*Original Research*

# Incidence of Idiopathic Immune Mediated Hemolytic Anaemia of Dogs in Chennai*

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**Abstract**

*Idiopathic immune-mediated hemolytic anemia (IMHA) is the most common autoimmune disease in dogs. The aim of the study was to study the incidence and clinicopathological changes of idiopathic IMHA, which is not having any secondary underlying triggering causes. The suspected anaemic dogs brought with clinical signs such as pale or icteric mucous membranes were screened for IMHA by saline agglutination and spherocyte count and confirmed by flow cytometry. The positive cases were then subjected to PCR for ruling out secondary underlying causes like Babesia spp and Ehrlichia canis (secondary IMHA). Of the 75 suspected anemic dogs, 32 cases were confirmed by flow cytometry. Among thirty two cases, thirteen were primary (Idiopathic) IMHA (17.3 %) and remaining nineteen cases were diagnosed as secondary IMHA. The most common clinicopathological findings were anaemia with PCV less than 15 per cent with regenerative changes, leucocytosis, thrombocytopenia, elevated PT and APTT, elevated BUN, creatinine, ALT, ALP, hypoalbuminemia and hyperbilirubinaemia.*

**Key words:** Autoagglutination, Dog, Idiopathic IMHA, Flow Cytometer, Spherocytes, PCR

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## Introduction

IMHA is one of the common types of anemia in small animals and considered to be the most common autoimmune disease in dogs. The disease is caused by immune-mediated destruction following activation of complement and/or extra vascular haemolysis of red blood cells (RBCs) and results in an accelerated decrease in the total RBC mass (Mitchell and Kruth, 2010). IMHA are of two main type *viz.*, primary and

secondary, based on the presence of underlying disease. Primary (idiopathic) IMHA is a classic example of an autoimmune disorder with no identifiable underlying cause and is the predominant form of IMHA. Normally, auto antibodies are prevented from reacting with host tissues by suppressor T cells. It is believed, however, that IMHA-affected animals have poorly regulated suppressor T-cell function or over stimulated immune systems that allow auto antibodies to attach to normal cells and trigger RBC destruction. Secondary IMHA is caused by an immunologic response to non-self-antigens that have modified or are associated with normal RBC membranes. Secondary IMHA can be caused by a number of underlying processes. Affected RBCs may become infected by pathogens or coated with foreign antigen. Documented or hypothesized causes of secondary IMHA include bacterial, viral, rickettsial, parasitic, protozoan, and neoplastic disorders (Giger, 2005). Clinical features of the disease are the result of a spontaneous destruction of erythrocyte leading to intravascular or extravascular hemolysis. These processes result in anemia and in some cases, prehepatic icterus due to accumulation of unconjugated bilirubin (Balch and Mackin, 2007). IMHA is considered to have a poor prognosis in dogs, with mortality rates of rates of 30%–40%, presumably reflecting improvements in awareness of disease, speed of diagnosis, and availability of supportive care and blood products (Goggs *et al*., 2015). The diagnosis of IMHA is based on the presence of anemia and a combination of clinical pathology findings which can include the presence of spherocytes (Weinkle *et al*., 2005), auto agglutination of erythrocytes resulting from anti-erythrocyte IgG and IgM and/or a positive Coomb's test which detects antibodies or complement on the erythrocyte surface. Flow cytometry for the detection of IgG on RBC has been proven to be highly sensitive and specific for the diagnosis of IMHA (Morley *et al*., 2008). Hence, the present study was undertaken with the objective to study the incidence of Idiopathic Immune mediated hemolytic anaemia of dogs in Chennai.

**Selection of Animals and Sampling**

Seventy five suspected anemic dogs with packed cell volume (PCV) <30% that were referred to the Small Animals Out Patient Unit of Madras Veterinary teaching Hospital and Critical Care Unit of the Department of Veterinary Clinical Medicine, Madras Veterinary College, Chennai during a period of 1 year from July 2016 to June 2017. An individual history of those dogs with pale or icteric mucous membranes were screened for IMHA by saline agglutination test, sphereocyte count and flow cytometry. Ten apparently healthy dogs brought for routine health checkup formed the source of control group and were subjected to a detailed clinical, haematological and various diagnostic tests to evaluate the health status of dogs.

