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# Application of Flow Cytometry for Early Diagnosis and Monitoring in Cats with Immune-mediated Hemolytic Anemia

Parkorn Limlengler<sup>1</sup> Anudep Rungsipipat<sup>2</sup> Rosama Pusoonthornthum<sup>3</sup>

## *Abstract*

The study aimed to apply flow cytometry (FC) for early detection and monitor of treated response cats with IMHA by measuring immunoglobulin-G (IgG) and immunoglobulin-M (IgM) on red blood cells. Coombs' test and FC method were performed in twenty-nine cats including: ten clinically normal client-owned cats and nineteen anemic cats with packed cell volume of less than 27%. Five anemic cats were positive for Coombs' test and had significantly high levels of IgG and IgM on the red blood cells with high level of mean fluorescence intensity (MFI) measured by the FC method. In seven out of the fourteen anemic cats with negative Coombs' results, the FC method demonstrated the increase of MFI values for red blood cell-bound feline IgG and IgM. These seven cats were suspected of having subclinical IMHA. The FC method can provide the MFI value of the red blood cell-bound feline IgG and IgM which can be used for early detection and monitoring of the treated response in cats with IMHA.

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**Keywords:** cat, flow cytometry, immune -mediated hemolytic anemia

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## บทคัดย่อ

### การประยุกต์ใช้เครื่อง Flow cytometry ในการวินิจฉัยระยะแรกและเฝ้าระวังภาวะ Immune-mediated hemolytic anemia ในแมว

ภากร ลี้มเล็กเลิศ<sup>1</sup> อนุเทพ รังสีพิพัฒน์<sup>2</sup> รสมา ภูสุนทรธรรม<sup>3\*</sup>

วัตถุประสงค์ในการศึกษาค้นคว้าครั้งนี้ เพื่อการประยุกต์ใช้เครื่อง flow cytometry ในการวินิจฉัยระยะแรกและเฝ้าระวังภาวะ immune-mediated hemolytic anemia ในแมวที่ได้รับการรักษา โดยการวัดภูมิคุ้มกันกลอบูลินชนิด G และ M บนผิวเซลล์เม็ดเลือดแดง วิธีการตรวจ Coomb's Test และเครื่อง flow cytometry นำมาใช้ในการตรวจแมว จำนวน 29 ตัว ซึ่งถูกแบ่งเป็น 2 กลุ่ม คือ แมวเลี้ยงที่มีอาการทางคลินิกปกติ จำนวน 10 ตัว และ แมวที่มีภาวะเลือดจาง (ค่าเม็ดเลือดแดงอัดแน่น น้อยกว่า ร้อยละ 27) จำนวน 19 ตัว ผลการศึกษาพบ แมวจำนวน 5 ตัวที่มีภาวะเลือดจางให้ผลทดสอบบวก ต่อ Coomb's Test ค่า IgM และ IgG บนผิวเซลล์เม็ดเลือดแดง มีค่าสูงอย่างมีนัยสำคัญ ร่วมกับค่า mean fluorescence intensity (MFI) ซึ่งวัดด้วยเครื่อง flow cytometry แมวจำนวน 7 ตัว ใน 14 ตัวให้ผลลบต่อ Coomb's Test แต่พบการเพิ่มขึ้นของค่า mean fluorescence intensity (MFI) เมื่อวัดด้วยเครื่อง flow cytometry ซึ่งจัดแมวดังกล่าวอยู่ในกลุ่มโรค IMHA ที่ไม่แสดงอาการ การประยุกต์ใช้เครื่อง flow cytometry ในการตรวจวิเคราะห์ค่า MFI นั้นสามารถนำมาใช้ในการวินิจฉัยระยะเริ่มต้นและเฝ้าระวังโรคในการรักษาแมวที่ป่วยด้วยโรค IMHA

