Short Paper

Immune-mediated hemolytic anemia in cats referring to Veterinary Teaching Hospital of Tehran (2006-2007)

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Summary

Immune-mediated hemolytic anemia (IMHA) is characterized by the destruction of erythrocytes or sometimes bone marrow erythroid precursors mediated by immunoglobulins (IgG, IgM), with or without complement (C_3). The main objectives of this study were to assess the laboratory test results of IMHA and to investigate its possible underlying causes in cats referring to the Veterinary Teaching Hospital of Tehran. The Coombs' test (CT) was performed in 74 cats with PCV below 0.35. The test was positive in 26 cats. These positive CT cats were categorized into four groups based on the PCV ranges and type of anemia including: Group A: 9 of 26 cats had nonregenerative anemia (PCV median, 0.22) (1 feline leukemia virus positive which had erythroleukemia, 1 feline infectious peritonitis positive, 1 with *Hemoplasma* spp., 3 with renal failure, 2 with inflammatory disease and 1 with no diagnosis). Group B: 4 cats (PCV median, 0.31) had a regenerative anemia with severe dehydration. Group C: the other 9 cats with a normal range of PCV (median, 0.34) involved with various conditions (vaccination, parturition, acetaminophen poisoning, osteoporosis, and renal failure). Group D: the remaining 4 cats with a marginal range of PCV (median, 0.30) had a history of inflammatory disease and drug therapy. The results of this study indicated that infectious diseases and drug therapy were the main factors associated with positive Coombs' test results.

Key words: Immune-mediated hemolytic anemia, Coombs' test, Cat

Introduction

Immune-mediated hemolytic anemia (IMHA) characterized by antibody production against red blood cells appears to occur much less frequently in cats than in dogs (Kohn *et al*., 2006). It may occur as a primary (idiopathic) disorder, or secondarily may be associated with other diseases or conditions such as bacterial, viral, rickettsial, or protozoan infections, hepatopathy, and other autoimmune disorders. It is characterized by reduced survival time of red blood cells. In cats, IMHA has been most commonly associated with *Hemoplasma* spp. infection, feline leukemia virus (FeLV), feline infectious peritonitis (FIP), lymphoproliferative and myeloproliferative disease, babesiosis, exposure to chemicals or toxins (e.g., acetaminophen, onion), drug therapy, severe hypophosphatemia or immune reactions against red blood cells (Thrall *et al*., 2004; Kohn *et al*., 2006). Immune-mediated hemolytic anemia has also been described in cats suffering from systemic lupus erythematosus, or after blood transfusion from incompatible donors (Auer and Bell, 1983; Lusson *et al*., 1999). In dogs, spherocytes are pathognomonic for IMHA. In cats, however, identification of spherocytes is difficult since feline red blood cells are much smaller and do not have a central pallor (Giger, 2000). Previous studies have shown the usefulness of the Coombs' test (CT) for detection of IMHA in cats as

well as in dogs (Dunn *et al*., 1984; Kohn *et al*., 2006).

The diagnosis of immune-mediated hemolytic anemia in our study is based on the detection of erythrocyte-bound immunoglobulin. The main purpose of this study was to examine the laboratory test results of IMHA and investigate its possible underlying causes in cats referring to the Veterinary Teaching Hospital of Tehran.

Materials and Methods

Preparation of anticat immuno-globulin

To produce rabbit anticat immunoglobulin, gammaglobulins of a feline serum sample were precipitated with 45% saturated ammonium sulfate and dialyzed against phosphate buffered solution (PBS) at 4°C for 48 h (Hay and Westwood, 2002; Nassiri *et al*., 2005). The protein of the dialyzed solution was measured using the Bradford method, and the dialyzed solution was then diluted with PBS until a concentration of 200 μg per 500 μl was achieved. The later diluted solution was mixed with 500 μl of the Ferund's complete adjuvant and was injected into a rabbit's thigh. Blood serum was collected from the rabbit two weeks later, and the rabbit anticat immunoglobulin was detected via double diffusion method (Hay and Westwood, 2002; Nassiri *et al*., 2005).

