

Immune Mediated Hemolytic Anemia Detection in Dogs

Maroudam Veerasami^{1a}, Chitra Karupannan^{1a}, Monisha Periyasamy¹, Saveedha Muralimanokaran¹,

Baranidaran Rengaswamy.G², Gopal Dhinakar Raj*

Scientist, TRPVB-TANUVAS, Chennai, India ^{1a}

Scholars, TRPVB-TANUVAS, Chennai, India¹

Professor, MVC, TANUVAS, Chennai, India²

a. Equal contribution to the work for first author

*Corresponding author

Director, TRPVB-TANUVAS, CAHS, Chennai, India*

ABSTRACT: Immune mediated haemolytic anemia (IMHA) is a common cause of anemia in dogs ,a condition in which the immune system destroys its own blood cells. IMHA detection by the conventional direct antiglobulin test (DAT) has been reported to be of very low sensitivity. In the present study a rapid and sensitive flowcytometric assay developed and compared with the conventional DAT and haematological parameters. IMHA suspected dog (n=56) packed cell volume and white blood cells were found to be significantly less than normal dog (n=25). The conventional coombs test which is of less sensitive than flow cytometry showing sensitivity of 16.07% where asflowcytometry with 96.42%. It is confirmed that flowcytometry analysis a more rapid and sensitive method as alternative to detect using low volume of cells than conventional technique.

KEYWORDS: Immune mediated haemolytic anemia, Flowcytometry Haematologicalparameters and direct antiglobulin test.

I. INTRODUCTION

Immune Mediated Haemolytic Anemia (IMHA) is a common cause of anemia in dogs and cats. IMHA is a disorder in which the RBCs get damaged in the blood vessel (22) and in tissues leading to intravascular and extravascular haemolysis [1, 2,21]. The RBCs were chronically stimulated by immunoglobulin (IgG and IgM) and complements binding on its membrane causing accelerated damage resulting in IMHA [3, 4,5,10]. IMHA can be classified as primary and secondary IMHA [5,19] Primary IMHA is a classic autoimmune disorder with no recognized underlying cause and is the most frequent form in the western countries but seems rare in India [6, 7].Secondary IMHA caused by antibody attacking membrane antigen is often witnessed in cases suffering from haemoprotozoan diseases such as *Ehrlichiosis*, *Babesiosis*, etc.[14,7,17] Direct Antiglobulin Test (DAT) using conventional tube technique/coombs test are the most commonly used methods and is still a gold standard test for the diagnosis of IMHA [8, 9]. Conventional coombs test has several disadvantages including false negative results, requirement of high quantity of antiserum, RBCs, time and high cost (6,11) and subjective interpretation (13). False negative results of coombs test has been attributed to the lower detecting capacity of bound IgG which responds as a minimum of 500 IgG molecules bound per RBC [10]. Due to its polyvalent character it lacks to identify the RBCs surface bound antibodies. In addition, the immunosuppressive medication will instantly alter DAT positive dogs to false-negative DAT outcome [3]. FC has the capability to measure simultaneously several criteria of single cell in composite mixture[12]. FC proved to be highly sensitive in detecting the RBC bound immunoglobulins for early detection of IMHA condition in various species like

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 8, Issue 1, January 2019

dog, cat and humans [2,11,20]. Membrane bound immunoglobulin on RBC is detected by fluorescein conjugated anti antibodies dog IgG and IgM and the quantity of bound fluorochromes is directly proportional to amount of membrane bound IgG and IgM. Present study was performed to optimize rapid FC assay for clinical diagnosis of IMHA in dogs with determined threshold level of MFI in comparison with conventional coombs test and hematological parameters.

II. MATERIALS AND METHODS

Study population and sample collection

Clinically IMHA suspected EDTA citrated blood samples (56) and apparently normal dogs (25) were collected from Department of Veterinary Clinical Medicine, Madras Veterinary College, and TANUVAS. The dogs with symptoms such as anemia with blanched mucous membranes, bloody urine and ecchymosis were clinically suspected for IMHA. The dogs came for vaccination were considered as apparently healthy dogs.

Heamotology parameters

Hematology parameters for the study population carried out as per standard protocol. Blood was collected from all the apparently healthy dogs (n=25) and clinically IMHA suspected dogs (n=56) for count of Heamoglobin (HB) content, Packed Cell Volume (PCV), White Blood Cells (WBCs) and Red Blood Cells (RBCs) .

