Secondary Immune Mediated Hemolytic Anaemia in Dogs in Chennai, Tamil Nadu

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ABSTRACT

Immune-mediated hemolytic anemia (IMHA) is the most common autoimmune disease in dogs. This study was conducted to study the incidence and clinicopathological changes of secondary IMHA, which is having any secondary underlying triggering causes. The 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 further subjected to haematology, biochemistry, coagulation profile, MAT and polymerase chain reaction (PCR) for the diagnosis of underlying secondary causes like *Babesia spp*, *Ehrlichia canis* and *Leptospira* spp. (secondary IMHA). Thirty two cases were positive for IMHA, out of which thirteen cases were primary IMHA (17.3 %) and remaining nineteen cases were secondary IMHA (82.7 %) due to underlying causes such as *Babesia gibsoni* (13), *Ehrlichia canis* (3) and *Leptospira* spp. (3) respectively. Highest incidence was observed in Labrador dogs with age group of 2-8 years in male dogs. The most common clinicopathological findings were anaemia with reduced Hb, PCV and RBC, leucocytosis, neutrophilia, monocytosis, thrombocytopenia, elevated PT, BUN, Creatinine, ALT, ALP, hypoalbuminemia and hyperbilirubinaemia.

Keywords: Dog, Secondary IMHA, Flow cytometer, PCR

Immune-mediated haemolytic anaemia (IMHA) is a common haematological disorder in dogs (Fenty *et al.,* 2011) with major therapeutic challenges related to complications and high mortality rates (Swann and Skelly, 2013). Idiopathic/ primary IMHA is immune mediated destruction of RBCs resulting in loss of self tolerance against endogenous (self) antigens and not associated with any cause, where if the immune reaction is associated with an exciting cause / foreign (non self) antigen such as infectious disease, chronic inflammation, neoplasia,

toxins, parasites, drugs, vaccines, the condition was termed as secondary IMHA (Balch and Mackin, 2007; Jutkowitz *et al.,* 2013).

Balch and Mackin (2007) reported that physical examination typically revealed pale mucous membranes, tachypnea, splenomegaly, hepatomegaly, icterus, pigmenturia (hemoglobinuria or bilirubinuria), fever, and lymphadenopathy and Jaundice were most easily observed abnormalities in IMHA. Authors also reported tachycardia,

S3 gallop and grade II or III of VI systolic murmur were common in anemic patients.

Lobetti (2012) stated that diagnosis of IMHA requires one or more of the following three hallmarks had to be presented to reach a definitive diagnosis of IMHA. It was included that marked spherocytosis, true autoagglutination and positive direct coombs' test. Especially, presence of marked spherocytosis and true autoagglutination along with thrombocytopenia in the dog with anemia were virtually pathognomic of IMHA. Flow cytometry for the detection of IgG on RBC has been proved 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 secondary immune mediated hemolytic anaemia of dogs in Chennai.

MATERIALS AND METHODS

Selection of animals and sampling

Seventy five anemic dogs were presented 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, spherocyte count and confirmed by 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. 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 (Balch and Mackin, 2007).

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 °C for 48 hr or less before flow cytometric 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 500µl 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 genus *Babesia* and VirB9 of *E. canis*

was employed following the procedure of Kledmanee *et al.* (2009).

Table 1: Multiplex PCR for the amplification of the 16s rRNA gene fragment of genus *Babesia* and VirB9 of *E. canis*

Pathogen	Primer	Sequence (5'-3')	Product Size
Babesia spp.	Ba103F	CCAATCCTGACACAGGGA GGTAGTGACA	619 bp
	Ba721R	CCCCAGAACCCAAAGAC TTTGATTTCTCTCAAG	
E. canis	Ehr1401F	CCATAAGCATAGCTGA TAACCCTGTTACAA	380 bp
	Ehr1780R	TGGATAATAAAACCGTA CTATGTATGCTAG	

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 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.

Nested PCR for the amplification of the 16s rRNA gene fragment of *E. canis* was employed following the procedure of Rajagopal *et al.* (2009).

