# Automated impedance-based and manual leukocyte differential counts in healthy equines

[*Contagem diferencial de leucócitos automatizada por impedância e manual em equinos saudáveis*]

## "Artigo Científico/Scientific Article"

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**Abstract**

The speed and convenience provided by automated cell counters are reasons for the increasing use of this technology in veterinary practice, with impedance methodology being one of the most commonly used in Brazil. In this regard, the objective of this study was to compare the automated differential leukocyte count obtained by impedance with the manual count obtained from the evaluation of blood smears in healthy horses. For this purpose, the automated differential leukocyte count by impedance using a veterinary cell counter (Abx Micros ESV 60, Horiba) was compared to the manual count using optical microscopy in 545 blood samples from healthy horses. Paired t-tests or Wilcoxon tests, Deming regression, Pearson or Spearman correlations, and Bland-Altman analysis were used, considering differences significant when p<0.05. All optical microscopy counts were carried out by the same veterinary clinical pathologist with ten years of previous experience in the area. The automated method showed higher concentrations of segmented neutrophils and monocytes, and lower concentrations of lymphocytes and eosinophils compared to the manual method. The mean error of the automated count was -10.26% for segmented neutrophils, 11.04% for lymphocytes, -41.39% for monocytes, and -10.84% for eosinophils, ranging from -200% to 161.4% depending on the cell type. There was a significant correlation between the methodologies only for the counts of segmented neutrophils, lymphocytes, and eosinophils. In conclusion, the manual differential leukocyte count in healthy horses cannot be replaced by the automated method, and the analysis of blood smears remains a fundamental tool for the proper interpretation of the leukogram in horses.

**Palavras-chave**: Horse; leukogram; impedance.

**Resumo**

A rapidez e praticidade garantidas por contadores de células automatizados são motivos para utilização crescente dessa tecnologia na rotina veterinária, sendo que a metodologia por impedância é uma das mais utilizadas atualmente no Brasil. Nesse aspecto, objetivou-se comparar a contagem diferencial de leucócitos de equinos saudáveis automatizada obtida por impedância com a manual obtida a partir da avaliação do esfregaço sanguíneo. Para tal, a contagem diferencial de leucócitos automatizada por impedância em contador de células veterinário (Abx Micros ESV 60, Horiba) e manual utilizando microscopia óptica foram comparadas em 545 amostras de equinos saudáveis utilizando-se teste de t pareado ou Wilcoxon, regressão de Deming, correlações pelos coeficientes de Pearson ou Spearman e análise de Bland-Altman, sendo as diferenças consideradas significantes quando p<0,05. Todas as contagens em microscopia óptica foram realizadas pelo mesmo

patologista clínico veterinário com dez anos de experiência prévia na área. O método automatizado apesentou maiores concentrações de neutrófilos segmentados e monócitos, e menores concentrações de linfócitos e eosinófilos que o método manual. O erro médio da contagem automatizada foi de -10,26% para neutrófilos segmentados, 11,04% para linfócitos, -41,39% para monócitos e -10,84% para eosinófilos, variando de -200 a 161,4% dependendo do tipo celular. Houve correlação significativa entre as metodologias apenas para as contagens de neutrófilos segmentados, linfócitos e eosinófilos. Conclui-se que a contagem diferencial de leucócitos manual em equinos saudáveis não pode ser substituída pelo método automatizado, de forma que a análise do esfregaço sanguíneo ainda é uma ferramenta fundamental para adequada interpretação do leucograma em equinos.

**Keywords**: Cavalo; leucograma; impedância.

## Introduction

The complete blood count (CBC) is the most commonly requested laboratory test for evaluating the overall health status of patients. Therefore, obtaining results quickly during routine or emergency consultations is essential in veterinary medicine (Grotto, 2009). It is generally noted that the CBC is almost always indispensable in the diagnosis and evaluation of the clinical progression of pathological processes in veterinary medicine (González and Silva, 2008; Lilliehöök and Tvedten, 2009a; Siqueira and Bastos, 2020).

