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Short Paper

Introducing a combined Leishman-Giemsa stain as a new staining technique for avian blood smears

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Summary

Background: The use of conventional stains including Giemsa, Wright and Leishman have become an essential tool for differential diagnosis of cells in peripheral blood. **Aims:** The aim of the study was to develop a new combination of Leishman-Giemsa (L&G) stain for avian blood smears and to compare its efficacy with conventional staining methods. **Methods:** Three sets of peripheral blood smears, one smear for L&G stain and two other smears for Leishman and Giemsa stains, created from 50 broiler chickens blood samples. All the three sets of slides were blind screened by two expert clinical pathologists and scored based on the staining characteristics (4 parameters) such as nuclear features of red blood cell (RBC) and white blood cell (WBC), cytoplasmic features and cytoplasmic granularity of WBC. The average grading score assigned by two experts for each staining method were compared. **Results:** The average grading score of two conventional Leishman and Giemsa staining methods were significantly lower ($P<0.001$) than new L&G staining method in avian nuclear features of the RBC and WBC. The L&G stain gave a better clarity of nuclear features of avian RBC and WBC. The new L&G staining technique created significant differences ($P<0.001$) in cytoplasmic features of avian WBC compared to the other two methods. **Conclusions:** For the first time, the results of the present study showed that the avian blood cells are more desirable stained with a new combination of L&G stain. In addition, it gives a better nuclear and cytoplasmic differential staining than the conventional Giemsa and Leishman stains when used alone.

Key words: Avian, Blood smear, Leishman and Giemsa stain

Introduction

The peripheral blood and bone marrow smear examination for differential diagnosis of cells are associated with the staining characteristic and correct knowledge of the different blood cells morphology. Study of stained blood smear is considered as one of the most important methods in hematology analysis. A well-stained blood smear helps in the diagnosis of the avian leukocytes. Precise assessment of the morphological characteristics of the granulocytes depends on an adequately prepared blood film (Robertson and Maxwell, 1990). Most Romanowsky stains include Wright stain, Giemsa stain, Wright-Giemsa stain, Leishman stain, Wright-Leishman stain, May-Grunwald stain and May-Grunwald-Giemsa stain commonly used for staining avian blood films as well as human and mammalian blood film staining (Garbyal *et al*., 2006). In the literature it has been proven that Romanovsky stains give a better clarity of nucleus and cytoplasm characteristics. In general, Romanovsky stains contain two components that include blue azure and eosin Y. Blue azure tends to nucleic acids and the nuclear and cytoplasmic proteins, although eosin Y connects to the basic groups of hemoglobin (Kass *et al*., 2002). Leishman-Giemsa (L&G) cocktail as a relatively new technique has been previously used for cytology smear and so far only one study has shown the benefits of this method for staining of human peripheral blood/bone marrow smears (Belgaumi *et al*., 2013; Gajendra *et al*., 2015; Suryalakshmi *et al*., 2016; Doddagowda *et al*., 2017). It has been shown that a new modification of Wright-Giemsa stain gives results similar to the standard Wright-Giemsa stain and can reduce the staining time (Teerasaksilp *et al*., 2005; Kondo *et al*., 2011, 2012; Nakada *et al*., 2014). We developed for the first time a new combination of L&G stain for avian blood smears and compared the efficacy of this staining technique with conventional staining methods.

Materials and Methods

The present study was carried out in the Veterinary Hospital of Semnan University from September 2017 to November 2017. Fifty 30-day-old healthy Ross chickens without hematological abnormalities were included in the study. Three sets of peripheral blood smear, one smear for L&G stain and the other two smears for Leishman and Giemsa stains, were created from 50

broiler chicken blood samples. The slides were blind reviewed by two expert haematopathologists who evaluated and scored all three sets of slides, based on the staining characteristics such as the nuclear features, cytoplasmic features, degree of granularity of the cytoplasm and other morphological red blood cell (RBC) and white blood cell (WBC) characteristics as follows:

1- Staining criteria of mature bird RBC include: yellowish orange ovalocyte with a tinge of blue

2- Staining criteria of nucleus of mature WBC include: purplish blue, appropriate nucleus morphology according to the different WBC types

