

ORIGINAL ARTICLE

Detection of frequent neutrophil misclassification by the ProCyte Dx in sick dogs and how to avoid it

E. BERGSTRAND*, H. W. TVEDTEN† AND I. LILLIEHÖÖK‡,1

*University Animal Hospital, Swedish University of Agricultural Sciences, Box 7054, 75007, Uppsala, Sweden

†Clinical Pathology Laboratory, Swedish University of Agricultural Sciences, Box 7054, 75007, Uppsala, Sweden

‡Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Sciences, Swedish University of Agricultural Sciences, Uppsala 75007, Sweden

¹Corresponding author email: inger.lilliehook@slu.se

OBJECTIVES: A common and severe error in identifying neutrophils in feline blood samples by the IDEXX ProCyte Dx haematology analyser (ProCyte) has been reported. The hypothesis was that the same or similar error would be identified during analysis of canine blood samples and that white blood cell dot plot evaluation would be critical to detect and avoid erroneous results.

MATERIALS AND METHODS: Eighty-six canine blood samples collected for clinical diagnosis of hospital patients were evaluated. Differential leukocyte counts were determined by the ProCyte Dx, ADVIA 2120 and manual methods. ProCyte neutrophil percentage results were considered unacceptable if the result was 15% different than percentage results from both ADVIA 2120 and manual counts. ProCyte WBC dot plots and instrument flags were evaluated for correctness.

RESULTS: The ProCyte neutrophil counts were unacceptably lower than the ADVIA 2120 and manual neutrophil counts in 13 samples (15% of 86 samples). Neutrophils misclassified by the instrument were erroneously classified as monocytes and/or lymphocytes. All these samples were from patients with systemic inflammation. The error could be eliminated by rejecting results from samples with incorrect separation of cell clusters in the ProCyte WBC dot plots.

CLINICAL SIGNIFICANCE: The ProCyte neutrophil count error with canine blood samples is common, severe and might affect clinical decisions. Operators of the instrument must evaluate white blood cell dot plots for correctness to avoid the error.

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INTRODUCTION

ProCyte (IDEXX ProCyte Dx, IDEXX Laboratories, Westbrook, ME, USA) is an automated haematology analyser used by many veterinary clinics. There are two validation studies that have evaluated the performance of ProCyte for canine samples. One study described fair to excellent correlation between ProCyte and manual differential leukocyte count in 59 canine samples without comments about erroneous leukocyte results (Fujino *et al.* 2013). Except for monocytes, the other study indicated good to excellent correlation with manual differential counts for the canine leukocyte differential

count. However, this was after exclusion of samples with invalid separation of leukocyte populations in the white blood cell (WBC) dot plots. Data for ungrouped results were presented in a table and manual differential count was recommended in case of invalid separation of leukocyte cell populations. Misclassifications of immature neutrophils were suspected in the excluded samples, but these samples were not further discussed (Goldmann *et al.* 2014).

ProCyte is commonly used in clinics without veterinary clinical pathologists or medical technologists with veterinary training. Instrument operators may not always be aware of the frequency and consequences of instrument errors.

Severe errors in the classification of neutrophils in feline blood samples by the ProCyte have been described. With feline blood, especially in samples with immature and toxic neutrophils, the ProCyte misclassified neutrophils, mainly as lymphocytes, in about 13% of patient samples. The error could be avoided by rejecting automated differential leukocyte counts in samples with abnormal WBC dot plot patterns (Tvedten *et al.* 2017).

The aims of this study were to determine the frequency and severity of errors with the differential leukocyte counts in canine blood using the ProCyte. The hypothesis was that automated instrument leukocyte counts from sick dogs with immature and toxic neutrophils would have similar problems to those described in feline samples. Another aim was to determine if inspection of WBC dot plots and instrument flags would avoid these errors.

