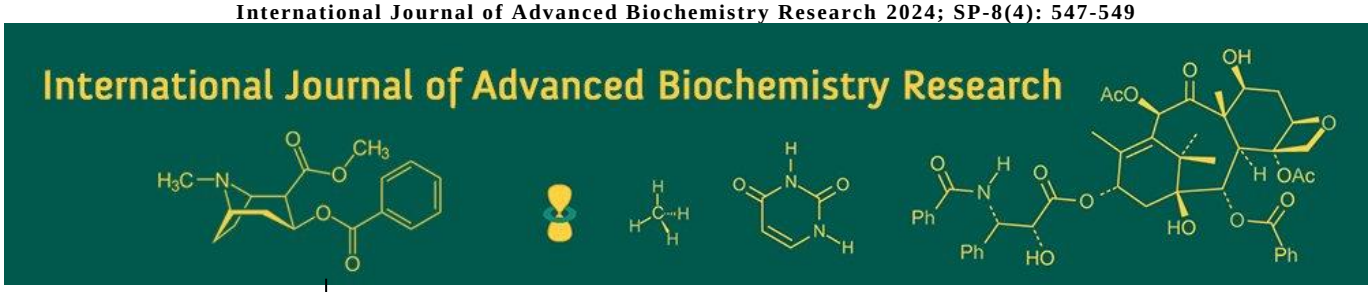

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Diagnosis of immune mediated haemolytic anemia in dogs affected with *B. gibsoni*

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Abstract
The present study was carried out to diagnose immune mediated hemolytic anemia associated with *Babesia gibsoni* in dogs. A total of 136 dogs suspected for *B. gibsoni* infection were examined by microscopy and screened by PCR, out of which 64 dogs were positive for *B. gibsoni* infection. Among 64 dogs, 28 dogs were positive for IMHA by Coombs test, 21 dogs were positive for IMHA by Saline agglutination test and 18 dogs had spherocytosis.

Keywords: Immune mediated haemolytic anemia, Coombs test

Introduction
The present study entitled was carried out to diagnose immune mediated hemolytic anemia associated with *Babesia gibsoni* in dogs. The study was conducted in dogs presented to small animal OPD, Department of Veterinary Medicine with clinical signs of anorexia, pyrexia, lethargy, pale mucous membrane, lymphadenopathy, ascites, vomiting, diarrhea, icterus, hematuria suggestive of canine babesiosis. A total of 136 dogs suspected for *B. gibsoni* infection were examined by microscopy and screened by PCR, out of which 64 dogs were positive for *B. gibsoni* infection. Among 64 dogs, 28 dogs were positive for IMHA by Coombs test, 21 dogs were positive for IMHA by Saline agglutination test and 18 dogs had spherocytosis.
Further these dogs were subjected to saline agglutination test, spherocytosis in blood smear examination and Coombs test for diagnosis of immune mediated haemolytic anaemia (IMHA).

Materials and Methods
Clinical cases
Dogs presented to the Department of Veterinary Medicine, Veterinary College Hospital, Hebbal, Bengaluru with a history of tick infestation, high fever, pale / icteric mucous membrane, inappetence, anaemia, lethargy, vomiting, diarrhoea, hematuria were screened by saline agglutination test, spherocytosis and Coombs test for diagnosing IMHA. These dogs were screened for *B. gibsoni* infection by microscopic examination of blood smears and PCR. Eighteen dogs infected with *B. gibsoni*, positive for saline agglutination test, spherocytosis and Coombs test were selected for the study.

In-saline agglutination test
One drop of EDTA blood was mixed with one to four drops of normal saline on a glass slide. The agglutination of RBCs was assessed macroscopically and then it was observed under the microscope for RBC agglutination which appears like cluster of grapes.

Examination of blood smear for B. *gibsoni* organisms and spherocytosis
Thin blood smears were prepared from the whole blood sample collected, which was stained with Giemsa stain. Smears were examined under the oil immersion 100X objective of the microscope to detect the presence of *B. gibsoni* organisms and spherocytosis.

