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Automated hematological cell count using sysmex XN-1000V in *Testudo hermanni*: Agreement with manual count

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ABSTRACT

Mediterranean area represents the main habitat of *Testudo hermanni*. Clinical signs of disease of these tortoises are non-specific, making the hematology results crucial in revealing underlying pathological conditions. However, accurate automated identification of blood cell populations is hampered by the presence of nucleated erythrocytes (NRBC) and thrombocytes (Thr), necessitating manual methods such as counting chambers. The aim of the study was to assess the performance of the novel automated hematology analyzer Sysmex XN-1000 V, which includes a a specific channel (WNR) for counting NRBC, in accurately identify and quantify the different blood cell populations of *Testudo hermanni*. Additionally, its agreement with manual counts was evaluated. Fifty heparinized blood samples were initially counted using the Neubauer improved chamber and then analysed twice with Sysmex XN-1000 V. Thirteen out of 50 samples were instrumentally counted again after 48 h to assess the inter-assay precision. All WNR scattergrams were re-analysed using an ad hoc gate panel to differentiate two populations: NRBCs (weak fluorescence signal) and WBC + Thr (high fluorescence signal). Sysmex XN-1000 V demonstrated optimal intra- and inter-assay precision for NRBCs (CV 0.98% ± 1.96; 1.31% ± 2.98) and moderate precision for WBC + Thr (CV 9.24% ± 16.61; 12.69% ± 10.35). No proportional nor constant errors were observed between the methods for both the populations. The instrumental NRBC counts were consistently slightly lower, while WBC + Thr counts were slightly higher compared to manual counts. These findings suggest that Sysmex XN-1000 V can be used for analyzing cell populations in heparinized blood of *Testudo hermanni*. However, specific instrumental reference intervals are suggested.

1. Introduction

Hermann's tortoise (*Testudo hermanni*) is considered as a near-threatened terrestrial chelonian by the International Union for Conservation of Nature (International Union for the Conservation of Nature, 2023). Hermann's tortoises include two subspecies: *Testudo hermanni hermanni* (also known as Western Hermann's tortoise) and *Testudo hermanni boettgeri* (also known as Eastern Hermann's tortoise). This species is widespread across Mediterranean Europe (Cheylan, 2001). Despite the apparent decline in its wild population, this species is commonly kept as a companion animal. Unlike conventional mammalian species, clinical signs of disease in tortoises are often subtle. Consequently, chelonians are considered among the most difficult vertebrates to evaluate clinically (Wilkinson, 2004). Blood biochemistry and hematological analysis are critical for assessing and monitoring their health conditions. Hematological evaluation is paramount for identifying a range of pathological conditions such as inflammation (evidenced by increased peripheral leukocyte counts), or anemia (indicated by decreased erythrocyte counts) and for monitoring pathophysiological states like dehydration and stress (Campbell, 2015). However, hematological and blood biochemical reference intervals are available for <5% of chelonian species (McArthur et al., 2004). The hematology of tortoises and other non-mammalian animals, generally does not rely on automated methods due to the presence of nucleated red blood cells (NRBC) and thrombocytes (Thr) which automated analyzers fail to accurately identify (Nardini et al., 2013). Therefore, the additional use of specific hemocytometer counting chambers is required for complete blood cell count (CBC) (Campbell, 2015). Several manual methods are recommended for hematology in these species (Sykes 4th and Klaphake, 2015). One of the most used involves the use of Neubauer chamber, following blood dilution with Natt and Herrick staining solution (Natt and Herrick, 1952). Despite its common use in clinical practice, this

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method is time consuming and, being operator-dependent, it may have an inherent imprecision up to 10% (Campbell, 2015). Moreover, differentiating small lymphocytes from thrombocytes is particularly challenging using the hemocytometer counting chambers due to their similar morphology and size in this species (Stacy et al., 2011; Sykes 4th and Klaphake, 2015). Semi-direct techniques like the Unopette method using phloxine B have been shown to severely underestimate leukocyte and thrombocyte counts in chelonians compared to the direct Natt and Herrick method, thus their use is not recommended in these species (Wilkinson, 2004). The use of an automated hematology analyzer, instead of the manual methods, for the evaluation of tortoise CBC could shorten turnaround times and potentially enhance accuracy and precision, given the higher number of cells counted.