Due to the high demand for CBC, speed and quality have become extremely important factors in delivering reports, as a correct diagnosis based on a reliable test allows for appropriate treatment. In many cases, in order to expedite results, veterinary laboratories have replaced manual counting methods with automated counts using veterinary cell counters, with impedance-based technology being the most widely used nowadays (Moritz et al., 2004; Borges and Siqueira, 2009). Furthermore, there has been a noticeable increase in the routine of veterinary clinics and hospitals acquiring these instruments for performing global counts of blood cells, without performing the differential count and morphological analysis of blood smears using optical microscopy by a veterinary clinical pathologist. This can lead to unreliable results.

Although automated impedance-based counts are fast, they measure and count cells based mainly on particle size, differentiating platelets and erythrocytes in an isotonic dilution and leukocytes in a lysing dilution. For this reason, animals with morphological abnormalities in these cells, especially size alterations, may present unreliable counts, particularly if there are overlaps in cell sizes. Therefore, the evaluation of blood smears plays an important and often vital role in hematological diagnoses, as it allows for the detection and quantification of morphological changes and the presence of hemoparasites (Welles et al., 2009).

In veterinary medicine, some studies have compared manual and automated leukocyte counts using various equipment, with few studies comparing these counts in horses. Riond et al. (2011) found in equine samples that the automated leukocyte differential count was able to adequately detect the lymphopenia, lymphocytosis, and neutropenia detected by the manual count, while neutrophilia was not detected in a significant number of samples. Perez-Ecija et al. (2014) compared leukocytes in donkeys using flow cytometry and impedance methods, and differences were observed for almost all variables, except for absolute counts of monocytes and eosinophils. Giordano et al. (2008) and Bauer et al. (2012) observed poor agreement between impedance and manual methods on blood smears for monocyte counts in cats, dogs and horses. Lilliehöök and Tvedten (2009b) observed that impedance tends to overestimate lymphocyte counts and underestimate neutrophil and eosinophil counts in horses, while in canine and feline species, the equipment overestimated eosinophil concentrations. Thus, significant discrepancies between counts according to methodology and equipment used are evident, and so far, we have not found studies that have used a significant number of samples and the equipment used in the present study.

In this context, the present study aimed to compare the automated differential leukocyte count obtained by impedance with the manual count obtained through optical microscopy in healthy horses.

## Materials and Methods

A total of 545 equine blood samples were included in the study, all of which were obtained from the routine of the Veterinary Clinical

Laboratory at the Roque Quagliato Veterinary Hospital of Unifio in Ourinhos, SP, Brazil. The 545 samples were collected from healthy horses, a condition determined through clinical evaluation (rectal temperature, respiratory and heart rates, capillary refill time, mucous membrane color, and intestinal movements) and laboratory tests (complete blood count, albumin, aspartate aminotransferase - AST, gamma-glutamyltransferase - GGT, globulins, total protein, and urea) within the reference range for the species. The samples were collected between August 2021 and July 2022, with the aim of confirming the routine health status of these animals for participation in equestrian events of hot-blooded' horses. All horses were adults ranging in age from 2 to 15 years and had up-to-date deworming according to information provided by the owners. Gender and racial pattern were not criterion for inclusion in this study.

For the hematological analysis, all blood samples (2 mL) were obtained after local antisepsis by jugular venipuncture using a sterile hypodermic needle and syringe, and were subsequently placed in tubes with K2EDTA (BD Vacutainer®, Becton-Dickson, New Jersey, USA). The samples were transported to the laboratory in a refrigerated isothermal box and analyzed within a maximum period of two hours. The automated leukocyte count was performed using a veterinary automated cell counter (ABX Micros ESV 60, Paris, France) that was calibrated and twice weekly verified with low, normal, and high-level controls (ABX Minotrol 16, Paris, France) to determine total leukocytes, eosinophils, lymphocytes, monocytes, and segmented neutrophils (segmented neutrophil count was obtained by subtracting eosinophils from the granulocyte count). It should be noted that the horses included in the study were healthy and did not have left-shifted neutrophils or basophil concentrations exceeding 100 basophils per microliter of blood, therefore, the basophil count was not considered in the present study.

