Original Article

Comparison of Giemsa, Leishman, and Leishman-Giemsa Staining Methods for Evaluating Fish Blood Smears

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How to Cite This Article Arvan, E., Mehdizadeh Mood, S., & Ahmadi-Hamedani, M. (2025). Comparison Giemsa, Leishman, and Leishman-giemsa Staining Methods for Evaluating Fish Blood Smears. *Iranian Journal of Veterinary Medicine, 19*(3), 571-578. http://dx.doi.org/10.32598/ijvm.19.3.1005575

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ABSTRACT

Background: The hematological panel provides valuable information about the physiologic status and health of fish in aquatic environments through optimal staining of the nucleus, cytoplasm, and cytoplasmic granules.

Objectives: Our research focused on creating a new Leishman-Giemsa dye mixture specifically for fish blood smears and evaluating its effectiveness compared to standard staining techniques.

Methods: Blood samples were taken from 20 healthy common carp to produce three groups of peripheral blood smears: One for Leishman-Giemsa dye and two for Leishman's and Giemsa's stains. Two experienced clinical pathologists extensively examined all three types of blood smears based on four staining characteristics: Nuclear characteristics of erythrocytes and leukocytes, cytoplasmic characteristics, and leukocyte granulation. The mean ratings from the two experts for each staining technique were compared.

Results: Regarding the characteristics of RBC and WBC nuclei, the two conventional staining methods, Leishman and Giemsa, yielded substantially lower mean scores than the new Leishman-Giemsa staining method ($P<0.05$). Leishman-Giemsa staining enhanced the clarity of RBC and WBC nuclear characteristics. The new Leishman-Giemsa staining technique resulted in a statistically significant ($P<0.05$) difference in the cytoplasmic characteristics of fish WBC compared to the other two methods.

Conclusion: The results of this study demonstrated for the first time that fish blood cells stained with the novel Leishman-Giemsa method are more desirable. In addition, its nuclear and cytoplasmic staining is superior to that of conventional Giemsa and Leishman stains when used separately. The Leishman-Giemsa cocktail has a high index for air-dried smear discoloration.

Keywords: Common carp, Cytoplasmic granules, Hematological assessment, Nuclear features, Leishman-Giemsa staining

Article info:
Received: 30 Jun 2024
Accepted: 17 Sep 2024
Available Online: 01 Jul 2025

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Introduction

Aquaculture has grown faster than capture fisheries, especially in Asia, in recent years (Abdulrahman, 2022) and is expected to expand further over the next decade (Naylor et al., 2021; Mousavi et al., 2024). Overcrowding affects the growth, health, and susceptibility of fish to disease (Li et al., 2021). It sounds necessary to use a fast applicable, inexpensive approach to monitor health status and urgent disease diagnosis (Chen & Luo, 2023). Blood evaluation can provide predictive information about the clinical status of fish (Shahjahan et al., 2022).

Most automated mammalian blood count analyzers cannot be used because of the presence of nucleated RBC in fish. Therefore, the common method is the manual evaluation of stained blood smears. The most important aspect of a fish's hematological examination may be the evaluation of blood cell morphology in stained blood films. The blood film provides essential information regarding RBC abnormalities, including altered cell color and shape, the presence of inclusions, and altered nucleus position. In addition to revealing significant hematological characteristics of fish thrombocytes, stained blood film provides information regarding variations in leukocyte number and morphology. Also, blood parasite detection and identification require a blood film (Grant, 2015).

The routine method for staining fish blood smears is Romanowsky (Wright/Giemsa/Leishman stains) (Doddagowda, 2017). Wright-Giemsa staining is a classical staining method to identify and classify blood cell types (Fanous et al., 2021). Fish RBCs are rich in smooth eosinophilic cytoplasm and a central, oval-shaped condensed nucleus (Witeska, 2022). Fish WBC granules refer to small, spherical structures found within white blood cells (WBC) in fish. The routine method for staining fish blood smears is Romanowsky (Wright/Giemsa/Leishman stains) (Doddagowda, 2017).