The new hematology analyzer Sysmex XN-1000 V, equipped 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) due to a weaker fluorescence signal (Tantanate and Klinbua, 2015). This new automated instrument has been successfully validated in dogs (Grebert et al., 2021), but not yet in non-mammalian species.

Therefore, this study hypothesizes that the new channel WNR can accurately count different cell populations in heparinized blood samples from *Testudo hermanni.* The aims of this study were to assess the feasibility of obtaining a partial instrumental CBC based on WNR results from Sysmex XN-1000 V and to evaluate the agreement between Sysmex XN-1000 V data and the manual counts in *Testudo hermanni*.

2. Materials and methods

2.1. Caseload

Fifty adults *Testudo hermanni*, privately owned or living in rescue shelters 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 occipital sinus was collected using a 2.5 mL syringe and a 22G needle (Rays) from each animal for routine health check or diagnostic purposes. All the blood withdrawals were performed at ambient temperature (15–30 °C), with the animals being manually restrained, without need of sedation. None of the samples used in this study was macroscopically lymph diluted or clotted. From each animal, 1 mL of blood was withdrawn (corresponding to a maximum of 0.5% of the body weight) and immediately put in lithium heparin test tube (FL Medical) and stored refrigerated in a thermal bag until reaching the lab after a maximum time of 3 h. In the lab, all the samples were refrigerated at 4 °C for a maximum of 48 h from withdrawal. 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 h, while 13 out of 50 samples were stored up to 48 h and used for the assessment of inter-assay reproducibility.

2.2. Manual count

Within 24 h from withdrawal, all the samples were manually counted using the Neubauer improved hemocytometer chamber (Blau Brand) by a single operator (SM, third year ECVCP resident) (Fig. 1). To this aim, whole blood samples were diluted 1:200 with Natt and Herrick's staining solution, as already described in literature (Bielli et al., 2015). Specifically, WBC and Thr were counted together (WBC + Thr), due to the inherent difficulties in distinguishing small lymphocytes from thrombocytes in the chamber. The WBC + Thr were counted in the nine large square of the grid designed on the counting chamber (in order to improve the accuracy of the count) and the result was adjusted based on the counting chamber area (9 mm^2), the chamber depth (0.1 mm) and the dilution used as suggested by the manufacturer (Fig. 1), in order to obtain the absolute number per microliter. The NRBC were counted in five small squares of the central big square, following the Neubauer chamber's instructions (Fig. 1), and the result was then divided by 100 to obtain the absolute number of cells per microliter.

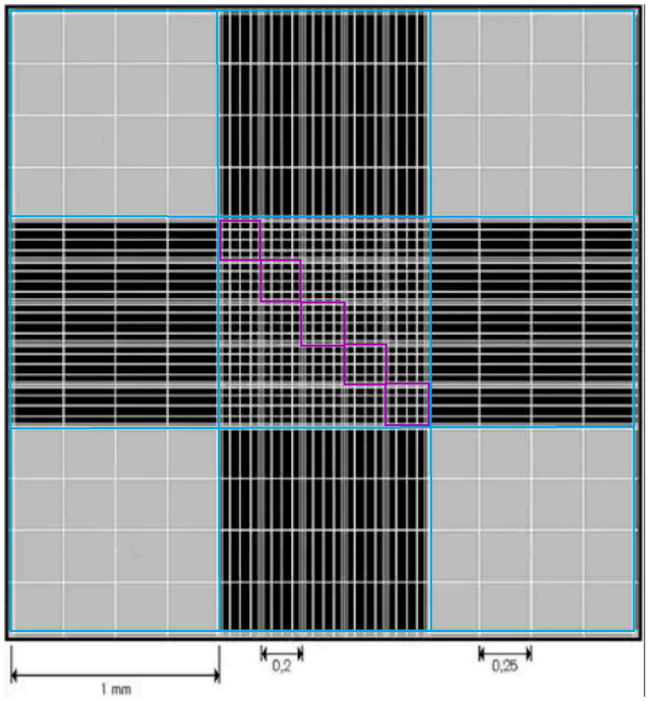

Fig. 1. Example of Neubauer hemocytometer microscopic grid. The nine large squares (in light blue) were used to count leukocytes and thrombocytes and the value obtained was then divided by 4.5. Erythrocytes were counted in five small squares inside the central large square (in purple) and the value obtained was then divided by 100 (according to manufacturer instructions). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Automated count

Sysmex XN-1000 V performances were checked on daily basis using three level of quality control material, provided by the manufacturer (Sysmex Corporation).