MATERIALS AND METHODS

Samples

In this prospective study, 86 canine blood samples were collected in K₃ ethylenediaminetetraacetic acid tubes in the period between March and September in 2018. The blood samples were collected as a part of the clinical investigations of canine patients at the “University Animal Hospital at the Swedish University of Agricultural Sciences.” Most samples were collected from dogs with various ongoing diseases during hospitalisation, but some samples came from patients at the polyclinic. There was no selection criteria based on breed, age, sex or that the dogs had any certain disease or other condition. Dog owners agreed to the use of collected blood for blinded research providing written or oral consent. Each sample was analysed routinely by the automated haematology analyser ADVIA (ADVIA 2120, Siemens Healthcare GmbH, Ashburn, Germany). To be included in this study, each sample also needed to be analysed with ProCyte within 4 hours from collection. There was no other strategy for selection or randomisation of samples other than the availability for the samples to be handled within this time limit. An extra blood smear was prepared and stained with May-Grunewald Giemsa to be used for a manual differential leukocyte count. Before being analysed, the samples were stored in room temperature.

Instruments

ProCyte Dx (software version 00-33_51) reports a five-cell automated canine differential leukocyte count based on a laser-based flow cytometer technique after staining with a fluorescent polymethine dye that stains nucleic acids and cytoplasmic organelles. The separation of leukocytes into leukocyte subpopulations is based on two parameters: side scatter (SSC) and side fluorescent light (SFL). Each cell is presented on a WBC dot plot. The leukocytes that stain most strongly with the fluorescent dye, such as monocytes, are presented highest along the y axis of the WBC dot plot. SSC reflects the cell complexity and the most complex leukocytes, such as eosinophils, are presented to the right along the x axis. ProCyte WBC flags indicate the presence of abnormal cell populations or difficulties in the leukocyte count. The “WBC abnormal distribution” flag indicates indistinct separation of leukocyte clusters and

the user is advised to perform a manual blood film review. ProCyte also flags if band neutrophils are suspected (Anonymous 2014).

ADVIA 2120 (software version 5.9.0-MS) is a laser-based flow cytometry instrument. The differential leukocyte count is performed in two separate channels. The peroxidase channel is the default method for the automated differential count. Leukocytes are assessed based on peroxidase staining intensity along the x axis and size by low angle light scatter along the y axis. ADVIA has a unique leukocyte classification of large unstained cells (LUC) which are large cells placed high along the y axis and with little or no peroxidase staining placing them to the left on the dot plot. LUC in a canine blood sample can include various leukocytes such as monocytes, blast cells, basophils and other large peroxidase negative cells. Peroxidase positive cells as neutrophils and eosinophils are placed more to the right along the x axis. The BASO-channel [leukocyte counting channel (basophil channel) in the ADVIA 2120 haematology instrument] analyses cells in which cytoplasm is removed by a reagent. The BASO-channel WBC count is the ADVIA default total leukocyte count. ADVIA has several instrument WBC flags. White blood cell comparison error (WBC-CE) indicates a disagreement in total leukocyte count between the two channels. PX-NV indicates an inadequate separation of lymphocytes and debris in the peroxidase channel (Anonymous 2006).

ADVIA has been used as reference method in previous studies (Tvedten & Lilliehöök 2011, Bauer *et al.* 2012, Goldmann *et al.* 2014). The ADVIA's techniques are based on modifications from the Technichon H-1E, which has been validated for canine samples (Tvedten & Haines 1994).

Manual methods

A 100-cell manual differential leukocyte count (M-diff) was performed by one of the authors (HT) for each sample. Morphological changes such as toxic neutrophils and reactive lymphocytes were also assessed. In the comparison between methods, only total neutrophils were evaluated because the ADVIA 2120 and ProCyte do not report non-segmented neutrophils.