The granules contain various proteins and enzymes involved in the fish's immune response, including the recognition and destruction of foreign pathogens, such as bacteria, viruses, and parasites (Naqid, 2024; Talazadeh et al., 2024). In bacterial infections in fish, such as bacterial septicemia, it is possible to detect the presence of bacteria through the examination of blood smears. For example, cocci of *Streptococcus iniae* were reported in the plasma and intracellularly within a neutrophil in a yellow tang (*Zebrasoma flavescens*) (Clauss, 2008).

The granules in WBCs may contain enzymes. The presence and quantity of these granules can provide valuable information about the health and immune status of the fish. Fish WBC granules can be visualized using various laboratory techniques, such as staining. Giemsa stain is a type of histological stain that is commonly used to stain chromatin (Hasankhani et al., 2023). Leishman stain generally makes the colors of the nucleus and neutrophil granules violet, making the differential count more convenient due to the better contrast between the nucleus and cytoplasm (Sareen et al., 2018).

The staining solution contains a mixture of methylene blue and eosin, which stain cytoplasmic granules of WBCs a dark purple color. By examining the stained cells under a microscope, researchers can identify the presence and characteristics of granules in WBCs, which can provide important information about the immune response of fish and the type of bacterial infection. For the first time, we used and evaluated the effectiveness of a novel Leishman-Giemsa (L&G) stain for fish blood smears in comparison to traditional staining techniques.

Materials and Methods

This study was conducted in the Aquatic Animal Laboratory of the Faculty of Veterinary Medicine at Semnan University. Blood samples were taken from 20 healthy common carp (*Cyprinus carpio*) (450.00±20.00 g) to produce three groups of peripheral blood smears: One for Leishman-Giemsa dye and two for Leishman's and Giemsa's stains. Smears were fixed in absolute methanol for 1 minute, air dried, and stained with three different stains according to the following protocol:

A) Leishman stain: The Leishman stain was applied to the slide for two minutes. Then, a double volume of Sorensen buffer with a pH of 6.8 was added and mixed for 15 minutes. After rinsing the smears with tap water, five minutes were spent drying the slides (Gajendra et al., 2015).

B) Giemsa stain: In order to fix the smear, absolute methyl alcohol was applied for five minutes. The alcohol was removed, and then, the smears were stained with freshly diluted Giemsa stain (1:10 with Sorensen buffer, pH 6.8) for 20 minutes. Subsequently, the prepared smears were rinsed with tap water and allowed to drain for five minutes (Gajendra et al., 2015).

C-Leishman-Giemsa stain

The Leishman stain was applied to the smears for two minutes, followed by a thorough washing with running

Table 1. Evaluation of the mean grading scores of four parameters under study in three different staining methods

Parameters	Mean±SD		
	Leishman	Giemsa	Leishman-Giemsa
Nuclear characteristics of erythrocytes	3.25±0.91	3.5±0.6	3.9±0.3
Nuclear characteristics of leukocytes	3±0.79	3.9±0.36	3.8±0.36
Cytoplasmic characteristics of leukocytes	3±0.89	2.9±0.55	3.7±0.44
Leukocyte granulation	2.6±0.75	2.3±0.74	3.6±0.59

water. Subsequently, freshly diluted Giemsa stain (1:10 with Sorensen buffer, pH 6.8) was added and allowed to sit for 15 minutes. Finally, the smears were washed with flowing water and air-dried for 5 minutes (Gajendra et al., 2015).

Two experienced clinical pathologists extensively examined all three types of blood smears based on four staining characteristics: Nuclear characteristics of erythrocytes and leukocytes, cytoplasmic characteristics, and leukocyte granulation.

1) Staining criteria for erythrocytes include ovalocytes that appear yellow-orange with pale blue; 2) Staining criteria for the mature leukocyte nucleus include a violet-blue coloration and the proper nucleus morphology for various WBC types.

3) The cytoplasmic staining criteria for granulocytes, lymphocytes, and monocytes are respectively pale pink, sky blue, and grey-blue; 4) The criteria for staining the granules of leukocytes and eosinophils are as follows: Rod-shaped or dark orange to brown-red, and round or oval, orange to red.

