Original Research Article

THE CYTOLOGY OF RESTING AND REACTIVE NK CELLS OF CHICKENS

ABSTRACT

Lymphoid cells are composed of 3 distinct series; those developing in the bursa of Fabricius are B-cells, and those of the thymus are T-cells. The former concern humoral immunity and the latter cell-mediated immunity (CMI). A third lineage, the natural killer cells (**NK**) develop independently. A study of chicken blood films stained with Wright-Giemsa obtained from a Marek's disease project provided an opportunity to study NK cells. These lymphoid cells were recognized because of their characteristic cytoplasmic granules. NK size varied from small cells similar in radius (**R_C**) to resting T-cells (R_C ~ 3 µm) to larger types (R_C ~ 5 µm). Granules also varied in size and number. NKs were often in fields also containing bacteria either free swimming or attached to RBC or other cells. The total white cell count (**TWBC**) of the study population ranged from leukopenia (very low) to leukemoid reaction (very high) levels (TWBC 5K/µL - 200K/µL; ave. ~50K/µL. NKs comprised 380/45134 (~ 1.7 %) of all leukocytes. The study population heterophil/lymphocyte ratio (**H/L**) ave. (~ 0.4) appears to give a false impression of a low stress status. It is concluded as would be the case for plasmacytes or other forms of reactive cells, multiple forms of NK cells appearing in a hemogram indicate inflammation or infection. Therefore, the simple H/L ratio is an inappropriate stress indicator when accompanied by a high TWBC.

Comment [C21]: Write briefly in one sentence.

Key words: NK cell, lymphocyte, stress, H/L ratio

Abbreviations: cm cell membrane, CMI cell-mediated immunity, CD cluster of differentiation, BCR B cell receptor, TCR T cell receptor, IFN interferon, H/L heterophil/lymphocyte ratio, L_S/L_M small and medium lymphoid cells, NK natural killer cell, Ls/NK small NK cell, Lm/NK medium and large NK cell, MD Marek's disease, MDV Marek's disease virus, R_C cell radius, PR ploidy ratio,

INTRODUCTION

Lymphoid cells are composed of 3 distinct series; those developing in the bursa of Fabricius are B-cells, and those of the thymus are T-cells. The former concern humoral immunity and the latter are concerned with cell-mediated immunity (CMI). A third lineage, the natural killer cells (NK) develop independently (Lam and Linna, 1979). Chicken NK cells of the spleen and intestinal epithelium express CD8 while lacking BCR and TCR. NK cells of chicken blood express Fc receptors, CD3, low levels of CD4, and are capable of releasing IFNγ (Neulen, et al, 2015). Functionally, NK cells are innate immune cells capable of destroying virally infected or transformed cells (Straub, et al 2013).

Marek's disease, an important cause of morbidity and mortality of chickens, is caused by infection with a ~~is a~~ highly contagious alphaherpesvirus (MDV). T-cell tumors are a hallmark of MDV infection. Resistance to MD can depend on NK cell activity (Sharma, 1981). Recently it has been shown *in vitro* that NK cells are readily infected by MDV (Bertzbach, et al. 2019). The same authors demonstrated an increased expression of CD107 of a small portion of MDV infected NK cells and hypothesized that successful infection of NK cells is a consequence of their activation.

Thus, NK cells, like other lymphocytes, may become activated under an appropriate stimulus. For example, members of the B-cell derived plasmacyte series have been shown to display an array of forms described as "reactive plasmacytosis" (Cotter, 2022a). These were found in the blood and bone marrow of lame ducklings from which *Streptococcus* and *E. coli* were isolated.

Here the purpose is to describe an array of NK cells in experimental chickens from a study of Marek's disease. Blood smears from four groups, controls, vaccinated, challenged, and both vaccinated and challenged were studied. For the purpose of this description, NK cells are defined as lymphocytes containing cytoplasmic azurophilic granules detected with light microscopy in Wright-Giemsa stained samples. The presumption is that azurophilic granules correspond to the electron-dense granules of NK cells detected by electron microscopy (Gobel, et al 2015). Active NK cells are recognized by cytology as as well as CD expression. When activated NK cells are in hemograms they indicate stress, inflammation, and disease.

MATERIALS AND METHODS

Experimental Chickens

The specific pathogen-free chickens (**SPF**) in this study were from two highly inbred lines of chickens. Line 6.3 is MD-resistant and line 7.2 is MD-susceptible. The study chickens were from unvaccinated breeder hens and carried no maternal antibodies to MDV or herpesvirus of turkeys.