Definition of unacceptable results and data analysis

ProCyte canine differential leukocyte counts in percent were compared with percentage results from ADVIA and the M-diff. ADVIA and M-diff were considered reference methods. Unacceptable errors with the ProCyte differential leukocyte count were defined as neutrophil results (in percentage), which deviated more than 15% in absolute deviation from both reference methods. Lymphocyte and monocyte counts from ProCyte and both reference methods were also evaluated in the samples with unacceptably erroneous neutrophil counts. Results are compared as difference plots and Bland–Altman method comparison analysis (Analyze-it Software, Leeds, UK).

The ProCyte WBC dot plots were manually assessed by one author (EB) based on the separation of cell clusters with a grading system from 1 (excellent) to 5 (poor). WBC dot plots with indistinct separation of cell clusters were given the higher grades and the WBC dot plots with correct separation of cell clusters were given the lower grades. Grades 1 to 2 were defined as acceptable WBC dot plots and

WBC dot plots with grades 3 to 5 were defined as not acceptable. A groomed data group was formed which included results with only acceptable dot plots. The separation of cell clusters in the dot plots from ADVIA peroxidase channel were also evaluated. The presence of instrument flags for leukocytes was recorded and evaluated.

C-reactive protein

C-reactive protein (CRP) results was retrospectively retrieved from the patients with unacceptable ProCyte errors. CRP of eight samples was analysed during routine work day hours in serum with an immunoturbidimetric method on an automated biochemistry instrument (Architect c4000, Abbott Laboratories, Abbott Park, IL, USA) with reagent from Gentian AS (Moss, Norway), (Hillström *et al.* 2014). An additional five samples were analysed after routine work hours with an immunoturbidimetric method with Eurolyser cCRP-VET on an Eurolyser SOLO (Eurolyser Diagnostica GmbH, Salzburg, Austria) (Jasensky *et al.* 2015). Reference intervals were less than 7 and 10 mg/L, respectively.

RESULTS

Frequency of unacceptable error

ProCyte neutrophil counts often had good agreement with the two reference methods (Fig 1). However, in 13 of the 86 canine samples (15%), the ProCyte had unacceptable discrepancy from both reference method results, as previously defined. In these 13 samples, the ProCyte neutrophil results (%) were 17.5 to 64.5 lower than percentage results from both reference methods. In two additional samples, the difference of the ProCyte neutrophil count exceeded 15% compared to only one of the reference

methods, but not both and therefore these results were not classified as unacceptable in this study.

The ProCyte error was reflected by inadequate separation of leukocyte clusters. All or a large portion of the neutrophils had moved higher up in the WBC dot plot. This higher fluorescence staining indicates a higher content of nucleic acids in the cytoplasm. The 13 samples with unacceptable ProCyte results had both a left shift (16 to 38% band neutrophils) and moderate to severe toxic change in neutrophils seen on blood smear evaluation. Serum CRP concentrations in these 13 patients were markedly elevated with values from 73 mg/L and above.

The error in the ProCyte leukocyte counts in the 13 samples with falsely decreased neutrophil counts also induced erroneously increased monocyte and/or lymphocyte counts (Fig 1). The effects of these errors on absolute counts ($10^9/L$) for neutrophils, lymphocytes and monocytes for the 13 cases are shown in Fig 2. In 12 blood samples, a false clinical conclusion for at least one type of leukocyte occurred based on comparison of incorrect results to laboratory-specific reference intervals. For example, the ProCyte neutrophil count falsely indicated severe neutropenia in two cases.

Evaluation of ProCyte dot plots and flags

All ProCyte WBC dot plots were evaluated and classified into five grades. Well-separated cell populations were classified as 1 to 2, while the presence of varying degrees of overlapping leukocyte cell populations were classified as 3 to 5. Sixty-two of 86 (72%) ProCyte WBC dot plots were considered acceptable, while 24 had incorrect separation of cell clusters and were not acceptable (Table 1). Examples of dot plots with different grades are presented in Fig 3. The 13 samples with unacceptable neutrophil errors had poor separation of leukocyte clusters in the ProCyte WBC dot