Coombs Test
Coombs test was carried out in suspected cases of IMHA in dogs. The collected blood sample was centrifuged at 1500 rpm for 5 minutes at room temperature. 4.9 ml of normal saline was added to 0.1 ml of packed RBCs. The red blood cells were washed 4 times in normal saline and finally 4.9 ml of normal saline was added to make 2% suspension.

Coombs test procedure
100 µL of normal saline was pipetted into 4 test tubes. Two fold dilutions of the Coombs reagent was prepared by Pippeting 100 µL of Coombs reagent (MP biomedicals) to the first tube, mixed and transferred 100 µL from the 1st tube to the 2nd tube, additionally 100 µL of the mixture was transferred from 2nd tube to the 3rd tube. 100 µL was discarded from 3rd tube. The control 4th test tube had only normal saline. Furthermore 100 µL of washed 2% RBCs were added to all the test tubes, gently mixed and was incubated at 37 ºC for 30 minutes. After the incubation time was complete, all the four test tubes were centrifuged for 1 minute.

Interpretation
The contents of each tube were evaluated by placing a few drops of solution on a clean glass slide, covered with glass coverslip and examined at a magnification of 100X. Presence of clumps and large aggregates of RBCs was interpreted as positive result of Coombs test while absence of clumping was considered as negative. The clumps should not be present in control cells.

Results and Discussion
Diagnosis of IMHA
Out of 64 dogs positive for *B. gibsoni*, 28 dogs showed positive for IMHA by Coombs test, 21 dogs were positive by saline agglutination test and 18 dogs had spherocytosis.

Saline agglutination test
Out of 64 dogs positive for *B. gibsoni*, 21 dogs showed positive for IMHA by saline agglutination test.

Sphericity count
Out of 64 dogs positive for *B. gibsoni*, 18 dogs had spherocytosis.

Coombs test
Out of 64 dogs positive for *B. gibsoni*, 28 dogs showed positive for IMHA by Coombs test accounting to 43.75 percent.

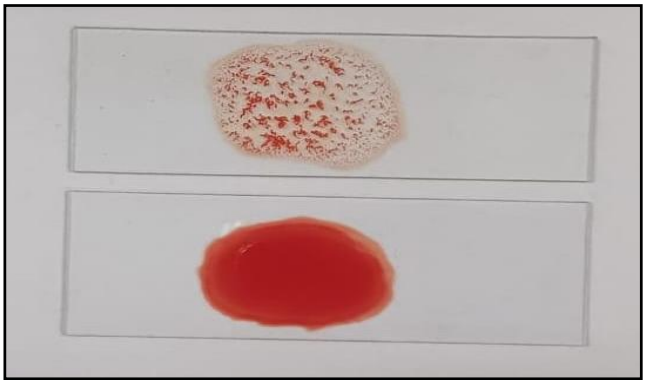

Plate 1: Comparision of positive and negative saline agglutination test

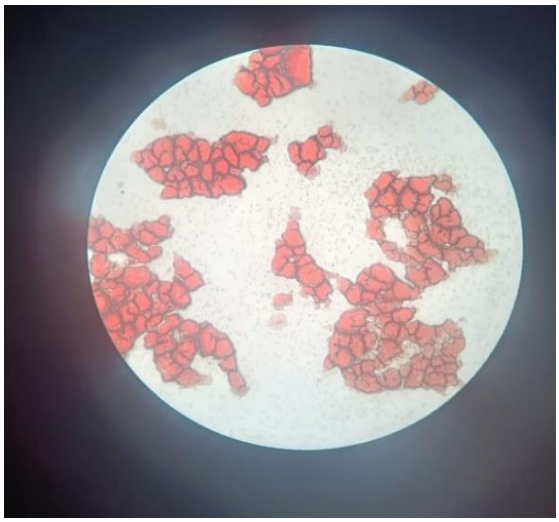

Plate 2: Positive saline agglutination test under the microscope (100X)

