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AUTOMATED HEMATOLOGICAL CELL COUNT USING SYSMEX XN-V IN TESTUDO HERMANNI: AGREEMENT WITH MANUAL COUNT

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Original Article

AUTOMATED HEMATOLOGICAL CELL COUNT USING SYSMEX XN-V IN TESTUDO HERMANNI: AGREEMENT WITH MANUAL COUNT

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Abstract (word count to be adapted based on journal)

Testudo hermanni is a tortoise diffused in the Mediterranean area both in the wild and as a pet. Being clinical signs of these animals very non-specific, the complete blood count could reveal possible non-overt pathological conditions. Automated blood cell counts in non-mammalian species is challenging, given the presence in peripheral blood of nucleated erythrocytes (NRBC) and thrombocytes (Thr). These peculiar features hamper an accurate identification of the blood cell populations and the quantification of leukocytes (WBC) using instruments, thus making necessary the use of manual methods, including counting chambers. Aim of the study was to assess the performance of the novel automated hematology analyzer Sysmex XN-V which offers the possibility to count NRBC into a white and nucleated red blood cells channel (WNR) in accurately identify and quantify the different blood cell populations of *Testudo hermanni* and its agreement with manual counts. Fifty heparinized blood samples were counted using the Neubauer improved chamber and then counted twice with Sysmex XN-V. Thirteen out of fifty samples were counted again after 48hrs. All the WNR scattergrams were re-analysed using a gate panel created *ad hoc* to separate two main populations: NRBCs (weak fluorescence signal) and WBC + Thr (high fluorescence signal). Intra - and inter-assay precision of Sysmex XN-V was optimal for NRBCs (CV 0.98% ± 1.96; 1.31% ± 2.98) and moderate for WBC + Thr (CV 9.24% ± 16.61; 12.69% ± 10.35). No proportional nor constant errors were observed between the methods for NRBC and WBC +Thr, with the automated measurements always slightly lower compared to the manual count for NRBC and slightly higher for WBC + Thr. These results suggest that Sysmex XN-V can be used to analyse blood in *Testudo hermanni*, but the use of specific instrumental reference intervals should be adopted.

Keywords: Tortoises; Automated blood cell count; Sysmex XN-V.

Introduction

Hermann's tortoise (*Testudo hermanni*) is considered as a near threatened terrestrial chelonian by the International Union for Conservation of Nature (IUCN, 2004). It is widespread in all the Mediterranean Europe area (Cheylan, 2001). Despite the apparent decrease in wild population, this species is commonly kept as a pet. Compared to conventional mammalian species, clinical signs of disease in tortoises are less evident, thus both blood biochemistry and hematological analyses are an important part of the routine panel of investigations that allow to assess and monitor their health conditions. The hematology of non-mammalian animals, including tortoises, generally does not rely on automated methods due to the presence of nucleated red blood cells (NRBC) and thrombocytes (Thr) that are not accurately identified by automated analyzers (Nardini et al, 2013). Thus, the use of specific hemocytometer counting chambers is required for complete blood cell count (CBC) (Campbell, 2015). One of the most used methods involves the use of Neubauer chamber, after blood dilution with Natt and Herrick staining solution (Natt and Herrick, 1952). Even if it is commonly used in clinical practice, this method is time consuming and, being operator-dependent, it may have an inherent imprecision up to 10% (Campbell, 2015). Moreover, the differentiation between small lymphocytes and thrombocytes is challenging using the hemocytometer counting chambers because of their similar morphology and size (Stacy et al, 2011). The use of an automated hematology analyzer, instead of the manual methods, for the evaluation of tortoise CBC would allow a shorter turnaround time together with a possible increased accuracy and precision, thanks to the higher number of cells counted.

The new hematology analyzer Sysmex XN-V with the WNR channel offers the possibility to count and differentiate the total white blood cells and NRBC. In this channel, whole blood reacts with an acidic hemolyzing reagent and a nucleic acid dye. In mammalian

blood samples, NRBC are here differentiated from leukocytes (WBC) thanks to a weaker fluorescence signal (Tantanate and Klinbua, 2015). This new automated instrument has been already validated in dogs (Grebert et al., 2012), but not in non-mammalian species.

Thus, the aims of this study were to evaluate the possibility to perform a CBC based on WNR results from Sysmex XN-V and to evaluate the agreement of Sysmex XN-V data with the manual counts in *Testudo hermanni*.