Study Location

The chicks were housed in modified Horsfall-Bauer isolation units located at the ADOL (USDA) Laboratory, 3606 E Mt Hope Rd., East Lansing, MI 48823 (USA).

Blood Samples

Wing-vein blood was drawn at day of age 19, 24, 34, and 49. Vaccination (Rispens) was on day 7 and MD challenge (rMd5, a virulent strain) was on day 14. Slides were prepared immediately, air-dried, and post-fixed in 100% MeOH. Staining was done with Wright-Giemsa.

Standard Differential Count

Two counts of 200 leukocytes/slide were sorted using criteria as described by Lucas and Jamroz (1961) and Cotter (2015a). The designation "typical heterophil" (HT) as used here was assigned to the most frequent type seen in earlier studies (Cotter, 2015a, b, c). Classic heterophils (HC) resemble those most often illustrated in the literature. Rare variant heterophils (HV) are distinct from both HT and HV, Cotter and Heller (2016). Ls and Lm are small and medium sized lymphocytes. Total white blood counts (TWBC) were determined by a modified microscopic method as described in Campbell and Ellis (p.26, 2007). Standard Differential Count (SDC) was determined at 40x magnification.

Heterophil/Lymphocyte Ratios (H/L 1; H/L 2) and Total White Blood Count (TWBC)

Two counts of 200 leukocytes gave the total white count (TWBC) by the method of Campbell and Ellis (2007) and 2 heterophil/- lymphocyte ratios. H/L 1 = (HT + HC +HV) / Ls; and H/L 2 = (HT + HC + HV) / (Ls + Lm).

Light Microscopy and Photomicrographs

Olympus CX-41(Olympus America, Center Valley, PA 18034-0610) equipped with Plan N 40x, 0.65 numerical aperture dry, and Plan N, 1.25 numerical aperture 100x oil objectives. All images were captured at 100x with an Infinity-2 1.4-megapixel charge-coupled device Universal Serial Bus 2.0 Camera, and processed with Infinity Analyze software that was also used for pixelation (Release 6.5) (Lumenera, Inc., Ottawa, ON, Canada)

Welfare

These experiments were approved in accordance with the guidelines set forth by the ADOL Institutional Animal Care and Use Committee and the Guidelines for Care and Use of Laboratory Animals published by the Institute for Laboratory Animal Research.

RESULTS

The strategy for presentation will begin with examples of small and medium size lymphocytes as companions to NK cells. Some cytological features of NK cells are shown with the aid of differential pixelation. Descriptions of reactive NK cells will follow. The SDCs for the study figures are given as % in Table 1.

NK Cells of Non-Vaccinated Non-Challenged (NV/NC) Controls

Insert figure 1 A here; figure 1B here

Figure 1A. shows 2 examples of NK cells in a NV/NC control chicken of the susceptible line (7.2) obtained at 49d. The small cell (*Ls/NK) at the lower left is likely a resting NK; its few small azurophilic granules are restricted to the cytoplasm immediately internal to the cm. The dimensions [A_C ~ 48 µm^2; A_N ~ 30 µm^2] represent diploid conditions [PR 1.0], and the N/C of 0.6 represents an early reactive cell. The cytoplasm located immediately outside of the slightly indented (reniform) nucleus is clearer than the cytoplasm near the cm edge suggesting a Hof (Golgi). The patchy chromatin is typical of an NK at this stage of activation. The larger cell (Lm/NK) at the top right [A_C ~ 67 µm^2] is at a further reactive stage, and the slightly larger eccentric nucleus [A_N ~ 34 µm^2] is also diploid [PR 1.1]. The greater number and size of the cytoplasmic granules is clear evidence of heightened reactivity. As is the further elaboration of the ER. The chromatin is patchy, and a few azurophilic granules are located directly over the nucleus. Free swimming and cell-associated bacteria (CAB) are encircled and a *Hemomycetes avium* (Ha) shard (mycelium fragment) (Cotter, 2015c, 2022c) is located at the arrow. Although a study control, this chicken appears to be infected with both bacteria and a fungus. The smear in which these cells were found was “thin” an indication of rheological changes in plasma were in progress when the blood was drawn. Thus, the TWBC was estimated at 37K/µL and H/L ratios (H/L 1 0.23; H/L 2 0.19) were affected by plasma quality.

