Type of Article: Original Research Article

Title: Aviary caged hens with unusual lymphocytes and normal H/L ratios

ABSTRACT

The aim is to describe hemograms of hens that bear upon interpretation of the traditional H/L stress determination method. Hemograms from aviary caged (AV) commercial hens sampled at 18 wk with low to moderate heterophil/lymphocyte ratios (H/L, range 0.02 – 0.66) were accompanied by slightly elevated total white cell counts (TWBC ~ 30K/µL) to leukocytosis (TWBC ~ 120K/µL) levels. Atypical cells were commonly seen in Wright-Giemsa stained blood by standard differential counts (SDC). Heterophils exhibited signs of toxicity affecting both the nucleus, the cytoplasmic granules, and cell membranes. Many forms of atypical lymphocytes were also detected. These were aggregated small lymphocytes (Ls), medium sized reactive lymphocytes (Lm) plasmacytes (PC) including Mott cells, and other atypical forms. Some PCs contained pink cytoplasmic vacuoles (Russell bodies) an indicating they are IgA types. Developmental PCs rarely found in the circulation of homeostatic avians were also present. Bacteria were commonly associated with the atypical cells. These were both free swimming and cell associated (CAB) types. Fungal forms were also present including yeast-like conidia and hyphae. When microorganisms were in a field the background erythrocytes were in Rouleaux formation, an indication of an inflammatory milieu. In conclusion, it is demonstrated that in the absence of detailed cytological descriptions the H/L ratio can be misleading. Given its wide usage in stress and welfare determinations these observations emphasize some of the difficulties of the simple H/L method. They reinforce earlier observations and draw attention to the necessity of cytological detail before the computed H/L ratio can be accepted as a stress measure.

Key words: commercial hens, heterophil lymphocyte ratio, atypical lymphocytes, plasmacytes

1. INTRODUCTION

Hemogram data derived from either hemocytometer or standard differential counts (**SDC**) are widely used to estimate stress. Attention is directed toward the ratio of heterophils to lymphocytes (**H/L**). The theory is based on a series of studies by Davidson and Rowell (1) and Gross and Siegel (2, 3, 4) who observed increased H/Ls in experimental chickens treated with corticosterone, exposed to social stress, or injected with bacteria. H/L values about 0.5 accompanied by total white blood counts (TWBC) about 30K/µL would indicate homeostasis. However, in many instances H/Ls are reported without detailed descriptions of the cells used for its computation or the total white blood count (**TWBC**) (5). A low H/L accompanied by a high TWBC cannot receive the same physiological interpretation as one with a normal TWBC (6,7).

As neither heterophils nor lymphocytes are homogeneous (7, 8, 9) it is crucial to define which are included in the H/L computation and which cells should be excluded. Heterophils of caged hens and ducks were sorted into 3 categories based on cytoplasmic granules and nuclear configuration. Typical heterophils (**HT**) with faint granules, the most frequent type, are distinct from classic heterophils (**HC**) with deep red spindle shaped granules. A third heterophil (variant type, HV) with orange spherical

granules also occurs (9,10,11) Therefore, it is possible that the same H/L value may be associated with distinct heterophil spectra.

Lymphocyte counts are used as the H/L denominator often without regard to their origin or if they are reactive. In an SDC from a homeostatic source most lymphocytes should be small resting (T-cell) types. Reactive types or plasmacytes are not suitable for use in the H/L. Therefore, the aim of this manuscript is to expand observations of leukocytes whose cytology would render the simple H/L an inappropriate stress measure. The source are commercial hens housed in aviaries and sampled at 18 wk. The information should be of interest to those who rely on the H/L computation to assess stress and establish welfare status.

2. MATERIALS AND METHODS

2.1 Chickens and Welfare

Chicks of a Lohmann White Egg commercial type (LSL) were housed in aviaries (**AV)** at 850 to 1,700 hens per compartment. They were vaccinated at the hatchery for laryngotracheitis and Marek's disease. They were managed according to a typical program designed for commercial poultry. The experimental protocol was approved by the Michigan State University Institutional Animal Care and Use Committee. A detailed description of the housing systems and management practices is found in Jones et al (12).