Materials and methods

Caseload

Fifty adult Hermann's tortoises (*Testudo hermannii*) were sampled. Since this was an analytical study not focused on the diagnostic potential of the new instrument for any given disease, animals were included irrespective of their health status. A blood sample from the cervical plexus was collected from each animal for routine health check or diagnostic purposes and stored in a lithium heparin test tube for a maximum of 48 hrs. According to the Ethical Committee decision of the University of Milan, residual aliquots of samples or tissues collected during routine visit, can be employed for research purposes without any supplementary authorization (EC decision n° 02-2016), under owners' informed consent. All the collected samples were used to assess intra-assay repeatability within 24 hours, while thirteen out of fifty samples were stored up to 48 hours and used for the assessment of inter-assay reproducibility.

Manual count

Within 24 hrs from withdrawal, all the samples were manually counted using the Neubauer improved hemocytometer chamber (Blau Brand) by a single operator (SM, third

92 year ECVCP resident) (Fig. 1). To this aim, whole blood samples were diluted 1:200 with
93 Natt and Herrick's staining solution, as already described in literature (Bielli et al., 2015).
94 Specifically, WBC and Thr were counted together (WBC + Thr), due to the inherent
95 difficulties in distinguishing small lymphocytes from thrombocytes in the chamber. The WBC
96 + Thr were counted in the nine large square of the grid designed on the counting chamber (in
97 order to improve the accuracy of the count) and the result was adjusted based on the counting
98 chamber area (9 mm^2), the chamber depth (0.1 mm) and the dilution used as suggested by the
99 manufacturer (Fig. 1), in order to obtain the absolute number per microliter. The NRBC were
100 counted in five small squares of the central big square, following the Neubauer chamber's
101 instructions (Fig. 1), and the result was then divided by 100 to obtain the absolute number of
102 cells per microliter.
103
104 *Automated count*
105 Sysmex XN-V performances were checked on daily basis using three level of quality
106 control material, provided by the manufacturer (Sysmex Corporation).
107 The very first sample was acquired with "Dolphin" animal species setting, which is
108 fitted to count erythrocytes with larger size. However, the Dolphin WNR scattergram is
109 divided into three different population (namely NRBC, basophils and other leukocytes) and
110 the pre-set gates did not fit with cell clouds obtained with turtle sample. For this reason, ad-
111 hoc gates were set into the WNR channel, based on the two clouds grossly discriminable in
112 the scattergram and the sample was re-analysed. Based on the results of the manual count on
113 the same sample, the concentration of cells in the two clouds were compatible with NRBC
114 and WBC+Thr, respectively. Finally, the customed gate setting was saved as "Turtle" species
115 and used to acquire all the following samples (Fig. 2).

To assess the intra – assay precision, all the samples were analysed in duplicate, within 24 hrs from the withdrawal. To assess the inter – assay precision, thirteen out of fifty samples were additionally stored at 4°C for further 24 hrs, then brought at room temperature and resuspended before being re-analysed with Sysmex XN-V.

Statistical analysis

Statistical analysis was performed using the specific software Analyse-it for Microsoft Excel (Analyse-it Software Ltd). Specifically, the intra and inter – assay precisions of the automated method were assessed through the measurement of imprecision (coefficient of variation = standard deviation/mean x 100). The comparison between manual and automated count was assessed using the Wilcoxon signed rank test for paired data comparing the manual count with the mean of the automated counts. Finally, the agreement between methods was assessed using Passing & Bablok regression analysis and the Bland - Altman difference plot test. Statistical significance was set at $P < 0.05$ for all tests.

Results

Animals

Among the fifty adult tortoises sampled, 13 were male and 29 were female (sex was not reported for eight animals). Descriptive statistics for both manual and automated counts are reported in Table 1.

Intra and inter – assay precision of Sysmex XN-V

Both intra- and inter-assay imprecision were slightly higher for WBC + Thr than for NRBC, with inter-assay imprecision being higher than intra-assay one for both parameters (Table 2). Still, no significant difference between the two automated counts at 24hrs and between the

mean of the two 24hrs counts and the 48hrs count was found, either for NRBC and WBC + Thr ($p>0.05$ for all analyses).

