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ORIGINAL RESEARCH

# Spurious reticulocyte profiles in dogs with large form babesiosis: a retrospective study

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*Babesia*, dot plots, pseudoreticulocytosis, reticulocyte fluorescence ratios

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**Background:** Erroneously high reticulocyte counts (pseudoreticulocytosis) have been reported in dogs with leukemia. Pseudoreticulocytosis and an abnormal reticulocyte profile were observed in a dog with large form babesiosis presented at our institution.
**Objectives:** The aims of this retrospective study were to determine if dogs with babesiosis and other dogs had abnormal reticulocyte profiles, and to correlate these profiles with the primary diagnosis.
**Methods:** All canine CBCs obtained with the Sysmex XT-2000iV or Procyte DX were reviewed. Cases of large form babesiosis were identified and their reticulocyte dot plots were analyzed. Dogs with abnormal reticulocyte profiles but without microscopically apparent intraerythrocytic *Babesia* piroplasms were identified. The reticulocyte profiles and fluorescence ratios of dogs with and without babesiosis were compared.
**Results:** Twenty of 92 dogs with babesiosis had abnormal reticulocyte profiles, including 8 with a separation between the reticulocyte and mature RBC plots or a continuum of reticulocytes from the RBC plot but with a higher density of dots in the middle of the "comet tail" than in the left quarter of the dot plot. Thirteen of 6980 dogs without *Babesia* on the blood smear had abnormal reticulocyte profiles, including 3 with leukemia. The medium-fluorescence reticulocyte ratios tended to be higher in dogs with babesiosis and abnormal dot plots than in other dogs, whereas the high-fluorescence ratio was higher in one dog with leukemia.
**Conclusion:** Abnormal reticulocyte dot plots and atypical reticulocyte fluorescence ratios may occur in dogs with babesiosis and alert clinical pathologists to consider this diagnosis.

## Introduction

Canine babesiosis is a tick-borne disease distributed worldwide and caused by hemoprotozoan parasites of the genus *Babesia*. The classical presentation is associated with a febrile and hemolytic syndrome, but numerous atypical manifestations, including locomotor, cerebral, ocular, gastrointestinal, and vascular forms, make the etiologic diagnosis challenging. Because the disease is potentially severe and life-threatening, early diagnosis is essential.[1]

Diagnosis is based on epidemiologic data, especially the prevalence of *Babesia* sp. infection in the geographic area, and clinicopathologic findings such as fever, anemia, and thrombocytopenia, and is confirmed by the microscopic observation of intraerythrocytic parasites on blood smears. However, microscopic diagnosis can be time-consuming and tedious, and recognition of the parasites requires experience. Moreover, false negatives can occur in early and late stages of the disease. Serologic tests are not useful in the diagnosis of acute canine babesiosis because seroconversion occurs one to 2 weeks after infection and seroprevalence in endemic areas is high. Polymerase chain reaction can be used to confirm the diagnosis, but testing usually has to be performed at a reference laboratory and the results are not immediately available.

The interpretation of anemia requires reticulocyte enumeration. The Sysmex XT-2000iV automated hematology analyzer identifies reticulocytes by an RNA-binding fluorescent stain and quantification by flow cytometry. In addition, the Sysmex classifies the reticulocytes according to their RNA content into low- (LFR), medium- (MFR), and high-fluorescence ratio (HFR) reticulocytes. Both mature RBC and reticulocytes are plotted in a gate where the *X*-axis represents increasing fluorescent intensity and RNA content, and the *Y*-axis represents the size or volume of the cells. Mature RBC, per definition lacking RNA and being nonfluorescent appear on the left of the dot plot. In the presence of reticulocytosis, there are increased numbers of dots in the LFR, MFR, and HFR regions which extend and taper away from the mature RBC toward the right of the plot, resembling a "comet tail."

