

Response to letter regarding “Effect of dilution of canine blood samples on the specificity of saline agglutination tests for immune-mediated hemolysis”

Dear Editor,

We thank Dr Giger for his interest in our study, and this opportunity to re-emphasize several points made in the original manuscript.

Our primary aim was to determine if increasing dilution improved the specificity of saline agglutination tests (SATs).¹ As specificity is the proportion of animals without the disease that test negative, our a priori sample size calculation focused on the number of dogs without immune-mediated hemolysis (IMH) needed to determine if increasing dilution reduced false positive rates.¹ Based on this sample size calculation, at least 111 anemic dogs without IMH were required to achieve adequate study power.¹ As the study included 138 dogs without IMH, we are confident that case numbers were acceptable for assessment of the effect of dilution on false positive SATs. We believe this is supported by the relatively tight confidence intervals for our estimates of specificity, for example, at a 49 : 1 ratio of saline to blood, the 95% confidence interval is 93% to 99%.¹ We acknowledge that the number of dogs with IMH is low, and therefore the estimates of sensitivity are imprecise. However, this study was designed to focus on specificity rather than sensitivity because (a) it is already well established that not all dogs with IMH will have a positive SAT² and (b) both our clinical experience and Dr Giger's previous study suggested that, when performed at a 1 : 1 dilution, false positive SATs are common in dogs without IMH.³

We are somewhat confused by Dr Giger's suggestion that 29 dogs were excluded from the study. As stated in abstract and results, only 3 of the enrolled 150 dogs were subsequently excluded from calculation of specificity, sensitivity, and diagnostic accuracy.¹ The supplementary materials provide clinical details for these 3 animals, which we believe support our assessment that we could not clearly determine if IMH was a component of their anemias.¹ However, we recognize that this is subjective, and the supplementary materials therefore also provide results of SATs at each dilution for these cases.¹ Should readers disagree with their exclusion, this information allows recalculation of specificity, sensitivity, and diagnostic accuracy including these dogs. However, we do not believe that this would alter the conclusions of the study.

Dr Giger raises concerns about the criteria used to classify dogs as unaffected by IMH. Specifically, he suggests that it is without precedent to consider dogs with a positive direct antiglobulin test (DAT) but no evidence of hemolysis to be unaffected by IMH. We strongly dispute this, as positive DAT test results can occur in the absence of anemia, as has been reported in low numbers of human blood donors, and as a nonspecific finding in sick patients, for example, in association with hypergammaglobulinemia.^{4,5} To quote directly from a recent review of autoimmune hemolytic anemia (AIHA) in humans, “thus, AIHA cannot be diagnosed from DAT positivity alone; there should be evidence of hemolysis, and other congenital or acquired hemolytic disorders should be excluded in complex cases.”⁶ Similarly, the British Society of Haematology's guidelines for diagnosis of AIHA in humans begin with determining if hemolysis is present before DAT testing is performed.⁷ In our study, all dogs with a positive DAT but no evidence of hemolysis had a plausible nonimmune-mediated cause of anemia, and none were considered to be affected by IMH by their attending clinician.¹ Therefore, we are comfortable that our classification of dogs as affected or unaffected by IMH was in line with current clinical practice.

Regarding the methodology used for SATs, we agree that this test lacks formal validation, and our study is 1 of multiple steps necessary to develop a more standardized protocol. It is therefore unsurprising that there is variability in how the test is performed. Because the SAT is intended as a patient-side screening test, we opted to use a simple protocol which we believe, based on text book descriptions and anecdotal experience, is consistent with that used in many clinical pathology laboratories and by veterinarians in primary care and emergency practice. Specifically, we did not attempt to adjust samples to achieve a standardized PCV and used room temperature blood and reagents. However, as discussed in the manuscript, we agree that warming blood and saline to body temperature can be useful when clinically irrelevant cold agglutinins are suspected to be causing agglutination.

We opted to focus on microscopic agglutination because based on clinical experience, we considered this to be the most common source of false positive results. In a recent survey of veterinarians and technicians, 34/70 performed microscopic evaluation alone and 29/70 both microscopic and macroscopic evaluation, so we would

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suggest that our use of microscopic evaluation was not inappropriate or inconsistent with clinical norms.² Similarly, 16/28 surveyed individuals performed the SAT on unwashed erythrocytes.² While we agree that washing cells can be helpful when there is a suspicion that a SAT is a false positive, given only 4/138 dogs had a false positive SAT at the 49 : 1 dilution, our results suggest washing is not routinely necessary provided an adequate saline to blood ratio is used.

We opted to use a recently released point-of-care DAT test. This was partly driven by practical considerations. This test requires only a very small volume of blood and its use allowed us to include dogs with a wide range of causes of anemia, even when little remnant blood was available after clinical testing. Another reason for its selection was the variable performance of laboratory DAT tests in the veterinary literature.⁸ This likely reflects, at least in part, the lack of a standardized protocol in veterinary medicine for DAT testing. In our study, the point-of-care gel kit test was performed according to the manufacturer's instructions and using a centrifuge preapproved by the manufacturer, and could be readily adopted by other investigators, potentially helping to increase the external validity of studies reporting veterinary DAT testing.

Last, we would like to stress that the aim of our study was not to compare the relative performance of SAT vs DAT. In fact, we would advocate for the use of DAT as a confirmatory test in most cases of suspected immune-mediated hemolytic anemia (IMHA), but we recognize many veterinarians cannot obtain a laboratory DAT result at initial presentation. Given the rapid progression of IMHA in many dogs, waiting 24 to 48 hours for a laboratory DAT result before starting immunosuppression is often not an option. As the 49 : 1 dilution achieved 97% specificity, we stand by our conclusion that use of an adequately diluted SAT as a rapid, inexpensive cage-side screening test for IMHA is justified. However, we would emphasize that while the 49 : 1 dilution achieved high specificity for IMHA, occasional (4/138) false positives did occur. Therefore, as for any diagnostic test for IMHA,

SAT results should be evaluated as part of the full clinical picture, with a particular emphasis on establishing if there is evidence of hemolysis. We would also stress that performing a SAT does not preclude subsequent DAT testing to help confirm the diagnosis of IMHA.

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REFERENCES

1. Sun PL, Jeffery U. Effect of dilution of canine blood samples on the specificity of saline agglutination tests for immune-mediated hemolysis. *J Vet Intern Med.* 2020;34:2374-2383.
2. MacNeill AL, Dandrieux J, Lubas G, et al. The utility of diagnostic tests for immune-mediated hemolytic anemia. *Vet Clin Pathol.* 2019;48:7-16.
3. 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;28:583-591.
4. Heddle NM, Kelton JG, Turchyn KL, et al. Hypergammaglobulinemia can be associated with a positive direct antiglobulin test, a nonreactive eluate, and no evidence of hemolysis. *Transfusion.* 1988;28:29-33.
5. Parker V, Tormey CA. The direct antiglobulin test: indications, interpretation, and pitfalls. *Arch Pathol Lab Med.* 2017;141:305-310.
6. Barcellini W, Fattizzo B. How I treat warm autoimmune hemolytic anemia. *Blood.* 2021;137:1283-1294.
7. Hill QA, Stamps R, Massey E, et al. The diagnosis and management of primary autoimmune haemolytic anaemia. *Br J Haematol.* 2017;176:395-411.
8. Garden OA, Kidd L, Mexas AM, et al. ACVIM consensus statement on the diagnosis of immune-mediated hemolytic anemia in dogs and cats. *J Vet Intern Med.* 2019;33:313-334.