Comparison the Efficiency of Three Staining Methods Including Giemsa, Leishman and Leishman-Giemsa for Evaluation Fish Blood Smear

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Abstract

Backgrounds: The haematological panel provides valuable information about the physiologic status and health of the fish in the aquatic expert's test through the 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. Two experts' mean ratings for each staining technique were compared.

Results: Regarding RBC and WBC nuclei characteristics, the two conventional Leishman and Giemsa staining methods 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: This study's results 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 conventional Giemsa and Leishman stains when used separately. The Leishman-Giemsa cocktail has a high index for air-dried smear discolouration.

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

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). Over-density affects their growth, health and susceptibility to disease (Li *et al.,* 2021). It sounds necessary to use a fast applicable, inexpensive approach in order to monitor health status and urgent disease diagnosis (Chen and 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 nucleated red blood cells in fish. Therefore, the common method is the manual evaluation of stained blood smears. The most important aspect of a fish's haematological examination may be the evaluation of blood cell morphology in stained blood films. The blood film provides essential information regarding red blood cell abnormalities, including altered cell colour and shape, the presence of inclusions, and altered nucleus position. In addition to revealing significant haematological 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 red blood cells are rich in smooth eosinophilic cytoplasm and a central, oval-shaped condensed nucleus (Witeska, 2022). Fish white blood cell granules refer to small, spherical structures found within white blood cells in fish.

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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's possible to detect the presence of bacteria through investigation of the blood smear (e.g. Cocci of *Streptococcus iniae* , in a yellow tang (*Zebrasoma flavescens*) was reported in the plasma and intracellular within a neutrophil (Clauss, 2008).

The granules in white blood cells may contain enzymes. The presence and quantity of these granules can provide valuable information about the health and immune status of the fish. Fish white blood cell 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 colour of the nucleus and neutrophil granules violet, and the differential count becomes convenient due to 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 white blood cells as a dark purple colour. By examining the stained cell under a microscope, researchers can identify the presence and characteristics of granules in white blood cells 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 L&G stain for fish blood smears effectiveness 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 gr weight) 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, after air dried, and stained with three different stains as following protocol:

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

B- Giemsa stain: In order to fix the smear, absolute methyl alcohol was applied to it for five minutes. The alcohol was removed, then the smears was 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 let them 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 water. Subsequently, freshly diluted Giemsa stain (1:10 with Sorensen buffer, pH=6.8) was added and left for 15 minutes. Finally, washing with flowing water and air-drying 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 colouration and the proper nucleus morphology for the various WBC varieties.
 3- The granulocyte, lymphocyte, and monocyte cytoplasmic staining criteria are respectively pale pink, sky blue, and grey blue.
 4- The criteria for staining 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 0 (worst) to 4 (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, 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.

Table1- Evaluation of the average grading scores of four parameters under study in three different staining methods.

Parameters	Leishman)mean±SD(	Giemsa)mean±SD(	Leishman-Giemsa)mean±SD(
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

3-1-Characteristics of RBC nucleus colour

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 indicated that Leishman-Giemsa staining, the nucleus and chromatin staining are more detailed rather than Leishman (Figure 2), but the difference in nuclear staining is not significant compared to the Giemsa group, because in the Giemsa group Also, the nuclear chromatin is clear and does not differ much from the Leishman-Giemsa group.
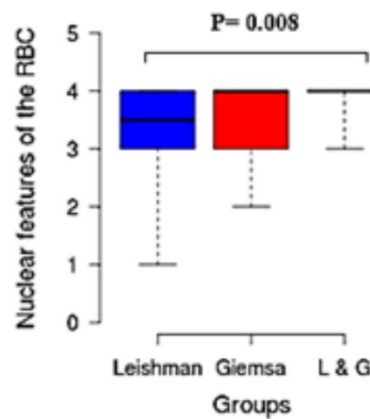

Figure1. Boxplot of Nuclear characteristics of erythrocytes in Leishman, Giemsa and Leishman-Giemsa staining methods, Central lines show the medians; box limits indicate the 25th and 75th percentiles. n=20 sample points.
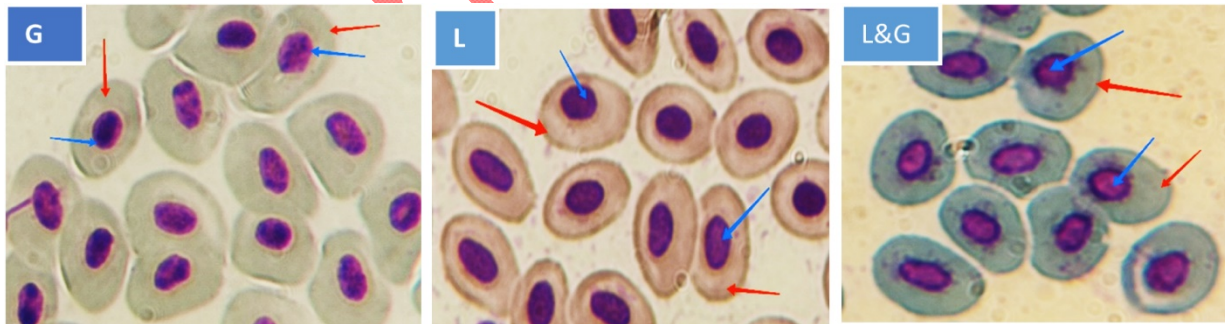
Figure 2. Comparison of Common carp (*Cyprinus carpio)* RBC nucleus (blue arrows) and cytoplasmic (red arrows) features using different stains: G) Giemsa, L) Leishman , L&G) Leishman-Giemsa stain (X1000)

