



Efficacy of Microtiter Method of Direct Antiglobulin Test in diagnosis of Immune Mediated Haemolytic Anaemia in Dogs

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ABSTRACT

The present study was carried out to assess the efficacy of microtiter method of direct antiglobulin test in diagnosis of Immune Mediated Haemolytic Anaemia in Dogs. Two hundred and fifty eight anaemic dogs with pale/icteric mucous membrane were used for the present study. Initial screening of these dogs was done with saline agglutination test. Forty seven dogs were found positive for saline agglutination test. Whole blood samples of these dogs were used for direct antiglobulin test (DAT). The test was performed using polyvalent antiserum in a 96-well microtiter plate allowing multiple dilutions of antisera. Twenty five dogs were found to be positive for DAT at various titers. The use of microtiter method helped in detecting 8 additional Coombs' positive dogs which would be negative in standard tube method dilution. The use of this method increases the sensitivity of DAT by overcoming prozone effect.

Keywords: Immune-mediated haemolytic anaemia, DAT- Microtiter method, Prozone effect, Coomb's test

Immune-mediated haemolytic anaemia (IMHA) is one of the most common immune-mediated diseases of dogs (Klag *et al.*, 1993). It is a type II immunological response involving destruction of erythrocytes by immunoglobulins IgG, IgM and complement C₃ (Wilkerson *et al.*, 2000). Immune-mediated haemolytic anaemia has a high morbidity and mortality (Kidd and Mackman, 2013).

Direct antiglobulin test (DAT) also known as Coombs' test is used to detect antibodies on the surface of the erythrocytes. The test was introduced first by Dr. Robin Coombs in human practice in detecting incomplete Rh antigens (Coombs *et al.*, 1945). This principle is used in the diagnosis of IMHA (Slappendel, 1979; Wardrop, 2005; Warman *et al.*, 2008). Direct antiglobulin test is not considered a standard test for diagnosing IMHA but most commonly used. The tube method was considered standard in the past (Slappendel, 1979) but false negative results are very common because of low sensitivity of test

ranging from 37 to 89% (Overmann *et al.*, 2007). One reason for false negative result being improper dilution of antiserum (prozone effect). The prozone effect is caused due to excess of antiglobulin in relation to antigen at lower dilutions, this results in decrease cross linking of erythrocytes and failure of agglutination at lower dilutions. The tube test according to manufacturer guidelines allows dilution upto 1:8, but a 96-well microtiter plate allows additional dilutions of antisera in economical way. The objective of present study was to study the advantage of using 96-well microtiter method over tube method.

MATERIALS AND METHODS

Selection of cases

Two hundred and fifty eight anaemic dogs with pale/icteric mucous membrane presented to the Medicine Unit of

Veterinary Hospital Mannuthy and Kokkalai over a period of ten months were used for the study. Positive saline agglutination test and DAT were considered as diagnostic criteria for confirmation of Immune mediated haemolytic anaemia. Initial screening of the dogs was done with saline agglutination test. Forty seven dogs (18.21%) that were found positive for saline agglutination test were chosen for further evaluation of DAT by microtiter method.

Saline agglutination test

A drop of EDTA-anticoagulated whole blood was placed on clean grease free slide and equal quantity of normal saline (0.9% NaCl) was added to it and mixed. The slide was evaluated for the presence of macroscopic agglutination. A cover slip was placed on the slide and also observed for microscopic agglutination (Fig. 1).

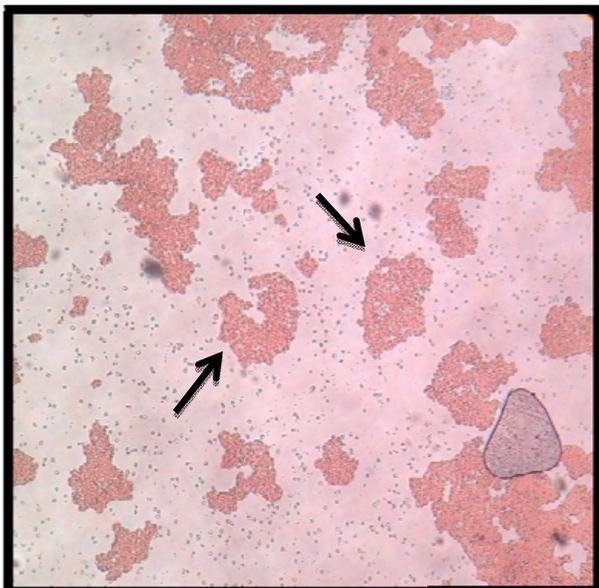


Fig. 1: Microscopic saline agglutination test in IMHA dog

DAT or Coombs' test protocol

Microtiter method described by Jacobs *et al.* 1998; and Overmann *et al.* 2007 was used in the study. In brief, two ml of EDTA-anticoagulated blood was collected from all forty seven dogs giving positive saline agglutination test. The blood was centrifuged at 2500 rpm for 5 minutes and 100µl of packed RBCs were transferred to 15ml centrifuge tube. The RBCs were washed using 4.9 ml of PBS for