Table 2: Nested PCR for the amplification of the 16s rRNA gene fragment of *E. canis*

Ehrlichia spp. (16s r RNA)		
Primer	**Sequence**	**Size**
ECC Outer forward	5' AGA ACG GCT GGC GGC MG C 3''	1st cycle 380 bp
ECB Outer reverse	5' CGT ATT ACC GCG GCT GCT GGC A 3'	
ECAN5 Inner forward	5' CAA TTA TTT ATA GCC TCT GGC TAT AGG A 3'	2nd cycle
HE3 Inner reverse	5' TAT AGG TAC CGT CAT TAT CTT CCC TAT 3'	

Thermal cycling for PCR amplification was as follows:

$$\frac{94°C}{3\,min} \rightarrow \left[\frac{94°C}{1\,min} \rightarrow \frac{65°C}{2\,min} \rightarrow \frac{72°C}{2\,min} \right] \times$$
$$30\ cycles \rightarrow \frac{72°C}{5\,min} \rightarrow \frac{4°C}{\infty}$$

The amplicons were separated by electrophoresis in 1.5 per cent agarose gel in 40 mM Tris-acetic acetate pH of 8.4, 1 mM EDTA, stained with ethidium bromide (0.5μg/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 taken for the 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 AND DISCUSSION

Out of the 75 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 taken for the study.

In the present study incidence of secondary IMHA at Madras Veterinary College Teaching Hospital was comparatively higher (59.37 per cent) than primary IMHA (40.63). Piek *et al.* (2008) recorded incidence of secondary IMHA as 20 to 25 per cent. In the present study this finding was in contrast to Piek *et al.* (2008). The reasons might be due to

the breeds which are said to have genetic predisposition to primary IMHA are unpopular and rare in Chennai, India where the study was carried out and endemic nature of the haemoprotozaon parasites.

Sixty five per cent of the IMHA dogs had *Babesia gibsoni* infection alone as the triggering factor. The higher prevalence of *Babesia gibsoni* infection might be one of the reasons for increased incidence of secondary IMHA in the present study. *Ehrlichia canis* as an etiology for secondary IMHA was seen in 16 per cent of cases. In the present study, these haemoparasites acted as a trigger for IMHA in concurrence with the observations made by Archer and Mackin (2013) and Kidd *et al.* (2015).

Incidence of secondary IMHA was found higher in 4-8 years (36.84 per cent) followed by 2-4 years (31.57 per cent), above 8 years (15.78 per cent) and 1-2 years (15.78 per cent) age groups and more common in male (52.7 per cent) when compared to female dogs (47.3 per cent). Result obtained in the present study is in agreement with the observations made by Archer and Mackin (2013), who reported highest incidence of IMHA in middle aged dogs. Higher incidence of male dogs in the present study which might be due to small size of population or over representation of male dogs to the hospitals which is in contrast to Weinkle *et al.* (2005) who stated that males are less susceptible to IMHA and this might be due to protective nature of androgen.

Breed wise incidence was found highest in Labrador Retriever (26.31 per cent) followed by Spitz, Terrier, Cocker Spaniel, German Shephard with 10.52 per cent each. While the incidence in Bull Mastiff, Cross breed, Mongrel, Golden Retriever, Rottweiler, Pug, Dalmatian and Beagle was 5.63 per cent each. The highest incidence in secondary IMHA was found in Labrador breeds which might be be due to over representation of the breeds to the hospital. The present study was contradictory to Balch and Mackin (2007) who stated that the greater prevalence of the disease in particular breeds like Old English sheepdog, Collie, Poodle, Cocker Spaniel, English Springer Spaniel and Irish Setter.

Clinical signs in secondary IMHA dogs were anorexia (78.9 per cent), lethargy (89.4 per cent), vomiting (36.8 per cent), pyrexia (84.2 per cent), dehydration (68.4 per cent), tachypnoea (63.1 per cent), tachycardia (26.3 per cent), pale mucosa (73.6 per cent), icteric (26.3 per cent),

hemoglobinuria (36.8 per cent), epistaxis (26.4 per cent) and ecchymosis (31.5 per cent).

In present study, non specific clinical signs recorded in secondary IMHA dogs were anorexia, lethargy, vomiting, pyrexia, dehydration and specific signs caused by severe anemia were tachypnoea, tachycardia, pale mucosa, icteric, hemoglobinuria and ecchymosis (Balch and Mackin, 2007).

More specific signs of haemolysis were recorded in the present study, where intravascular haemolysis causes release of free haemoglobin into the circulation further leading to pigmenturia (Haematuria / haemoglobinuria). Haemolysis is more seen in IgM mediated disease as IgM is better in fixing complement than IgG. The complement bind to the erythrocyte surface and form a membrane attack complex leading to osmotic lysis of RBC (Balch and Mackin, 2007).