Animals and primary laboratory evaluation

Seventy four cats with or without anemia (with PCV below 35%) were tested in this study. These cats were referred to the University of Tehran Teaching Hospital during a 1-year period (2006-2007). The individual characteristics and history of these cats were recorded, and after clinical examination, blood samples were taken and initial evaluation (complete blood count (CBC), microscopic autoagglutination, clinical chemistry) was performed (Tables 1 and 2). Infectious diseases, such as FeLV, feline immunodeficiency virus (FIV), and FIP were diagnosed by rapid serological test. Tests for FeLV antigen and FIV antibodies were conducted in most cats (FASTTest[R]

FeLV and FASTTest[R] FIV). The presence of *Hemoplasma* spp. was evaluated on a blood smear stained with Giemsa (Kohn *et al*., 2006).

Direct Coombs' test

For performing the direct Coombs' test, red blood cells were washed three times with PBS. During each washing, the red blood cells were carefully and thoroughly mixed with PBS and then centrifuged for 3 min at $1200 \times g$. After the final washing, a 2% suspension of red blood cells in PBS was prepared. The Coombs' test was performed in a 96-well microplate. 50 μl of rabbit anticat antisera was added to 50 μl of PBS, and then additional dilutions were prepared with a 1:1 dilution of antisera in PBS. Afterwards, 50 μl of red blood cell suspension was added to each well and incubated at 37°C for 45 min, then at room temperature for 45 min. Afterwards, agglutination was microscopically determined (Nassiri *et al*., 2005; Kohn *et al*., 2006). Ten healthy-non anemic cats (in addition to the 74 cases) were used to verify the specificity of CT and defining the cut off calibration controls. Descriptive statistics were calculated using SPSS 12.0 for Windows.

Results

Successful production of rabbit anticat immunoglobulin was detected via double diffusion method (Hay and Westwood, 2002). The cutoff level of ≥1:4 was proposed for this assay. None of the 10 control cats had positive CT results. During the study period (2006-2007), the CT was performed in 74 cats with PCV below 0.35. The results were positive in 26 cases. The age of the positive cats varied between 0.5 to 12 years average. Ten Cats were male (38.5%) and 16 of them female (61.5%). These positive CT cats were categorized into four groups based on the PCV ranges and type of anemia including: group A: 9 cats had nonregenerative anemia due to low reticulocytosis (Aggregated reticulocytes $<40 \times 10^{12}$ /L; median packed cell volume (PCV): 0.22) including; 1 with FIP, 1 with FeLV which had erythroleukemia, 1 with

Hemoplasma spp., 3 with renal failure, 1 with enteritis, 1 with dental abscess (FeLV positive) (secondary IMHA), and 1 with no underlying cause (primary IMHA). Group B: 4 cats (PCV median, 0.31) had a regenerative anemia- due to high reticulocytosis- with severe dehydration including: 1 with *Hemoplasma* spp. infection (PCV 0.30; aggregated reticulocytes >40 × 10^{12} /L; dehydration, hyperbilirubinemia, and severe bilirubinuria, left shift, nucleated red blood cell (NRBC) 2%), 1 with pneumonia and FeLV positive (PCV, 0.31; severe dehydration, hyperbilirubinemia, leukemoid reaction, reticulocytosis), 1 was febrile (>39.5°C, PCV, 0.29; dehydration, hyperbilirubinemia, toxic neutrophils, polychromasia, hypochromia, NRBC 1%), 1 with a history of trauma (PCV, 0.34; severe dehydration, hyperbilirubinemia, hyperglubolinemia, polychromasia, macrocytosis hypochromia). Group C: the other 9 cats with a normal range of PCV (median, 0.34) had various histories such as vaccination, parturition, poisoning with acetaminophen, osteoporosis with severe hypophosphatemia (n = 1 each), 2 with a history of surgery and 3 with renal failure and severe azotemia (blood urea nitrogen (BUN) 17.85-53.55 mmol urea/L, creatinine (Cr) 159.12-265.2 μmol/L). Group D: the remaining 4 cats with a marginal range of PCV (median, 0.30) had a history of drug therapy with various antibiotics (e.g., cotrimoxazol, cefazolin).