2.2 Blood, Stain Procedure, H/L and TWBC Computations

Blood samples were collected from brachial veins into EDTA tubes and stained later using Wright-Giemsa. Differential counts (SDC): a minimum of 200 leukocytes per slide were sorted into categories: small or medium lymphocytes, monocytes, heterophils, basophils, or eosinophils. Morphological criteria for sorting were as described in (9 -11). Division of the sum of all heterophil types (typical, variant, and classic) by the number of small "resting" lymphocytes is H/L 1. Division of the same heterophil value by the sum of all lymphocyte types, (resting, reactive, and atypical) is H/L 2. The difference, H/L 1 – H/L 2 is expresses as ΔH/L. The average number of white blood cells in five 40× microscopic fields multiplied by 4,000 provides the total white blood cell count (TWBC/ µL) estimate, after a modification of a method described by Campbell and Ellis (13).

2.3 Light Microscopy and Photomicrographs

An Olympus CX-41(Olympus America, Center Valley, PA) fitted with Plan N 40×, 0.65 Numerical Aperture dry, and Plan N, 1.25 N.A. 100× (oil) objectives. The images were captured by an Infinity2 1.4 Megapixel CCD USB 2.0 Camera and processed with Infinity Analyze software (Release 6.5, Lumenera Inc., Ottawa, Ontario, Canada). Magnification was 100× (oil).

3. RESULTS

Standard differential counts (%) total white cell counts (**TWBC**), and H/L ratios for the hens of Figures 1-5 and the average values for all hens in the study are in Table 1. For simplicity all heterophil types (typical, classic and variant) are combined into one category "H" and "Bst" represents a combination of granuloblasts (mesomyelocytes and metamyelocytes).

Insert Table 1 here.

As heterophils represent the numerator of the H/L computation examples of types seen in the source population are in Figure 1. The cells of both panels A and B are standard size band type (young) cells [HT, R_C 6.7 µm, A_C 141 µm^2] with a single lobe usually the youngest types. The cytoplasmic granules of each are poorly stained suggesting toxicity. The HT of panel C has 3 well-stained lobe are the youngest cell types but its cell membrane (**cm)** is irregular. The variant heterophil of panel D is smaller [HV, R_C 4.5 µm, A_C 64 µm^2]. It has 3 faint nuclear lobes and contains orange spherical cytoplasmic granules. Collectively each cell of figure 1 exhibits some form of atypia.

Insert Figure 1 here

Reactive lymphocytes and plasmacytes are given in Figure 2. Panel A. Binuclear bi/PCs with patchy cytoplasm, a cell type suggesting stress and possible viremia (14) and more traditional PCs with well developed Hofs (Golgi). Panel B. Reactive small lymphocytes (**Ls)** have formed multicellular aggregates. Panel C. Ls with scanty cytoplasm and larger reactive Lm cells with vacuolated cytoplasm; is likely a plasmacyte. Panel D. An atypical plasmacyte (*PC) with paracrystalline Russell bodies; an indication of a defective antibody. Either free or cell associated bacteria (CAB) are at the arrows of panels B, C, and D.

Insert Figure 2 here

Additional examples of reactive lymphocytes are in Figure 3. Panel A. a reactive lymphocyte (Lm) with plasmacyte cytoplasmic characteristics (Hof) is in a field with a Ls whose cm contains blebs (zeiosis) an indication of toxicity. Panel B. A second example of a plasmacytoid Lm in a field contrasts with a (resting) Ls (inset). The background RBC of both panels A and B are Rouleaux (adhering) forms indicate inflammation. Panel C. An Ls is adhering to a Lm/PC whose cytoplasm is fenestrated with small and large clear vacuoles also indicate inflammation. Remnants of 2 lysed nuclei (possibly RBC) are also present (lower right). Panel D. A solitary Lm/PC (right arrow) is attached to RBCs (left and down arrows).