**คำสำคัญ:** แมว การตรวจวัดเซลล์วิทยา ภาวะโลหิตจางเนื่องจากภูมิคุ้มกันบกพร่อง

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## Introduction

Anemia is one of the most common abnormalities in cats. The primary causes of anemia are blood parasites, trauma, chronic renal failure and viral infection. One of the causes of feline anemia is immune-mediated hemolytic anemia (IMHA) (Gunn-Moore et al., 1999; Day, 2000, Husbands et al., 2002; Mackin, 2002; Nassiri et al., 2005; Kohn et al., 2006; Zini et al., 2007; Weiss, 2008). IMHA is an abnormality which red blood cells are destroyed in the blood vessels (intravascular hemolysis) and in tissue (extra vascular hemolysis) (Barker, 2000; Giger, 2001; Mackin, 2002). Chronic stimulation of the red blood cells by the immunoglobulin and complements causes IMHA resulting in red blood cell breakdown (Day, 2000; Mackin, 2002; Kohn et al., 2006). This abnormality causes acute or chronic anemia in cats and leads to multiple organ dysfunction and death. Besides the destruction of the red blood cells, immunoglobulin and complements from IMHA may damage the bone marrow and cause a reduction in production of the red blood cells (non-regenerative anemia) (Day, 2000; Mackin, 2002; Kohn et al., 2006; Zini et al., 2007; Weiss, 2008). Early, fast and precise diagnosis of IMHA is needed in feline medicine to

reduce the number of death caused by IMHA. The objective of this study was to test the flow cytometry (FC) as a possible diagnostic method for early detection of immunoglobulin-bounded on red blood cells in anemic cats with suspected IMHA and monitor treated response cats with IMHA.

## Materials and Methods

The studied animals were divided into 2 groups. Group A consisted of ten clinically healthy client-owned cats presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and animal hospitals in the Bangkok metropolitan with normal completed blood count (CBC) and blood chemistry results. The cats were included with no gender or breed preference aging between 12-120 months old. Group B were anemic cats consisting of nineteen cats with anemia presented at the Small Animal Hospital, Faculty of Veterinary Science, Chulalongkorn University and animal hospitals in the Bangkok metropolitan with no gender or breed preference within the same age. Cats with chronic renal failure, blood loss anemia and/or those receiving immunosuppressive treatment within one month were excluded. Cats with hematocrit lower than 27% were assigned into 2 subgroups (Couto,

2001). Group B1, the regenerative anemia group, consisted of cats with an absolute aggregated reticulocyte count level of 42,000/ $\mu$ l and/or corrected reticulocyte count  $\geq$  0.4% (Cowgill et al., 2003). Group B2, the non regenerative anemia group, consisted of all anemic cats that had been anemic for at least 5 days with an absolute aggregated reticulocyte count of less than 15,000/ $\mu$ l and/or corrected reticulocyte count  $\leq$  0.4%.

**Sample collection:** Peripheral blood was collected from the cephalic or saphenous vein and then placed into an EDTA tube.

**Diagnostic method:** The blood samples were tested for packed cell volume (PCV), completed blood count (CBC), reticulocyte count (manual count), total protein (refractometer) and blood chemistry by colorimetric method including alanine aminotransferase (ALT), alkaline phosphatase (ALP) (Audit Diagnostic, Ireland), blood urea nitrogen (BUN) (Merck, Germany) and creatinine.

**Auto-agglutination test:** Macroscopic agglutination was achieved by visible inspection of multiple red speckles on a glass slide (Mackin, 2002). Microscopic agglutination was performed with a saline dilution on a glass slide (one drop of RBCs to two drops of saline) and inspected under light microscope (Mackin, 2002).

**Direct agglutination test (DAT, Coombs' test) (Quigley et al. 2001):** One ml of EDTA blood was centrifuged at 3000 rpm for 5 min to separate the red blood cells from other components, and then 0.1 ml of red blood cell pellet was mixed with 4.9 ml of phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 5 min. Washed red blood cells in the volume of 0.1 ml were mixed with 4.9 ml of PBS (2% red blood cell solution). For blocking the antigen-antibody reaction, 0.1% bovine serum albumin was added. Each of the twelve wells of a 96 well microtiter plate was filled with 0.1 ml of PBS and added with Feline Coombs' reagent (Antiserum feline IgG, IgM and C3 from VMRD Coombs' test, USA). Then, the solution in the 1st well was properly mixed and 0.1 ml was transferred into the next well. This step