Piek *et al.* (2011) reported most of the dogs with IMHA develop anaemia rapidly and clinical signs such as lethargy, loss of appetite, vomiting and diarrhoea occured in 15–30 per cent of cases. Petechiation and ecchymosis as a result of concurrent severe thrombocytopenia is reported by Piek *et al.* (2008) and could be due to concurrent immune-mediated thrombocytopenia (ITP).

In the present study pyrexia (84.2 per cent) was recorded in secondary IMHA dogs. This was in agreement with Piek *et al.* (2008) who reported secondary IMHA caused by *Babesia* spp. organisms that can stimulate production of endogenous pyrogens which inturn releasing co PGE_2 resulting in the increase of set point in hypothalamus.

The hematological, coagulation profile and biochemical findings of dogs with secondary IMHA are presented in Table 3. A significant reduction in mean Hb, mean RBC, PCV value was observed between control and secondary IMHA group. A highly significant increase in the values of WBC, neutrophil, mopnocyte was observed between control and secondary IMHA group. A highly significant decrease in the values of lymphocyte and platelet was observed between control and secondary IMHA group. A significant increase in the values of PT and APTT was observed between control and secondary IMHA group.

Dogs with secondary IMHA showed significant reduction in PCV, Hb and values when compared to control dogs indicating a state of severe anaemia. Swann and Skelly

Table 3: Comparison of hematology, serum biochemistry and coagulation profile of healthy dogs with Secondary IMHA

Parameter	Secondary IMHA (n=19)	Control (n=10)	F-value
Hb (g/dl)	4.93±0.45b [b]	12.61±0.46[a]	108.94**
RBC(mill/uL)	2.59±0.27 [b]	6.01±0.14[a]	84.30**
PCV (%)	16.25±1.38 [b]	36.15± 1.14 [a]	58.62**
WBC (/ ul)	16715.38±2261.43 [b]	10709.90±783.79 [a]	10.54**
Neutrophil (%)	77.08±1.41 [ab]	74.20±0.29 [a]	5.22*
Lymphocyte (%)	15.61±0.96 [b]	21.20±0.71 [a]	9.02**
Monocyte (%)	6.15±0.76 [b]	3.60±0.37 [a]	3.96*
Eosinophil (%)	1.00±0.27	1.20±0.32 [a]	0.12 [NS]
Platelets (/cmm)	89461.54±5087.13 [b]	228300.10± 22668.65 [a]	2.41**
PT (sec)	31.75±3.39 [b]	11.50±0.92 [a]	4.09*
APTT (sec)	42.13±3.83 [ab]	30.80±1.51 [a]	2.87*
BUN (mg/dl)	49.12±3.05[b]	24.29±1.33[a]	31.58**
Creatinine (mg/dl)	1.24±0.16[b]	0.5±0.09[a]	9.77**
Total Protein (g/dl)	6.53±0.26	6.90±0.09	1.68 [NS]
Albumin (g/dl)	2.13±0.10[c]	3.43±0.05 [a]	43.64**
ALT (IU//l)	125±5.56[b]	59.67±4.03[a]	49.67**
ALP (IU//l)	373±61.50 [b]	117.00±14.35 [a]	6.26**
Total Bilirubin (IU/L)	0.87±0.18 [a b]	0.53±0.07 [a]	3.96*
Direct Bilirubin (IU/L)	0.73±0.15	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.

(2013) reported that IMHA was predominantly a type II immune hypersensitivity reaction in which the immune system produces autoantibodies that bind to the host's own erythrocytes, causing both intravascular and extravascular haemolysis.

Dogs with secondary IMHA showed significant increase in WBC, monocyte and significant decrease in lymphocyte and platelets (Table 3). The alterations in the leucogram values in the present study were in agreement with the reports of the Piek *et al*. (2008), McAlees (2010) and Piek *et al*. (2011). In the present study, leukocytosis might be due to severe tissue injury (Archer and Mackin 2013).

The present study revealed thrombocytopenia and increase PT in cases of secondary IMHA (Table 3). Majority of the cases in the present study were secondary IMHA caused by vector borne haemoparasites or consumptive coagulopathy as cause of thrombocytopenia (Jutkowitz *et al*., 2013).

A highly significant increase in mean BUN, creatinine, ALT, ALP and total bilirubin value was observed between control and secondary IMHA group (Table 1). There was no significant difference in mean total protein and direct bilirubin value between control and secondary IMHA group. A highly significant decrease in mean albumin value was observed between control and secondary IMHA group.

Swann and Skelly (2013) reported that an increase in BUN levels in dogs might be due to prerenal, renal or gastrointestinal origin resulting from hypoxia or thromboembolism.