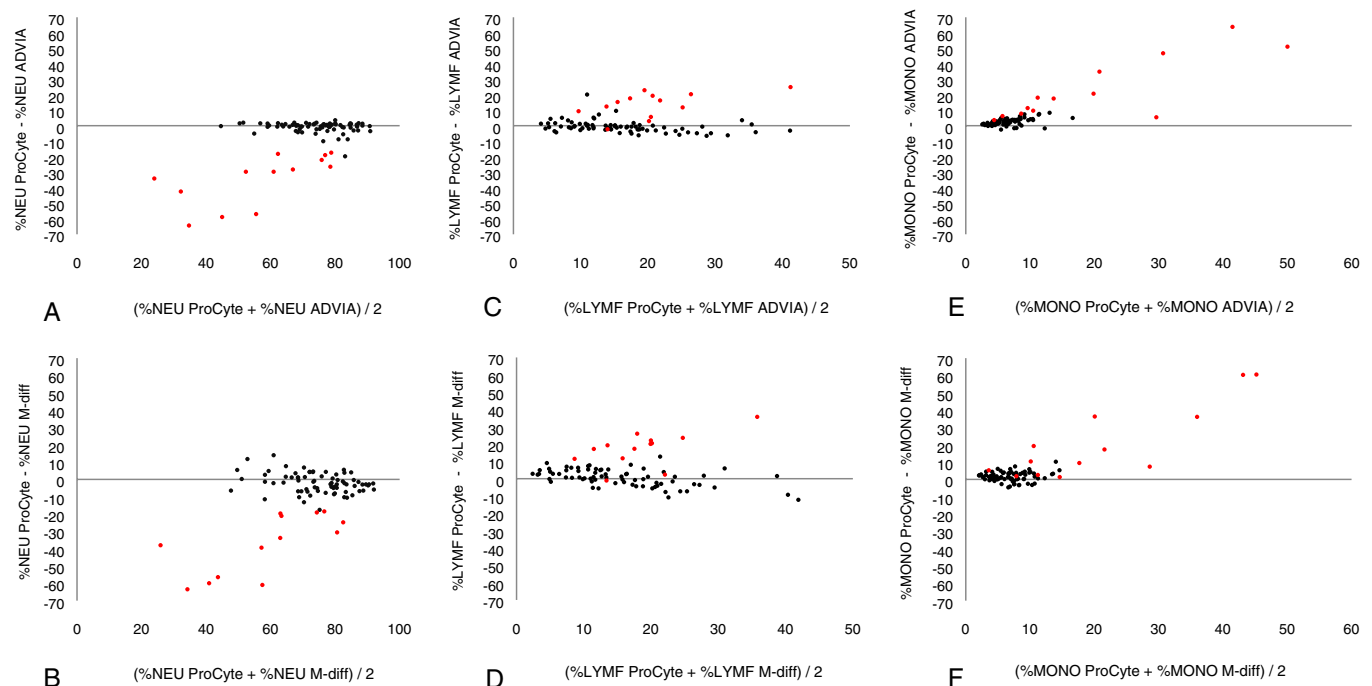


FIG 1. Difference dot plots presenting difference in differential leukocyte counts (%) for neutrophils (A + B), lymphocytes (C + D) and monocytes (E + F) when comparing ProCyte Dx with both reference methods (ADVIA 2120 and manual leukocyte differential count, M-diff) in 86 canine samples. Red dots indicate results from the 13 samples with unacceptably erroneous neutrophil counts