was repeated until well number 11 (two fold dilution). This made a serial dilution from 1:1, 1:2... 1:1024. The negative control was in the well number 12 (PBS only). Two percentage of red blood cell solution was added to each well, which was incubated at 37°C for 30 min and then kept at room temperature for 30 min. Result interpretation was evaluated by a negative result containing a button of RBCs that would stream when the microtiter plate was tilted. A positive result exhibited a matte formation that did not stream when tilted. The results were reported as negative or positive IMHA. The highest dilution with a complete agglutination titer would be reported as part of all positive results.

**Flow cytometry test (Quigley et al., 2001):** Two percentage of red blood cell solution derived from Coombs' test sample was diluted with PBS at 1:1 ratio called 1% RBC solution. Fluorescein isothiocyanate (FITC)-labeled goat anti-cat IgG (heavy chain specific) (Serotec, USA) and FITC-labeled goat anti-cat IgM (m chain specific) (Serotec, USA) were prepared at the dilution 1:30. Fifty  $\mu$ l of the prepared sample were mixed with 50  $\mu$ l (FITC)-labeled goat anti-cat IgG (polyclonal) and/or (FITC)-labeled goat anti-cat IgM (polyclonal) and incubated at 4°C in the dark for 45 min. The RBCs were washed twice and resuspended in 0.2 ml of isotonic PBS with 0.1% bovine serum albumin and 0.1 ml of 10% buffered formalin. Samples were analyzed by flow cytometry (BD FACSCaliburTM, USA). Forward and side (orthogonal) scattering signals and fluorescein-generated fluorescence (488 nm) signals were collected in list mode. Specific gates were set to identify red blood cells. For each sample, the median fluorescence channel was recorded for 10,000 red cells. BD CellQuest Pro software was used for the calculation of mean fluorescence intensity (MFI). The MFI values from ten clinically normal client-owned cats were used to establish a reference interval. For the clinically normal cats, a test sample was considered positive if the MFI was more than 2 SD above the mean and was considered negative if the MFI fell below this value.

**Table 1** Comparison of the packed red blood cell volume, corrected reticulocytes percentage, red blood cell, white blood cell and platelet in the clinically normal, IMHA and non-IMHA cats.

Parameter	Reference Ranges	Clinically normal Group (n=10)	Anemic group	
			IMHA (n=5)	NON-IMHA (n=14)
Red blood cell (cell/ $\mu$ l)	6.56-11.20x10 <sup>6</sup>	6.7 $\pm$ 0.98x10 <sup>6</sup>	2.84 $\pm$ 1.25x10 <sup>6</sup> aa	3.29 $\pm$ 1.73x10 <sup>6</sup> bb
PCV (%)	28-45	41.30 $\pm$ 6.02	17.20 $\pm$ 5.80 aa	16.93 $\pm$ 6.10 bb
Corrected reticulocyte (%)	0-0.4	0.08 $\pm$ 0.11	0.24 $\pm$ 0.32	0.34 $\pm$ 0.50
Platelet count (cell/ $\mu$ l)	1.75-5.00x10 <sup>5</sup>	2.59 $\pm$ 1.38x10 <sup>5</sup>	0.84 $\pm$ 0.46x10 <sup>5</sup> a	1.12 $\pm$ 1.10x10 <sup>5</sup> b
White blood cell (cell/ $\mu$ l)	4.04-18.70x10 <sup>3</sup>	10.72 $\pm$ 3.40x10 <sup>3</sup>	15.16 $\pm$ 8,37x10 <sup>3</sup>	13.82 $\pm$ 12.00x10 <sup>3</sup>

Note a:  $p < 0.05$  when compared between controls and IMHA cats, b:  $p < 0.05$  when compared between non IMHA and control cats, aa:  $p < 0.01$  when compared between controls and IMHA cats, bb:  $p < 0.01$  when compared between non IMHA and control cats, IMHA: Immune-mediated hemolytic anemia, Non-IMHA: Non-immune mediated hemolytic anemia

**Data analysis:** The sensitivity of the tests were defined by the number of cats with IMHA that tested positive with gold standard (DAT) and specificity was defined by the number of healthy cats that tested negative with gold standard (DAT). Positive predictive value was defined by the number of cats that tested positive that had IMHA, and negative predictive value was defined by the number of cats that tested negative that did not have IMHA.