3- Staining criteria of cytoplasm of granulocyte, lymphocyte and monocyte include: pale pink, sky blue and gray blue, respectively

4- Staining criteria of granules of heterophiles and eosinophils include: rod or speculated dark orange to brown-red shaped and round or oval orange to red shaped, respectively

The slides were compared by giving scores for staining criteria of four parameters of each staining methods as, score 0 (worst) to score 4 (best). The derived data were collected and compiled for further statistical analysis to compare the staining efficacy of each method. The average grading score for each staining method from the two experts was compared to test the interpersonal variability, and analysis of variance (ANOVA) was used to assess the difference among the average grading scores by each expert (Table 1).

1- Staining procedures of slides with Leishman stain:

The smear was covered with Leishman stain for 2 min. Double volume of Sorensen buffer (pH=6.8) was added to the slide and mixed gently for 15 min. Washed off with running water and dried (5 min) (Gajendra *et al*., 2015).

2- Staining procedures of slides with Giemsa stain:

The smear was covered with absolute methyl alcohol for 5 min (fixation). The alcohol was drained off and the slide was covered with freshly diluted Giemsa stain (1:10 with Sorensen buffer, pH=6.8) for 20 min. Washed off with running water and dried (5 min) (Gajendra *et al*., 2015).

3- Staining procedures of slides with Leishman and Giemsa stain:

The slide was covered with Leishman stain for 2 min. The slide was washed in running water. Fresh diluted Giemsa stain (1:10 with Sorensen buffer, pH=6.8) was poured for 15 min. Washed off with running water and dried for 5 min (Gajendra *et al*., 2015).

Results

The comparison of average grading scores of four parameters such as nuclear features of RBC, nuclear and cytoplasmic features of WBC and degree of granularity of the cytoplasm among three staining methods was presented in Table 1. The average grading score of two conventional Leishman and Giemsa staining methods were significantly lower ($P<0.001$) than new L&G staining method in avian nuclear features of the RBC and WBC (Table 1). The L&G stain gave a better clarity of nuclear features of avian RBC and WBC (Figs. 1A-C). The new L&G staining technique created significant differences ($P<0.001$) in cytoplasmic features and cytoplasmic granulations of avian WBC (Figs. 1B and C) compared to the other two methods (Figs. 2B and C and 3B and C).

Table 1: Comparison of average grading scores of four parameters for three staining methods

Parameters	Leishman stain (A)	Giemsa stain (B)	Leishman-Giemsa stain (C)
		Grading (mean±SD)	
Nuclear features of the RBC	3.27 ± 0.25	3.17 ± 0.24	3.70 ± 0.25 $^{**, \#\#}$
Nuclear features of the WBC	3.32 ± 0.24	3.15 ± 0.23	3.77 ± 0.25 $^{**, \#\#}$
Cytoplasm features of the WBC	2.88 ± 0.36	3.28 ± 0.37	3.74 ± 0.29 $^{**, \#\#}$
Granules of the WBCs	3.08 ± 0.24	2.96 ± 0.24	3.80 ± 0.24 $^{**, \#\#}$

P-values: *<0.01 and **<0.001 when group C compared with group A; $^{\#}$<0.01 and $^{\#\#}$<0.001 when group C compared with group B