The manual differential leukocyte count was performed on blood smears stained with a commercial hematological stain (Panótico Rápido, Laborclin, Pinhais, PR, Brazil) using the optical microscopy at a magnification of 1,000X, following the protocol described by Jain (1986). For this, in the final third of the blood smear in the monolayer region of cells along one of the edges of the smear, 100 leukocytes were counted per slide.

The counting was always performed by the same veterinary clinical pathologist with ten years of previous experience in the area and was unaware of the results obtained by the automated method. In the laboratory routine, blood smear slides are numbered for the differential leukocyte count and morphological analysis without the results of the impedance-based differential count, only including the total leukocyte count. None of the animals had more than 100 band neutrophils per microliter of blood, so the band neutrophil count was not considered in this study. Basophils are not differentiated by the automated count, none of the animals showed basophilia and this count was also not considered in this study. The results of the differential counts were expressed as absolute values.

The variables were tested for normality using the Shapiro-Wilk test, and differences between groups were assessed using paired t-tests or Wilcoxon tests. Deming regression was applied, and correlations were determined using Pearson or Spearman coefficients, with positive/negative correlations considered weak (r = 0.2000 to 0.3999), moderate (r = 0.4000 to 0.6900), strong (r = 0.7000 to 0.8999), or very strong (r = 0.9000 to 1.000). Bland-Altman analysis was performed by comparing the percentage differences between the methods (Manual - Automated) with their respective means. All statistical analyses were performed using a computer program (GraphPad Prism, v.6.00 for Windows, GraphPad Software, La Jolla, CA, USA, www.graphpad.com), with p < 0.05 considered statistically significant.

**Results**

When comparing manual and impedance-based leukocyte counts in healthy horses, we observed that the automated count showed higher counts than the manual counts for segmented neutrophils (Figure 1A) and monocytes (Figure 1C), while it was lower for lymphocytes (Figure 1B) and eosinophils (Figure 1D).