Cytoplasmic features of the small *Ls/NK cell of Figure 1A are enhanced by differential pixelation in Figure 1B. Cytoplasmic and nuclear granule locations are enhanced with fuchsia pixels (panel B; vacuole locations are in yellow (panel C). The reactive Lm/NK is enlarged without pixelation in Panel C, and its vacuoles' locations are enhanced by red pixels in panel D.

A Lm/NK found in an NV/NC of the susceptible line (7.2) at 49d cell is in Figure 2A. It has an eccentric nucleus and characteristic azure cytoplasmic granules and clear vacuoles. A Ls in the same field is likely a B-cell and is diploid because of the dimensions [A_C ~ 53 μm^2; A_N ~ 41 μm^2] and ploidy [PR 1.3]. The patchy chromatin arrangement of the Ls contrasts with the finer chromatin of the Lm/NK whose dimensions [A_C ~ 113 μm^2; A_N ~ 55 μm^2] and ploidy [PR 1.8] represent a > diploid condition (2N+). About 20 large granules, ~10 small granules, and ~6 clear vacuoles are distributed throughout the cytoplasm of the Lm/NK, and a few are located directly over the nucleus. A group of 3 bacteria and separate encapsulated bacteria are located by arrows. The TWBC of this sample, at 50K/µL, indicates (mild) leukocytosis. In contrast the H/L 1 (~0.2) and H/L 2 (~0.15) suggest homeostasis.

Insert Figure 2A, B here

A Lm/NK with an eccentric nucleus and condensed patchy chromatin is from a resistant line (6.3) at 27d is in Figure 2B. The cytoplasm contains many clear vacuoles and others with a faint pink substance; possibly incomplete or degraded perforin. A few magenta granules are at the cm edge (arrow). A group of 3 encapsulated bacteria is circled. Several of the RBCs are not fully hemoglobinized and are irregularly shaped (pRBC). A solitary thrombocyte (Th) containing 6 specific granules a reactive cell (Cotter, 2022c).

NK cells of NV/NC resistant chickens (Line 6.3) at 24d are in Figure 3. A Lm/NK cell with an eccentric nucleus and characteristic azure cytoplasmic granules and clear vacuoles is in a field with a Ls; likely a B-cell [A_C ~ 32 μm^2; A_N ~ 28 μm^2; PR 1.0]. The patchy chromatin arrangement of the Ls contrasts with the finer chromatin of the Lm/NK whose size [A_C ~ 66 μm^2; A_N ~ 36 μm^2] and ploidy ratio [PR 1.3] represent a diploid condition (2N). About 20 large granules, ~ 10 small granules, and ~6 clear vacuoles are distributed throughout the cytoplasm of the Lm/NK, and a few are located directly over the nucleus. A group of 3 bacteria and separate encapsulated bacteria are located by arrows. The TWBC of this sample, at 50K/µL, indicates (mild) leukocytosis.

Enhancement of the cytoplasmic features of a reactive Lm/NK of panel A by differential pixelation is in Figure 3B and C. Cytoplasmic and nuclear granule locations are enhanced with fuchsia pixels (panel B) vacuole locations are in yellow pixels (panel C).

Insert figure 3 here

An array of NK variants of the MD resistant line (6.3) is in Figure 4. Panel A. A large granular Lm/NK has a half-moon eccentric nucleus with patchy chromatin. It contains many magenta granules and has clear cytoplasmic vacuoles. The ruffled cm (zeiosis) indicates heightened reactivity. Its overall dimensions suggest polyploidy [A_C ~ 99μm^2, A_N ~ 43μm^2, N/C 0.4, PR 1.7]. Panel B. A small Ls/NK with a patchy nucleus [A_C ~ 67μm^2, A_N ~ 41μm^2, N/C 0.6, PR 1.3] is diploid at an early reactive state. A Ls (T-cell) is at the left of Panel C whose dimensions [A_C ~ 36μm^2, A_N ~ 30μm^2, N/C 0.8] are of a resting diploid cell. 3 NK cells at various reactive states are in panel D. The NK cells appear somewhat distorted possibly due

to damage from toxins released by many small encapsulated bacteria located throughout the field. Some bacteria have formed clusters (arrows).