Agreement between automated and manual count

Slightly lower NRBC values were obtained with the automated count than with the manual one, but the difference was not statistically significant ($P = 0.560$, Table 1). No constant or proportional error for the NRBC count was found with the Passing & Bablok regression analysis (intercept: 0.052; 95% CI: -0.027 – 0.108; slope: 0.85; 95% CI: 0.70 – 1.02). Conversely, the Bland-Altman plot highlighted a significant negative bias for manual count of NRBC compared to automated one (-0.031; 95% CI: -0.057 – -0.004; $P = 0.0206$) (Fig. 3).

For WBC + Thr, results were significantly higher using the automated count compared to the manual count ($P = 0.027$). The Passing & Bablok regression analysis did not reveal the presence of constant nor proportional errors (intercept: -0.34; 95% CI: -2.22 – 0.86; slope: 1.17; 95% CI: 0.88 – 1.51) with manual counts. The Bland-Altman plot highlighted a significant positive bias for manual WBC + Thr count compared to automated one (1.04; 95% CI: 0.29 – 1.79; $P = 0.0072$) (Fig. 3).

Discussion

The present study aimed to assess the possibility to get accurate and reliable data using an automated hematological analyzer for the analysis of blood in a non-mammalian species. To this aim, we evaluated the agreement between a commonly used manual technique and a new fluorescence flow cytometry analyzer in hematological cell counts of Herman's tortoises.

Tortoises are free–ranging animals, but they are also diffused as pets. Specifically,

Herman's tortoise is one of the most common pet tortoises in Italy. The correct management of these animals requires a specialized clinician, with a peculiar expertise in exotic animals, due to their disguised clinical signs and peculiar blood sampling technique. Interpretation of clinical-pathological results is also difficult in these animals, due to the paucity of data on healthy animals and intrinsic laboratory variability. Manual hemocytometer chambers are commonly used for the whole blood evaluation in this species, similarly to other animals of veterinary interest characterized by physiologically nucleated erythrocytes and thrombocytes (namely birds, other reptiles and fishes) (Campbell, 2015). Different techniques have been proposed for manual hematological evaluation in tortoises (Brenn-White et al., 2022; Nardini et al., 2013). The most commonly used is the evaluation of a whole blood sample diluted with the Natt and Herrick' staining solution. Due to the lack of a more standardized method, chamber count was applied in the present study as a reference standard to assess the performances of Sysmex XN-V. Nevertheless, the manual method is time consuming compared to the automated evaluation used for the other companion animals whose blood does not contain measurable NRBC in normal conditions. Indeed, the manual count requires at least 12-15 minutes of sample incubation time with staining solution, followed by the microscopical count, whose turnaround time is operator dependent (and generally not shorter than five minutes). On the other hand, automated count using Sysmex XN-V analyzer, requires less than one minute to be performed. Moreover, due to the low number of cells counted by the operator, the manual method is affected by a low accuracy, with an inherent error reported to range from 10% (Campbell, 2015) to 20 – 40% (Ammersbach et al., 2015). For these reasons, the turnaround time for reporting hematology results in lower vertebrates is longer, compared to mammalians, and results might be less accurate.

The new hematology analyzer Sysmex-XN-V has recently been validated in the canine

species (Grebert et al., 2021). The main difference compared with the previous Sysmex XT series concerns the presence of an optimized fluorescent optical channel for platelet (PLT-F, that use an oxazine – based fluorescent dye) and the possibility to count NRBC in a dedicated channel (WNR channel) and differentiate these cells from the other nucleated blood cells (WBC). In this latter channel, whole blood is treated with an acidic haemolysing reagent (Lysercell® WNR) that lyses RBC, while it perforates WBC and NRBC membranes. (Sysmex XN manual). Then, a fluorescent nucleic acid dye (Fluorocell® WNR) enters the cells and binds to nucleic acids and organelles. Each cell is hit by an incident laser light and the optical data generated are recorded by specific detectors. The data are finally shown in a scattergram with side fluorescence light (SFL) on the x-axis (which is proportional to the amount of nucleic acids content) and the forward scattered light (FSC) on the y-axis (which is proportional to the cell size). In human blood samples, different clouds are easily discriminable, with WBC having small size and high fluorescence intensity, basophils large size and fluorescence intensity, and NRBC small size and low fluorescence intensity. The enumeration of NRBC in human blood seems to have a good precision and could replace the traditional blood smear count (Kaido et al., 2017; Tantanate and Klinbua, 2015).