Pseudoreticulocytosis has been reported in human beings with malaria, and in people and dogs with leukemia.[2–6] Pseudoreticulocytosis is defined as an erroneous automated reticulocyte count, resulting from cells or fragments incorrectly counted as reticulocytes based on size and RNA content resembling reticulocytes. We recently noted pseudoreticulocytosis and a noncharacteristic "comet tail" reticulocyte dot plot in a Sysmex plot RBC in a dog presenting with large form babesiosis at our institution. We hypothesized that pseudoreticulocytosis and abnormal reticulocyte dot plots might represent a characteristic and previously unnoticed finding in dogs with large form babesiosis. Therefore, the specific aims of this study were to (1) retrospectively examine the reticulocyte dot plots of all dogs with large form babesiosis to determine whether this was a consistent finding, and (2) retrospectively examine all abnormal reticulocyte dot plots in dogs and determine a possible correlation with the primary diagnosis. We were hoping that reticulocyte dot plots available from modern hematology analyzers would have early diagnostic potential in clinical cases of large form babesiosis.

## Materials and Methods

This retrospective, descriptive study was conducted in the Laboratory of Clinical Pathology at the National Veterinary School of Toulouse. All canine CBCs determined with the Sysmex XT-2000iV (Sysmex, Kobe, Japan) or Procyte DX (IDEXX Laboratories, Westbrook, ME, USA) hematology analyzers between March 2008 and May 2014 were reviewed. Cases of large form babesiosis diagnosed by microscopic observation of intra-erythrocytic parasites were selected. The CBC included total and differential WBC and platelet counts, but in this study the main focus was on the erythroid profile, including RBC count, HGB concentration, HCT or PCV, and relative and absolute reticulocyte count. Both analyzers provided a dot plot of mature RBC and reticulocytes based on polymethine-mediated RNA-fluorescence and flow cytometric quantification. In addition, the Sysmex also provides LFR, MFR, and HFR, and the immature reticulocyte fraction (IRF), representing the sum of MFR and HFR. In addition, blood smears were assessed for RBC morphology including subjective grading of the degree of polychromasia (absent, slight, moderate, or marked), as documented on the CBC report. When RBC-related variables were below the RI and the reticulocyte count above the upper limit of the RI ($19.0–150.0 \times 10^9$/L), the anemia was classified as regenerative. These cases were all confirmed by evaluation of WG-stained blood smears and reticulocyte counting in New Methylene Blue-stained specimens, according to the Standard Operating Procedures of the laboratory.

All analyzer-generated reticulocyte dot plots from dogs with large form babesiosis were assessed twice by a veterinary clinical pathologist (ECVCP diplomate) and a resident in clinical pathology with experience in analyzing optical dot plots. Reticulocyte dot plots were defined as abnormal if there was a complete separation between the reticulocyte and mature RBC plots, or a continuum of reticulocytes from the mature RBC plot but with a higher density of dots in the middle of the area of the "comet tail" corresponding to the LFR reticulocytes. Reticulocyte dot plots deemed abnormal were examined for distinct patterns.

When available and assessable, blood smears were examined microscopically at high magnification (400×). Each individual RBC was examined for intra-erythrocytic piroplasms and the number of parasitized RBC/smear was quantified. When parasitemia was high, counting was arbitrarily stopped at 1000 parasitized RBC. If there were < 1000 parasitized RBC, all RBC on the smear were examined. As the distribution of parasitized erythrocytes was highly heterogeneous, the percentage of parasitized RBC was not considered representative for the degree of parasitemia, and was therefore not calculated.

Finally, all canine CBC obtained with the Sysmex and Procyte analyzers between March 2008 and May 2014 were reviewed a second time to identify abnormal reticulocyte dot plots in dogs excluding dogs with large form babesiosis. Specifically, in these cases babesiosis was excluded by microscopic analysis of the blood smear, in addition an etiologic diagnosis was

made based on the available information in the medical records. The CBC including RBC variables and reticulocyte dot plots were analyzed as in dogs with large form babesiosis.

## Results

### Dogs with large form babesiosis

Between March 2008 and May 2014, 6980 canine CBCs were reviewed. During this period, 92 cases of large form babesiosis (1.32%) were diagnosed. Most of these dogs (92%) were thrombocytopenic, 2 dogs of which had numerous platelet clumps, suggesting pseudothrombocytopenia. Sixty percent of the dogs were leukopenic due to neutropenia, lymphopenia, or both. Five dogs had lymphocytosis or monocytosis.