Blood samples were initially evaluated for autoagglutination and spherocytosis, complete blood count and serum biochemistry was taken. Two ml of whole blood was collected in a dry vial containing 10 per cent Ethylene Diamine Tetra Acetic Acid (EDTA) for complete blood count. About 5.0 ml of blood was collected in a vacutainer without anticoagulant and left undisturbed in slanting position until complete





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clotting occurred and serum was then separated by centrifugation used for biochemistry. Thirty-two dogs positive by flow cytometry were selected for the study. The saline agglutination test was performed by mixing a drop of whole blood collected in EDTA vacutainer with drop of saline on a glass slide. Microscopic agglutination test was performed with a saline dilution on a glass slide (one drop of blood to two drops of saline) and inspected under light microscope. The positive result was manifested by clumping of red blood cells (Day, 2008).

An air dried thin blood smear was made from capillary blood obtained from the anterior edge of the hairless ventral surface of the ear, stained with Leishman-Giemsa stain and examined microscopically for *Babesia* species and *Ehrlichia canis* organism, differential leucocyte count, spherocyte count and blood picture analysis.

**Flow Cytometer**

One milliliter of whole blood collected aseptically from each dog by venepuncture of cephalic, saphenous or jugular vein in vacutainers coated with 10% Ethylene Diamine Tetra Acetic Acid (EDTA) as anticoagulant. Samples were incubated at $4^0$ C for 48 hr or less before flowcytometric analysis. 1 ml RBC from each sample were washed twice in 0.9% saline at 37 C and diluted the cells to 2% ice chilled in isotonic phosphate-buffered saline (PBS), pH 7.4, molarity 0.01. Fifty microliters of washed 1% red cells were incubated in the dark at 4 C for 45 min with a 1:30 dilution (PBS diluent) of fluorescein isothiocyanate (FITC)-labeled sheep anti-dog IgGd (heavy chain specific), FITC-labeled goat anti-dog IgMd (m chain specific). Cells were washed twice and resuspended in 200 ml of isotonic PBS with 2% heat treated fetal bovine serum and 100 ml of 10% buffered formalin. Then incubated RBCs were washed twice with ice chilled phosphate buffered saline for 5 minutes, 1700 rpm to remove the unbound flurochormes. The washed stained RBCs were diluted 500ul of 1X sheath buffer and analyzed in flow cytometry (Kucinskiene *et al*., 2005). The IMHA flurochrome stained RBCs were acquired using MoFlow XDP flow cytometery (Beckman Coulter, USA) and data were analyzed using the summit software.

**Multiplex PCR**

DNA isolation kit (QIAamp DNA Mini Kit®, Qiagen) was used for the extraction of parasite DNA from 200µl of blood collected in EDTA vacutainers according to the manufacturer's instructions. Genomic DNA isolated from the whole blood of healthy dog was used as a negative control. Multiplex PCR for the amplification of the 16s rRNA gene fragment of molecular length 619 bp in genus *Babesia* and VirB9 of *E. canis* with a molecular length 380 bp was employed following the procedure of Kledmanee *et al. (*2009). Thermocycling consisted of initial denaturation step of 15 min at 94°C followed by 30 cycles of 45 sec at 94°C, 45 sec at 65°C, and 90 sec at 72°C with a final extension step of 10 min at 72°C. The amplicons were





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separated by electro-phoresis in 1.5% agarose gel in 40 mM Tris-acetic acetate of pH 8.4, 1 mM EDTA, stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

**Microscopic Agglutination Test (MAT)**

A battery of live leptospira serovars (*L. australis, L. autumnalis, L. ballum, L. bataviae, L. canicola, L. grippotyphosa, L. hebdomadis, L. icterohaemorrhagiae, L. javanica, L. pomona and L. pyrogenes*) were employed. The antigen antibody reaction / agglutination observed at > 1: 200 serum dilutions were considered positive. Positive samples for hemoprotozoan parasite like *Babesia spp* and *Ehrlichia canis* screened by multiplex PCR and leptospirosis by MAT were excluded from study. The results are expressed as mean ± SE. Data are classified with descriptive statistics and P values <0.05 are considered statistically significant. Data analysis was performed with the SPSS 20.