This is the first study, to the author's knowledge, that evaluate the performance of Sysmex XN-V in analyzing whole blood of chelonians. Intra- and inter-assay precision resulted optimal for NRBC, whereas less satisfying results were obtained for the assessment of WBC + Thr, with CV values being comparable to those of the manual methods (Campbell, 2015; Ammersbach et al., 2015). This result may be related to the lower proportion of leukocytes and thrombocytes compared with the number of erythrocytes. Moreover, the delay needed to perform inter-assay evaluation may have caused artifactual storage-induced changes, such as platelet clumping, that may have affected the count. Irrespective of the

causes underlying the lower precision in the WBC + Thr count after 48 hours, the results here obtained suggest that it is advisable to perform the automated blood cell count within 24 hrs from the withdrawal, to reduce the probability of artifacts.

In the agreement evaluation between manual and automated counts, no constant, nor proportional errors were observed for both NRBC and WBC + Thr. Nevertheless, there is a significant overestimation in WBC + Thr and a significant underestimation of NRBC in the automated measurement compared to the manual count. This may be due to the high inherent inaccuracy of manual count, or to a limit of the customed gate in the WNR channel. Calculation of analyzer specific reference intervals for the considered species would overcome this issue.

One limitation of this study is that the automated count using the WNR channel only allows to count leukocytes and thrombocytes together, but from a clinical perspective, this information may have limited usefulness. Nevertheless, even using the manual hemocytometer count, the differentiation between leukocytes and thrombocytes may be difficult. Thus, an estimation of these two populations from the blood smear microscopical observation is needed to obtain the absolute number of leukocytes and thrombocytes separately. Another possible limitation is that there is no certainty that Sysmex XN-V counted NRBC accurately, and some other nucleated cells may have been included in the gate. However, the good agreement between instrumental and manual counts for NRBC and the higher counts obtained for WBC+Thr with the instrumental method suggest that the gating strategy applied is most likely consistent with the actual blood populations.

Finally, there was no information about the clinical status of the animals enrolled in this study. Nevertheless, this is a purely analytical study, and clinical implications of the results obtained with automated count remain to be addressed.

Based on the results of this study, future perspectives are open for the measurement of

241 species – specific hematologic reference intervals. Moreover, it would be interesting to
242 evaluate the performances of Sysmex XN-V on other species of reptiles using the same gating
243 strategy here proposed (eventually with some small adjustments, if needed, based on possible
244 species differences).
245
246 **Conclusions**
247 Based on the results obtained in this study, the new hematology analyzer Sysmex XN-
248 V seems to be able to replace the manual count for the evaluation of *Testudo hermanni* blood
249 samples. However, the two methods are not perfectly superimposable, thus a calculation of
250 instrument specific reference interval is advisable. The use of Sysmex XN-V would markedly
251 reduce turnaround time for the analysis of tortoises' blood, compared with the manual count,
252 possibly increasing the accuracy of results (associated with the higher number of cells counted
253 by the automated instrument).
254
255 **Conflict of interest statement**
256 Sysmex corporation supplied the automated hematology instruments and reagents
257 used in this study. Sysmex corporation played no role in the study design nor in the collection,
258 analysis and interpretation of data, nor in the decision to submit the manuscript for
259 publication. None of the authors has any other financial or personal relationships that could
260 inappropriately influence or bias the content of the paper.
261
262
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Table 1

Descriptive statistics for the samples enrolled in this study. In the table are reported, for both NRBC and WBC+Thr populations, the mean ± standard deviation and median (minimum – maximum) values for manual count (Man) and automated count (XN-V) (performed in duplicate at 24 hrs and expressed also as a mean of the two measurement, as well as the measurements performed at 48 hrs).

	NRBC ($x10^9$/L)				
	Man (n = 50)	XN-V 24h 1 (n = 50)	XN-V 24h 2 (n = 50)	XN-V 24h Mean (n = 50)	XN-V 48h (n = 13)
Mean ± S.D.	0.48 ± 0.17	0.44 ± 0.14	0.44 ± 0.14	0.44 ± 0.14	0.52 ± 0.11
Median (Min - Max)	0.46 (0.22 - 1.04)	0.42 (0.22 - 0.76)	0.43 (0.22 - 0.76)	0.43 (0.22 - 0.76)	0.55 (0.33 – 0.74)
	WBC + Thr ($x10^6$/L)				
	Man (n = 50)	XN-V 24h 1 (n = 50)	XN-V 24h 2 (n = 50)	XN-V 24h Mean (n = 50)	XN-V 48h (n = 13)
Mean ± S.D.	6.14 ± 3.10	6.81 ± 4.33	6.94 ± 4.03	6.88 ± 4.05	11.41 ± 3.32
Median (Min - Max)	6.1 (1.5 – 15.5)	6.13 (1.97 - 19.03)	6.53 (1.88 - 19.48)	6.61 (1.97 - 19.25)	10.9 (7.21 - 17.87)

Table 2

Intra and inter - assay imprecision of Sysmex XN-V expressed as coefficients of variation (CV) of nucleated red blood cells (NRBC) and white blood cells and thrombocytes (WBC + Thr) populations.