Seventy-two of the 92 cases of large form babesiosis had normal reticulocyte dot plots, and 34 were anemic. In 3 cases, anemia was regenerative with absolute reticulocyte counts of $167.0 \times 10^9$/L, $196.0 \times 10^9$/L, and $232.0 \times 10^9$/L. For these 72 dogs, 34 blood smears were available. The smears from 26 dogs showed < 1000 (8–603) parasitized RBC/smear. The smears from the other 8 dogs had a high number of parasitized cells (> 1000 parasitized RBC/smear).

Twenty out of the 92 cases of large form babesiosis had abnormal reticulocyte dot plots (Figure 1) with 3 distinct patterns: (1) 8 dot plots were characterized by a complete separation between the reticulocyte and mature RBC plots or a continuum of reticulocytes from the RBC plot but with a higher density of dots in the middle of the "comet tail" than at the beginning (Group 1); (2) 3 plots were similar to Group 1 plots but were less dense (Group 2); and (3) 9 plots were considered ambiguous or suspected to be abnormal due to the higher density of dots in the middle of the comet tail compared to the left quarter (Group 3). Both observers similarly characterized the Group 1 and Group 2 plots, but required some discussion to reach a consensus concerning the Group 3 plots. For all dogs with large form babesiosis and abnormal reticulocyte profiles, the abnormal reticulocyte cluster was observed in the middle part of the plot or, in one case, slightly toward the right.

Fourteen of the 20 dogs with abnormal dot plots were anemic. Blood smears were available for 15 of these 20 dogs, and all but one (ie, 7 Group 1 dogs, one Group 2 dog, and 6 Group 3 dogs) had numerous parasitized cells with > 1000 parasitized RBCs/smear. The other smear (from a Group 2 dog), showed only 315 parasitized RBCs but was of poor quality.