The very first sample was acquired with "Dolphin" animal species setting, which is fitted to count erythrocytes with large size (>80 fL). However, the Dolphin WNR scattergram is divided into three different population (namely NRBC, basophils and other leukocytes) and the preset gates did not fit with cell clouds obtained with tortoise sample. For this reason, ad hoc gates were set into the WNR channel, based on the two clouds grossly discriminable in the scattergram, and the sample was re-analysed using this gating strategy. Based on the results of the manual count on the same sample, the concentration of cells in the two clouds were compatible with NRBC and WBC + Thr, respectively. Finally, the customed gate setting was saved as "Tortoise" species 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 h from the withdrawal. To assess the inter – assay precision, 13 out of 50 samples were additionally stored at 4 °C for further 24 h, then brought at room temperature and gently resuspended using a blood mixer (Asal) before being re-analysed with Sysmex XN-1000 V.

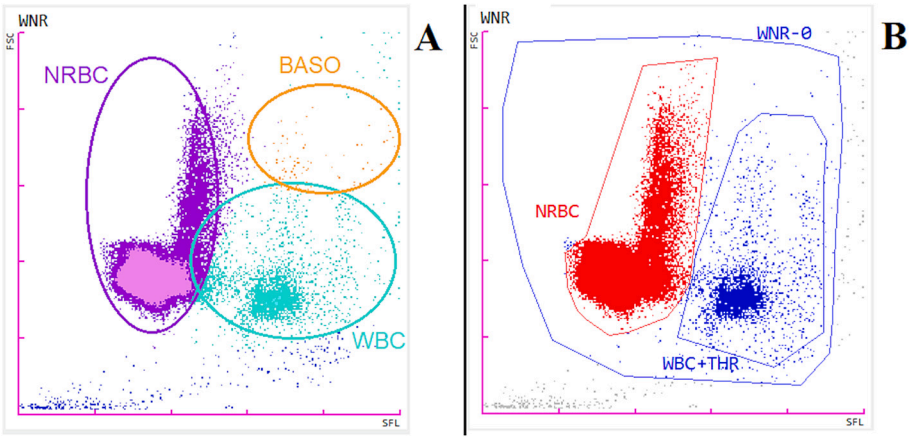

Fig. 2. Example of a dot plot scattergram obtained using the "Dolphin" species (A). The cells were divided into three populations: NRBC in purple on the left, WBC (including neutrophils, monocytes, eosinophils and lymphocytes) in light blue on the bottom right and basophils (BASO) in yellow, on the upper right. Ad-hoc "Tortoise" 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. WNR-0 is the gate that includes the entire population of blood cells counted in the WNR channel (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.4. Statistical analysis

Statistical analysis was performed using the specific software Analyse-it for Microsoft Excel (Analyse-it Software Ltd). Data distribution was assessed with Kolmogorov-Smirnov analysis. Intra and inter – assay precisions of the automated method were assessed through the measurement of imprecision (coefficient of variation = standard deviation/mean x 100). Based on data distribution the comparison between manual and automated counts was assessed using the Wilcoxon signed rank test for paired data comparing the manual count with the mean of the automated counts at 24 h. 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.

3. Results

3.1. Animals

Among the 50 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. Data distribution was normal for manual and instrumental NRBC ($P = 1.0$ for each method) and for manual WBC + Thr counts ($P = 0.07$), whereas it was non-normal for instrumental WBC + Thr count ($P = 0.006$).

3.2. Intra and inter – Assay precision of Sysmex XN-1000 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 24 h and between the mean of the two 24 h counts and the 48 h count was found, either for NRBC and WBC + Thr ($P > 0.05$ for all analyses).

3.3. 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 ($P = 0.560$). No constant or proportional error for the NRBC count was found with the Passing & Bablok regression analysis

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.

Table 1
Descriptive statistics for the samples enrolled in this study. In the table are reported, for both NRBC and WBC + Thr populations, the descriptive statistics for both manual count (Man) and automated count (XN-V) performed in duplicate at 24 h and expressed also as a mean of the two measurements, as well as the measurements performed at 48 h. The *P* value obtained with Wilcoxon signed rank test for paired data in the comparison between manual and instrumental counts is reported.