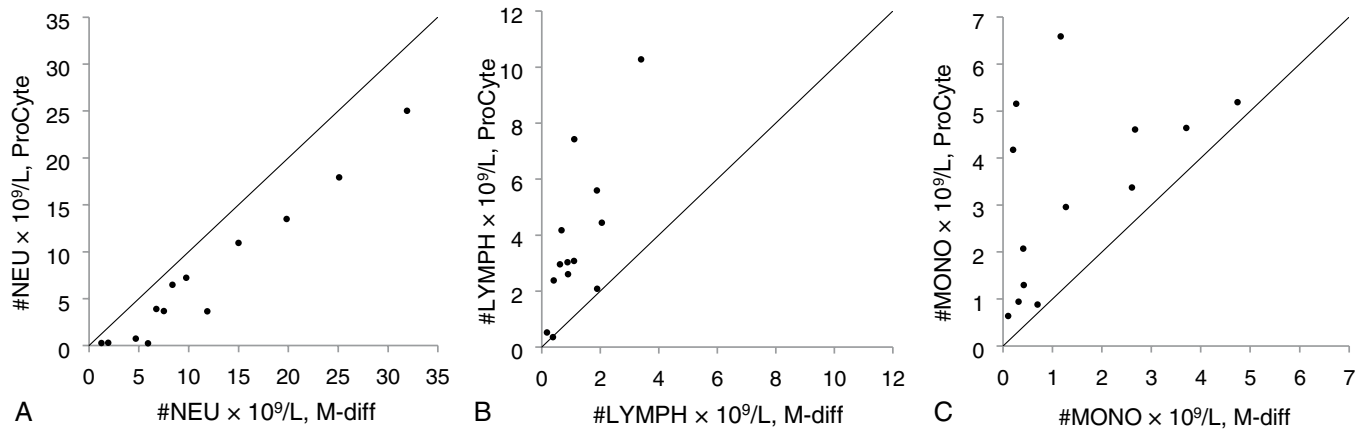


FIG 2. Comparisons of ProCyte Dx neutrophil (A), lymphocyte (B) and monocyte (C) absolute numbers ($10^9/L$) with manual leukocyte differential counts (M-diff) in the 13 samples with unacceptable errors. Total leukocyte count results from ProCyte were used to calculate the results in absolute numbers for M-diff. Black line represents $x=y$. Dots below the line indicate false low results with ProCyte, and dots above the line indicate false high results

plots. The neutrophil cell cluster had moved higher along the y axis in these samples and had moved to the counting area of lymphocytes and/or monocytes. In two cases, the neutrophils merged mainly with the lymphocytes, and in three cases, the neutrophils merged mainly with the monocytes on the ProCyte WBC dot plot. In eight cases, neutrophils merged with both lymphocytes and monocytes.

ProCyte had two flags to indicate potential errors with the leukocyte analysis. The “WBC abnormal distribution” flag was reported in 13 samples and the “Band neutrophils suspected” flag was reported in 28 of the 86 samples in the study. This was 50% (12 of 24) and 83% (20 of 24), respectively, of the samples with unacceptable WBC dot plots. All the 13 samples with unacceptable ProCyte neutrophil errors had a “Band neutrophils suspected” flag, whereas the “WBC abnormal distribution” flag was reported in 10 of these samples (77%).

Agreement after exclusion of samples with unacceptable ProCyte WBC dot plots

A subgroup of samples (groomed) with ProCyte WBC dot plots which were considered reliable was formed to compare to a group containing all samples. The groomed group was made by rejecting samples with incorrect separation of cell clusters in the ProCyte WBC dot plots. The level of agreement between ProCyte and ADVIA neutrophil counts in the samples with acceptable dot plots became very good to excellent after this rejection. The Bland–Altman difference plots comparing ProCyte and ADVIA neutrophil counts are shown in Fig 4. For the group containing all results, mean bias was -6.1% and limits of agreement was -33.3 to 21.1% . This indicates erroneously low neutrophils counts. After rejection of the 24 samples with unapproved ProCyte WBC dot plots, mean bias for the groomed data group improved to -0.5% and the limits of agreement was -5.2 to 4.1% .