Insert Figure 3 here.

Examples of atypical plasmacytes with pink vacuoles are in Figure 4. Panel A. Solitary PC with an irregular cm and cytoplasm with pink and orange vacuoles (Russell bodies) in a field with aggregated thrombocytes (Th) and RBCs (15). Panel B. Two mutually adhering cells are plasmacytes with pink Hofs (IgA flame cell types) and patchy cytoplasm. Panel C. A PC with a pink Hof is in a field with an elongated bacillus containing a terminal endospore (arrow).Panel D. A proplasmacyte (Türk cell) has a giant nucleolus. A pseudopod is extending from its cytoplasm is at the lower left. The inset is a 2.5x enlargement of a mixed species microcolony seen to the left of the Türk cell.

Insert Figure 4 here

Additional examples of reactive cells from the study flock are in Figure 5. Panel A. A large plasmacytoid cell (**Lm/PC**) is associated with mesomyelocytes; developmental cells of the granulocytic series. Small lymphocytes (Ls) and a lysed nucleus of uncertain origin are nearby. Panel B. A pair of monocytoid cells (Mn) with reactive cytoplasmic characteristics. Panel C. A (mixed) bacterial microcolony composed of bacilli of varying lengths that are attached to a RBC, itself attached to another RBC. The predominantly magenta color of the bacteria suggests these are (Gram negative) Mollicutes, bacteria with thin cell walls. Panel D. Conidia at different stages of development are attached to a Lm.

Insert Figure 5 here

The scatter plot of H/L ratios suggest low stress for the AV hens of the flock (Figure 6). The large data points are from the hens providing the figures. The open circles are the other hens also housed in aviaries. Reference lines, H/L 1 at 0.5, and H/L 2 at 0.4 represent theoretical homeostasis (low or no stress) values.

Insert Figure 6 here

4. DISCUSSION

The objective of this study is to expand on observations of the robustness of traditional H/L stress detection method. In earlier work the H/L was divided into two categories H/L 1 and H/L 2 as an attempt to recognize the importance of cytology in establishing stress levels (5,8,10,11,14-16). The value of H/L 1 is determined by using all heterophil categories as the numerator. Only small lymphocytes (Ls) are used in the denominator as these should be more common in the absence of high stress levels. In contrast H/L 2 is computed using both small (Ls) and larger lymphocytes (Lm) (see Cotter, 5). If there is minimal stress Lm cells are few and there is little difference in the two measurements. Therefore, the ΔH/L would be $\leq$ 0.1; as was true for the hens of this study (Table 1). However, in many reports the H/L value is given in the absence of the total white blood count (TWBC) and too few cells counted for the SDC (see Archer, as recent examples (17,18). Here the TWBC ranged from the homeostatic level (~30k/µL) to well into leukocytosis (TWBC > 100k/µL; Table 1). Remarkably atypical cells of all series were found in the subject hens independent of either H/L or TWBC values (Figure 6).

Furthermore, many authors do not realize that multiple heterophil types exist (Lucas and Jamroz, 1961, (9) as do lymphocytes. This is despite their possession of multiple granule types and physiologic functions (20). This deficiency includes some who take extraordinary care in the experimental protocol but neglect cytology (21). Therefore, some of their H/L values could be contaminated with toxic and atypical cells. A situation that immediately suggests stress, inflammation, and disease.

Reactive and apoptotic heterophils were described in hens from the same population as the subjects of this study but housed in conventional cages (10). Such cells indicate inflammation and infection (22). Here bacteria and fungi were present in many of the blood films (Figures 2-5) at levels high enough to indicate bacteremia/fungemia rather than as casual contaminants of blood. Not only were microorganisms found in the blood but many were attached to RBCs and other cells. These types are

referred to as cell associated bacteria (**CAB**) are indications that erythrocytes should be given consideration in interpretation of hemograms.