		NRBC (%)	WBC + Thr (%)
Intra – Assay	CV_{mean} ± St.Dev	0.98 ± 1.96	9.24 ± 16.61
(n = 50)	CV_{median} (Min – Max)	0.00 (0.00 – 10.25)	3.99 (0.16 – 90.61)
Inter – Assay	CV_{mean} ± St.Dev	1.33 ± 3.10	12.86 ± 10.73
(n = 13)	CV_{median} (Min – Max)	0.00 (0.00 – 11.38)	13.01 (1.48 – 39.81)

St.Dev, standard deviation; Min – Max, minimum – maximum

Figure legends

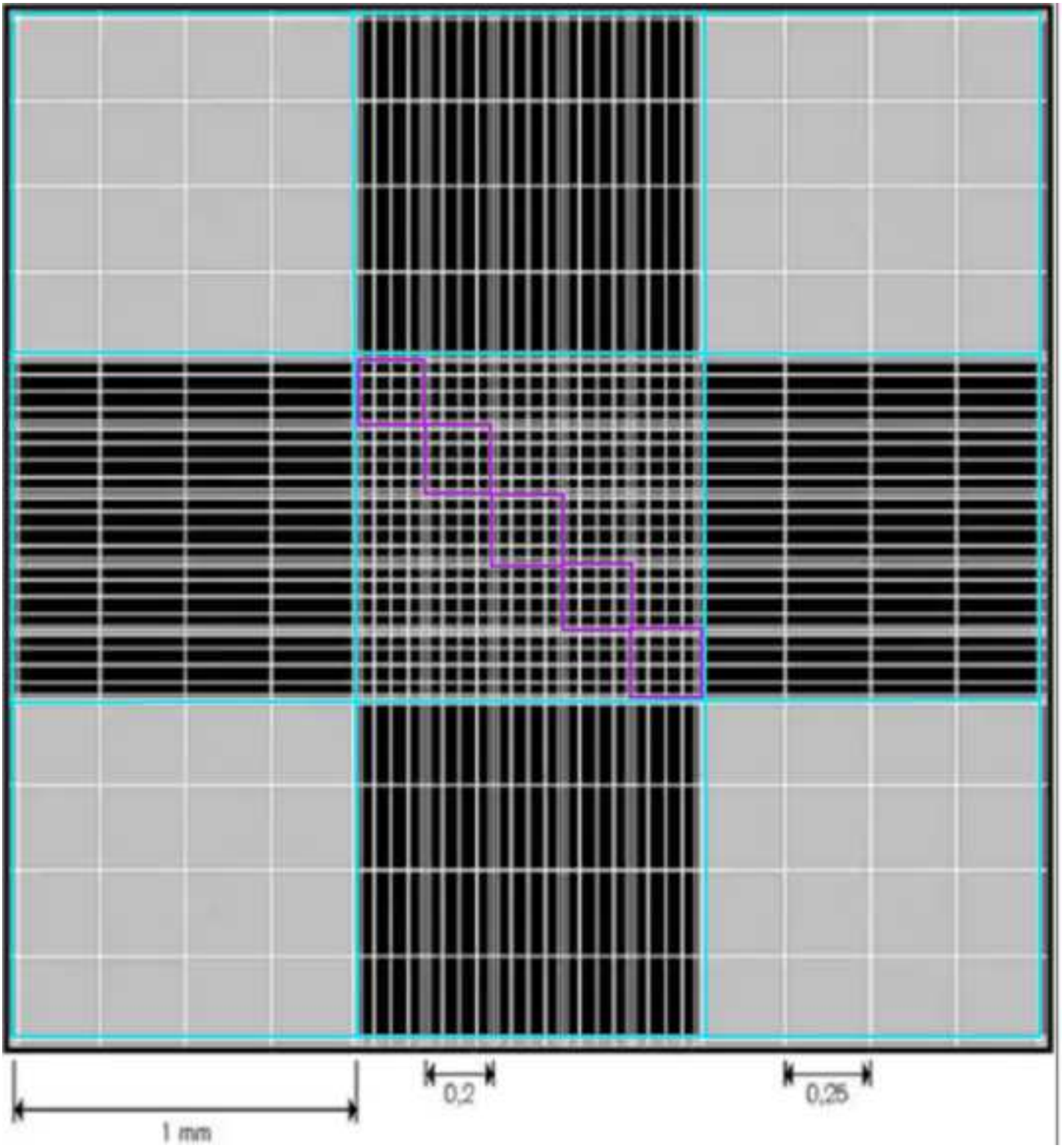
Fig.1. Example of Neubauer hemocytometer microscopic grid. The nine large squares (in light blue) were used to count leukocytes and thrombocytes and the value obtained was then divided by 4.5. Erythrocytes were counted in five small squares inside the central large square (in purple) and the value obtained was then divided by 100 (according to manufacturer instructions).