**Results**

Out of the 75 suspected anemic dogs, flow cytometric analysis results were positive for IMHA in 32 cases. Out of thirty two dogs, thirteen (40.63 per cent) dogs were of primary (idiopathic) IMHA, nineteen (59.37 per cent) dogs were secondary IMHA due to underlying causes like Babesiosis (13), Ehrlichiosis (3) and Leptospirosis (3). Positive samples for hemoprotozoan parasite like *Babesia spp* and *Ehrlichia canis* screened by multiplex PCR and Leptospirosis by MAT were excluded from study.

Incidence of Idiopathic IMHA was more common in 2-8 years (30.76 per cent) when compared to 1-2 years (23.07 per cent) and above 8 years (15.38 per cent) age groups and more common in female (61.5 per cent) when compared to male dogs (38.5 per cent). Breed wise incidence was highest in Labrador retriever (30.75 per cent) followed by Cocker Spaniel, Terrier and Spitz with 15.38 per cent each. Other breeds with 7.69 per cent occurrence were recorded in Golden Retriever, Shih Tzu and Pomeranian. Clinical signs in primary IMHA dogs were anorexia (92.3 per cent), lethargy (84.6 per cent), vomiting (38.4 per cent), pyrexia (53.8 per cent), dehydration (84.6 per cent), tachypnoea (69.2 per cent), tachycardia (53.8 per cent), pale mucosa (46.1 per cent), icteric (53.2 per cent), hemoglobinuria(38.1 per cent), epistaxis (7.6 per cent) and ecchymosis (15.3 per cent). The hematological, coagulation profile and biochemical findings of dogs with idiopathic IMHA are presented in Table 1.

In idiopathic IMHA (n=13), spherocytosis was observed in four cases (30.78 per cent) and saline agglutination test was positive in six cases (46 per cent). Apart from spherocytes, anemic changes like poikilocytosis in three cases (23.1 per cent), anisocytosis in six cases (46.1 per cent) and hypochromasia in six cases (46. 1 per cent) were also seen in primary IMHA. In the present study, thirteen cases out of seventy five suspected anemic cases (17.33 per cent) were diagnosed by flow cytometric analysis. Flow cytometry,





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RBC of seven dogs (53.8 per cent) were bound with both IgG and IgM, four dogs (30.8 per cent) RBC bound with IgM only and two dogs RBC (15.4 per cent) were only binding with IgG.

**Table 1:** CBC, coagulation profile and serum biochemistry of Idiopathic IMHA

| Parameter | Primary IMHA (n=13) | Control (n=10) | F-value |
|---|---|---|---|
| Hb (g/dl) | 4.53±0.33[b] | 12.61±0.46[a] | 108.94** |
| RBC(mill/uL) | 2.37±0.29[b] | 6.01±0.14[a] | 84.30** |
| PCV (%) | 13.22±1.07[b] | 36.15± 1.14[a] | 58.62** |
| WBC (/ ul) | 25569.23±2742.42[b] | 10709.90±783.79[a] | 10.54** |
| Neutrophil (%) | 80.69±1.69[b] | 74.20±0.29[a] | 5.22* |
| Platelets (/cmm) | 74897.54±2939.13[b] | 228300.10± 22668.65[a] | 2.41** |
| PT (sec) | 33.80±8.69[b] | 11.50±0.92[a] | 4.09* |
| APTT (sec) | 63.63±14.93[b] | 30.80±1.51[a] | 2.87* |
| BUN (mg/dl) | 53.24±2.32[b] | 24.29±1.33[a] | 31.58** |
| Creatinine (mg/dl) | 1.33±0.12[b] | 0.5±0.09[a] | 9.77** |
| Total Protein (g/dl) | 6.23±0.28 | 6.90±0.09 | 1.68[NS] |
| Albumin (g/dl) | 2.46±0.10[b] | 3.43±0.05[a] | 43.64** |
| ALT (IU//l) | 113.64±3.16[b] | 59.67±4.03[a] | 49.67** |
| ALP (IU//l) | 541.00±115.04[b] | 117.00±14.35[a] | 6.26** |
| Total Bilirubin (IU/L) | 1.33±0.24[b] | 0.53±0.07[a] | 3.96* |
| Direct Bilirubin (IU/L) | 1.14±0.23 | 0.49±0.07 | 3.22[NS] |