Insert Figure 4 here

A Lm/NK with an eccentric nucleus with patchy chromatin is in Figure 5, Panel A. The cytoplasm contains granules and clear vacuoles. The dimensions [A_C ~ 121µm^2, A_N ~ 64µm^2, N/C 0.5, PR 2.1] indicate a tetraploid cell. A pair of toxic heterophils (HT) and reactive thrombocytes (Th) are at the lower right (Cotter, 2022). A smaller Ls/NK cell in a field with bacteria (arrows) is in panel B. The dimensions [A_C ~ 69µm^2, A_N ~ 34µm^2, N/C 0.5, PR 1.1] indicate a diploid reactive type. A giant NK cell with a reniform nucleus is surrounded by cytoplasm differentiated into an endoplasmic zone immediately surrounding the nucleus and a deeper stained ectoplasmic zone. An accumulation of clear vacuoles at the edge of the nucleus suggests the formation of a Hof (Golgi) an indication of a secretory potential. Magenta granules are also scattered throughout the cytoplasm. The giant cell dimensions [A_C ~ 113µm^2, A_N ~ 43 µm^2, N/C 0.4, PR 1.7] indicate a tetraploid. A solitary Lm/NK with an irregular cm is in a field containing many small encapsulated bacteria enhanced by fuchsia pixelation. Some of the bacteria have attached to the deep folds of the cm also fuchsia enhanced. The giant cell dimensions [A_C ~ 85µm^2, A_N ~ 34µm^2, N/C 0.5, PR 1.1] indicate a diploid. A few bacteria have attached to cytoplasmic streams emanating from the cm (arrows).

Insert Figure 5 here

Hemomycetes avium (Ha)

The fungus *Hemomycetes avium* (Ha) was initially described in a blood smear from a laying hen (LSL 56 wk) housed in an enriched cage whose TWBC was in the leukemoid reaction range (~ 200 K/µL) with low (~ 0.25) H/L ratios (Cotter, 2015c). Bacteria and atypical leukocytes were also observed in the same sample. A more detailed description of the microscopic characteristics of Ha appears in (Cotter 2021). This fungus was detected at all sample dates and both chicken lines in blood smears from the current study. 3 (Line 6.3) examples seen in blood and 1 bone marrow observation (Line 7.2) are in Figure 6. Bacteria also were commonly observed in the same fields as Ha.

Insert Figure 6 here

DISCUSSION

Control of Marek's disease remains an important concern to the poultry industry despite the availability of vaccines for nearly a half-century. Certain chickens are resistant others are susceptible as a result of their peculiar genetics. It has long been established that the expression of genes at the major histocompatibility locus (B-complex) contributes to differential resistance (Bacon and Witter, 1993). Early *in vitro* studies implicated the role of NK cells in MD resistance (Sharma and Okazaki, 1981). NK cells are known for anti-viral and anti-tumor activity by directly killing target cells through the release of several cytokines. Therefore, the study of NK cell features should be of interest to Marek's disease investigators and those concerned with other infectious diseases. The occurrence of NK cells along with bacteria (Figures 1, 2, 4, 5) and fungi (Figure 6) reinforces this notion.

Here the variety of NK cells is demonstrated. NK forms differ in size and cytoplasmic condition. It is presumed that some of the variations described here represent the transition from small resting

cells with few granules to larger reactive types with more granules and vacuoles. Some NKs may be phagocytic (Figure 5D) but this possibility needs further study

It is also demonstrated that the reniform nuclear shape and patchy chromatin of some NK's coupled with granulation aids in their differentiation from other lymphocytes, especially plasmacytes. Even Mott cells, atypical plasmacytes, whose cytoplasm is filled with immune globulin (Ig) containing vacuoles should not be confused with NK's (Cotter, 2022; Cotter and Bakst, 2017).

NKs are typically rare in the blood (Straub et al. 2013) here they represented ~ 1.7% of all leukocytes in this study (380/~45,000). They were as frequent in control chickens as in the other groups. A maximum of 19 NKs were in 1 blood smear but no NKs were found in ~ 46% (127/277) of the SDCs obviating the ability to associate NK forms with individual treatments. Therefore, the focus is placed on a description of variation in NK cytology.

The occurrence of the fungus *Hemomycetes avium* (Ha) is a remarkable feature of these samples. Ha in blood and bone marrow is a clear sign of immunosuppression. Surprisingly Ha and bacteria occurred in control samples as well as vaccinated or challenged chickens. This observation suggests the possibility of superinfection with an unidentified virus. The nature of the responsible agent remains unknown.

An indirect objective of this study is to suggest that when diverse NK forms occur in a hemogram they should be given similar weight as other atypia in deciding the appropriateness of a simple H/L ratio as a stress determinant (Cotter, 2015a, 2022c). This aspect is clearly indicated when reactive NKs occur simultaneously with low H/Ls. It is also obvious that when low H/Ls are accompanied by high TWBC the stress question had already been decided.