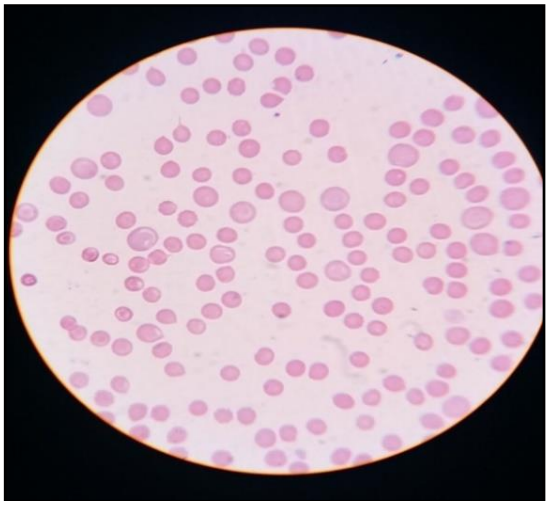

Plate 3: Presence of spherocytes in giemsa stained blood smear

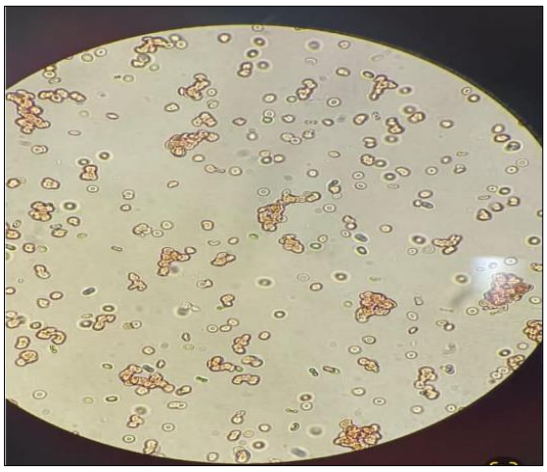

Plate 4: Positive coombs test (40X)

Saline agglutination test
In the present study, 32.812 percent (21 dogs) of dogs were positive for IMHA caused by *B. gibsoni* by saline agglutination test. Weinkle *et al*. (2005) [5] reported that 40 to 89 percent of dogs with IMHA had positive saline agglutination test. Lachungpa *et al.* (2020) [4] reported that 21 percent of dogs were positive for IMHA by saline agglutination test. The reason for in-saline slide

agglutination was to break up rouleaux formation but not erythrocyte aggregates using saline. This variation in the results could be because a positive saline agglutination is seen when a high level of anti-RBC antibodies are present that are attached to more than one cell.

Spherocytosis

Spherocytosis was observed in 18 dogs (28.125%) with IMHA caused by *B. gibsoni* in the present study. Partial phagocytosis of RBC results in removal of a part of RBC membrane, these RBCs form spherocytes. Spherocytosis is the hallmark of IMHA (Caviezel *et al.* 2014) [1]. A spherocyte count of 2+ or greater was highly suggestive of IMHA (Nelson and Couto, 2009) [3]. While spherocytosis is recognized as a hallmark of IMHA, accurate evaluation requires meticulous examination and identification due to potential discrepancies among observers which could result in false negative results (Caviezel *et al.* 2014) [1].

Coombs test

Out of 64 dogs positive for *B. gibsoni*, 28 dogs (43.75 percent) were positive by Coombs test.

Our study is in accordance with Farewell *et al*. (1982) [2] who reported that out of 37 *B. gibsoni* and *B. canis* infected dogs tested, 31 were Coombs test-positive.

Interpretation

Coombs test was more effective in the diagnosis of IMHA as compared to Saline agglutination test or spherocytosis in the blood smear examination.

Conclusion

Study highlights the utility of various diagnostic methods in detecting immune-mediated hemolytic anemia (IMHA) in dogs infected with Babesia gibsoni. The saline agglutination test demonstrated a positivity rate of 32.812%, aligning with previous findings, albeit showing variability likely attributed to differing antibody levels. Spherocytosis, a hallmark of IMHA, was present in 28.125% of cases, emphasizing its diagnostic significance despite potential observer variability. Furthermore, the Coombs test revealed a positivity rate of 43.75%, consistent with prior research. These diagnostic tools collectively contribute to the accurate identification and management of IMHA in Babesia gibsoni-infected dogs, emphasizing the importance of comprehensive evaluation in clinical practice.

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