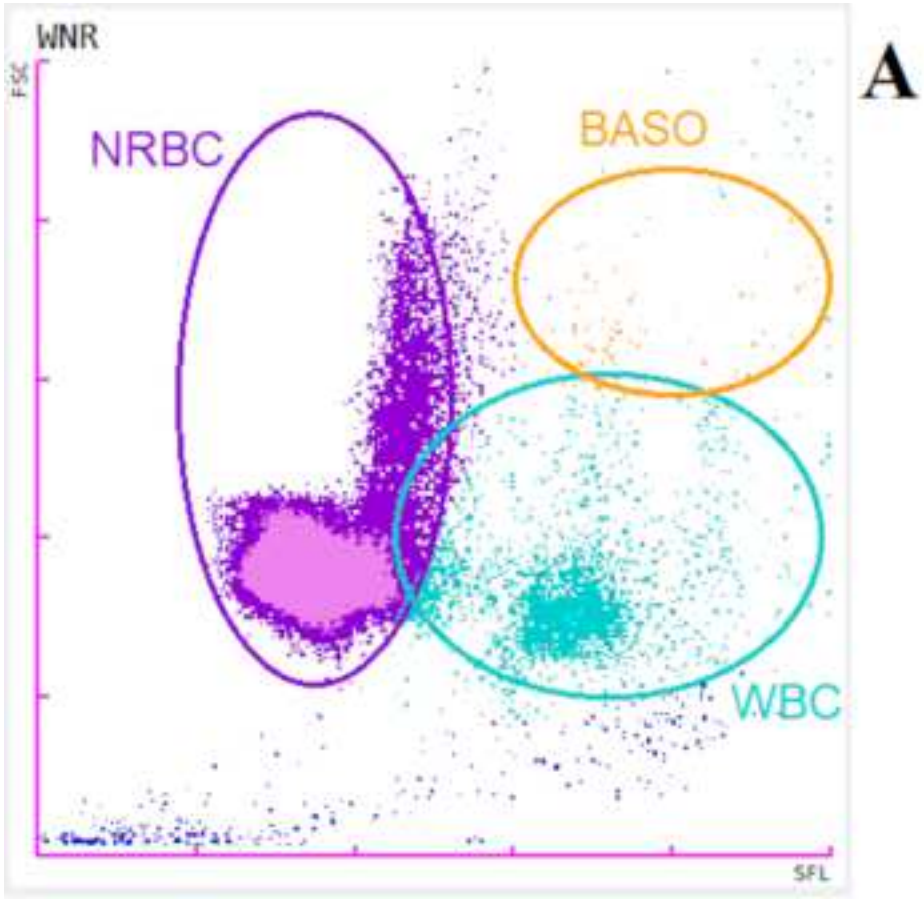
Fig. 2. Example of a dot plot scattergram obtained using the "Dolphin" species (A). The cells are divided into three populations: NRBC in purple on the left, WBC (including neutrophils, monocytes, eosinophils and lymphocytes) in light blue on the bottom right and basophils (BASO) in yellow, on the upper right. Ad-hoc "turtle" gates were created in order to distinguish two main populations of tortoise's samples: the erythrocytes (NRBC) in red, on the left, and the leukocytes and thrombocytes (WBC + THR) in blue, on the right (B).