In the Bland-Altman comparison, the automated count had a mean error of -10.26% for segmented neutrophils, with a range of -64% to 32% (Figure 2A). For lymphocytes, the mean error was 11.04% with a range of -82.7% to 84.2% (Figure 2B). The mean error for monocytes was -41.39%, ranging from -200% to 109.4% (Figure 2C), and for eosinophils, the mean error was -10.84%, ranging from -200% to 161.4% (Figure 2D).
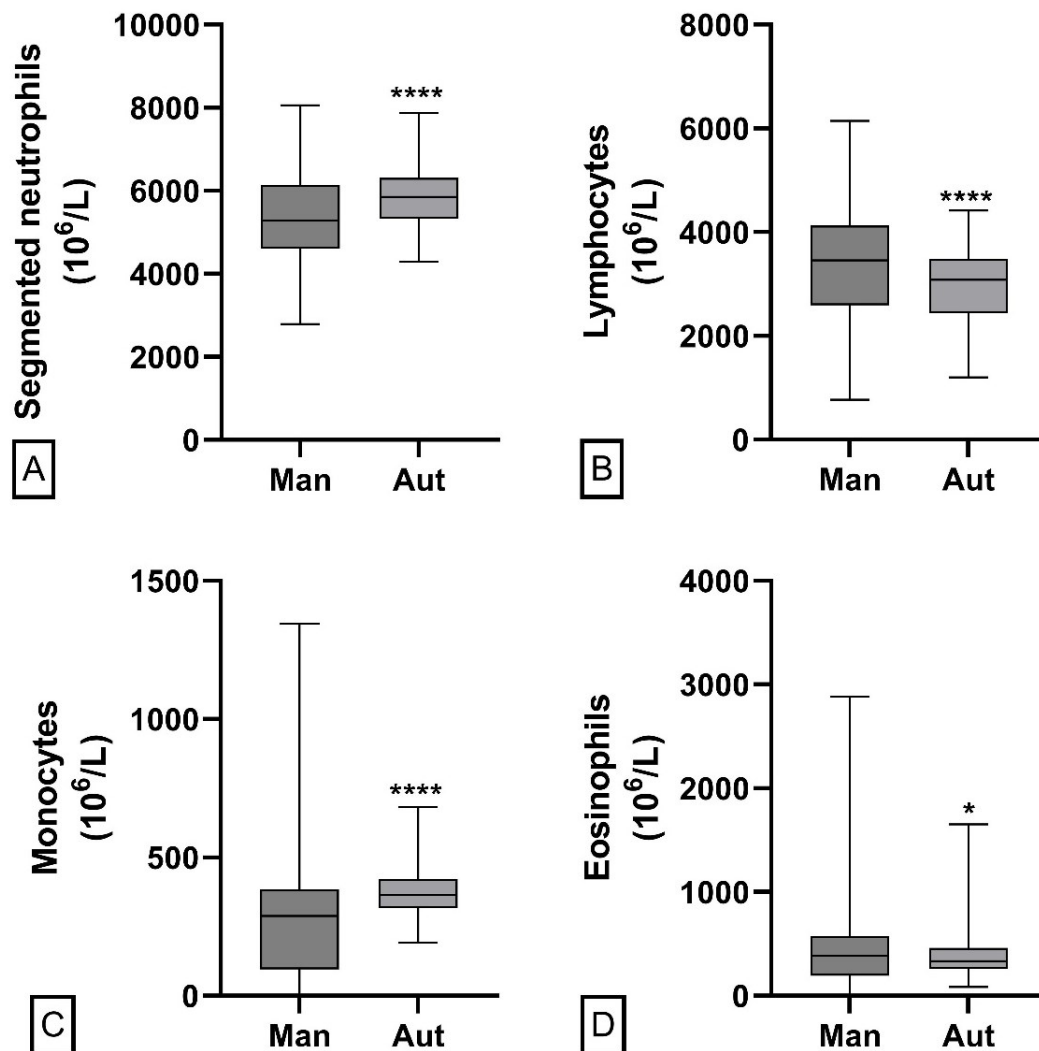
**Figure 1.** Comparison of leukocyte differential counts by manual method using blood smear under optical microscopy (Man) and automated method using impedance (Aut) in healthy horses (n=545). Bars indicate minimum and maximum values and boxes represent the first and third quartiles. Statistically significant difference is indicated by * (p<0.05), ** (p<0.01), *** (p<0.001) or **** (p<0.0001).

Deming regression analysis with determination of correlation between manual and automated differential leukocyte counts showed a significant correlation for segmented neutrophils, lymphocytes, and eosinophils, with a strong correlation for lymphocytes and a moderate correlation for the other cell types. There was no significant correlation between the methods for monocyte counts (Figure 3).

## Discussion

The present study aimed to compare the leukocyte differential count in healthy horses in order to determine the reliability of the automated impedance method for differential leukocyte count in comparison to the conventional manual method, considered the gold standard (Freeman et al., 2022). Significant differences were observed between the methods in the counts of segmented neutrophils, lymphocytes, monocytes and eosinophils. Underestimation or overestimation of values by the automated method resulted in falsely decreased or elevated counts, leading to errors ranging from -200% to 161.4%, depending on the cell type, although some cell types showed moderate to strong correlation.

The comparison between automated impedance-based leukocyte differential count and manual count using optical microscopy revealed that the automated methodology overestimated the counts of segmented neutrophils and monocytes, while underestimating the counts of lymphocytes and eosinophils.