*Mean bearing same manuscript in the row do not differ significantly; **Highly significant (P≤0.01); *Significant (P>0.05) [NS] – Non significant; Hb Hemoglobin, RBC Red blood cell, WBC white blood cell, PT prothrombin time, APTT activated partial thromboplastin time, BUN Blood urea nitrogen, ALT alanine aminotransferase, ALP alkaline phosphatase*

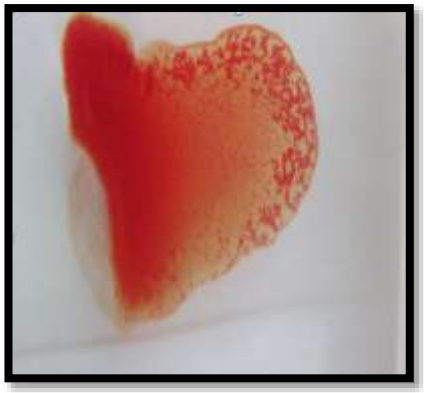

**Fig.1:** Positive saline agglutination test

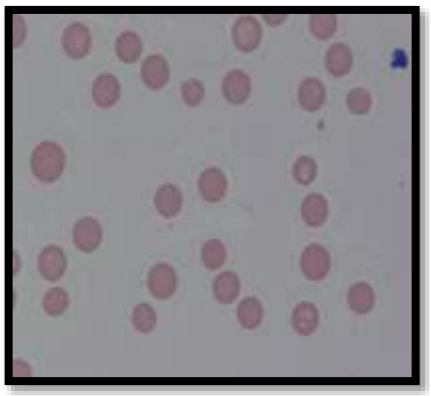

**Fig. 2:** Spherocytes





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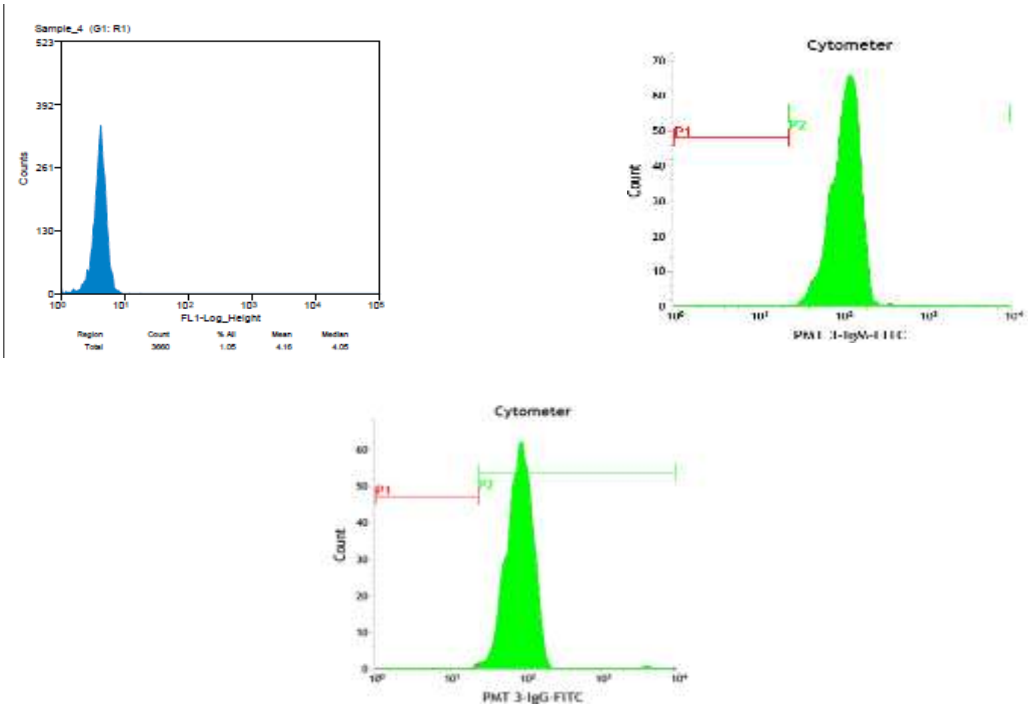


**Fig. 3:** The Flow cytometer histogram of positive IMHA dogs using optimized Flow Cytometeric assay indicating the achieved threshold Mean fluorescence intensity (MFI)

**Discussion**

McCullough (2003) reported that the incidence of primary IMHA was about 60 to 75 per cent. In the present study incidence of primary IMHA was comparatively lower than secondary IMHA which might be due to the endemic nature of the haemoprotozaon parasites. Highest incidence of idiopathic IMHA was observed in middle aged, female dogs (Archer and Mackin, 2013). The highest incidence was found in Labrador breeds which might be due to over representation of these breeds in the population.