Other findings

ADVIA peroxidase dot plots were also assessed. The neutrophil cell cluster was generally well separated. However, in one of the 13 samples with unacceptable ProCyte errors, the neutrophil

Table 1. Evaluation of ProCyte WBC dot plots based on correctness of leukocyte cell cluster separation

Dot plot grade	Samples with unacceptable neutrophil count error (n=13)	All samples (n=86)
<i>Acceptable</i>		
1	0	51
2	0	11
<i>Not acceptable</i>		
3	2	10
4	8	11
5	3	3

The number of samples with each grade is presented for all 86 samples and for the 13 samples with unacceptable ProCyte neutrophil counts

population was indistinctly separated from monocytes in the ADVIA dot plot suggesting slightly falsely low neutrophil count. Even though this caused a lesser difference in this sample between ProCyte and ADVIA, the ProCyte neutrophil result still was much lower than ADVIA and manual count.

An unexpected finding was that the ProCyte total leukocyte counts were higher than ADVIA total leukocyte counts, which were determined from the instrument’s BASO-channel. Mean bias was $1.6 \times 10^9/L$ and limits of agreement were -0.4 to $3.5 \times 10^9/L$ (Fig 5).

DISCUSSION

The results from the current study with canine hospital patient blood samples are similar to an earlier study of feline hospital patient samples (Tvedten *et al.* 2017). Approximately 15% of the ProCyte results in the current canine study had unacceptable errors with falsely low neutrophil counts and falsely increased lymphocyte and/or monocyte counts. This emphasises the importance for ProCyte operators to be able to identify adequate separation of leukocyte clusters on dot plots before accepting results. Rejecting samples with abnormal ProCyte WBC dot plots can eliminate unacceptable errors. Rejection of ProCyte results based solely on the presence of the WBC abnormal distribution flag was not sufficient to avoid unacceptable results. A manual differential

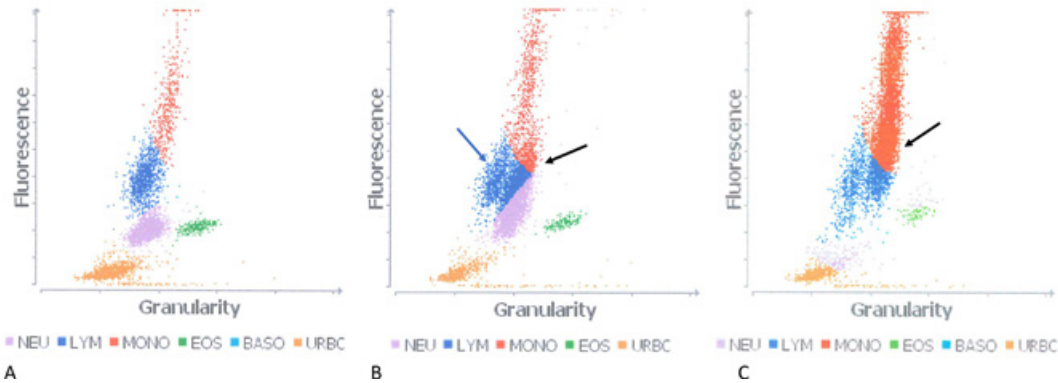


FIG 3. Examples of ProCyte WBC dot plots with different grades. (A) Grade 1 (acceptable) where the individual leukocyte clusters are distinctly separated. Dot plot (B) was graded as 3 (not acceptable) with an oval cluster of neutrophils (black arrow) to the right of the correct lymphocyte cluster (blue arrow). The neutrophils were incorrectly divided into neutrophils (purple), lymphocytes (blue) and monocytes (red) with straight lines drawn through the cell cluster. (C) Grade 5 (not acceptable). This dot plot had a long oval cluster of neutrophils extending to the top of the dot plot (black arrow). Almost all neutrophils were erroneously classified as lymphocytes (blue) and monocytes (red)