Variables (means of PCV, red blood cell count, corrected reticulocytes percentage, platelet count, white blood cell count, total protein, ALT, ALP, BUN, creatinine, MFI of IgG and IgM) were compared among groups of the clinically normal, non-IMHA and IMHA cats with ANOVA. Ninety-five percent confidence intervals (95% CI) were reported along with sensitivity, specificity, positive/negative predictive values, and ANOVA.

### Results

The direct flow cytometry erythrocyte immunofluorescence assay and Coombs' test were applied to detect immunoglobulin bounded with red blood cells. Blood samples from ten clinically normal client-owned cats were used as the controls (PCV 33-52 %, mean 41.30±6.02%, median 42%, red blood cell count 5.50-8.66×10<sup>6</sup> cell/μl, mean 6.75±0.98×10<sup>6</sup> cell/μl, median 6.65×10<sup>6</sup> cell/μl). Nineteen anemic cats with results from the whole blood (EDTA) test (PCV 8-26%, mean 17.00±5.86%, median 17%, red blood cell count 1.0-6.07×10<sup>6</sup> cell/μl, mean 3.17±1.60×10<sup>6</sup> cell/μl, median 3.50×10<sup>6</sup> cell/μl) were clinically diagnosed as IMHA over a study period of 11 months. The breeds of the nineteen anemic cats consisted of one Siamese, one Persian and seventeen domestic short hair cats. These nineteen cats had previously suffered from viral diseases and other symptoms.

The IMHA positive cats had the RBC range

of 1.48-4.0×10<sup>6</sup> cell/μl, mean 2.84±1.25×10<sup>6</sup> cell/μl, median 3.55×10<sup>6</sup> cell/μl and PCV range between 12-26%, mean 17.20±5.80%, median 17% with evidence of regeneration in only one cat. Thrombocytopenia (mean platelet value was 0.84±0.46×10<sup>5</sup> cell/μl) was found in all cats with the IMHA ranging from 49,000 to 148,000 cell/μl (median, 53,000 cell/μl) (Table 1). The IMHA cats consisted of two FeLV infected cats, two FIV infected cats, one cat with FeLV and FIP co-infection (Table 1). The mean age of the cats with IMHA was 4.4 years old.