Direct antiglobulin test (DAT)/coombs test

DAT was performed as described earlier by (18). Briefly 1ml of blood sample was centrifuged at 1008 RCF for 5 minutes for RBC separation. The 0.1ml RBC pellet was mixed with 4.9 ml of ice cold PBS and centrifuged at 1008 RCF for 5 minutes. The RBCs pellet was washed twice and 0.1 ml of 2% RBC suspension was prepared and allowed to incubate with the 0.1ml of Coombs reagents at three dilutions neat/2, neat/3, and neat/4 along with negative reaction with PBS control for 30 minutes at 37°C, followed by centrifugation for 1 minute. Then the mixtures was added to the 96 well titre plate and incubated at 4 °C to visualize the agglutination or can be viewed directly under microscope at 100x magnification to declare as positive.

Optimization of fluorochromes

RBC cell were separated from the EDTA citrated whole blood and washed twice with PBS, then the packed RBC was diluted 1:40(v/v) with RPMI 1640 with 2% FBS. The fluorochromes and RBC titration were carried out using the RBCs separated from blood sample of clinically proven IMHA dog and apparently healthy dog. The FITC conjugated sheep anti-dog IgG heavy chain and FITC conjugated goat anti-dog IgM were tested at 1 in 5, 1 in 10, 1 in 20 and 1 in 40 dilutions against 1, 2, 3 and 4 millions of RBCs. The fluorochromes at different dilutions were added to different counted RBC and incubated for 4 hours at 4°C. To take out the unbound immunoglobulins the cells were washed twice with PBS for 324 RCF 5 minutes. Washed stained RBCs were diluted 500ul of 1X sheath buffer and analyzed in flow cytometry.

III. EXPERIMENTAL RESULTS AND DISCUSSION

Flow cytometry (FC) analysis on study samples

The optimized fluoro-chromes dilutions and RBCs counts were used for routine fluoro-chromes staining of study blood samples. The fluoro-chrome stained RBCs were acquired using MoFlow XDP flow cytometry (Beckman Coulter, USA) and data were analyzed using the summit software. A total of 30,000 events were recorded for all clinical samples as unstained, anti-dog IgG heavy chain FITC stained and goat anti-dog IgM FITC stained. The apparent healthy dogs Mean +3SD of Mean fluorescent intensity (MFI) in unstained, anti-dog IgG heavy chain FITC stained and goat anti-dog IgM FITC stained were determined and used as cut-off/threshold for clinical diagnosis.

Clinical investigation and hematological parameters

Based on the clinical signs of the suspected dogs (n=56); 48 % of the animals were shown any one of the clinical signs such as icterus, ecchymosis, and epistaxis (Fig 1) and 51% of animals were confirmed anemia and suspected for

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 8, Issue 1, January 2019

Ehrlichiosis and *Babesiosis* by haematology and 1 % animals shown anemia without any obvious clinical signs. The hematology reports of apparently healthy dogs in comparison with clinically suspected dogs in mean \pm standard deviation showed significant reduction in the following parameters such as Hemoglobin (HB) content, Packed Cell Volume (PCV), White Blood Cells (WBCs) and Red Blood Cells (RBCs).

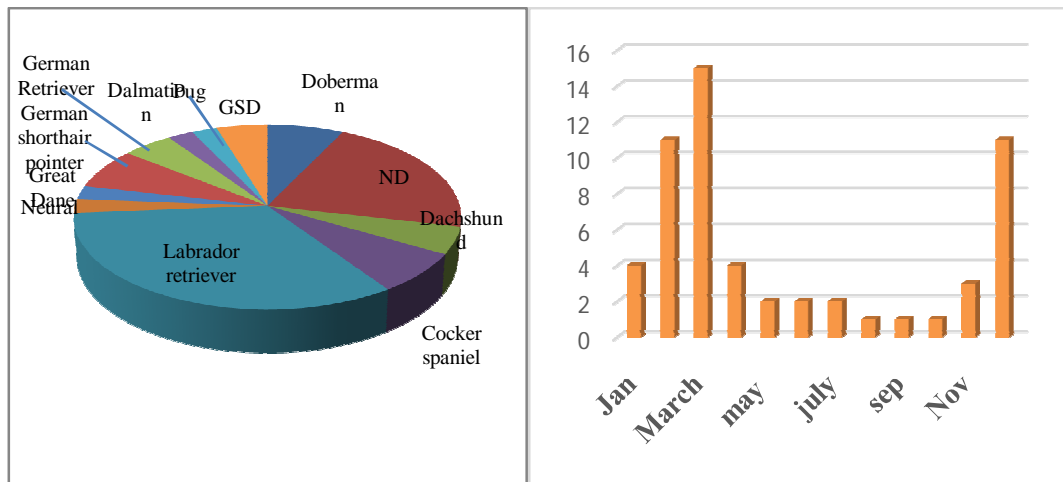


Fig 1:Prevalence of breed and seasonal variation

Flow cytometry optimization:

The gradient shift of MFI during various dilution of anti-dog IgG/IgM heavy chain FITC stained 2 million RBC count of clinically positive dog was observed. The titration of fluoro-chromes and RBC count indicated that 40 μ l and 20 μ l of fluoro-chromes corresponding to 1 million RBCs count showed maximum mean fluorescent intensity in clinically known positive dog without any background fluorescent signal in the clinically known negative dog. The apparent healthy dogs (n=25) Mean +3SD threshold MFI in unstained, anti-dog IgG heavy chain FITC stained and goat anti-dog IgM FITC stained were obtained as 3.94, 5.8 and 5.7 respectively (Fig 2). The achieved threshold MFI of 6 MFI was applied for the clinical diagnosis of clinically suspected dogs (n=56) (Table 1).

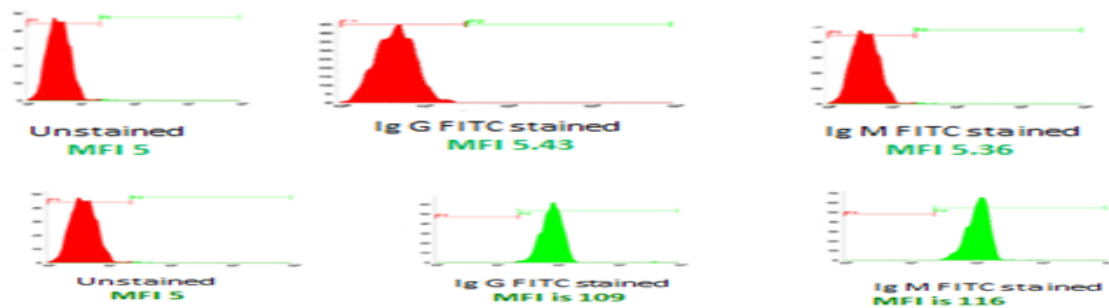


Fig 2: Mean fluorescent intensity of Normal and suspected dogs.

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 8, Issue 1, January 2019

MFI of normal unsuspected dogs				MFI of IMHA Suspected dogs			
ID	Unstained	IgG bound RBC	IgM bound RBC	ID	Unstained	IgG bound RBC	IgM bound RBC
N1	3.81	4.47	4.47	S1	4.19	10.06	18.65
N2	2.33	2.15	2.23	S2	4.23	5.83	39.14
N3	1.8	2.1	2.37	S3	4.56	66.73	15.4
N4	2.31	2.35	2.37	S4	4.21	93.3	96.09
N5	2.26	2.54	2.54	S5	5	109	116
N6	2.17	2.19	2.45	S6	5	103	140
N7	2.14	2.87	2.81	S7	1.5	3.81	13.92
N8	2.12	2.2	2.44	S8	3.2	14.35	22.87
N9	3.07	2.67	2.21	S9	2.92	13.54	34.53
N10	2.05	2.45	2.65	S10	4	36	22.7
N11	2.05	2.45	2.26	S11	1.68	6.88	19.12
N12	2.33	2.65	2.4	S12	4.99	6.6	17.18
N13	2	1.67	1.98	S13	4.67	27.71	20.82
N14	3.2	4.35	2.87	S14	4.5	17.76	12.84
N15	1.58	1.75	2.34	S15	4.5	30.42	35.09
N16	1.66	1.87	1.91	S16	1.72	37.21	29.74
N17	1.69	3.52	4.11	S17	1.31	27.43	5.56
N18	1.58	3.53	3.71	S18	1.52	30.55	11.49

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 8, Issue 1, January 2019

N19	1.59	3.4	4.27	S19	1.64	51.72	10.21
N20	1	4.41	5.53	S20	1.54	29.97	21.57
N21	2.26	3.19	1.98	S21	2.08	4.73	4.28
N22	1.45	3.72	4.08	S22	3.12	30.9	23.85
N23	2.24	1.59	4.04	S23	3.14	52	143.04
N24	1.54	3.59	2.58	S24	1.96	4.33	4.84
N25	1.51	2.4	4.7	S25	1.48	28.55	28.45
				S26	3	54.72	30.52
				S27	3	7.5	30.51
				S28	2.71	26.28	16.37
				S29	2.5	697.78	99.77
				S30	1.86	13.52	12.59
				S31	2.48	8.4	7.88

Table 1: Mean fluorescent intensity of normal and suspected dogs.