In present study, nonspecific clinical signs recorded in primary IMHA dogs were anorexia, lethargy, vomiting, pyrexia, dehydration and specific signs caused by severe anemia were tachypnoea, tachycardia, pale mucosa, icteric, hemoglobinuria and ecchymosis (McCullough, 2003). A rise in the total plasma bilirubin concentration imparts icterus to tissues greater than 2 mg/dl (Meyer and Harvey, 2004). Dogs with primary IMHA showed significant reduction in PCV, Hb and RBC values when compared to control dogs indicating a state of severe anaemia (Day 2010). In the present study leukocytosis with a neutrophilic left shift (leukemoid response) might be due to tissue necrosis as a consequence of anaemic hypoxia and thromboembolic condition or might be due to inflammation, stress response or sepsis (Chen *et al.*, 2009).

In the present study, increased blood urea nitrogen (BUN) and serum creatinine (Cr) values were observed. Archer and Mackin (2013) reported that azotemia in IMHA might be due to haemoglobin induced renal





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damage or pre renal due to dehydration. Hypoalbuminemia and hypoproteinemia was recorded in the present study which was attributed to decreased synthesis by liver or loss through haemorrhage, as most IMHA dogs have thrombocytopenia and are hypercoagulable (Ishihara *et al.*, 2010). The ALP and ALT values were significantly high indicates a liver dysfunction due to hypoxia resulting from severe anaemia (Archer and Mackin, 2013). Balch and Mackin (2007) stated that an elevated bilirubin level was common in patients with IMHA and could be result from either hemolytic or hepatobiliary. Acute hemolysis caused prehepatic accumulation of bilirubin, resulting from excessive tissue breakdown of RBC hemoglobin.

The present study revealed moderate to severe thrombocytopenia, increase in PT and APTT in primary IMHA. Piek *et al*. (2008) stated that hypercoagulable state and thrombocytopenia suggest the presence of diffuse intravascular coagulation in many dogs, which was supported by finding of low fibrinogen concentration in dogs and increase of D-dimer and decrease anti thrombin activity. In the present study, spherocytosis was observed in 30.78 per cent and saline agglutination test was positive in 46 per cent. Similar findings of spherocytosis and autoagglutination were reported by Weinkle *et al*. (2005). Giger (2005) stated that diagnosis of IMHA requires one or more of the following three hallmarks presented to reach a definitive diagnosis of IMHA which include marked spherocytosis, true autoagglutination and positive direct Coombs's test, especially, presence of marked spherocytosis and true autoagglutination along with thrombocytopenia in the dog with anemia were virtually pathognomonic of IMHA. Patients with IMHA commonly have regenerative anemia characterized by reticulocytosis, polychromasia and anisocytosis. IMHA may also be non regenerative if antibodies or complement is directed against erythrocyte precursors in the bone marrow. Thus, the absence of reticulocytosis should not be ruled out as a diagnosis for IMHA (Giger, 2000).

Kucinskiene *et al.* (2005) reported that it was more rapid, cost-effective, sensitive, objective method to determine erythrocytes-bound immunoglobulins when compared with other assays as direct antiglobulin test. Specificity and sensitivity of direct Coombs' test compared with that of flow cytometry was reported to be 100 versus 100% and 53 versus 92%, respectively. Morley *et al*. (2008) reported that flow cytometry for the detection of IgG on RBC was highly sensitive and specific for the diagnosis of IMHA.

**Conclusion**

Idiopathic IMHA is the most common autoimmune disease in dogs. Highest incidence was observed in females, Labrador dogs with age group of 2-8 years. The clinicopathological changes specific for IMHA are due to the rapid breakdown of the RBCs. Flow cytometer plays a vital role in diagnosis of IMHA. Spherocytes and saline agglutination is hallmark of IMHA which is present in 30- 40 per cent of IMHA dogs.





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**Conflicts of Interest**

The authors declare that they have no competing interests.

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