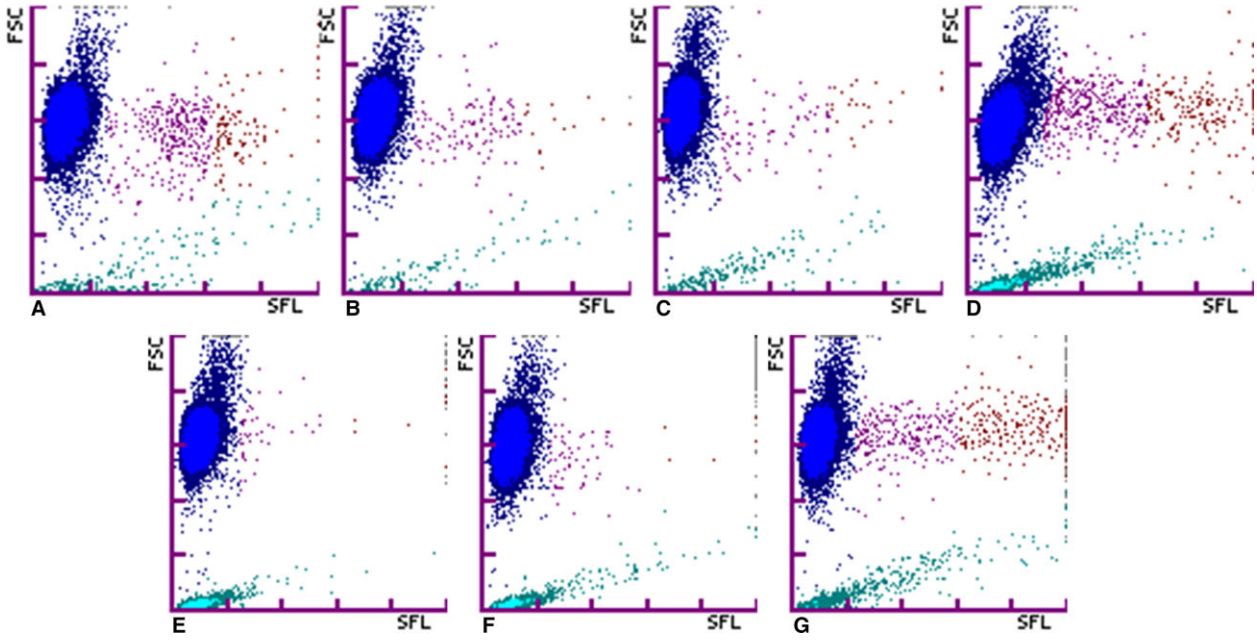


**Figure 1.** Abnormal reticulocyte profiles obtained with the Sysmex from dogs with large form babesiosis and nonregenerative anemia, one representative dog from Group 1 (**A**); one representative dog from Group 2 (**B**); one representative dog from Group 3 (**C**), a dog with large form babesiosis and regenerative anemia with a normal reticulocyte plot resembling the comet tail (**D**), a normal dog (**E**), a dog without babesiosis and with nonregenerative anemia (**F**) and a dog without babesiosis on the blood smear and with regenerative anemia indicated by reticulocytes dotted in the comet tail (**G**). Blue dots indicate mature RBC; red dots, reticulocytes with medium-fluorescence ratio; purple dots, reticulocytes with low-fluorescence ratio (high-fluorescence ratio reticulocytes are not represented in this scattergram).

### Dogs with a diagnosis other than large form babesiosis

For 13 out of 6980 dogs, the reticulocyte dot plot was abnormal but no large form *Babesia* was observed on the blood smear. Based on the above-defined criteria, the plots for these 13 dogs were categorized as Group 1 (4 dogs), Group 2 (3 dogs), or Group 3 (6 dogs), respectively. Three of the 4 Group 1 dogs had leukemia with circulating blast cells (Figure 2), and the dots were concentrated at the far right of the plot. The 4$^{th}$ dog had B-cell centroblastic lymphoma with marked leukocytosis (35.35 $\times$ 10$^6$/L) due to neutrophilia with a left shift, but had no circulating blast cells. No explanation was found for the abnormal reticulocyte profiles of the other 9 dogs: 2 had been diagnosed with cardiac failure, others had a mast cell tumor, malignant melanoma, hepatopathy, duodenitis, gastroenteritis, or hyperadrenocorticism, and the 7$^{th}$ dog was a healthy blood donor. A PCR was not performed to rule out babesiosis.

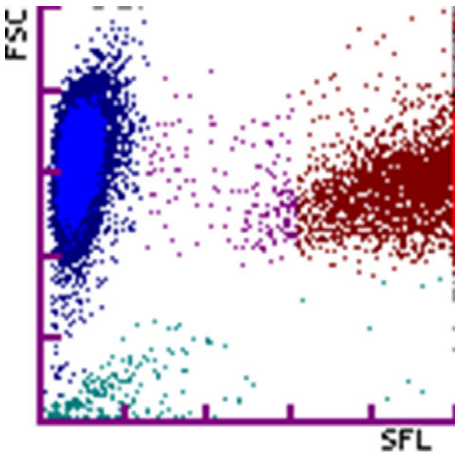


**Figure 2.** Abnormal reticulocyte profile from a dog with leukemia and nonregenerative anemia, obtained with the Sysmex. Compare this profile with profiles from dogs with large form babesiosis and either pseudoreticulocytosis (Figure 1A) or regenerative anemia and a normal reticulocyte profile (Figure 1D).

### Fluorescence ratios for dogs with and without large form Babesia on the blood smear

In dogs in Group 1 with large form babesiosis and abnormal plots, the MFR tended to be higher than for dogs in Groups 2 and 3, dogs with regenerative anemia, and dogs with abnormal plots and diseases other than babesiosis (Figure 3). Statistical analysis was precluded by the low number of dogs in some groups. The HFR in one dog with leukemia was higher than in all the other dogs. The IRF was high in all dogs that had either high MFR or HFR.

## Discussion

In this study, we showed that dogs with large form babesiosis and a high degree of parasitemia may display abnormal reticulocyte profiles and pseudoreticulocytosis. In addition, we showed that reticulocyte fluorescence ratios may be helpful in distinguishing the causes of pseudoreticulocytosis, as dogs with large form babesiosis and abnormal reticulocyte profiles tended to have a higher MFR index. In human medicine, pseudoreticulocytosis has been reported in patients with *Plasmodium falciparum* malaria.[2–4] In one person with a high parasite density, the reticulocytes as identified by the hematology analyzer represented 21.6% of the RBCs (absolute reticulocyte count of 92.0 $\times$ 10$^9$/L), whereas the reticulocyte count obtained by supravital New Methylene Blue staining