	NRBC ($x10^9$/L)					
	Man (n = 50)	XN-V 24 h 1 (n = 50)	XN-V 24 h 2 (n = 50)	XN-V 24 h Mean (n = 50)	XN-V 48 h (n = 13)	Man vs XN-V 24 h Mean
Mean ± S.D.	0.48 ± 0.17	0.44 ± 0.14	0.44 ± 0.14	0.44 ± 0.14	0.52 ± 0.11	$P = 0.560$
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 24 h 1 (n = 50)	XN-V 24 h 2 (n = 50)	XN-V 24 h Mean (n = 50)	XN-V 48 h (*n* = 13)	Man vs XN-V 24 h Mean
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)	$P = 0.027$

(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).

4. Discussion

The present study aimed to assess the feasibility of obtaining accurate and reliable data through the utilization of an automated hematological analyzer for the analysis of blood in a non-mammalian species. To this aim, the investigation focused on assessing the agreement between a conventional manual technique and a novel fluorescence flow cytometry analyzer in hematological cell counts of *Testudo hermanni*.

Tortoises are free–ranging animals, but they are also diffused as companion animals. Specifically, *Testudo hermanni* is one of the most common pet tortoises in Italy. The referral to a specialized clinician, with a peculiar expertise in exotic animals, is a pivotal part of the correct management of these animals. This is mostly due to their subtle clinical signs and peculiar blood sampling techniques. Interpretation of clinical-pathological results is also difficult in these animals, due to the paucity of data on healthy animals and the intrinsic laboratory variability. Manual hemocytometer chambers are frequently employed for the whole blood evaluation in this species, similarly to other non-mammalian species physiologically characterized by nucleated erythrocytes and thrombocytes (namely birds, other reptiles, amphibians 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, this manual count was applied in the present study as a reference standard to assess the performances of Sysmex XN-1000 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 min 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-1000 V analyzer, requires less than one minute to be performed. Moreover, due to the low number of cells

