 1:4 in isotonic saline (0.9% NaCl). A drop of the mixture was placed

653 on a glass slide, and a coverslip was placed over the drop. Grape-like aggregates of erythrocytes can be

654 observed (unstained, 200× magnification).

655

656 **Figure 3.** Survey responses indicating the post-transfusion time-frame in days (d) during which

657 respondents would cautiously interpret hematologic test results that support a diagnosis of IMHA

658 (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 <u>as part of a complete blood count</u> 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

- o   Remove the supernatant

- o   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

  - o   Agglutination appears as grape-like clusters of RBCs (Fig. 2) and must be distinguished from rouleaux where erythrocytes are arranged in linear stacks.

  - o   If agglutination is present, the result is positive and supports a diagnosis of IMHA

1.   Harvey J. Hematology Procedures. In: Harvey J, ed. *Veterinary Hematology: A Diagnostic Guide and Color Atlas*. St. Louis: Elsevier Saunders; 2012:13-14.

2.   Caviezel LL, Raj K, Giger U. Comparison of 4 direct coombs' test methods with polyclonal antiglobulins in anemic and nonanemic dogs for in-clinic or laboratory use. *J Vet Intern Med*. 2014;28(2):583-591. doi:10.1111/jvim.12292

3.   Stockham S, Scott M. *Fundamentals of Veterinary Clinical Pathology*. 2nd ed. Ames, Iowa: Blackwell Publishing; 2008:136.

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

1. MP_Biomedicals. Coombs' Tests. https://www.mpbio.com/catalogsearch/result/?q=coombs.

Accessed May 10, 2019.

2.   ALVEDIA. Alice Veterinary Diagnostics. http://www.alvedia.com/quicktest-dat-canine/. Accessed
     May 15, 2019.

3.   VMRD. Veterinary Medical Research and Development.
     https://www.vmrd.com/reagents/detail/canine-coombs-reagent-5-ml/. Accessed May 15, 2019.

4.   Caviezel LL, Raj K, Giger U. Comparison of 4 direct coombs' test methods with polyclonal
     antiglobulins in anemic and nonanemic dogs for in-clinic or laboratory use. *J Vet Intern Med*.
     2014;28(2):583-591. doi:10.1111/jvim.12292

5.   Piek CJ, Teske E, van Leeuwen MW, Day MJ. Good agreement of conventional and gel-based
     direct agglutination test in immune-mediated haemolytic anaemia. *Acta Vet Scand*.
     2012;54(1):10. doi:10.1186/1751-0147-54-10