Fig. 3. Passing & Bablok regression analysis and Bland – Altman plot of the automated count compared to the manual count for both the nucleated red blood cells (NRBC, A - upper part of the figure) and the white blood cells and thrombocytes (WBC + Thr, B – lower part of the figure) populations. Bland–Altman plots show the mean values obtained with Sysmex XN-V and manual counts on the x-axes and the difference between the results of the 2 methods on the y-axes. Dotted lines indicate bias; dot-dashed lines, 95% limits of agreement. In the Passing & Bablok regression analysis red dotted lines indicate 95% confidence intervals around the line of best fit.

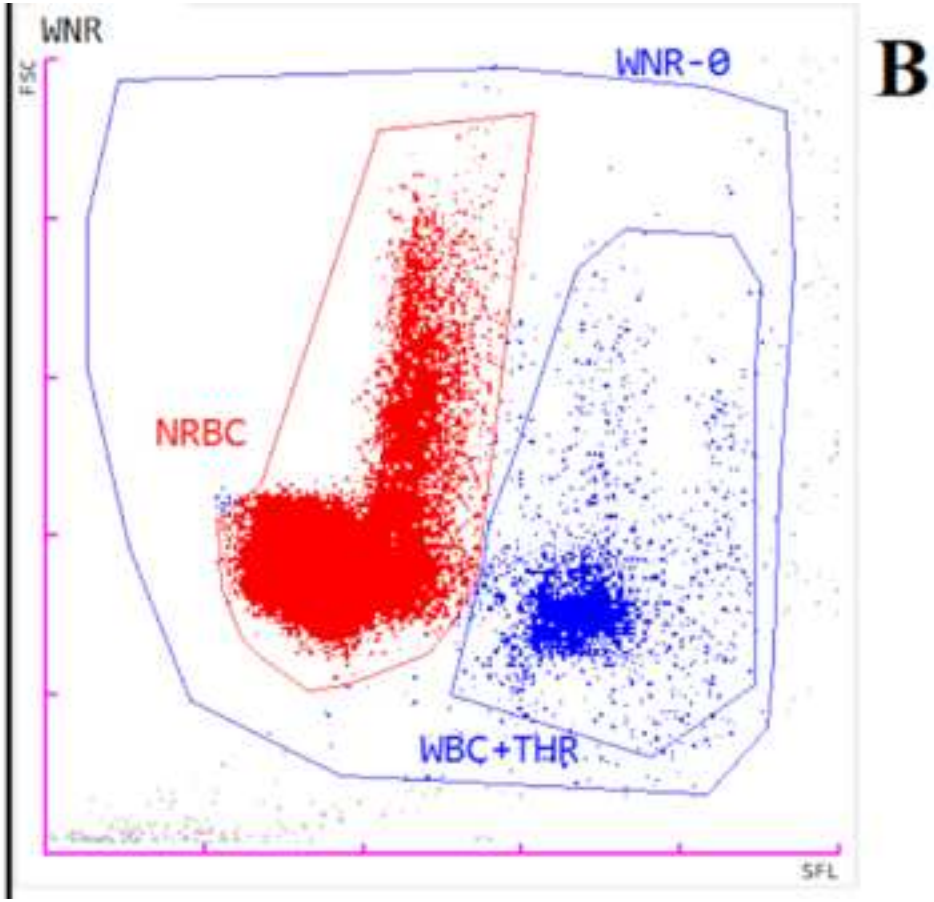
Figure1

Click here to access/download;Figure;Fig.1.tif





A



B

Fig. 2.tif

Figure 3

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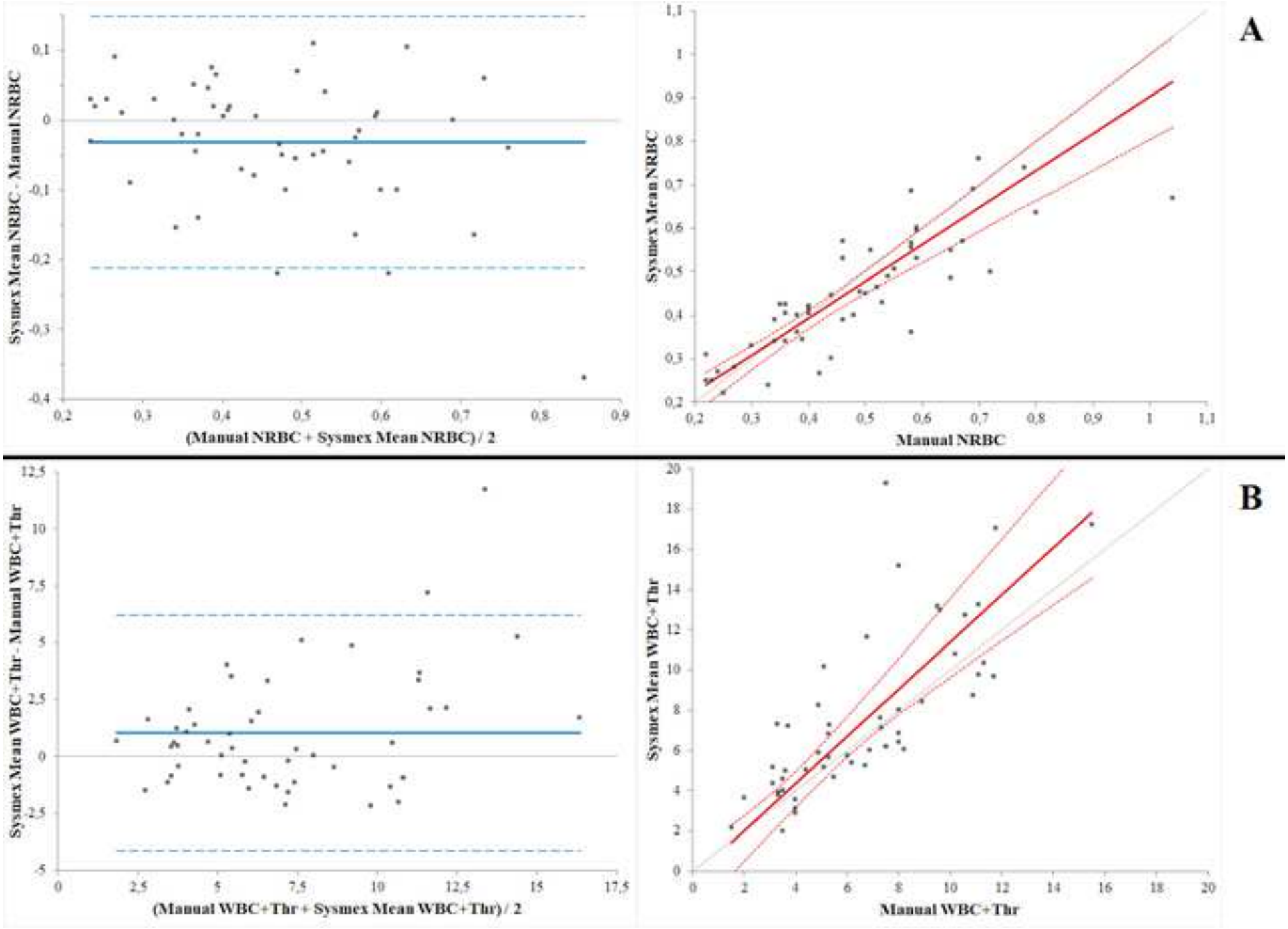

Fig.3.tif

Highlights

- Hematology of tortoises is usually performed manually due to nucleated blood cells
- The Sysmex XN-V hematology analyzer has a new channel for the NRBC identification
- The tortoises' blood automated count reduces turnaround time and increases accuracy
- The agreement between manual and instrumental count was good for NRBC
- The instrumental count gave slightly higher WBC + Thr counts

Dear Editor,

the manuscript here enclosed and titled ‘Automated hematological cell count using Sysmex XN-V in *Testudo hermanni*: agreement with manual count’ is submitted to be considered for publication on *The Veterinary Journal*. The relevance of the study relies on the very limited number of publications about instrumental hematology in reptilians and the concurrent diffusion of these animals as pets. Moreover, the number of reports about the new hematological instrument released by Sysmex corporation (Sysmex XN-V) in veterinary medicine is still very scarce and needs to be widen given the upcoming diffusion of this instrument in the veterinary laboratories.

The manuscript has been reviewed by an English-speaking scientist and a thorough format editing has been performed before submission in order to comply with journal guidelines.

I would certify that the manuscript has not be submitted to other Journals. Preliminary results of this study were presented at the 24th ESVCP (European Society for Veterinary Clinical Pathology) annual congress in Belgrad, 5-8th October, 2022.

Sincerely,
Alessia Giordano