In addition, plasmacytes were common. These cells are designed to secrete antibody but are rarely found among circulating leukocytes. Not only were PCs frequent but many were atypical; including Mott cells, a PC known to be defective (8, 23).

5. CONCLUSION

The cells of a homeostatic SDC should display a standard non-reactive appearance. Size, shape, N/C ratio, and an Romanowsky-Giemsa effect (RGE, staining) must be appropriate for each series. This is a critical issue in evaluating the H/L. Developmental stages and dividing cells may be found among the circulating community but these should be rarely seen in a sample from an avian at homeostasis. Atypia, especially heterophils, indicate a complex hemogram, already beyond the quiescent (homeostatic) stage. The question of the relation of stress to excess monocytes, is not considered here, or are reactive basophils (23) and both cell types need further attention.

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.

ACKNOWLEDGEMENTS

The blood samples were part of a larger study "The Coalition for a Sustainable Egg Supply" project as described by Swanson et al. (25).

COMPETING INTERESTS

Author has declared that no competing interests exist

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Table 1.

Source	No.	H[1]	Ls	Lm	Bst	Mn	Ba	Eo	TWBC(K)	H/L 1	H/L 2	ΔH/L
Figures	7	14.1	67.8	9.3	0.2	3.3	4.3	0.9	81.1	0.27	0.20	0.06
Flock	41	14.3	73.7	4.4	0.2	3.6	3.4	0.5	69.8	0.21	0.19	0.01

1. Cells: H, heterophil (∑ [HT typical, HC classic, HV variant,] Ls small lymphocyte ~6 µm diameter, Lm medium, large (diameter 8–10 µm) Bst, granulocyte developmental cell, Mn, monocyte, Ba, basophil, Eo, eosinophil. TWBC (K), total white blood cells per cubic µL 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 legends:

Figure 1. Examples of atypical (A, B band stages; C, 3 lobe stage) heterophils (HT) characteristic of the SDC from the subject hens. A variant heterophil (HV) is in panel D.

Figure 2. Panel A. Binuclear bi/PC with patchy cytoplasm and a large reactive lymphocyte (Lm/PC). Panel B. A group of 9 Ls and a larger reactive lymphoid cell. The remnants of a lysed basophil (Ba) are among the Ls. A solitary encapsulated bacterium is at the arrow.

Figure 3 Panel A. An example of a reactive lymphocyte with plasmacyte cytoplasmic characteristics. Arrows locate bacteria. Panel B. A second example of a plasmacytoid Lm in a field with a (resting) Ls (inset). Panel C. A Ls is adhering to a Lm/PC. Remnants of 2 lysed nuclei (?RBC) are also present (lower right). Panel D. A solitary Lm/PC (right arrow) are attached to RBCs (left and down arrows). Additional descriptions are given in the text.

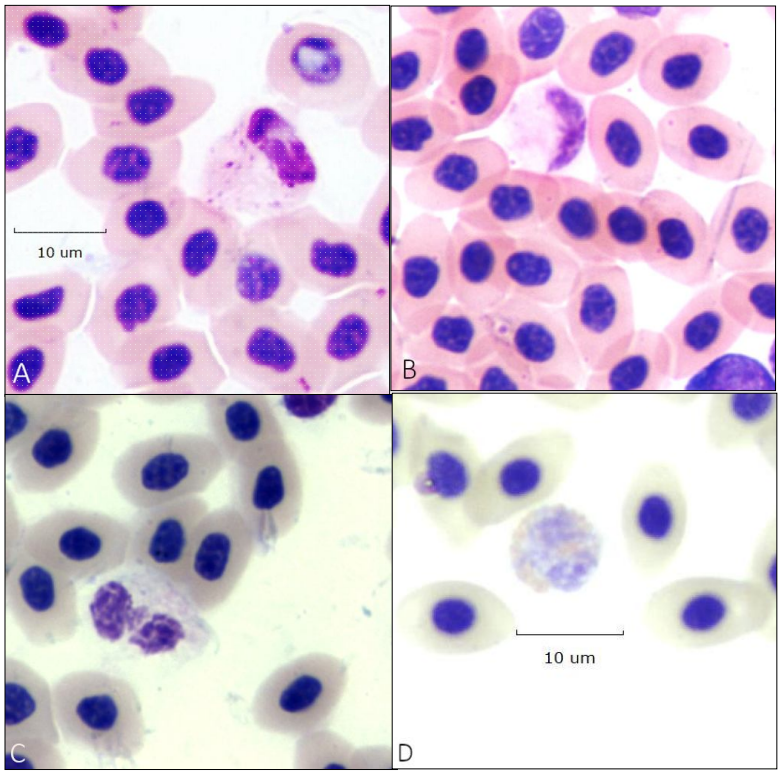
Figure 4. Panel A. Solitary PC with an irregular cm and cytoplasm with pink and orange vacuoles (Russell bodies) thrombocytes (Th) and RBCs. Panel B. Two adhering plasmacytes. Panel C. Plasmacyte with a pink Hof in a field with an elongated bacillus containing a terminal endospore (arrow). Panel D. Proplasmacyte (Türk cell). Inset a 2.5x enlargement of a mixed species microcolony.