Serum biochemical abnormalities (Table 1) included hyperbilirubinemia (1.88-34.2 μmol/L) in 7 cats, hyperglubolinemia (45-58 g/L) in 9 and hyperproteinemia (77-99 g/L) in 11 cats. Alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotranseferase (AST) were

Table 1: Clinical chemistry results in 26 cats with immune-mediated hemolytic anemia

Parameters	Min-Max	Median	Reference range
Blood urea nitrogen (mmol urea/L)	2.8-54	7.8	6.7-12.1
Creatinine (μmol/L)	17.7-265	62	80-195
Total protein (g/L)	30-99	68	60-75
Albumin (g/L)	20-49	30	28-39
Globulin (g/L)	20-58	39	28-45
Alanine aminotransferase (U/L)	80-350	88	25-97
Alkaline phosphatase (U/L)	0-101	10	0-45
Aspartate aminotransferase (U/L)	35-150	43	7-38
Bilirubin (μmol/L)	0.3-34	1.2	0.00-1.71

Table 2: Complete blood count in 26 cats with immune-mediated hemolytic anemia

Parameters	Min-Max	Median	Reference range
PCV (L/L)	0.12-0.35	0.30	0.31-0.45
Hemoglobin (g/L)	30-150	110	98-154
Red blood cells (×10^{12}/L)	1-11.6	5.8	5-10
MCV (fl)	39-67	57	39-55
MCHC (g/L)	260-420	343	300-360
Aggr reticulocytes (×10^9/L)	0-155	45	<40
White blood cells (×10^9/L)	5.1-99.5	18	5.5-19.5
Bands (×10^9/L)	0.1-19.9	0.47	0-0.3
Segmented (×10^9/L)	3.36-75.6	12.5	2.5-12.5
Lymphocytes (×10^9/L)	0.6-10.5	2.7	1.5-7
Monocytes (×10^9/L)	0.1-1.6	0.3	0-0.9
Platelets (×10^9/L)	35-420	205	300-800

PCV: packed cell volume, MCV: mean corpuscular volume, MCHC: mean corpuscular hemoglobin concentration and Aggr: aggregated

increased in 1 (101 IU/L), 4 (80-350 IU/L) and 3 (35-150 IU/L) cats, respectively.

Discussion

Numerous studies have evaluated the CT in the diagnosis of immune-mediated hemolysis in dogs (Engelbrecht *et al*., 2002; Nassiri *et al*., 2005), but only two studies have evaluated its diagnostic value in cats (Dunn *et al*., 1984; Kohn *et al*., 2006). The results of these studies showed that CT is a very useful method in diagnosing immune-mediated anemia in these animals. Therefore, this method was applied for diagnosis of IMHA in 74 cats with or without other diseases over 1 year in the Veterinary Teaching Hospital of Tehran.

Dunn *et al*. (1984) reported a positive result of the direct Coombs' test using polyvalent antiserum in 16 of 20 (80%) anemic cats, whereas we reported 26 CT positive among 74 cats (35%). The age of the cats varied between 0.5 to 12 years average. In another study, the average age of cats examined for hemolysis was 3.2 years (Kohn *et al*., 2006). A higher incidence of IMHA was reported in male cats (Scott *et al*., 1973; Kohn *et al*., 2006), but was not confirmed in our study (male 38.5%, female 61.5%).

In the present study, one of the cats had been vaccinated 1 month before initial presentation. Recent vaccination in dogs has been implicated as a trigger for immune-mediated disease, such as IMHA (Duval and Giger, 1996) and polyarthritis (Kohn *et al*., 2003). Vaccination against calcivirus has resulted in polyarthritis in cats (Dawson *et*

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al., 1993), whereas an association between vaccination and IMHA has not been described in cats. The vaccinated cat in our study (PCV, 0.33) had several clinical signs such as inappetence, lethargy and submandibular lymphadenopathy. Since this cat had not been re-vaccinated during the study period, it is unknown if vaccination would have triggered a recurrence of disease. A mean hematocrit of 0.12 was reported in cats with primary IMHA (Person *et al*., 1997). In another study (Kohn *et al*., 2006), 79% of cats had severe anemia (PCV<0.15). In our study, 9 of the positive tested cats (35%) had a nonregenerative anemia (PCV, 0.12-0.24), and 4 cats (15%) had regenerative anemia and dehydrated status (PCV median, 0.305).