In contrast to the present study, Miranda (2014) found no significant differences between manual and automated impedance-based methods in the counts of granulocytes and monocytes in 30 healthy mules.
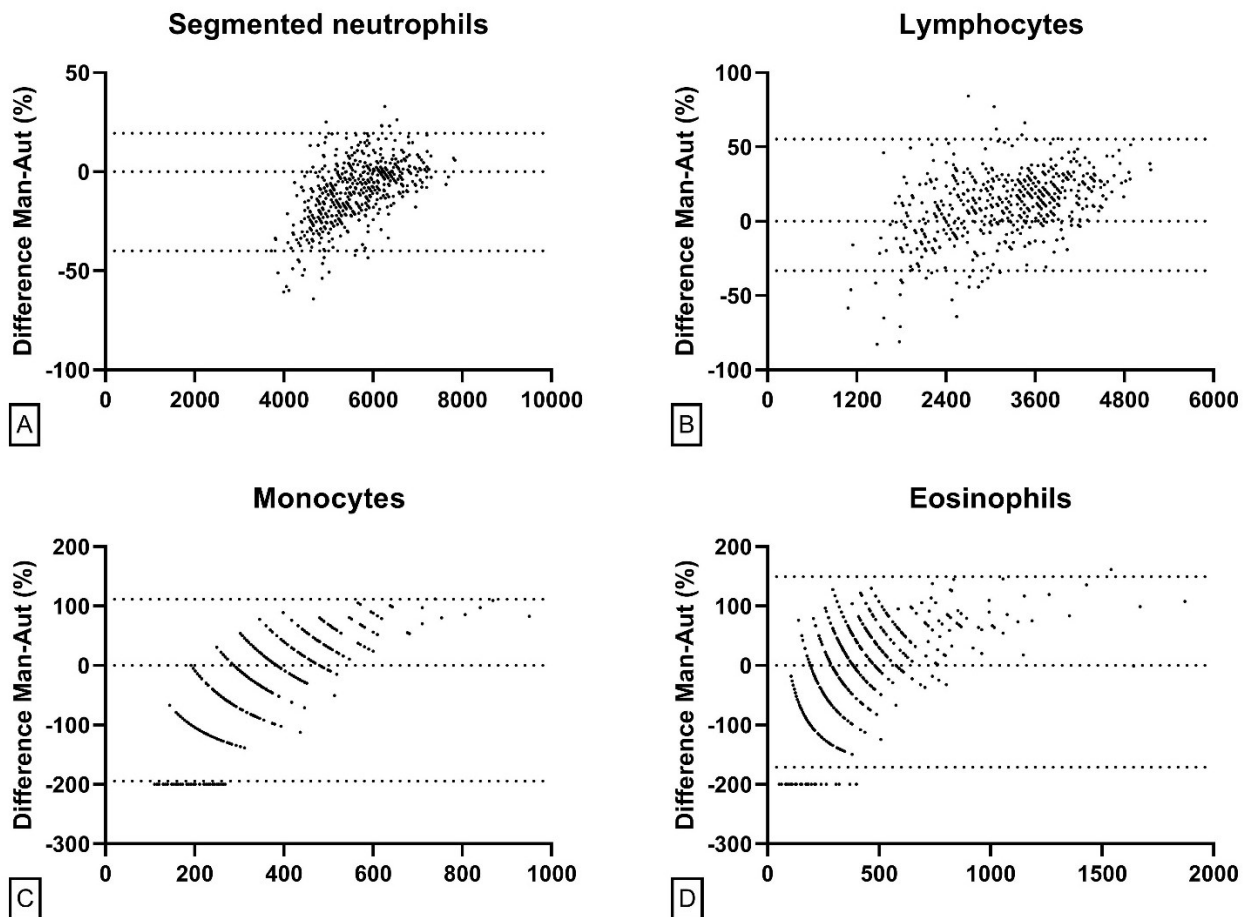
**Figure 2.** Bland-Altman plot for comparison of leukocyte differential counts by manual method using blood smear under optical microscopy (Man) and automated method using impedance (Aut) in healthy horses (n=545).
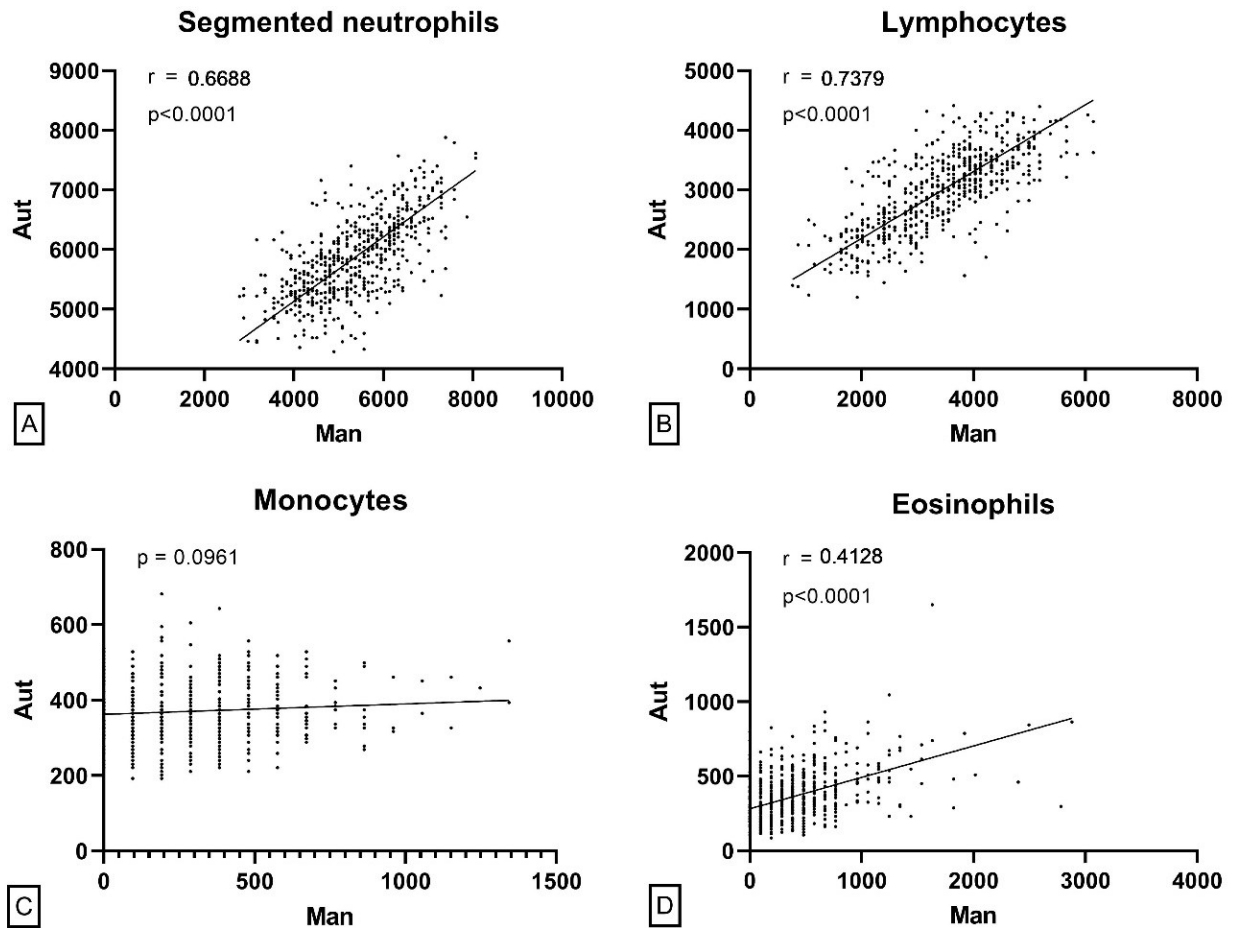
Furthermore, the authors observed that impedance underestimated lymphocyte counts, similar to the results of the present study. On the other hand, Riond et al. (2011), comparing manual and impedance-based counts with hydrodynamic focusing in 40 horses, found excellent correlation (r>0.95) for lymphocytes and other parameters, where authors combined the values of neutrophils, basophils, and monocytes. The authors did not perform statistical comparisons between groups, and similar to the present study, lymphocytes showed the strongest correlation, despite working with a smaller sample size.