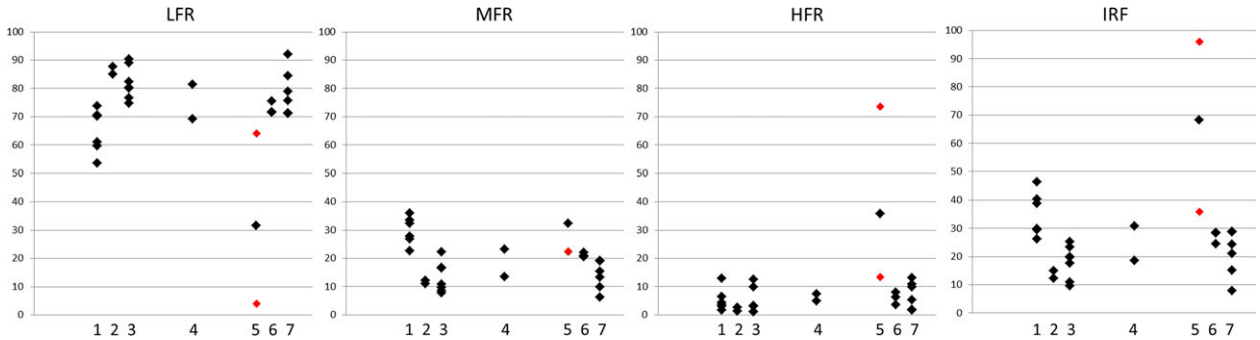


**Figure 3.** Scatter plots illustrating the percentages of low-fluorescence reticulocytes (LFR), medium-fluorescence reticulocytes (MFR), high-fluorescence reticulocytes (HFR), and the immature reticulocyte fraction (IRF, sum of MFR and HFR). (**1**, large form babesiosis Group 1; **2**, large form babesiosis Group 2; **3**, large form babesiosis Group 3; **4**, large form babesiosis group with regenerative anemia and normal reticulocyte dot plot; **5**, non-babesiosis Group 1; red dots are from leukemic dogs; **6**, non-babesiosis Group 2; **7**, non-babesiosis Group 3).

and manual microscopic counting indicated a much smaller reticulocyte proportion of 4% of RBCs (absolute count of $16.0 \times 10^9$/L).[3] The nucleic acid from the parasites was likely identified by the analyzer as nucleic acid from reticulocytes. Similarly, pseudoreticulocytosis due to the presence of nuclear material from intra-erythrocytic malaria parasites was detected by the Cell-Dyn 4000 hematology analyzer in 23 out of 46 patients with *P falciparum* malaria and almost exclusively in patients with a parasitemia of $\geq 0.5\%$.[4] Data obtained with the Cell-Dyn 4000 hematology analyzer may thus contribute to the diagnosis of malaria and decrease the delay in diagnosis, particularly in medical centers without expertise in tropical diseases.

Other causes for discrepancies between automated and manual reticulocyte analyses include the presence of giant platelets, platelet clumps, abnormal WBC, leukocytosis, and WBC fragments.[5,7–10] Heinz bodies, Howell–Jolly bodies, Pappenheimer bodies, or basophilic stippling may also interfere with reticulocyte evaluation by hematology analyzers using flow cytometry.[8,11] In our study, none of these abnormalities were found in dogs with abnormal reticulocyte dot plots and large form babesiosis, and only parasitized RBCs could explain the abnormal profile. Previous studies have shown that in some leukemic human patients and in a leukemic dog, the reticulocyte count was erroneously increased, with an abnormal reticulocyte profile in which the reticulocyte region was completely separated from the RBC region[5–7], as observed in some leukemic dogs in this study.

Reticulocytes are subclassified as LFR, MFR, and HFR reticulocytes. Reticulocytes with LFR and MFR are localized in the left and middle sections of the reticulocyte dot plot, and LFR, the most mature reticulocytes, are typically the most numerous. Reticulocytes with HFR are farthest to the right and represent the most immature reticulocytes, those with the highest concentration of RNA.[12] In human leukemia, WBC fragments and abnormal WBC overlap the reticulocyte regions, especially the IRF, which can be abnormally high. As leukemic cells have been speculated to stain less strongly than normal WBC with polymethine, resulting in lower fluorescence intensity, they may be displayed in the reticulocyte region. In some cases, a flag appears with the message "RET Abn Scattergram." Similarly, a discrepancy between the Sysmex XT-2000iV reticulocyte count and polychromasia was reported in a dog with acute leukemia.[6] in our study, the LFR, MFR, and HFR suggested an error with an atypical distribution of the high-fluorescence cells, which accounted for 66% of the reticulocyte fraction. Similarly, in one dog with leukemia, we observed an abnormal reticulocyte scattergram, and a high percentage of HFR, a high IRF, and low LFR. The CBC for one leukemic dog was performed with the Procyte analyzer, and so no fluorescence ratios were available. However, the reticulocyte profile revealed an abnormal cluster of dots to the far right of the scattergram.

