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LETTER TO THE EDITOR

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Letter regarding "Effect of dilution of canine blood samples on the specificity of saline agglutination tests for immune-mediated hemolysis," original and modified saline agglutination tests vs direct Coombs' tests

Dear Editor,

Agglutination of red blood cells (RBCs) has been studied for more than a century, but with the exception of dermatological cold agglutinin disease, represents an in vitro phenomenon.[1] Indeed, because agglutinated RBCs cannot pass through capillaries, circulating agglutinates would not be compatible with life. Robin Coombs, a veterinarian, is credited for development of the direct and indirect antiglobulin tests (DAT and IAT) in 1945, which detects subagglutinating auto- and alloantibodies bound to (washed) RBCs. The Coombs' tests continue to be important diagnostic immunohematologic tools and in human (also blood banking) and veterinary medicine.[2-8]

Macroscopic agglutination of canine blood may be driven by agglutinating IgM and IgG on the surface of erythrocytes, cold agglutinins, EDTA-anticoagulant, plasma proteins, or other plasma components. A saline agglutination test (SAT) was introduced by my residency mentors at the University of Florida around 1980 intended to break up unspecific macroscopic agglutination in domestic animals by adding 1 drop of saline to 1 drop of blood.[9] That presumably original description of SAT showed that saline dilution of a macroscopically autoagglutinating blood sample revealed a positive DAT result after washing.[9] Thereafter, some veterinarians modified the SAT by incorporating a 1 : 4 and higher blood to saline dilution step, adding microscopic examination, and thereafter utilized a positive result to replace DAT or "overrule" a negative DAT result to diagnose immune-mediated hemolytic anemia (IMHA) in veterinary practice.[5] Indeed, without any formal evaluation, the SAT made it into the recommendations of the recent ACVIM consensus statement on the diagnosis of IMHA.

In contrast, I have questioned the value of the SAT at both 1 : 1 and 1 : 4 dilution for decades, raising concerns about unspecific autoagglutination reactions.[3,4,7] Moreover, I have shown that washing of RBCs 3 times with saline reveals true/persistent autoagglutination that may actually preclude interpreting a DAT. In a recent study in *Journal of Veterinary Internal Medicine*, Sun and Jeffery[8] evaluated the SAT among 150 anemic dogs and confirmed that the SAT at blood to saline dilutions of 1 : 1 up to 1 : 9 is not specific and is not predictive of a diagnosis of IMHA. However, they state that the presence of microscopic agglutinins at a 1 : 49 dilution is specific in the diagnosis of IMHA. I would like to make a few remarks of caution:

Sun and Jeffery used microscopic rather than the originally intended gross visual examination in their SAT. They used EDTA-anticoagulated, refrigerated blood, and phosphate buffered saline instead of physiological saline, and test components that were not warmed to body temperature. Their comparison is based on a single gel minitube in-clinic DAT kit, which has not yet been studied, was performed with a centrifuge that was not recommended, and was carried out in part without an autoagglutination control test. While the DAT kit does not require washing RBCs, they did not account for the varied degrees of anemia and did not compare to the standard 3x washing with saline to exclude the presence of unspecific autoagglutination. Furthermore, there is no evidence that rouleaux cause macroscopic agglutination in dogs, which can be readily differentiated microscopically.

Sun and Jeffery excluded 27 anemic dogs with positive DAT results (75% of their DAT positive dogs) claiming they were false positives, because the diagnosis of IMHA did not fit clinically. Moreover, they excluded another 2 DAT positive dogs as having unclassified anemia. Although many clinicians in the past have "overruled" a negative DAT result or for that matter skipped performing a DAT, these authors dismissed most of their DAT positive results without precedent, causing further uncertainty. Their clinically based selection left only 9 IMHA cases with a positive DAT result and hemolytic anemia, which included 1 case after transfusion. Their small number of selected DAT positive cases led to large confidence intervals. In my view, the statistical analyses and sensitivity, specificity, and diagnostic accuracy of the DAT kit and SAT provided in their small study of a potentially skewed population of DAT positive dogs are premature and likely, as in most other studies of IMHA in dogs faulty due to many misdiagnoses of IMHA in dogs tested. Without a definitive diagnosis of IMHA, claims of false positive and false negative results should be avoided.

While Sun and Jeffery's study refutes the ACVIM consensus recommendation and usefulness of the regular SAT (1 : 1, 1 : 4, and even 1 : 9 dilution) for making a diagnosis of IMHA in dogs, and thereby confirms my prior statements over the past,[3,4,7] they now recommend a drastically modified SAT (1 : 49, which is more similar to 3x washing in my clinical diagnostic approach) on the basis of their very limited study with few cases of IMHA that excludes many DAT positive anemic dogs. More importantly, they do not endorse the general use of established laboratory DAT methods, which in my view should remain the key diagnostic tool for IMHA in dogs as is practiced in human medicine. Fortunately, the frequently observed unspecific spontaneous autoagglutination of canine blood, which can interfere with performing hematological tests and interpreting DAT, blood typing, and crossmatching results, can in nearly all cases be overcome with simple 3x washing with saline,[4] a standard process that is also used in human medicine.

Finally, my veterinary doctoral fellow Nadine Idalan has recently carried out a comparative study of immunodiagnostics, including SAT, spherocytosis, and 6 DATs (also the gel minitube kit) in a large cohort of dogs. This study reveals that the various DAT methods are superior over SAT in the diagnosis of IMHA. This manuscript is submitted and will be presented at the upcoming virtual ACVIM Forum 2021. I encourage everyone to perform a DAT to directly document antibody and/or complement bound to erythrocytes for diagnosis of IMHA rather than relying on any SAT methods; the Coombs' test is also the standard used in human medicine.

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REFERENCES

1. Jervell F. The influence of temperature upon agglutination of red blood corpuscles. *J Immunol*. 1921;4:445-451.
2. Wardrop KJ. Coombs' testing and its diagnostic significance in dogs and cats. *Vet Clin North Am Small Anim Pract*. 2012;42:43-51.
3. Giger U. Regenerative anemias caused by blood loss or hemolysis. In: Ettinger SJ, Feldman EC, eds. *Textbook of Veterinary Internal Medicine*. St. Louis, MO: Elsevier Saunders; 2005:1886-1908.
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;28:583-591.
5. Members of the Veterinary and Comparative Clinical Immunology Society Diagnostic Task Force, AL MN, Dandrieux J, Lubas G, et al. The utility of diagnostic tests for immune-mediated hemolytic anemia. *Vet Clin Pathol*. 2019;48:7-16.
6. 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.
7. Giger U. Diagnosis of immune-mediated hemolytic anemia. Proceedings of ACVIM Forum 2017 (and other conference proceedings and earlier years).
8. 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.
9. Werner LL. Coombs' positive anemias in dogs and cats. *Compend Cont Educ Vet Pract*. 1980;2:96-111.