The comparison between methods in other studies also demonstrated variation in results between manually obtained counts and those obtained by flow cytometry. Perez-Ecija et al. (2014), evaluating 84 healthy donkeys, observed that flow cytometry overestimated the counts of granulocytes + monocytes compared to impedance. When flow cytometry was compared to the manual method, differences were observed for almost all variables, except for monocyte and eosinophil counts. Lilliehöök and Tvedten (2009b), evaluating

73 ill horses, found that leukocyte populations were well separated in equine species when flow cytometry was used. The authors observed high correlations between flow cytometry and the manual method for neutrophils, with flow cytometry overestimating lymphocytes by 10-26% and underestimating monocyte counts. The lowest correlations were mainly observed for cell types present in low numbers, such as equine eosinophils and monocytes. These results demonstrate that the manual method often differs from automated methods, as observed in the present study when comparing impedance with the manual method.

In the present study, we observed that the mean error for the counts ranged from -41.39% for monocytes to 11.04% for lymphocytes, with individual errors ranging from -200% to 161.4%. An important factor to consider is that the present study only evaluated healthy horses without evident inflammatory conditions. In pathological conditions, lymphocytes may become larger than normal when reactive or neoplastic, monocytes may change in size and shape when activated, and neutrophils may be larger than normal when toxic.

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**Figure 3.** Correlations and regression between leukocyte differential counts by manual method using blood smear under optical microscopy (Man) and automated method using impedance (Aut) in healthy horses (n=545).

These changes could lead to errors in automated impedance-based counts (DeNicola, 2011). In fact, Riond et al. (2011) point out that neutrophilia was lost in a significant number of equine samples when considering neutrophilia determined by manual count. Souza et al. (2021) also observed higher errors in differential counts obtained by impedance in sick dogs, with discrepancies between the methodologies reaching -140% for segmented neutrophils, -447% for lymphocytes, -410% for monocytes, and -651% for eosinophils in the sick dog group. These results indicate that in ill animals, errors can be even greater than those observed in the present study, which could compromise the diagnosis of these animals.

Nabity et al. (2018) determined that if the observed errors exceed the acceptable total error stipulated by the American Society for Veterinary Clinical Pathology, an incorrect diagnosis may occur. The acceptable error for neutrophils and lymphocytes is up to 15%, up to 90% for eosinophils, and up to 60% for monocytes. In this study, considering these established limits, the automated count would have errors outside the permissible range for all cell types, as the error for segmented neutrophils ranged from -64% to 32%, lymphocytes from -82.7% to 84.2%, monocytes from -200% to 109.4%, and eosinophils from -200% to 161.4%.

Despite such high errors, we observed that the correlations between the automated impedance-based method and the manual method were significant for lymphocytes, segmented neutrophils, and eosinophils, being strong for lymphocytes and moderate for segmented neutrophils and eosinophils, with no significant correlation for monocytes. Riond et al. (2011) also found that the best correlation in horses was for lymphocytes. Bauer et al. (2012) observed poor agreement between methods for monocyte counts in cats, dogs and horses. However, the same author observed good correlation between the methods for canine eosinophils and moderate correlation for cats and horses. Excellent agreement was found for

canine neutrophil counts, and good agreement for cats and horses.

Taken together, these results demonstrate that the differential leukocyte count obtained by the automated impedance method should not be considered for an accurate interpretation of the leukogram in horses, especially when considering reference intervals obtained by the standard method of differential count in blood smears. Variations could be even greater when considering diseased animals in which cellular morphological changes would lead to errors in impedance-based counts. Additionally, the present study did not include horses with left-shifted neutrophils, an information that is frequently missed when the clinician uses exclusively automated impedance-based differential counts. Therefore, DeNicola (2011) emphasizes that microscopic examination of blood smears, when combined with data collection from the hematology analyzer, serves as a quality assurance measure for instrument performance and provides critical morphological information not provided by the hematology analyzer alone. The review of blood smears is a very important component of the complete blood count.

## Conclusion

The comparison of leukocyte differential count using manual and automated methods reveals that manual counts in optical microscopy cannot be replaced by automated counts using impedance in healthy horses, as significant errors could compromise the quality of the examination. It is imperative to underscore that exclusive employment of automated impedance counting might jeopardize diagnostic accuracy due to analytical and post-analytical errors, while also failing to enable cellular morphological evaluation and the detection of cellular inclusions, both of which are vital components of diagnosis.

## Conflict of Interest

The authors declare that there is no conflict of interest.

## Ethics Committee

This is a retrospective scientific study that utilized data from previously conducted laboratory tests, thereby exempting it from the need for Ethics Committee authorization.

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