3-2- Characteristics of WBC nucleus colour

Figure 3 shows the comparison of three different stainings related to the nucleus and cytoplasm of WBC in fish. Scoring of all three types of staining showed both Leishman and Giemsa groups had a significant difference ($P<0.05$) with the Leishman-Giemsa group, but both groups alone have no significant difference in terms of nuclear characteristics (Figure 4). The characteristics of Leishman-Giemsa staining of the nucleus, chromatin, and cytoplasmic granules have a higher score than the others, as shown in Figure 4.
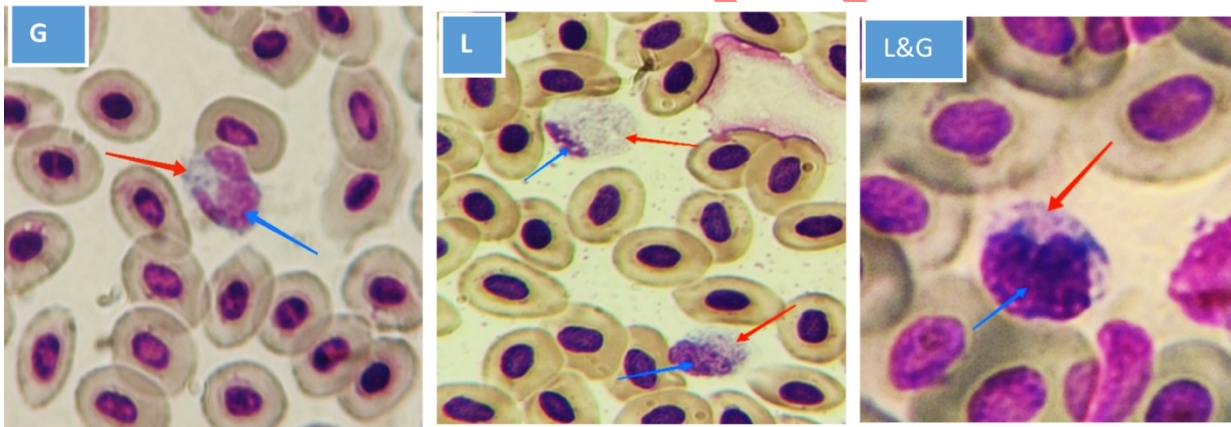

Figure 3. Comparison of Common carp (*Cyprinus carpio)* WBC nucleus (blue arrows) and cytoplasmic (red arrows) features using different stains: G) Giemsa, L) Leishman , L&G) Leishman-Giemsa stain (X1000)
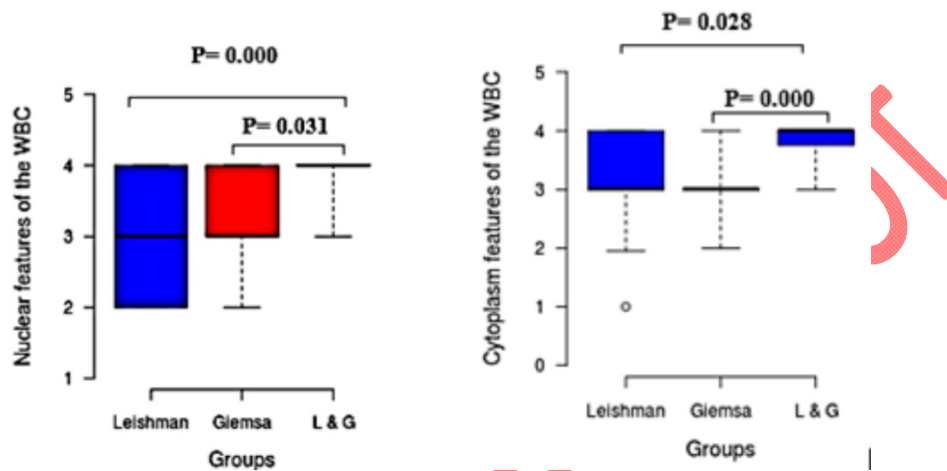
Figure 4. Boxplot of Nuclear characteristics of leukocytes in Leishman, Giemsa and Leishman-Giemsa staining methods, Central lines show the medians; box limits indicate the 25th and 75th percentiles.n=20 sample points. Figure 5. Boxplot of cytoplasmic characteristics of leukocytes in Leishman, Giemsa and Leishman-Giemsa staining methods, Central lines show the medians; box limits indicate the 25th and 75th percentiles. n=20 sample points.