The staining procedures were evaluated using scores ranging from zero (worst) to four (best) for four parameters and were statistically analyzed to compare effectiveness. The variability in average ratings provided by two experts for each method was compared. Then, the analysis of variance (ANOVA) was employed to determine the degree of disparity between the two experts' mean grading scores (Table 1).

Results

Table 1 provides a comparison of the average grading scores for four different characteristics, including nuclear characteristics of erythrocytes and leukocytes, cytoplasmic characteristics, and leukocyte granulation, among the three staining procedures.

Characteristics of RBC nucleus color

According to the scoring of all three types of staining (Figure 1), only a significant difference ($P<0.05$) was observed between Leishman and Leishman-Giemsa staining. Also, it was indicated that Leishman-Giemsa staining provides more detailed staining of the nucleus and chromatin compared to Leishman (Figure 2). However, the difference in nuclear staining is not significant when compared to the Giemsa group, as the nuclear chromatin in the Giemsa group is also clear and does not differ much from that in the Leishman-Giemsa group.

Characteristics of WBC nucleus color

Figure 3 shows the comparison of three different staining methods related to the nucleus and cytoplasm of WBCs in fish. Scoring of all three types of staining indicated that both the Leishman and Giemsa groups had a significant difference ($P<0.05$) when compared to the Leishman-Giemsa group; however, there was no significant difference between the two groups regarding nuclear characteristics (Figure 4). The characteristics of Leishman-Giemsa staining of the nucleus, chromatin, and cytoplasmic granules received higher scores than the others, as shown in Figure 4.

Characteristics of WBC cytoplasm color

Figure 5 demonstrates that based on the scoring of all three staining methods, the Leishman and Giemsa groups differed significantly ($P<0.05$) from the Leishman-Giemsa group. The staining of the nucleus, chromatin, and cytoplasmic granules received higher scores than the other two groups (Figure 5).

Discussion

In each fish species, variations in the hematological profile could indicate environmental changes, infections, or even parasitic infestations.

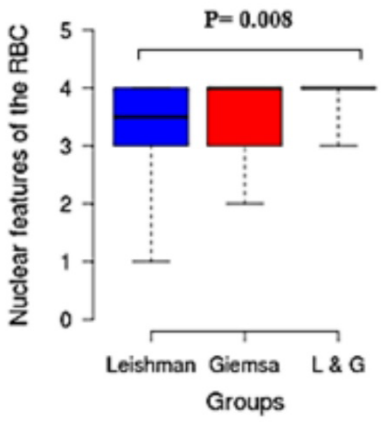

Figure 1. Boxplot of nuclear characteristics of erythrocytes in Leishman, Giemsa and Leishman-Giemsa staining methods

Note: Central lines show the medians; box limits indicate the 25th and 75th percentiles. n=20 sample points

Initially, Garbyal et al. (Garbyal et al., 2006) recommended the Leishman-Giemsa cocktail for staining cytologic smears. Later in 2015, Gajendra et al., 2015) introduced this method as a new reliable technique for staining human blood and bone marrow. According to Akhlaghi and Ahmadi-Hamedani (Akhlaghi & Ahmadi-Hamedani, 2019), the Leishman- Giemsa stain possesses nearly all the characteristics of an excellent stain for the morphological evaluation of avian blood cells.