Figure 5. Panel A. A large plasmacytoid cell (Lm/PC) mesomyelocytes, small lymphocytes (Ls) and a lysed nucleus. Panel B. Monocytoid cells (Mn). Panel C. A (?mixed) bacterial microcolony attached to a RBC. Panel D. Conidia at different stages of development are attached to a Lm.

Figure 6. Scatter plot of H/L 2 vs H/L 1 ratios for all AV housed hens in the study. Filled circles locate hens of hematology figures. Broken lines are expected values for homeostasis. Reference lines (H/L 1 = 0.5; H/L 2 = 0.4) are expected values of homeostatic samples.

FIGURES

Figure 1.



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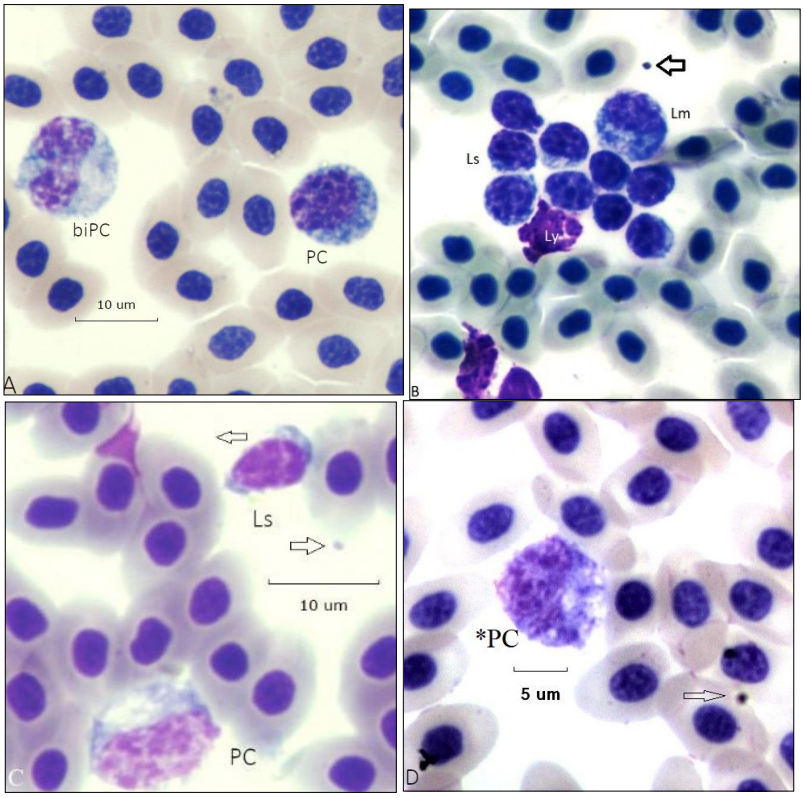