Figure Legends:

Figure 1A. Examples of NK cells in control chickens. Additional descriptions are in the text.

Figure 1B. Enhancement of the cytoplasmic features of a reactive Lm/NK of panel A by differential pixelation.

Figure 2A. A reactive NK in a field with a small lymphocyte (Ls) in a control 49 d MD susceptible chick. Panel B. A large reactive NK with small faint cytoplasmic granules from the MD resistant line at 24 d.

Figure 3. NK cells of NV/NC resistant chickens (Line 6.3) at 24d.

Figure 4. Examples of NK cells from MD the resistant (Line 6.3) at 5d post MD challenge (Panels A, B) and 34 d post-challenge (Panels C, D).

Figure 5. Examples of NK cells from MD susceptible from a single chick of line 7.2 at 22d post MD challenge (A, B).

Figure 6. Examples of the fungus *Hemomycetes avium* (Ha) in 3 blood films (Panels A, B, C) and 1 bone marrow sample from the current study (D). Bacteria are located by arrows.

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DECLARATION

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Table 1. Standard differential counts (% SDC) based on 2 x 200 total cells for samples of Figures 1- 6

Figure	HT[1]	HV	HC	Ls	Lm	NK	Bst	Mn	Ba	Eo	TWBC(K)	H/L 1	H/L 2	ΔH/L
1	6.8	1.3	10.6	62.8	13.0	0.2	0.4	0.0	4.8	0.0	38.0	0.3	0.3	0.1
2	6.1	6.1	0.0	62.3	19.1	0.2	0.0	0.2	5.9	0.0	50.0	0.2	0.2	0.0
3	5.8	0.2	1.2	74.3	10.8	0.0	0.2	0.7	6.7	0.0	20.0	0.1	0.1	0.0
4A	5.1	0.0	1.9	71.0	18.1	0.0	0.0	0.5	3.4	0.0	15.0	0.1	0.1	0.0
4C	14.3	0.0	0.5	51.7	25.1	0.0	0.0	0.0	8.4	0.0	20.0	0.3	0.2	0.1
4D	39.4	0.2	1.2	40.8	15.5	0.0	1.0	0.2	1.7	0.0	40.0	1.1	0.7	0.3
5A, B	22.7	1.2	0.2	52.7	16.9	1.7	1.0	0.0	3.6	0.0	73.0	0.5	0.3	0.1
5C	9.0	0.0	19.1	54.5	13.1	1.3	1.5	0.2	1.3	0.0	100.0	0.5	0.4	0.1
5D	22.7	1.2	0.2	52.7	16.9	1.7	1.0	0.0	3.6	0.0	76.0	0.5	0.4	0.1
6B	17.5	0.5	0.0	65.5	12.1	0.0	1.5	1.0	1.9	0.0	40.0	0.3	0.2	0.0
6C	5.2	2.0	0.5	77.8	7.9	0.2	0.2	0.5	5.7	0.0	40.0	0.3	0.2	0.0
4B, 6A	No SDC													
6D	Bone marrow													

[1]Abbreviations: Cells: H, heterophil (HC, classic, HV, variant, HT, typical) Ls small lymphocyte ~6 µm diameter, Lm medium lymphocyte ~8 µm diameter, Mn, monocyte, NK natural killer cell, Bst granuloblast, Ba, basophil, Eo, eosinophil. TWBC(K), total white blood cells per cubic mL in thousands (K). H/L 1 = (HC + HT + HV) / Ls; H/L 2 = (HC + HT + HV) / (Ls + Lm); ΔH/L= H/L1 – H/L2.