In secondary IMHA there was significant reduction in albumin values but no significant difference in albumin and globulin ratio and total protein values. Similar findings of hypoalbuminemia and hypoproteinemia was recorded by Ishihara *et al.* (2010) which was attributed to decreased synthesis by liver or loss through haemorrhage,

as most IMHA dogs have thrombocytopenia and are hypercoagulable. The high levels of globulin might be due to high immune response in IMHA. The ALP and ALT values were significantly high in secondary IMHA groups when compared to control healthy dogs. Archer and Mackin (2013) reported that an increase in ALT in IMHA dogs due to hypoxic damage of liver.

In the present study, there was significant increase in total bilirubin. Balch and Mackin (2007) stated that an elevated bilirubin level was common in patients with IMHA and could result from either hemolytic or hepatobiliary.

Spherocytosis was observed in three cases (15.78 per cent) and saline agglutination test was positive in six cases (21 per cent). Apart from spherocytes (Fig. 5), anemic changes like poikilocytosis in six cases (31.57 per cent), monocytosis in thirteen cases (68.4 per cent) and hypochromasia in seven cases (36.8 per cent)**.**
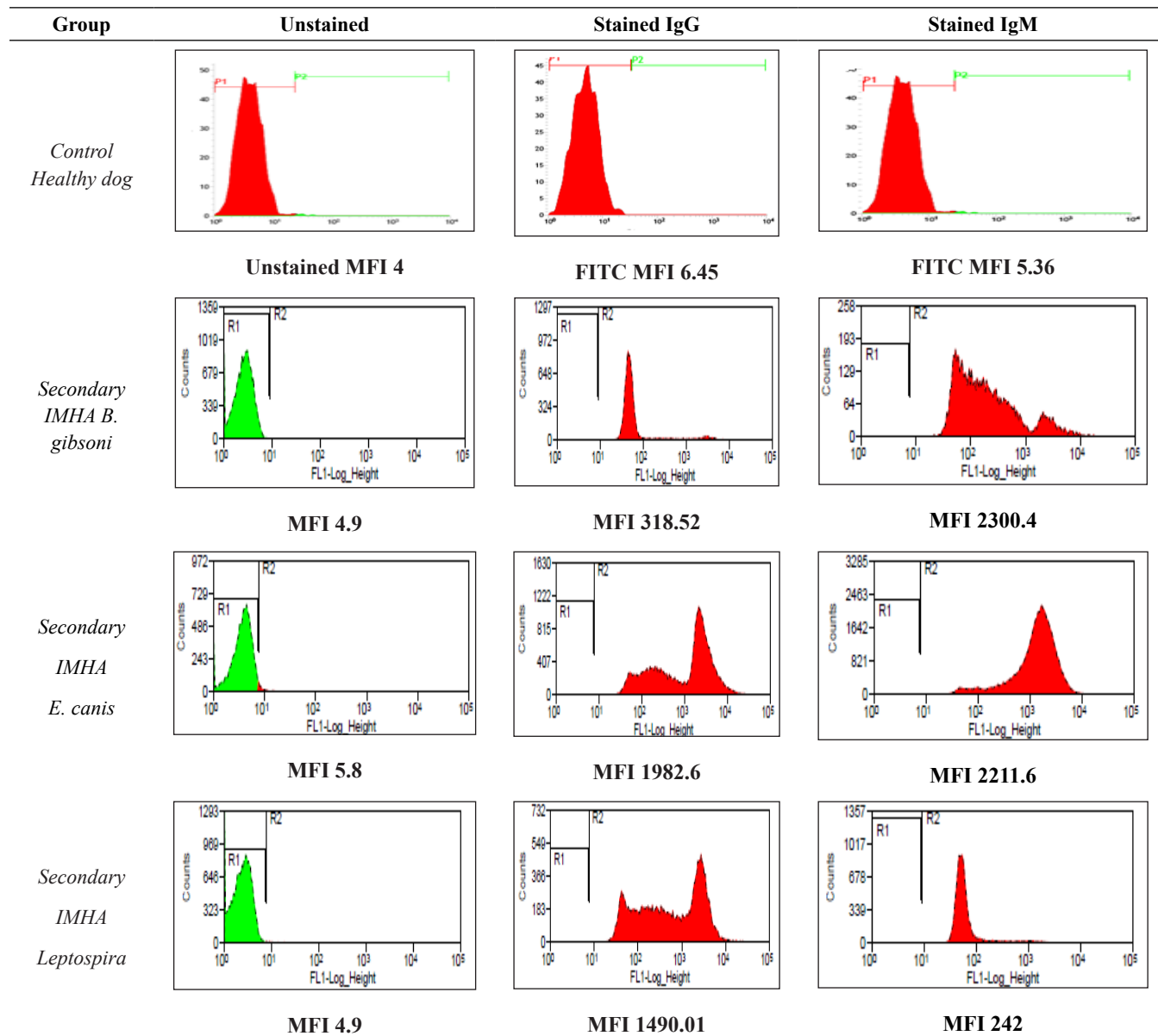