Dogs with large form babesiosis and a complete separation between the reticulocyte and mature RBC plots or a continuum of reticulocytes from the RBC plot but with a higher density of dots in the middle of the "comet tail" than in the right quarter tended to have higher MFR and lower HFR than dogs with leukemia. We propose that RBCs infected with large form *Babesia* organisms exhibit less intense fluorescence than WBC fragments or abnormal WBC associated with leukemia, as supported by the faint staining of intra-erythrocytic parasites with fluorescent dyes compared to the intense staining of WBC fragments or abnormal WBCs in leukemia.[8] We therefore suggest that the pattern of the abnormal reticulocyte dot plot, and localization of the dots, may vary according to the specific disease.

With one exception, all dogs with large form babesiosis and abnormal reticulocyte profiles had high numbers of parasitized RBC (> 1000), but 8 dogs with normal reticulocyte dot plots also had > 1000 parasitized RBC. Possible explanations are (1) true reticulocytes could have masked the parasitized RBC with reticulocyte counts within or above the RI and (2) the number of parasitized RBC required for an abnormal plot to be recognized might be much greater than 1000. One study in human beings with *P falciparum* malaria indicated that the cut-off percentage of parasitized RBC for pseudoreticulocytosis to appear was $\geq 0.5\%$.[4] Thus, it may be the percentage of parasitized RBC that is important in recognizing an abnormal reticulocyte profile. In human medicine, routine analysis for malaria consists of examining stained thick and thin blood smears, according to the World Health Organization, and quantitative buffy coat analysis.[4]

If malaria parasites are observed and the expected parasite density is $> 0.5\%$, the parasite density is expressed as the percentage of RBC infected by determining in duplicate the number of infected RBC/2000 RBC on a thin blood smear. In the case of an expected parasite density $< 0.5\%$, the parasite density is determined by counting in duplicate the number of parasites observed/100 WBC on a thick blood smear. Further investigations in dogs are needed to ascertain if it is the percentage of parasitized RBCs that is important and if a cut-off percentage for observing an abnormal reticulocyte profile can be determined.

This study had several limitations. The numbers of dogs (with or without babesiosis) with abnormal

reticulocyte profiles and pseudoreticulocytosis were low and subdividing the dogs into groups further reduced the numbers and precluded statistical analysis by group. In addition, the number and types of cases included in this study were dependent on the study's geographic location (south-west France) and the fact that it was carried out in a University veterinary hospital, a very different setting from general practice. This precludes any transferable calculation of diagnostic accuracy. For example, using the data reported in the results section and an estimated pretest probability of 20% that a dog might have large form babesiosis, the observation of an abnormal reticulocyte profile would have positive and negative predictive values (95% CI) of 96.6% (93.2–98.7) and 83.6% (82.2-85.7), respectively; however, this would be valid only in our hospital. A final limitation was that 2 different hematology analyzers were used, although the methodology used to identify reticulocytes was similar. However, one analyzer did not provide fluorescence ratios, and consequently these data were not available for all dogs.

The recognition of abnormal reticulocyte profiles, the detected reticulocyte patterns, and atypical reticulocyte fluorescence ratios may help clinical pathologists and clinicians who have developed expertise in examining the plots to suspect large form babesiosis in dogs with consistent hematologic findings, such as anemia with thrombocytopenia or leukopenia. Future studies are needed to see if a cut-off for the degree of parasitemia that is likely to result in an abnormal reticulocyte profile or pseudoreticulocytosis, can be established and if reticulocyte fluorescence ratios can be used to distinguish the causes of pseudoreticulocytosis. According to our quality procedures, the clinical pathologist must accept the dot plots and curves provided by the analyzers before validating the numerical CBC results. In addition, the manufacturers of hematology analyzers are advised to set flags to alert users when abnormal reticulocyte dots are observed.

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