3-3- Characteristics of WBC cytoplasm colour

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

Discussion

In each fish species, haematological profile variations could be a sign of environmental changes, infection or even parasitic infestation.

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

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 stain quality and is typically used to distinguish and identify leucocytes, is used in microscopy for staining blood smears. It is based on a methylene blue and eosin combination (Kulkarni,2020). It is a great stain for the nucleus, and using this dye alone causes the nucleus to be intensely stained while the cytoplasmic granules become less visible. Kulkarni(2020) used these two dyes for staining *Notopterus notopterus* blood smears.

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, Giemsa stain is a reliable cytoplasmic stain but offers a weaker color to the nucleus. When used alone, the Leishman-Giemsa recombinant dye staining approach was preferable to the other two traditional dyes, according to the results of experiments on human peripheral blood/bone marrow smear (Gajendra *et al.,* 2015). The modified L&G staining effectiveness on human peripheral blood/bone marrow smears demonstrates that when used alone, this approach is superior to the other two traditional stains (Gajendra *et al.*,2015).

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. The nucleus and cytoplasmic granules, on the other hand, showed poor coloration and less contrast when Leishman and Giemsa stain was used alone. Neutrophil granules were stained better with the new Leishman-Giemsa stain compared to the Leishman and Giemsa stains separately. On the

other hand, the new Leishman-Giemsa stain made a significant difference in the recognition of neutrophils (Table 1). In contrast to Giemsa, the Leishman stain has a methanol basis; 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 by the figures.

Although comparable to MGG, the LG staining protocol was less susceptible to batch-to-batch variation in staining quality. The MGG protocol required control of the differential staining pattern in the final washing phase via inspection of wet slides, whereas the LG 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 morphological differentiation of leukocytes.

Conclusion

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

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مقایسه سه روش رنگ آمیزی لشمن، گیمسا و لشمن-گیمسا جهت ارزیابی گسترش خونی در ماهی

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چکیده

زمینه مطالعه : تابلوی خون شناسی اطلاعات مفیدی را در خصوص وضعیت فیزبولوژیـک و سـلامت مـاهی در اختیـار متخصص آبزیان قرار می دهد. این مهم از طریق رنگ آمیزی مطلوب هسـته، سیتوپلاسـم و گرانـول هـای سیتوپلاسـمی صورت می پذیرد.

هدف: تحقیق حاضر با هدف ایجاد ترکیبی جدید از رنگ لیشمن-گیمسا برای نمونه‌های گسترش خون ماهی و مقایسه کارایی آن با تکنیک‌های رنگ‌آمیزی مرسوم انجام شد.

روش کار: از 20 ماهی کپور معمولی نمونه خون برای تهیه سه گروه گسترش خون محیطی: یک گسـترش بـرای رنـگ لیشمن-گیمسا و دو لام برای رنگ آمیزی لیشمن و گیمسا گرفته شد. دو پاتولوژیست بالینی متخصص، هـر سـه سـری گسترش خون را بر اساس چهار ویژگی رنگ‌آمیزی ارزیـابی کردنـد: ویژگـی‌هـای هسـته‌ای گلبول‌هـای قرمـز (RBC) و

گلبول‌های سفید (WBC)، ویژگی‌های سیتوپلاسمی، و گرانول‌های WBC. میانگین امتیـازات دو متخصـص بـرای هـر تکنیک رنگ آمیزی مقایسه شد.

نتایج: از نظر ویژگی‌های هسته‌های RBC و WBC، میانگین امتیازات دو روش رنگ‌آمیزی مرسوم لیشـمن و گیمسـا نسبت به روش جدید رنگ‌آمیزی لیشمن-گیمسا امتیاز پـایین تـری داشـتند (P<0.05).رنـگ‌آمیزی لیشـمن-گیمسـا شفافیت و وضوح ویژگی‌های هسته‌ای RBC و WBC را افزایش داد. روش جدید رنگ‌آمیزی لیشمن-گیمسا منجر بـه تفاوت معنی‌دار آماری (P<0.05) در ویژگی‌های سیتوپلاسمی WBC ماهی در مقایسه با دو روش دیگر شد.

نتیجه گیری نهایی: نتایج این مطالعه برای اولین بار نشان داد که سلول‌های خون ماهی رنگ‌آمیزی شده با روش جدید لیشمن-گیمسا مطلوب‌تر است. علاوه بر این، رنگ آمیزی هسـته ای و سیتوپلاسـمی آن نسـبت بـه رنـگ آمیـزی هـای معمولی گیمسا و لیشمن در صورت استفاده جداگانه برتری دارد. ترکیب لیشـمن - گیمسـا دارای شـاخص بالـایی بـرای تغییر رنگ لام خشک شده در هوا داشت.

کلید واژه ها: ماهی کپور، گرانول های سیتوپلاسمی، ارزیابی خون شناسی، ویژگی های هسـته، رنـگ آمیـزی لیشـمن-گیمسا

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