Flow cytometry and coombs test on study samples

The Figure 2a and 2b shows the flowcytometry histogram of clinically suspected dogs showing shift of 109 MFI for anti-dog IgG stained RBCs and 116 MFI for anti-dog IgM stained RBCs whereas normal dog showing shift of 5.43 MFI for anti-dog IgG stained RBCs and 5.36 MFI for anti-dog IgM stained RBCs. It indicates that achieved threshold MFI and optimized parameters were optimal for IMHA clinical diagnosis. Further testing indicates that suspected dogs 54 out of 56 were positive for IMHA by Flowcytometry with diagnostic sensitivity of 96.42 %. Whereas only 9 out 56 positive for IMHA by coombs test with diagnostic sensitivity 16.07%.

IV. DISCUSSION

Rapid flow cytometry method for detection of RBCs bound immunoglobins IgG and IgM in dogs for the clinical diagnosis of IMHA. The destruction of RBC by auto antibodies is the main feature of IMHA (3). The basal level of MFI elicited by fluoro-chromes used in the study upon reacting to the normal unbound RBCs from apparently healthy dogs were achieved as cut-off MFI. The optimal amount of fluoro-chromes to optimal RBC count was achieved by checker board titration of commercially procured fluoro-chromes and RBCs. Nonspecific binding antibodies as they were from different sources that the precise antibodies or the proportion of cells bound with IgM was less than IgG. The

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 8, Issue 1, January 2019

major isotype in the dogs was IgG and is recorded most normally in dogs with IMHA. In cases of canine autoimmune hemolytic anemia sequence of IgG and IgM antibodies have been diagnosed.(15)

About 25 apparently healthy dogs come for the vaccination to clinics were bled and tested for coombs test negative were used for threshold level MFI determination in flow cytometry assay. The threshold MFI obtained is 6 was used for the clinical diagnosis of suspected IMHA samples.

The known positive dog for IMHA was bled and antibody titration and RBC count titration were done for flow cytometry and Coombs test. Formerly flow cytometry has been used to identify anti-RBC antibodies in dogs with IMHA. The accord from these study is that flow cytometry is more precise than DAT for detection of RBC antibodies, and specificity as the similar to DAT (16). The flow cytometry showed good linear correlation between amount of bound RBCs and the mean fluorescence intensity. The fluochrome antibody dilutions 1 in 40 and 1 in 20 is optimal compared to 1 in 10 and 1 in 5 and RBC cell count of 1M is optimal than 2M, 3M. Among clinically suspected dogs (n=56) tested by flow cytometry and coombs tests in indicates the flow cytometry is high sensitivity of 96.42% than coombs test 16.07%. The present study results showed that coombs test is lesser sensitivity than FC whereas FC detects them at greater speed with lesser volume of RBCs.

V. CONCLUSION

The present study result show that coombs test is lesser sensitivity than Flowcytometry, whereas flowcytometry detects them at greater speed with lesser volume of RBC. The suspected Dog 54 out of 56 were positive for IMHA by flowcytometry with diagnostic sensitivity of 96.42% where as only 9 out of 56 positive for IMHA by coombs test with diagnostic sensitivity 16.07%.

VI. ACKNOWLEDGEMENT

We thank Director of Clinics, TANUVAS for providing necessary permission for blood samples collection. We Acknowledge TANUVAS for providing facility and Department of Biotechnology, Government of India for funding this work through grant number 102/IFD/DBT/SAN 2681/2011-2012 dated 29.09.2011. The author (s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