Cats appear to tolerate low hematocrit value better than dogs. Regeneration of red blood cells in patients with hemolytic anemia is characterized by macrocytic and either hypo- or normochromic erythrocytes (Thrall *et al*., 2004). In the initial evaluation, 14 cats had increased MCV, but only 4 had increased numbers of reticulocytes. Intravascular hemolysis was not documented in our study, nor in other studies on IMHA in cats (Dawson *et al*., 1993; Kohn *et al*., 2006). Intravascular hemolysis appears to occur less frequently in cats compared with dogs. It was described in 1 out of 23 tested dogs and in 4 out of 42 dogs, by Klag *et al*. (1993) and Engelbrecht *et al*. (2002), respectively.

Patients with IMHA commonly have regenerative anemia by reticulocytosis, polychromasia and anisocytosis. Production of antibodies against young erythrocyte in the bone marrow might cause a non regenerative immune-mediated hemolytic anemia, or pure red cell aplasia (Stokol *et al*., 2000). Therefore, some cases with IMHA may not have evidence of reticulocytosis and polychromasia (Halliwell and Gorman, 1989; Giger, 2000). In our study, 9 cats had nonregenerative anemia which was explained earlier. In addition, Feline leukemia virus (FeLV) infections may cause non-regenerative anemia in cats through its effect on bone marrow (Perkins, 2000).

In dogs with primary IMHA, approximately one third also suffered from immune-mediated thrombocytopenia (Evans' syndrome) (Engelbrecht *et al*., 2002). In one study (Kohn *et al*., 2006), 1 out of 19 and in our study 3 out of 26 tested cats suffered from both IMHA and thrombocytopenia. The combination of thrombocytopenia and IMHA was found in 6 of 18 cats (Husbands *et al*., 2002) in which platelet counts ranged from 13,000 to $100,000 \times 10^9$/L. Lymphocytosis was rare in our study (8%). Lymphocytosis was reported 52 and 32% by Husbands *et al*. (2002) and Kohn *et al*. (2006), respectively. In dogs, leukocytosis with a left shift is common in association with primary IMHA. In our study, leukocytosis was found in 11 cats (46%) which was rare in the study of Kohn *et al*. (2006) (10.5%).

In four cats with positive CT, liver enzyme activities, especially ALT, were increased. Cellular hypoxia due to low liver blood volume in anemia may induce hepatocyte injury and increase the level of serum enzyme activities (Lassen, 2004). Hyperbilirubinemia due to increased breakdown of hemoglobin was present in 7 of 26 cats. In 2 of these 7 cats with hyperbilirubinemia, the liver enzyme activity was also increased. The mean plasma bilirubin concentration was 2.73 µmol/L. In one study, 13 of 19 cats with IMHA had hyperbilirubinemia (Kohn *et al*., 2006). In another study, all cats suffering from IMHA had increased serum bilirubin concentration (Husbands *et al*., 2002).

In one case with acetaminophen toxicosis, PCV was 0.35 and it was presented to the hospital 4 h after exposure to this drug. This cat had nonspecific clinical signs (lethargy, depression, and anorexia), number of red blood cells (RBC); 5000,000 $\times 10^9$/L, white blood cells (WBC); 42000 $\times 10^9$/L, mean corpuscular volume (MCV) 58 fl mean corpuscular hemoglobin concentration (MCHC) 350 g/L. Unfortunately, after several days, we had no success in paging the owner for following up. In our study, the highest antibody titer that gave agglutination was observed in a cat with FIP (1/64 serum dilution). This is the first report on an erythroleukemia (M6) case with concurrent IMHA. Mortality rate was 7.7% in this study which was reported 23.5 and 24% in the studies of Kohn *et al*. (2006)

and Husbands *et al*. (2002), respectively. This study indicated a better prognosis for cats with IMHA than that reported by Husbands *et al*. (2002) and Kohn *et al*. (2006). The results of this study indicated that infectious diseases and drug therapy were the main factors which were associated with positive Coombs' test results.

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