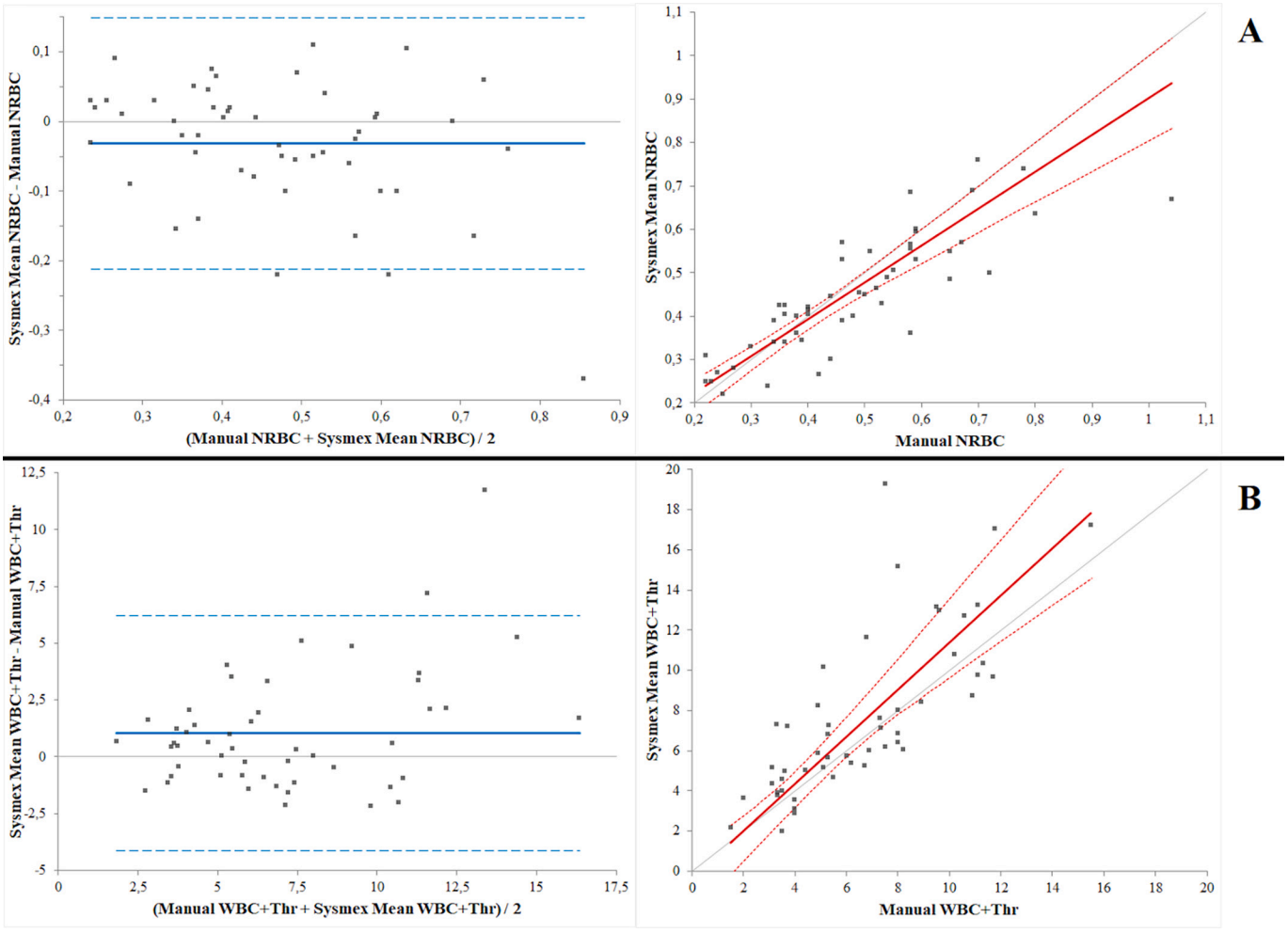

Fig. 3. Passing & Bablok regression analysis and Bland – Altman plot of the automated count compared to the manual count for both the nucleated red blood cells (NRBC, A - upper part of the figure) and the white blood cells and thrombocytes (WBC + Thr, B – lower part of the figure) populations. Bland–Altman plots show the mean values obtained with Sysmex XN-1000 V and manual counts on the x-axes and the difference between the results of the two 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 novel hematology analyzer Sysmex-XN-V has been recently 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 (namely WBC). In this latter channel, whole blood is treated with an acidic hemolysing reagent (Lysercell® WNR) that lyses RBC, while it perforates WBC and NRBC membranes (Sysmex XN-V series, 2019). 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-1000 V in analyzing heparinized 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 thrombocytes clumping, that may have affected the count. Irrespective of the causes underlying the lower precision in the WBC + Thr count after 48 h, the results here obtained suggest that it is advisable to perform the automated blood cell count within 24 h from the withdrawal, to reduce the occurrence 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 the inability of the automated count using the WNR channel to differentiate leukocytes and thrombocytes separately, and this represents a limitation from a clinical perspective. Nevertheless, even using the manual hemocytometer count, the differentiation between leukocytes and thrombocytes may be difficult. Therefore, 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-1000 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 consistency in the applied gating strategy. 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 species – specific hematologic reference intervals. Moreover, it would be interesting to evaluate the performances of Sysmex XN-1000 V on other reptile species using the same gating strategy here proposed (eventually with some small adjustments, if needed, based on possible species differences).

5. Conclusions

Based on the results obtained in this study, the new hematology analyzer Sysmex XN-1000 V shows a promise as a substitute for the manual counting for the evaluation of *Testudo hermanni* blood samples, particularly for NRBC and WBC + Thr counts. However, the two methods are not perfectly superimposable, thus a calculation of instrument specific reference interval is advisable. The use of Sysmex XN-1000 V could markedly reduce turnaround time for the analysis of tortoises' blood populations (NRBC and WBC + Thr), compared with the manual count, potentially enhancing the accuracy of results given the higher number of cells counted by the automated instrument.