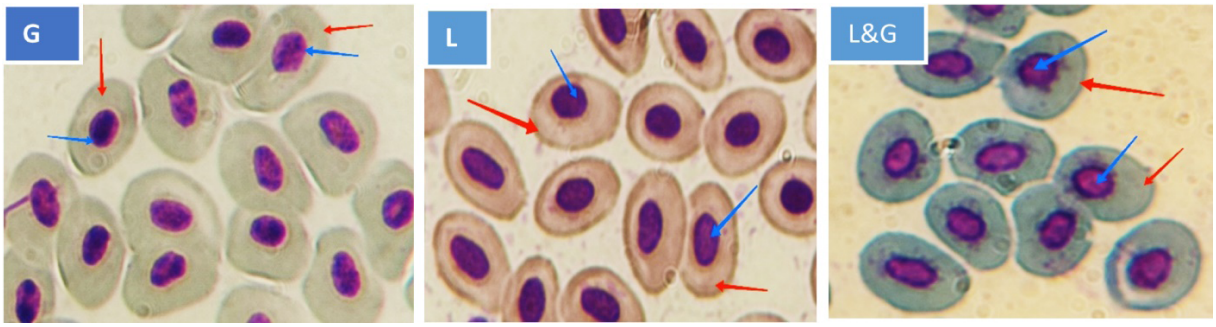

Figure 2. Comparison of common carp (*C. carpio*) RBC nucleus (blue arrows) and cytoplasmic (red arrows) features using different stains: G) Giemsa, L) Leishman, L & G) Leishman-Giemsa stain (×1000)

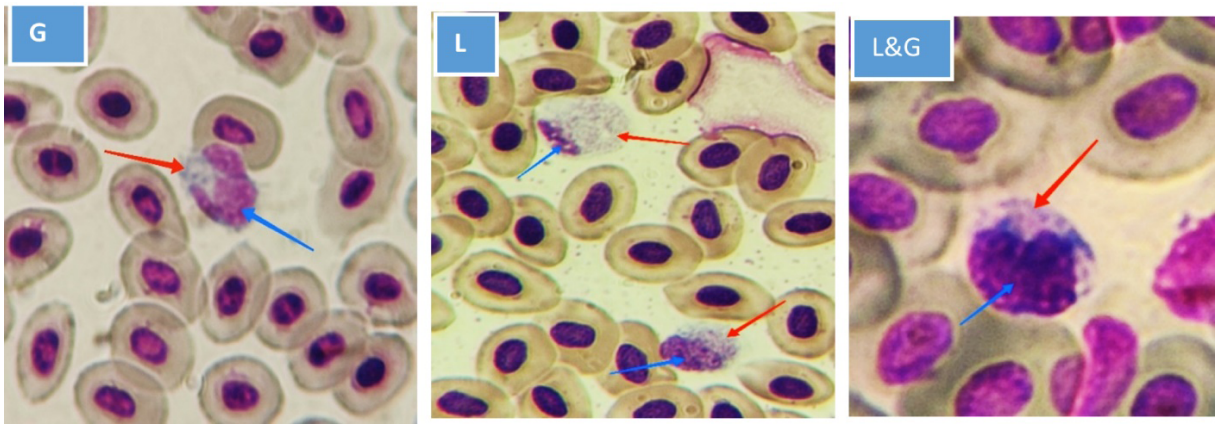

Figure 3. Comparison of common carp (*C. carpio*) WBC nucleus (blue arrows) and cytoplasmic (red arrows) features using different stains: G) Giemsa, L) Leishman, L & G) Leishman-Giemsa stain (×1000)

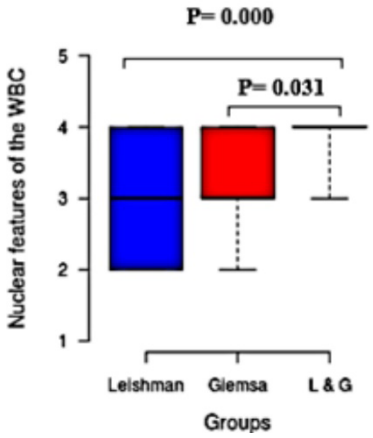

Figure 4. Boxplot of nuclear characteristics of leukocytes in Leishman, Giemsa, and Leishman-Giemsa staining methods

Note: Central lines show the medians; box limits indicate the 25th and 75th percentiles. n=20 sample points.

The present study aimed to evaluate the effectiveness of the Leishman-Giemsa stain, a new staining technique, on the characteristics of fish blood cells compared to the conventional Leishman and Giemsa stains. Two clinical pathologists, experts in their field, were asked to assess smears to minimize variability and produce more accurate results.