Figure 1 A

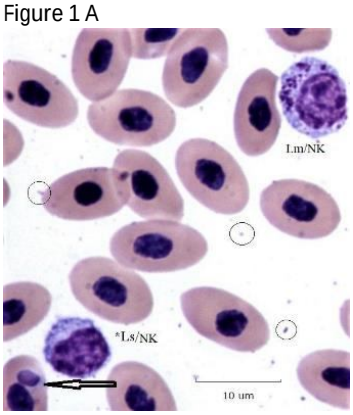


Figure 1 B

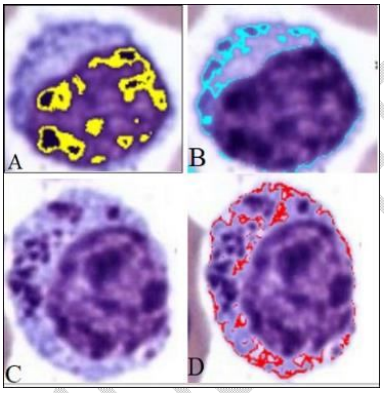
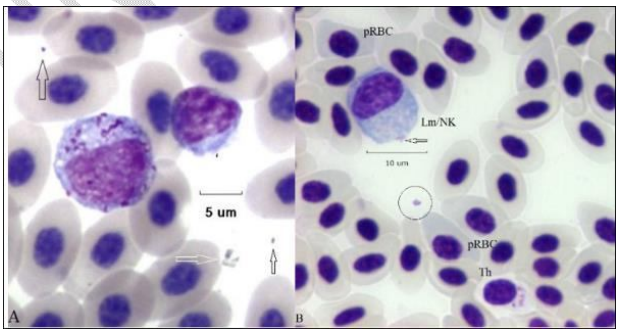


Figure 2A, B



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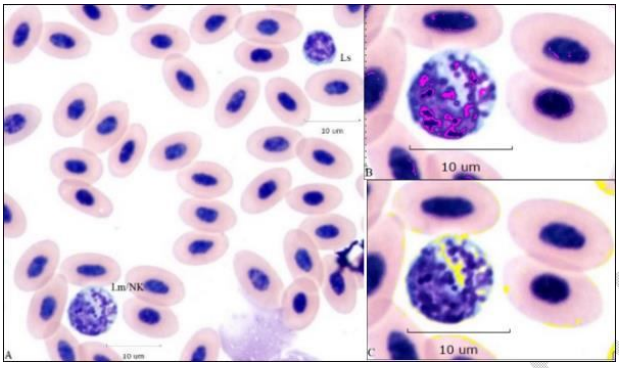

Figure 4.

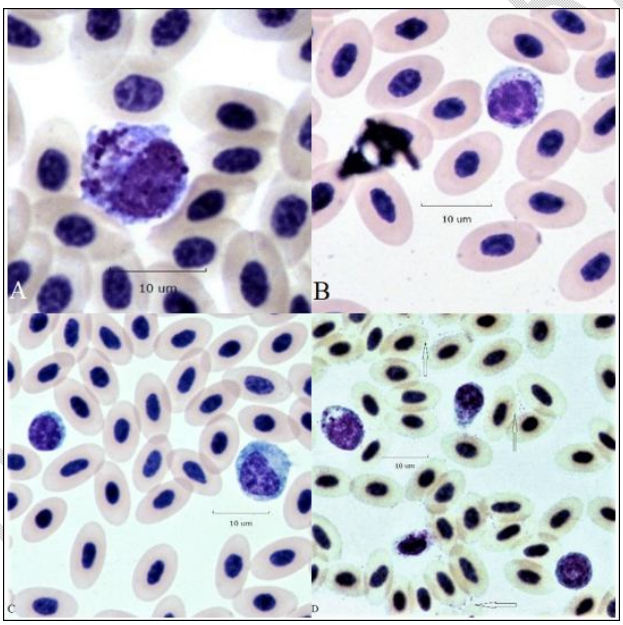

Figure 5

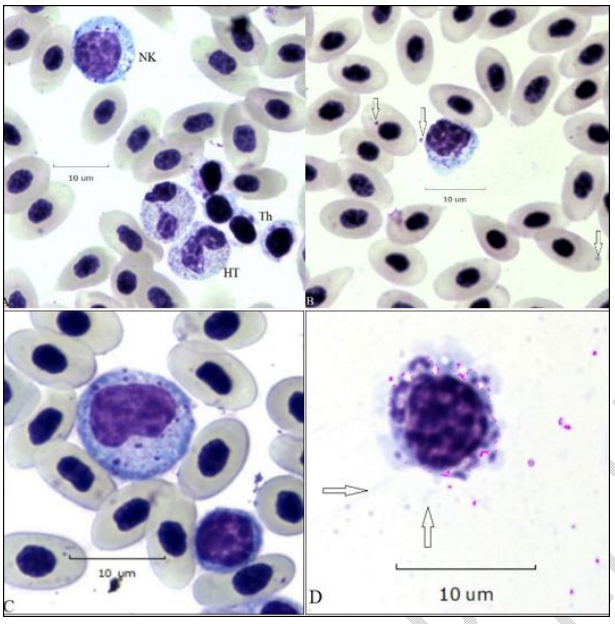

Figure 6