Fig. 1: Mean Fluorescent intensity (MFI) of unstained and stained anti dogIgF and anti dogIgM FITC in RBC of dog

Anaemic changes like anisocytosis, poikilocytosis and hypochromasia were seen in the present study. Spherocytosis is considered pathognomonic for IMHA (Piek, 2011). Blood smear examination also revealed haemoprotozoan parasites like *Babesia gibsoni* (65 per cent of total secondary IMHA caused by *Babesia spp.*) and *E. canis* (16 per cent).
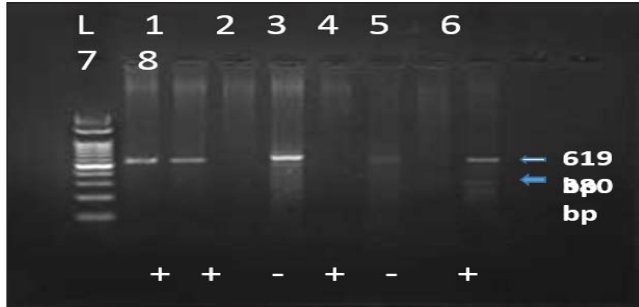
L = Ladder, sample 8 = positive control, 9 = negative control, Positive for Babesia spp. in sample no. 1,2, 4,6

Fig. 2: AGE showing PCR amplified product from 16s rRNA gene fragment of genus *Babesia* and VirB9 of *E. canis*
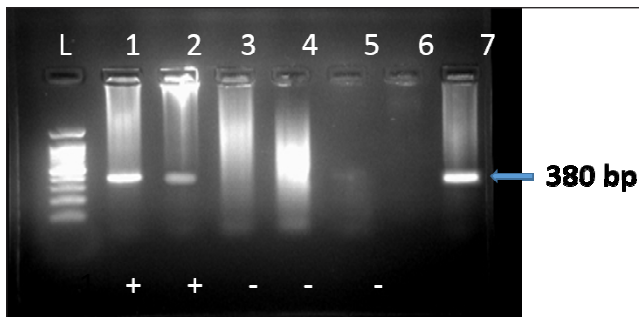
L = Ladder, sample 7 = positive control, 6 = negative control, Positive for Babesia spp. in sample no. 1 and 2

Fig. 3: AGE showing PCR amplified product from 16s rRNA gene fragment of *E. canis*

In the present study 21 per cent of secondary IMHA dogs had a positive saline agglutination test (Fig. 4). Weinkle *et al*. (2005) reported approximately 40 per cent to 89 per cent of dogs with IMHA had a positive saline agglutination test. The reason behind in-saline slide agglutination was to break up rouleaux formation but not erythrocyte aggregate using saline. The present study was in concurrence with the findings of Weinkle *et al*. (2005) and Vijayalakshmi (2011).

Out of 19 cases of secondary IMHA, three dog (15.78 per cent) was positive for microscopic agglutination test. The positive case had antibody titre more than 1/800 for *Leptospira pomona* and 1/400 antibody titre for *Leptospira autumnalis.*
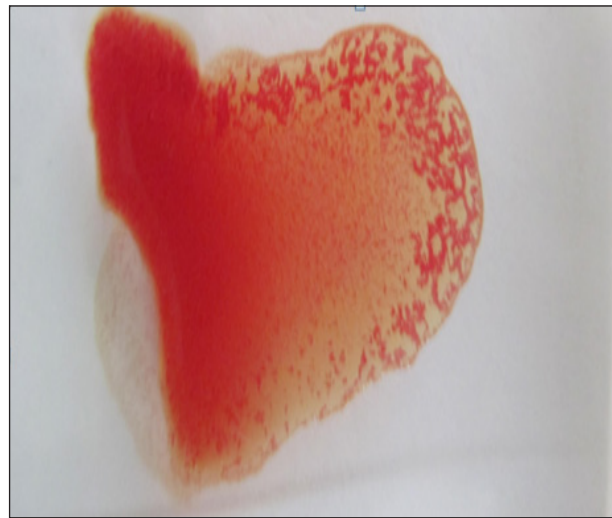