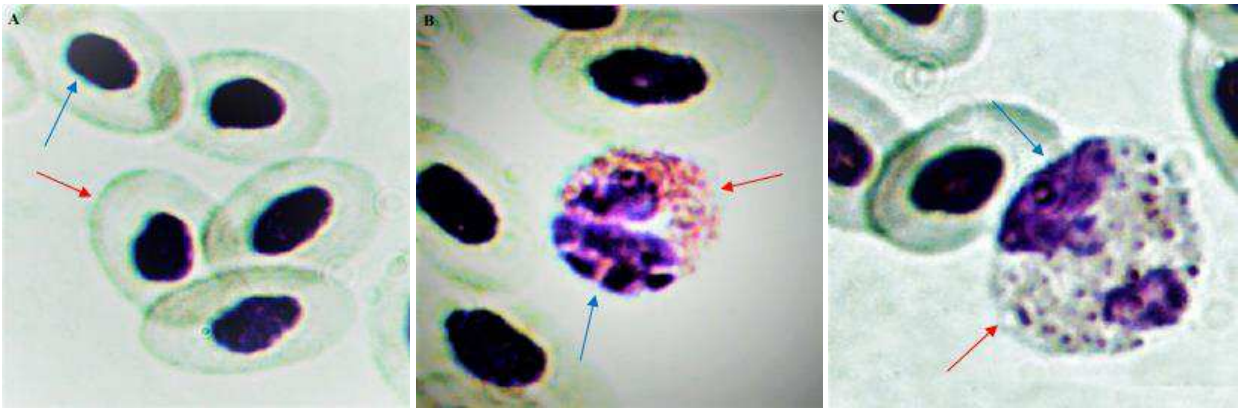

Fig. 1: The avian RBC and WBC. The nuclear (blue arrow) and cytoplasmic (red arrow) features of avian RBC (**A**), eosinophil (**B**), and heterophil (**C**) in Leishman-Giemsa stain (×1000)

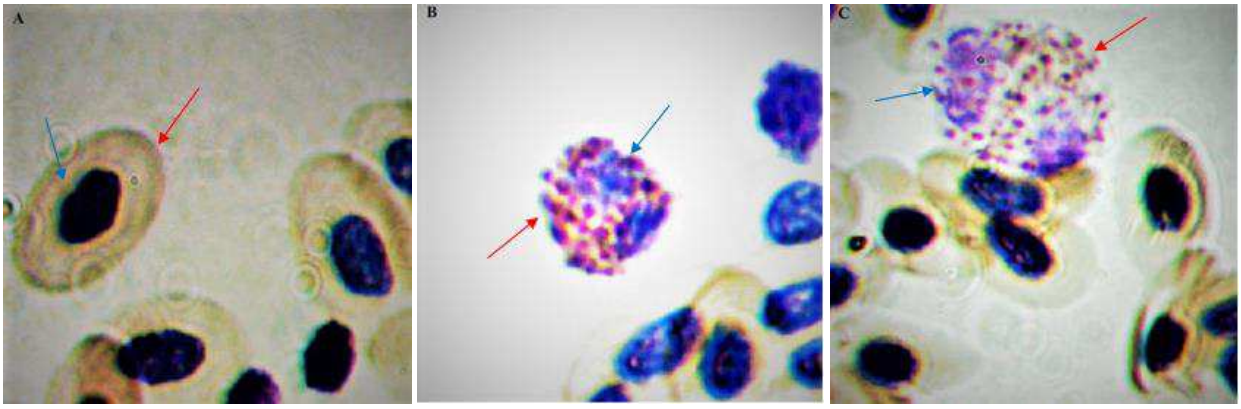

Fig. 2: The avian RBC and WBC. The nuclear (blue arrow) and cytoplasmic (red arrow) features of avian RBC (**A**), eosinophil (**B**), and heterophil (**C**) in Leishman stain (×1000)