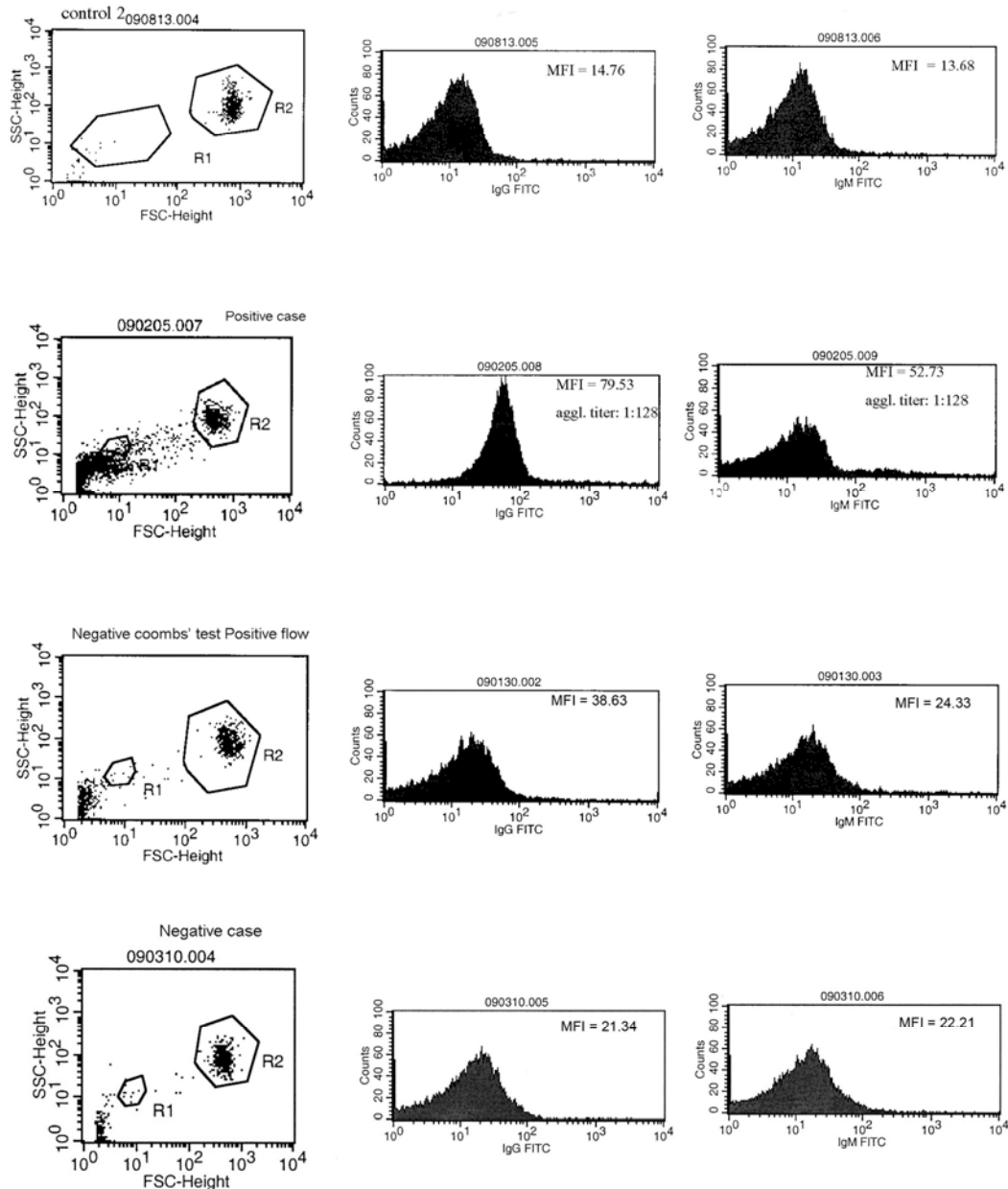
The PCV from the clinically normal cats, the IMHA and the anemic group were 41.30±6.02%, 17.20±5.80% and 16.93±6.10%, respectively (Table 1). The PCV and red blood cell count were significantly different between the clinically normal and the nineteen anemic cats ( $p<0.01$ ). Regenerative anemia was found in 20% of the IMHA cats (corrected reticulocytes count 0-0.8%, mean 0.24±0.32%, median 0.13%). The results showed lack of aggregated reticulocytes but found many punctuate reticulocytes which confirmed that the regenerative phase of IMHA had passed for 14-21 days. All of the IMHA cats had thrombocytopenia (platelet count 0.49-1.48×10<sup>5</sup>cell/μl, mean 0.84±0.46×10<sup>5</sup> cell/μl, median 0.53×10<sup>5</sup>cell/μl). Platelet numbers were significantly different between the clinically normal and the anemic cats ( $p<0.05$ ). The white blood cell counts (total white blood cell count 6,000-24,500 cell/μl, mean 15,160±8,371 cell/μl, median 18,700 cell/μl) were unremarkable.