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CRediT authorship contribution statement

Sara Meazzi: Conceptualization, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. **Valeria Martini:** Data curation, Formal analysis, Writing – original draft. **Amanda Moretti:** Data curation, Formal analysis, Writing – original draft, Writing – review & editing. **Emanuele Lubian:** Data curation, Investigation, Supervision. **Saverio Paltrinieri:** Data curation, Supervision, Writing – original draft. **Alessia Giordano:** Conceptualization, Data curation, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

Sysmex corporation supplied the automated hematology instruments and reagents used in this study. Sysmex corporation played no role in the study design nor in the collection, analysis and interpretation of data, nor in the decision to submit the manuscript for publication. None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

References

Ammersbach, M., Beaufrère, H., Gionet Rollick, A., Tully, T., 2015. Laboratory blood analysis in Strigiformes - Part I: hematologic reference intervals and agreement between manual blood cell counting techniques. Vet. Clin. Pathol. 44 (1), 94–108. https://doi.org/10.1111/vcp.12229.

Bielli, M., Nardini, G., Di Girolamo, N., Savarino, P., 2015. Hematological values for adult eastern Hermann's tortoise (*Testudo hermanni boettgeri*) in semi-natural conditions. J. Vet. Diagn. Investig. 27, 68–73. https://doi.org/10.1177/1040638714561251.

Brenn-White, M., Raphael, B.L., Rakotoarisoa, N.A.T., Deem, S.L., 2022. Hematology and biochemistry of critically endangered radiated tortoises (*Astrochelys radiata*): reference intervals in previously confiscated subadults and variability based on common techniques. PLoS One 17 (3), e0264111. https://doi.org/10.1371/journal.pone.0264111.

Campbell, T.W., 2015. Hematologic techniques in lower vertebrates. In: Exotic Animal Hematology and Cytology, 4th ed. Wiley Blackwell, UK, pp. 199–208.

Cheylan, M., 2001. *Testudo hermanni* Gmelin, 1789 – Griechishe Landschildkroete. In: Handbuch der Reptilien und Amphibien Europas [Band 3/IIIa] – Schildkroten (Testudines) I. Aula Verlag, Wiebelsheim, Germany, pp. 179–289.

Grebert, M., Granat, F., Braun, J.P., Leroy, Q., Bourgès-Abella, N., Trumel, C., 2021. Validation of the Sysmex XN-V hematology analyzer for canine specimens. Vet. Clin. Pathol. 50, 184–197. https://doi.org/10.1111/vcp.12936.

International Union for the Conservation of Nature, 2023. IUCN. https://www.iucnredlist.org/species/21648/176604335 (Accessed May, 2022).

Kaido, M., Takagi, Y., Kono, M., Nakazawa, F., Yamamoto, S., Wada, A., Morikawa, T., 2017. Investigation of morphological changes for the discrimination of nucleated red blood cells and other leukocytes in Sysmex XN hematology analyzer scattergrams using transmission electron microscopy. Pract. Lab. Med. 8, 70–76. https://doi.org/10.1016/j.plabm.2017.05.001.

McArthur, S., Wilkinson, R., Barrows, M., Meyer, J., 2004. Introduction. In: Medicine and Surgery of Tortoises and Turtles. Blackwell Publishing Ltd, Oxford UK, pp. 1–30.

Nardini, G., Leopardi, S., Bielli, M., 2013. Clinical hematology in reptilian species. Vet. Clin. North Am. Exot. Anim. Pract. 16, 1–30. https://doi.org/10.1016/j.cvex.2012.09.001.

Natt, M.P., Herrick, C.A., 1952. A new blood diluent for counting the erythrocytes and leucocytes of the chicken. Poult. Sci. 31, 735–738. https://doi.org/10.3382/ps.0310735.

Stacy, N.I., Alleman, A.R., Sayler, K.A., 2011. Diagnostic hematology of reptiles. Clin. Lab. Med. 31 (1), 87–108. https://doi.org/10.1016/j.cll.2010.10.006.

Sykes 4th, J.M., Klaphake, E., 2015 Jan. Reptile hematology. Vet. Clin. North Am. Exot. Anim. Pract. 18 (1), 63–82. https://doi.org/10.1016/j.cvex.2014.09.011.

Sysmex XN-V series, 2019. Manual: instructions for use (Sysmex corporation 2015–2018, Kobe Japan).

Tantanate, C., Klinbua, C., 2015. Performance evaluation of the automated nucleated red blood cell enumeration on Sysmex XN analyser. Int. J. Lab. Hematol. 37, 341–345. https://doi.org/10.1111/ijlh.12291.

Wilkinson, R., 2004. Clinical pathology. In: Medicine and Surgery of Tortoises and Turtles. Blackwell Publishing Ltd, Oxford UK, pp. 141–186.