- [1] M. Bohm, S. Basson, "Erosive rhinitis resembling granulomatosis with polyangiitis (Wegener's granulomatosis) in an Anatolian shepherd dog", *Journal of the South African Veterinary Association*, 86(1), pp. 2-7, 2015.
- [2] E. Carli, S. Tasca, M. Trotta, T. Furlanello, M. Caldin, L. Salano-Gallego, "Detection of erythrocyte binding IgM and IgG by flow cytometry in sick dogs with Babesia canis infection", *Veterinary Parasitology*, 162, pp. 51-57, 2009.
- [3] L.L. Caviezel, K. Raj, U. Giger, "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*, 28, pp. 583-591, 2014.
- [4] R.K. Chaudhary, S.S. Das, R. Gupta, D. Khetan, "Application of flow cytometry in detection of red cell bound IgG in Coombs negative AIHA", *Hematology*, 11, pp. 295-300, 2006.
- [5] D. Duval, U. Giger, "Vaccine-associated immune-mediated haemolytic anemia in the dog", *J Vet Intern Med*, 10, pp. 290-295, 1996.
- [6] U. Giger, "Idiopathic immune-mediated hemolytic anemia in dogs: 42 cases (1986-1990)", *Journal of the American Veterinary Medical Association*, 202, pp. 783-788, 1993.
- [7] R. Goggs, S.G. Dennis, A.D. Bella, K.R. Humm, G. McLauchlan, C. Mooney, A. Ridyard, S. Tappin, D. Walker, S. Warman, N.T. Whitley, D.C. Brodbelt, D.L. Chan, "Predicting Outcome in dogs with Primary Immune-Mediated Hemolytic Anemia: Results of a Multicenter Case Registry", *J Vet Intern Med*, 29, pp. 1603-1610, 2015.
- [8] J.L. Haynes, "Principles of flow cytometry", *Cytometry Suppl*, 3, pp. 7-17, 1988.
- [9] M.L. Jackson, S.A. Kruth, "Immune-mediated haemolytic anemia and thrombocytopenia in the dog: retrospective study of 55 cases diagnosed from 1969 through 1983 at the Western College of Veterinary Medicine", *Can Vet J*, 26, pp. 245-250, 1985.
- [10] M.T. Jacqueline, "The role of flow cytometry in companion animal diagnostic medicine", *The Veterinary Journal*, 170, pp. 278-288, 2004.
- [11] U. Jefferya, K. Kimuraa, R. Grayc, P. Luetha, B. Bellairea, D. LeVine, "Dogs cast NETs too: Canine neutrophil extracellular traps in health and immune-mediated hemolytic anemia", *Veterinary Immunology and Immunopathology*, 168, pp. 262-268, 2015.
- [12] D.R.E. Jones, T.J. Gruffydd-Jones, C.R. Stokes, F.J. Bourne, "Investigation of factors influencing performance of the canine antiglobulin test", *Res Vet Sci*, 48, pp. 53-58, 1990.
- [13] T.J. McAlees, "Immune-mediated haemolytic anemia in 110 dogs in Victoria, Australia", *Aust. Vet. J.*, 88(1-2), pp. 25-8, 2010.

International Journal of Innovative Research in Science, Engineering and Technology

(A High Impact Factor, Monthly, Peer Reviewed Journal)

Visit: www.ijirset.com

Vol. 8, Issue 1, January 2019

- [14] J.W. Melinda, D. Elizabeth, S. Wilma, H. Kenneth, C. Judy, R. Bonnie, "Isotype-Specific Antibodies in Horses and Dogs with Immune-Mediated Hemolytic Anemia", *J Vet Intern Med*, 14, pp. 190–196, 2000.
- [15] P. Morley, M. Mathes, A. Guth, S. Dow, "Anti-Erythrocyte Antibodies and Disease Associations in Anemic and Nonanemic Dogs", *J Vet Intern Med*, 22, pp. 886–892, 2008.
- [16] C.J. Pitek, "Prognosis in canine idiopathic immune-mediated haemolytic anemia Christine", *Veterinary Sciences Tomorrow*, pp. 1-8, 2012.
- [17] K.A. Quigley, B.J. Chelack, D.M. Haines, M.L. Jackson, "Application of a direct FC erythrocyte immunofluorescence assay in dogs with immune-mediated haemolytic anemia and comparison to the direct antiglobulin test", *J Vet Diag Invest*, 13(4), pp. 297-300, 2001.
- [18] A. Quinquenel, C. Al Nawakil, F. Baran-Marszak, V. Eclache, R. Letestu, M. Khalloufi, M Boubaya, C. Roy, N. Varin-Blank, A. Delmer, V. Levy, F. Ajchenbaum-Cymbalista, "Old data and new data: positive direct antiglobulin test identifies a subgroup with poor outcome among chronic lymphocytic leukemia stage a patients", *EPUB*, 95(1), pp. 296-305, 2015.
- [19] D.J. Recktenwald, "Introduction to flow cytometry: principles, fluorochromes, instrument set up calibration", *J Hematother*, 2, pp. 387–394, 1993.
- [20] J.W. Swann, B.J. Skelly, "Systematic Review of Prognostic Factors for Mortality in Dogs with Immune-mediated Hemolytic Anemia", *J Vet Intern Med*, 29, pp. 7–13, 2015.
- [21] J.W. Swann, B. Szladovits, B. Glanemann, "Demographic Characteristics, Survival and Prognostic Factors for Mortality in Cats with Primary Immune-Mediated Hemolytic Anemia", *J Vet Intern Med*, 30, pp. 147–156, 2016.
- [22] M. Yuki, "A case of non-regenerative immune-mediated anemia treated by combination therapy of human immune globulin and mycophenolatemofetil in a dog", *Open Veterinary Journal*, 1, pp. 46-49, 2011.