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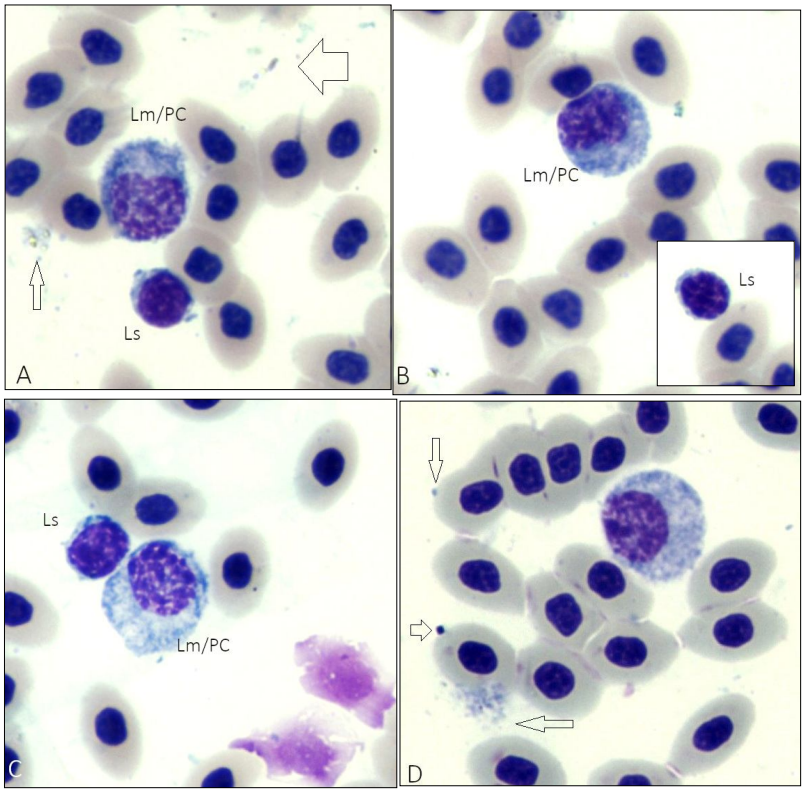

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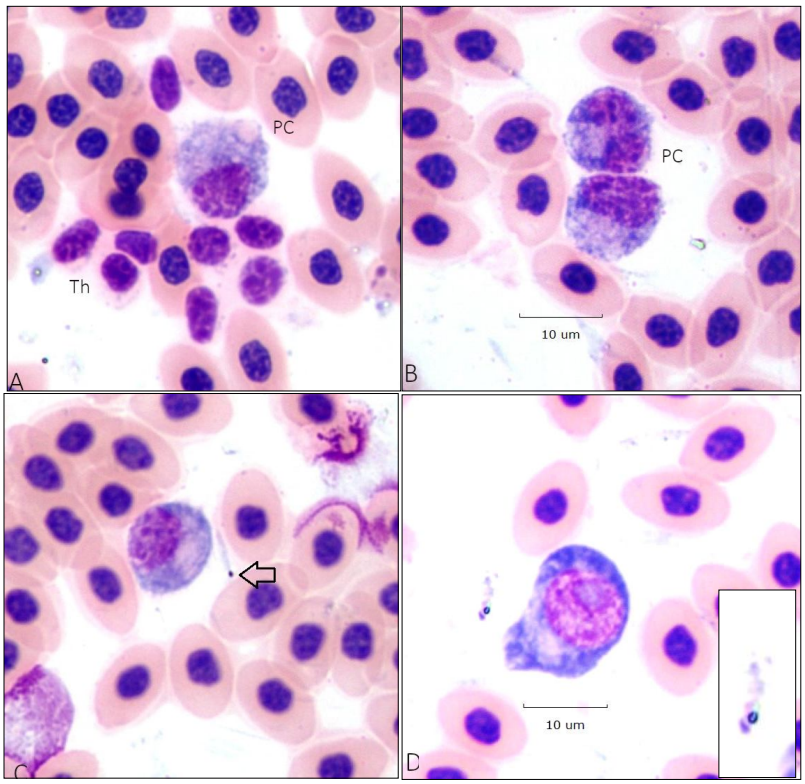