Blood chemistry values were unremarkable in cats with IMHA (ALT 25-98 U/l, mean 45.34±30.11 U/l, median 33 U/l, ALP 23.7-66 U/l, mean 44.85±29.91, median 44.85, BUN 14.2-31.4 mg/dl, mean 22.8±12.16 mg/dl, median 22.8 mg/dl, creatinine 0.7-1.74 mg/dl, mean 1.22±0.39 mg/dl, median 1.30 mg/dl). However, an abnormal blood morphology was observed in the cats with IMHA including stomatocytes, schistocytes, poikilocytosis, anisocytosis, polychromasia, and hypochromasia (this

**Table 2** Comparison of the results of auto-agglutination test, Coombs' test and mean fluorescence intensity (MFI) from the flow cytometry in the clinically normal, IMHA and non-IMHA cats

Parameter	Clinically normal group (n=10)	Anemic group (n=19)		
		IMHA (n=5)	NON-IMHA (n=14)	
Auto agglutination	0%	0%	7.69% (1/13)	
Coombs' Test	0%	100%	0%	
Flow cytometry (MFI)	IgG	19.20±4.00	61.01±13.98 <sup>aa</sup>	25.84±7.22 <sup>a</sup>
	Cut point	27.20		
	IgM	17.87±3.82	47.90±6.69 <sup>aa</sup>	22.72±6.51 <sup>a</sup>
	Cut point	25.51		

Note a:  $p<0.05$  when compared between the clinically normal and the non-IMHA cats, aa:  $p<0.01$  when compared between the clinically normal cats and the IMHA cats, MFI: Mean fluorescence intensity, IgG: Immunoglobulin-G, IMHA: Immune-mediated hemolytic anemia, IgM: Immunoglobulin-M, Non-IMHA: Non-immune mediated hemolytic anemia, Cut point: Mean+2SD of the clinically normal cats



**Figure 1** Flow cytometry evaluation of RBCs from healthy, non IMHA and IMHA cats after staining with IgG and IgM-specific secondary reagent. FSC/SSC dot-plots and fluorescence histogram are shown for one healthy control cat, one non IMHA anemic cat and five IMHA anemic cats after incubation with goat anti-cat IgG (1:30) and goat anti-cat IgM (1:30). Histogram of fluorescence profiles were generated after gating on RBC in FSC/SSC dot-plots (region R2). Mean fluorescence intensities (MFI) are noted within each histogram; in anemic cats agglutination titer (AT) is also shown.

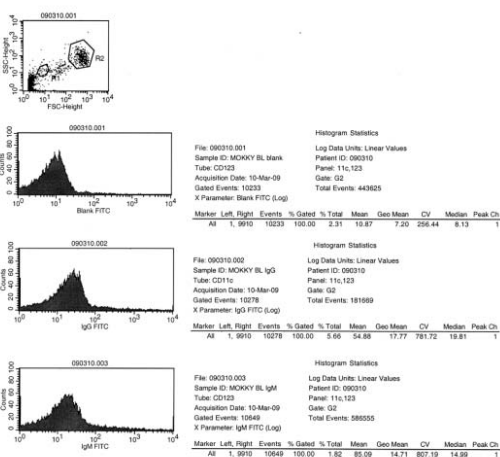
is normally found in felidae spp). Autoagglutination was negative in all five IMHA cats and positive in one of the fourteen non-IMHA anemic cats (7.14%) (Table 2). The DAT was positive in five IMHA cats and had the titer ranging from 1:1 to 1:128 in IMHA group. All of the non-IMHA cats were negative for DAT. Due to the low sensitivity of the DAT test, negative results were not expected to be truly negative.

The results of the FC method demonstrated that FC could detect IgG and IgM on the red blood cells (Fig 1). Total positive results of IMHA by flow cytometry were twelve out of nineteen anemic cats. All of the IMHA cats were positive by presence of both IgG and IgM on the red blood cell surface (MFI of IgG 43.46-79.53, mean 61.01±13.98, median 57.25,