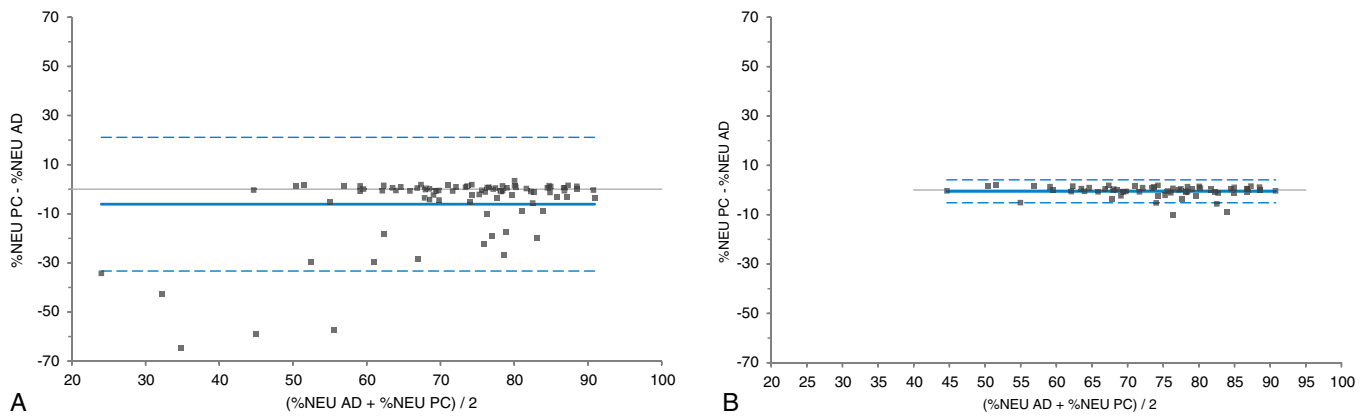


FIG 4. Difference plots comparing ProCyte Dx and ADVIA 2120 neutrophil counts (%) for all samples (A) and after 24 results with unapproved ProCyte Dx dot plots were excluded (B). Only two to a few ProCyte Dx neutrophil counts were slightly lower than results from ADVIA 2120 in the groomed data group. Mean difference (blue solid line) was -6.1% for the ungroomed data group (A) and -0.5% for the groomed data group (B). The dashed blue lines represent 95% limits of agreement that was -33.3 to 21.1% (A) and -5.2 to 4.1% (B)

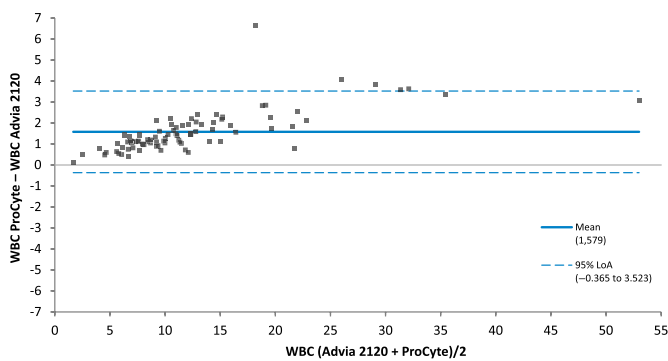


FIG 5. Bland-Altman difference dot plot presenting difference in total leukocyte counts ($10^9/L$) in 86 canine samples generated by ProCyte Dx and ADVIA 2120 (BASO-channel). The mean difference (blue solid line) was $1.6 \times 10^9/L$. The dashed blue lines represent 95% limits of agreement that was -0.4 to $3.5 \times 10^9/L$.

leukocyte count determination is needed for samples with questionable automated instrument results.

In feline samples, the neutrophil errors in ProCyte were mainly accompanied by falsely high lymphocyte counts (Tvedten *et al.* 2017). The canine neutrophils with greater polymethine

staining also moved upwards in the WBC dot plot but did not merge primarily with the lymphocyte cluster but also moved often up into the monocyte cluster. This error with the ProCyte for automated differential leukocyte counts was seen in dogs with severe inflammatory diseases. The leukocyte misclassification with the 13 (of 86) samples with unacceptable results were in patients that had evidence of inflammation. It was associated with the presence of a left shift, toxic change and increased CRP. Immature and toxic neutrophils have increased amount of RNA and thus increased polymethine staining causing these neutrophils to be found higher along the y axis in the ProCyte WBC dot plot where they are erroneously classified as lymphocytes and/or monocytes (Goldmann *et al.* 2014). It is most important to have correct leukocyte counts in blood samples from dogs with severe systemic inflammation. Studies on clinically normal dogs would likely not reveal the error.