100µl of RBC. The step was repeated three more times, finally the washed RBCs were diluted with PBS to prepare a 2% suspension. The DAT was performed in Tarsons 96 well, U-bottom microtiter plate and antiserum used was VMRD polyvalent Coombs' reagent, goat antiserum to canine IgG, IgM and C₃ obtained from Pullman, WA, USA. All the well in a row contained 50µl of PBS. About 50µl of antiserum was added to first well and serially diluted from 1:2 through 1:2048. (Manufacture guidelines instruct a tube method with dilution upto 1:8). The last well contained only PBS serving as negative control. 50µl of washed RBCs were added to each well and incubated at 37°C for 30 minutes. After incubation the plate was placed in room temperature for 30 minutes permitting RBCs to settle and to allow agglutination pattern to form.

The test was interpreted as positive if matte formation occurred and negative if RBCs formed button which would stream when the plate was tilted. The last well showing matte formation was recorded as the titer.

RESULTS AND DISCUSSION

Two hundred and fifty eight anaemic dogs showing signs such as pale/icteric mucous membrane were initially screened for Immune mediated haemolytic anaemia by saline agglutination test. Among them forty seven dogs were found positive for saline agglutination test. These dogs were chosen for further evaluation of DAT by microtiter method.

Out of the forty seven saline agglutination test positive dogs, twenty five dogs (53.20%) were positive for DAT at various titers, while twenty two (46.80%) were negative. Ten dogs (40%) were positive at a titer of 1:2, followed by 5 dogs (20%) at 1:4 titer. Four dogs (16%) were positive at 1:32 titer. At 1:8 and 1:64, two dogs (8%) were positive in each of these titer. While remaining 2 dogs one was positive at 1:16 (4%) and the other at 1:256 (4%).

Positive saline agglutination test indicates an immune response in IMHA, while Direct antiglobulin test detects the presence of antibodies on the surface of erythrocytes (Slappendal, 1979; Wardrop, 2005). Its clinical use was re-established by Caviezel *et al.* (2014). The sensitivity of DAT ranges from 37% to 89% (Slappendal, 1979; Wardrop, 2005; Overmann *et al.*, 2007). False negative results are common in DAT, which are mainly due

to improper washing of RBC, inadequate strength of antisera, excessive cold agglutinin, technical errors and prozone effect (Caviezel *et al.*, 2014). The tube method followed previously was a tedious process and prone to varying results (Overmann *et al.*, 2007). In the present study 96-well microtiter method was employed, which is often poorly described.

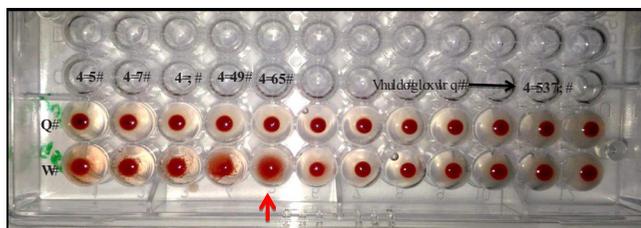


Fig. 2: Microtitre method of direct antiglobulin test or Coombs' test (N) Sample from healthy dog, (T) Sample of IMHA dog, note the first well showing button while Clear matte formation occurring at higher dilution 1:32 (Red arrow). Thus multiple dilution helping in overcoming prozone effect

In the present study the coombs' test was found to be positive at titers 1:2, 1:4, 1:8, 1:16, 1:32, 1:64 and 1:256. The serial dilution of the antisera beyond the manufacturer guidelines *i.e.* >1:8 dilutions up to 1:2048 was beneficial in detecting 8 additional (32%) positive cases. Similar results were obtained by previous study by Overmann *et al.* (2007) where microtiter method helped in detecting six more positive cases which were negative at lower dilutions. Prozone effect is where agglutination fails to occur at lower dilutions, because of excess antiglobulin compared to antigen resulting in decreased cross linking of erythrocytes and thus failure of agglutination. Higher dilutions reduce the level of antiglobulin and the level of antigen and antiglobulin becomes appropriate thus agglutination can be appreciated (Fig. 2). Thus Coombs' test performed by microtitre plate method helped in overcoming the prozone effect (Overmann *et al.*, 2007). Similar method of microtitre plate was described by Jacobs *et al.* (1984).

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CONCLUSION

The result of present study indicates that Microtiter plate method of DAT is more advantageous than tube method by detecting additional positive IMHA dogs.

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