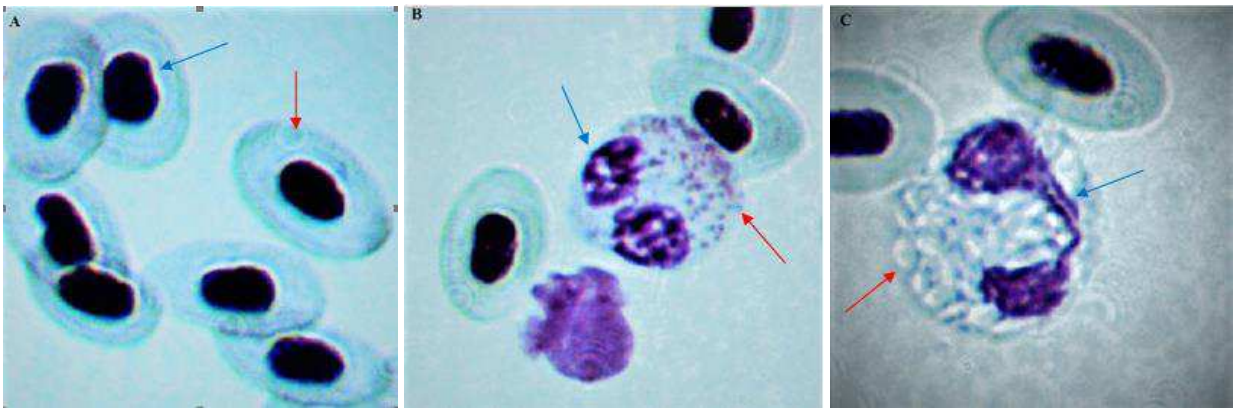

Fig. 3: The avian RBC and WBC. The nuclear (blue arrow) and cytoplasmic (red arrow) features of avian RBC (**A**), eosinophil (**B**), and heterophil (**C**) in Giemsa stain (×1000)

Discussion

In this pilot study, the efficacy of L&G stain, as a new staining technique, on the features of avian RBC and WBC included in the study was evaluated and compared to Leishman and Giemsa conventional stains. We selected an expert clinical pathologist to decrease Pearson's variability to smears evaluation and to optimize the results of reports. The evaluated parameters from each staining method were compared with the average grading score given by two experts. We found that the grading scores calculated by both experts were almost the same in the morphological evaluation of avian blood cells. The coefficient of variation (CV%) calculated for nuclear features of the RBC in Leishman, Giemsa and L&G stain were 0.076, 0.075 and 0.067, respectively. The CV% calculated for nuclear features of the WBC in Leishman, Giemsa and L&G stain were 0.072, 0.073 and 0.066, respectively. The CV% calculated for cytoplasm features of the WBC in Leishman, Giemsa and L&G stain were 0.125, 0.113 and 0.077, respectively. The CV% calculated for granules of the WBCs in Leishman, Giemsa and L&G stain were 0.078, 0.081 and 0.063, respectively.

Statistical analysis of average grading score from each staining method showed that the nucleus and cytoplasm of avian blood cells in L&G staining was much better staining than Leishman and Giemsa staining as presented in Table 1 ($P<0.001$).

Leishman stain is an excellent nuclear stain and using this stain alone leads to an intense staining nucleus and understained cytoplasmic granulations. Giemsa stain is a good cytoplasmic stain and when used alone gives fainter staining of nucleus and its combination with Leishman stain provides a proper staining of nucleus, cytoplasm with cytoplasmic granulations (Gajendra *et al*., 2015; Suryalakshmi *et al*., 2016). The results of the staining efficacy of the modified L&G stain on the human peripheral blood/bone marrow smears showed that this staining method was superior to the other two conventional stains when used alone (Gajendra *et al*., 2015).

We found that the new L&G stain is useful for morphological assessment of nucleus and cytoplasm in both the avian RBC and WBC (Figs. 1A-C), whereas Leishman and Giemsa stain alone created a poor staining of the nucleus and the cytoplasmic granules with fainter contrast between nuclear and cytoplasm (Figs. 2A-C and 3A-C). The granules of heterophils and eosinophils were better stained by the new L&G stain than Leishman and Giemsa stains as given in Table 1. Eosinophilic granules of heterophils and eosinophils are better visualized in L&G stain (Figs. 1B and C) than the two conventional stains (Figs. 2B and C and 3B and C). On the other hand,

the new L&G stain made a significant difference in heterophil detection (Table 1). As mentioned in the previous section Leishman stain unlike Giemsa stain, has a methanolic base and the new L&G stain does not need any further fixation (Gajendra *et al*., 2015). As is evident in certain figures, heterophiles in the figures of this manuscript are not degranulated, and these figures are themselves evidence of this claim.

During the study of avian blood it was found that this stain improves blood cell detection better than other formerly used stains such as Leishman and Giemsa. Finally, we found that the new L&G stain has almost all the features of a good stain for morphological assessment of the avian blood cells.

For the first time, the results of the present study showed that the avian blood cells are more desirable stained with a new combination of L&G stain. In addition, it gives a better nuclear and cytoplasmic differential staining than the conventional Giemsa and Leishman stains when used alone. Further studies should be undertaken to evaluate the staining technique abilities for identification of blast cells in avian bone marrow smears.

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Conflict of interest

We declare that there is no conflict of interest.

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