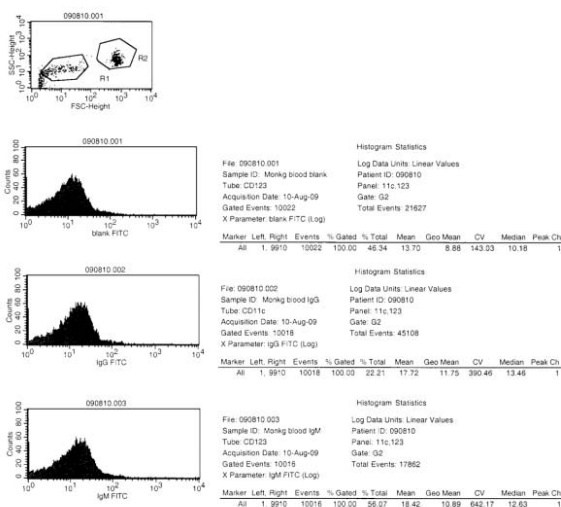
MFI of IgM 37.92-54.88, mean 47.90±6.69, median 48.71). After staining with (FITC)-labeled goat anti-cat IgG and IgM, MFI in the IMHA cats was more than mean+2SD of the clinically normal cats (cut point). The cut off point was 27.20 for IgG and 25.51 for IgM (Table 2). The difference between staining intensities of the IMHA group and the clinically normal group was statistically significant ( $p<0.01$ ) and also different between the IMHA group and the non IMHA ( $p<0.01$ ).

The sensitivity and specificity of the FC method was 100% and 50% compared with the DAT method. The positive predictive value was 41.66% and the negative predictive value was 100%. Three of the IMHA cats survived until the end of the study.

## Before treatment



## After treatment



**Figure 2** Comparison of flow cytometry results before and after treatment of the IMHA cat. MFI of IgG and IgM reduced from 54.99, 85.09 to 17.72 and 18.42 in 5 months of treatment.

One of the IMHA cats which were positive for FeLV infection (cat No. 5) was treated with prednisolone (2 mg/kg) and recovered from IMHA. The MFI of IgG and IgM of two cats with positive IMHA reduced from 54.99 and 85.09 on the first day of diagnosis to 17.72 and 18.42 after one month of treatment (Fig 2).

### Discussion

The results of this study demonstrated that cats with IMHA are sero-positive for FeLV or FIV. As in previous studies, most IMHA cases in cats were secondary IMHA (Day, 1996; Gunn-Moore et al., 1999). FeLV was reported to be the most common underlying cause in IMHA cats. Non self antigen from FeLV and FIV infection may coat the red blood cell surface and cause the immune system to attack the red blood cells. This is in contrast to canine patients where the primary IMHA is more commonly found than the secondary IMHA. An estimation of the primary canine IMHA is 60-75% (Piek et al., 2008). Feline IMHA has been reported in young male cats (McCullough, 2003) but the results of this study demonstrated neither age, gender nor breeds preferences for the feline IMHA.

Not only RBCs but also platelets decreased in the IMHA cats. This may be due to an immune system which not only attacks red blood cells but also causes damage to the platelet number. These results found that autoagglutination were negative in all IMHA cats and positive in one non-IMHA cat. This may suggest that autoagglutination is not a good screening test for feline IMHA due to the low sensitivity (0%).

The Coombs' test (DAT method) could detect five IMHA cats from nineteen anemic cats. Previous studies showed that the DAT test had low sensitivity due to the loss of antigens on red blood cell

surface during the red blood cells washing procedure (high false negative), but high specificity (true negative) (Quigley et al., 2001; Kucinskiene et al., 2005). Even though the fourteen anemic cats were negative for IMHA by DAT, the FC method could detect IgG and IgM on the red blood cells on seven out of fourteen anemic cats. These seven cats were classified as being in the early stage of IMHA and serial testing by the FC method were recommended. The FC method demonstrated a higher detection rate than the Coombs' test when the cut off point was set at mean+2SD of the clinically normal cats. All of the cats with MFI with more than 40 of IgG and 37 of IgM were suspected of having IMHA by the FC method. One important benefit of using MFI from the FC method is for monitoring the treatment response in cats with IMHA. IMHA cats has decreased MFI value by the FC method and better clinical sign after receiving prednisolone treatment.

Another main advantage of using the FC method in detecting IMHA in cats is that it needs much less blood samples than the DAT method. It is more cost effective than the DAT method due to the amount of anti-antigen used in each test. The results demonstrated that flow cytometry is a good early diagnostic tool for cats with suspected feline IMHA and can be used for monitoring patients. The only disadvantage of FC method is the availability of flow cytometry in only research facilities due to the high cost.

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