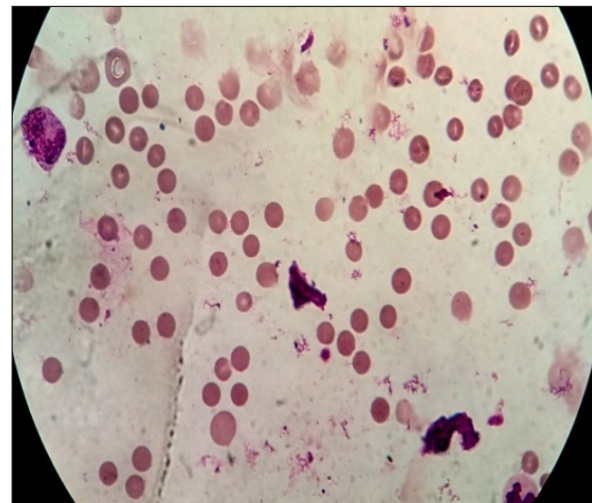**Fig. 4:** Positive saline agglutination test

Fig. 5: Spherocytes– Small RBCs with loss of central pallor

In present study, the case with Leptospira had positive antibody titers > 1:800 for *L. pomona,* and >1: 400 antibody titre for *L. autumnalis.* Barr *et al.* (2005) reported the sensitivity of a single MAT titer 800 for diagnosis was 22–67 per cent, depending on the laboratory used, and the specificity was 69–100 per cent. Leptospirosis as a cause

of secondary IMHA has been reported by earlier works of Shah *et al.* (2009) and Bovens *et al.* (2014).

In the present study, nineteen cases out of seventy five suspected anemic cases (25.33 per cent) were diagnosed by flow cytometric analysis. Flow cytometry, 11 dogs (58 per cent) RBC were binding with both IgG and IgM, two dogs RBC (10.5 per cent) bound with IgM and six dogs RBC (31.6 per cent) were bound with IgG.

In the present study, flow cytometry was effective in detecting Immunoglobulin IgG and IgM bound over the surface of RBC in both disease groups (Fig. 1). 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. 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.

In the present study, thirty two dogs (thirteen primary and nineteen secondary) were positive for IMHA by flow cytometry (100 per cent). In secondary IMHA spherocytosis was observed in 15.8 per cent and saline agglutination test was positive in 21 per cent respectively. Hence, flow cytometry was found to be effective in the confirmation of canine IMHA and in agreement with Morley *et al.* (2008).

Multiplex PCR was carried out for identification of genus specific for *Babesia* by amplifying 16s rRNA gene fragment and amplification of VirB9 gene of *E. canis*. Out of 32 cases subjected to multiplex PCR with genus specific primers for *Babesia* and *E. canis,* thirteen cases (40.6 per cent) were positive for *Babesia spp.* (Fig. 2) and negative for *E. canis*. Nested PCR for amplification of 16s rRNA gene fragment of *E.canis* was employed. After the second round of amplification, positive amplification of 380 bp amplicon was visualized on gel under UV light. Out of thirty two cases, three cases (9.4 per cent) were positive (Fig. 3).

The use of molecular characterization to identify *Babesia* species highlights the value of procedure of PCR as an adjuvant to current diagnostic methodology. The identification of genus specific *Babesia* in present study suggest that PCR has got a wider acceptance as a diagnostic tool for secondary IMHA (Kledmanee *et al.,*

2009). In the present study, multiplex PCR could only give one positive test out of thirty two samples which was not sensitive. Nested PCR was carried out to cross check the false negative test given by multiplex PCR (Rajagopal *et al.*, 2009).

CONCLUSION

Secondary IMHA is the most common autoimmune disease in dogs. Highest incidence was observed in males, Labrador dogs with age group of 2-8 years. Secondary IMHA (59.37 per cent) was major cause of immune mediated haemolytic anaemia in dogs than primary IMHA (40.63 per cent). *Babesia gibsoni* was the major underlying cause of secondary IMHA. Significant reduction in Hb, PCV and RBC, platelet and leucocytosis, neutrophilia, elevated PT, APTT were prominent findings of secondary IMHA. Significant increase in ALT, ALP activities, elevated levels of bilirubin and hypoalbuminemia were observed in secondary IMHA. 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.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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