The ADVIA and M-diff methods also have counting errors. For example, a systematic error with falsely low monocyte counts and falsely high lymphocyte/LUC counts in canine samples has been reported for ADVIA (Tvedten & Lilliehöök 2011). The monocyte cell population in ADVIA is often not restricted to

the monocyte counting area but partially in the LUC and lymphocyte areas. This may cause the falsely lower monocyte counts and high lymphocyte/LUC counts. Errors with neutrophil enumeration by ADVIA were rarely seen in the automated differential leukocyte count. This may be due to the different technique where leukocytes are stained based on peroxidase content instead of RNA concentration as in ProCyte. Severe inflammatory disease can lead to reduced myeloperoxidase staining of neutrophils in dogs. This can cause the ADVIA to count neutrophils as monocytes (Klenner *et al.* 2010). A disadvantage with manual leukocyte differential count is that a M-diff of 100 leukocytes is less precise than automated instrument methods (Kjeelgaard-Hansen & Lundorff Jensen 2006). Blood smear evaluation is also affected by the training and experience of the operator.

The threshold for considering a leukocyte count to be unacceptable in this study was 15% in absolute deviation from both reference methods. The American Society for Veterinary Clinical Pathology (ASVCP) chose a total allowable error for the neutrophil count to be up to 15% in relative difference (Nabity *et al.* 2018). The 13 samples with unacceptable errors (according to our criteria) had a relative difference in neutrophils varying from 20% up to 96%. This study's threshold of difference greater than 15% selected only the most severe errors in the canine automated differential leukocyte count performed by ProCyte. Less severe erroneous results probably occurred in more than the 13 samples based on the fact that 28% of ProCyte WBC dot plots were not approved. Only 10% of WBC dot plots were considered unacceptable in one validation study of ProCyte, based on both healthy and sick dogs. They excluded 25 of 263 (10%) samples when comparing neutrophil counts due to abnormal WBC dot plots (Goldmann *et al.* 2014). The higher percentage of dot plots with incorrect separation of cell clusters in our study could be that our hospitalised patient population more often had severe inflammation.

Total leukocyte counts with ProCyte were higher than total leukocyte counts with the ADVIA BASO-channel. This is in contrast to what was seen in a validation of ProCyte (Goldmann *et al.* 2014). The reason for this discrepancy in total leukocyte count or which instrument's total leukocyte count was more accurate was not determined. Because of the difference in total leukocyte counts between ADVIA and ProCyte, it was considered more appropriate to compare relative results as percent. For the comparison of absolute neutrophil counts, ProCyte results were compared with manual leukocyte differential count calculated from total leukocyte count from ProCyte.

It may be concluded that severely erroneous results in the automated differential leukocyte count with the ProCyte may be expected in some blood samples from severely ill dogs. Clinical decisions may be affected if automated instrument results are accepted without inspection of WBC dot plots or examination of blood smears. For example, ProCyte falsely indicated severe neutropenia in two

cases in our study. These results could lead to inappropriate use of antimicrobials due to concern for inadequate immune defence (Bisson *et al.* 2018). If instrument operators critically evaluate the WBC dot plot of every sample and reject results with abnormal dot plots, this risk of erroneous results can be essentially eliminated. ProCyte is an excellent haematology analyser for small animal haematology as previous validation studies have indicated (Fujino *et al.* 2013, Goldmann *et al.* 2014). All instruments and people make errors. Rejection of results from samples with abnormal dot plots is required to obtain accurate haematology results. Attention to instrument cyto-grams and flags, together with review of blood smear morphology in samples with suspect errors will provide better outcomes for critically ill patients.

Conflict of interest

None of the authors of this article has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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