Leishman's stain, which offers high staining quality and is typically used to distinguish and identify leukocytes, is employed in microscopy for staining blood smears. It is based on a combination of methylene blue and eosin (Kulkarni, 2020). This stain is excellent for nuclear visualization; using it alone results in an intensely stained nucleus while making the cytoplasmic granules less visible. Kulkarni used these two dyes for staining blood smears of *Notopterus notopterus* (Kulkarni, 2020).

The Giemsa stain, when combined with the Leishman stain, significantly enhances the staining of the nucleus and cytoplasm, resulting in pronounced cytoplasmic granulation (Gajendra et al., 2015; Suryalakshmi et al., 2016). When used alone, the Giemsa stain is a reliable cytoplasmic stain but offers a weaker coloration for the nucleus. According to the results of experiments on human peripheral blood and bone marrow smears, the Leishman-Giemsa recombinant dye staining approach was preferable to the other two traditional dyes (Gajendra et al., 2015). The modified Leishman-Giemsa staining effectiveness in human peripheral blood/bone marrow smears demonstrates that this approach is superior to the other two traditional stains when used alone (Gajendra et al., 2015).

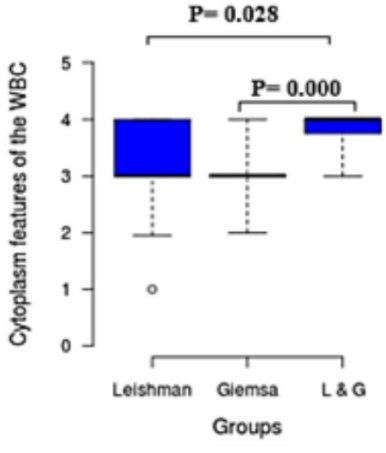

Figure 5. Boxplot of cytoplasmic characteristics of leukocytes in Leishman, Giemsa and Leishman-Giemsa staining methods

Note: Central lines show the medians; box limits indicate the 25th and 75th percentiles. n=20 sample points.

The morphological evaluation of the nucleus and cytoplasm in fish erythrocytes and leukocytes can be facilitated by the use of a recombinant Leishman-Giemsa stain. In contrast, the nucleus and cytoplasmic granules showed poor coloration and less contrast when the Leishman and Giemsa stains were used alone. Neutrophil granules were stained better with the new Leishman-Giemsa stain compared to the Leishman and Giemsa stains used separately. Furthermore, the new Leishman-Giemsa stain made a significant difference in the recognition of neutrophils (Table 1). Unlike Giemsa, the Leishman stain has a methanol base; thus, further fixation is not necessary for the novel Leishman-Giemsa stain (Gajendra et al., 2015). These images provide evidence that neutrophils were not degranulated, as shown in the figures.

Although comparable to May Grunwald-Giemsa (MGG), the Leishman-Giemsa staining protocol was less susceptible to batch-to-batch variation in staining quality. The MGG protocol required control of the differential staining pattern during the final washing phase through the inspection of wet slides, whereas the Leishman-Giemsa method included a final washing step with buffered water for a predetermined time period. Wright-Giemsa did not provide the cytoplasmic staining intensity required for the morphological differentiation of leukocytes.

Conclusion

The results of the present study demonstrated for the first time that fish blood cells were stained better and faster with the new combination of Leishman-Giemsa staining. In addition, compared to traditional Giemsa and Leishman stains used alone, it offers superior nuclear and cytoplasmic differential staining.

Ethical Considerations

Compliance with ethical guidelines

The present study was approved by the Vice-Chancellor for Education and Research of Semnan University, Semnan, Iran (Code: IR.SU.187).

Funding

This research did not receive any grant from funding agencies in the public, commercial, or non-profit sectors.

Authors' contributions

Conceptualization: Sara Mehdizadeh Mood, and Mahmood Ahmadi-Hamedani; Investigation and writing the original draft: Ehsan Arvan; Data curation, formal analysis and validation: Mahmood Ahmadi-Hamedani; Project administration, supervision, review and editing: Sara Mehdizadeh Mood.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

The authors would like to express their gratitude to Narges Nejabat who provided technical help in Laboratory.

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