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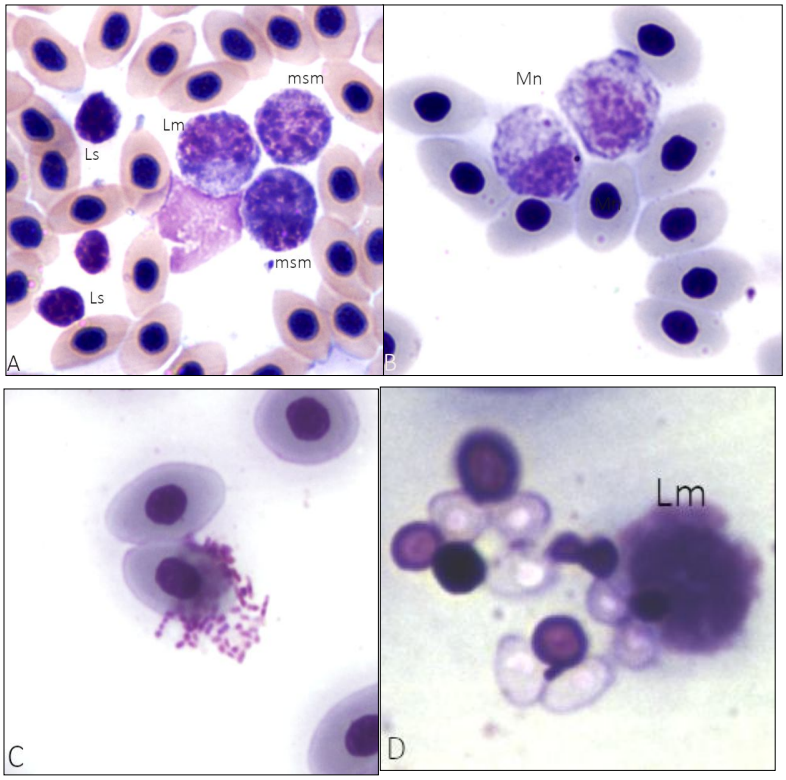
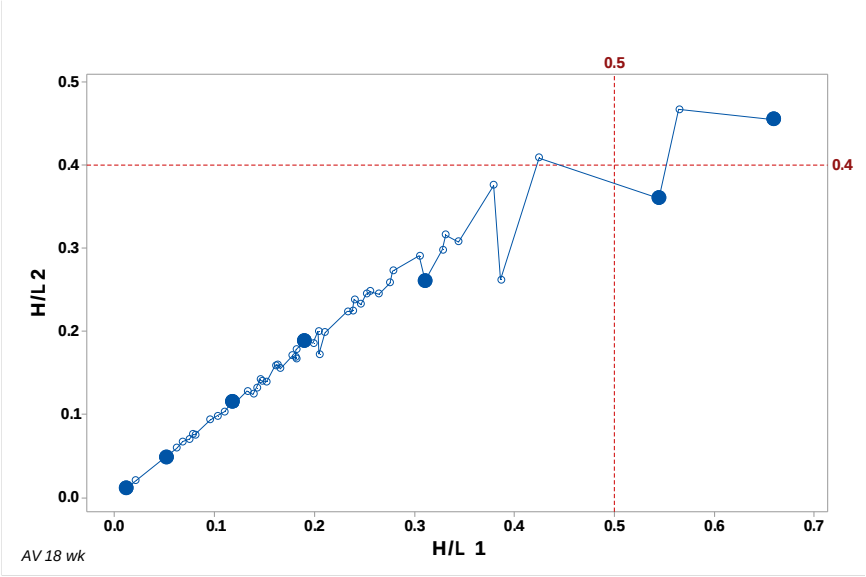


392 Figure 6.



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