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Diagnostic use of the reticulocyte maturity indices provided by the Sysmex XN-V analyser

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Canine anaemia: its differentiation and the importance of an accurate diagnosis

Anaemia (understood as the decrease in haematocrit [HCT], red blood cell [RBC] count and/or haemoglobin concentration) is one of the most common disorders in veterinary practice among small animals [1]. In the case of canines, anaemia can be classified according to multiple variables (see Table 1).

However, the classification of anaemia according to its cause is the most useful for veterinary clinicians, as it allows for the establishment of a treatment and prognosis that are much more personalised to the patient (see Table 2).

Given the high number of disorders that may cause anaemia in canines, it would be essential for the clinician to be able to narrow down the diagnosis early and precisely, according to the aetiology, thus guiding additional tests and reducing the time needed to establish a suitable treatment.

Table 1 *Different classifications of anaemia in canines, according to several criteria*

Classification according to severity	■ Mild anaemia –› HCT 30–37 % ■ Moderate anaemia –› HCT 20–29 % ■ Serious anaemia –› HCT 13–19 % ■ Severe anaemia –› HCT < 13 %
Classification according to the RBC morphology	■ Hypochromic or normochromic anaemia ■ Microcytic, normocytic or macrocytic anaemia
Classification according to bone marrow response	■ Regenerative anaemia (acute haemolytic and haemorrhagic) ■ Non-regenerative anaemia (chronic and peracute haemorrhagic, bone marrow failure or suppression)

Table 2 *Classification of canine anaemia based on its cause or aetiology, with examples of the main disorders for each subtype*

Haemorrhagic anaemia	■ Trauma ■ Gastrointestinal or external parasitic infections ■ Thrombocytopenia, thrombocytopathy	■ Cancer, ulcers or gastrointestinal perforations ■ Warfarin toxicity ■ Other coagulation disorders
Haemolytic anaemia	■ Idiopathic immunohaemolytic anaemia ■ Intoxication	■ Erythroparasites ■ Congenital RBC metabolic deficits
Anaemia due to failure or suppression of erythropoiesis	■ Bone marrow damage (infection, cancer, toxic products) ■ Anaemia of chronic disease	■ Renal disease ■ Iron deficiencies

The importance of determining reticulocytes

Erythropoiesis is the physiological process that allows the recycling and regeneration of circulating RBC in the body [2]. The last stage before the formation of mature circulating red blood cells is called a 'reticulocyte'. Reticulocytes are constantly produced in the bone marrow and, after a short storage period in the bone marrow, they are released (in low concentrations) into circulation, where they complete the last steps of maturation (thereby losing the remains of RNA, mitochondria and other cell organelles).

The main indication for a measurement of circulating reticulocytes is to allow differentiation between regenerative and non-regenerative anaemia [2]. An absolute increase in the circulating reticulocyte count usually detects the presence of a regenerative anaemia. However, it is not possible to distinguish between acute haemorrhagic and haemolytic anaemia, merely by assessing the reticulocyte count. In the same way, in non-regenerative disorders, simply determining the reticulocyte count, does not allow to distinguish between ineffective erythropoiesis due to bone marrow damage and those in which the condition originated outside the bone marrow (for example, in the case of anaemia of chronic disease).

Methods of measuring reticulocytes

The most appropriate test to determine reticulocytes in canines is to count them after staining [3], be it manually (using staining techniques such as new methylene blue or brilliant cresyl blue) or using automated analysers (which mark reticulocytes using specific fluorochromes to detect RNA remains). When comparing both techniques, the manual method reports higher statistical errors and relies much more on the skills of the operator, requiring at least 1,000 reticulocytes to be counted to obtain a reliable estimation. The use of automated analysers, such as the XN-V, allows the rapid detection (in less than one minute) of the fluorescence present in up to 30,000 RBC, obtaining far more reproducible and accurate data than by using manual counts [4].

Although, traditionally, the parameter that was used by clinicians to assess the regenerative response was the percentage of reticulocytes, it should be noted that this parameter presents serious flaws when assessing the power of the bone marrow response [2, 3]. For example, while in a healthy dog a 4% reticulocyte count is considered to be elevated, the same result in an anaemic dog with 2 million RBC is only 80,000 reticulocytes/µL (a normal value in a healthy animal, but extremely deficient in patients with this degree of anaemia).

Considering this, it is always much more effective to assess the degree of regeneration by studying the absolute reticulocyte count or, alternatively, one of the multiple regeneration assessment indices published for canines (see Table 3).

The objective of the preliminary investigation presented in this document was to determine the validity of haematological parameters in addition to the reticulocyte count in anaemic canine patients, in order to differentiate between haemorrhagic and haemolytic disorders and among the different processes that may result in non-regenerative anaemia.

Table 3 *Main methods to assess bone marrow regeneration in canines*

	Advantages	Disadvantages	Reference value in canines
Reticulocyte percentage $RET\% = \frac{RET\ [10^6/\mu L]}{RBC\ [10^6/\mu L]} \times 100$	✔ Data provided by basic automated analysers	✗ By not considering the level of anaemia, it is NOT useful in assessing anaemic patients.	■ Up to 1% in normal patients ■ In patients with anaemia it depends on the degree of anaemia.
Absolute reticulocyte count $RET\# = \frac{RET\ [\%] \times RBC\ [10^6/\mu L]}{100}$	✔ More useful than the relative count to assess the bone marrow response capacity	✗ Overlap between healthy animals and non-regenerative anaemia ✗ No differentiation between regeneration due to haemolysis or haemorrhage	■ In healthy subjects between 20,000 and 150,000/µL ■ Usually higher in regenerative conditions
Reticulocyte production index $RPI = \frac{RET\ [\%]}{\text{Reticulocyte maturation days}} \times \frac{HCT\ [\%]}{0.45}$	✔ More useful in anaemic subjects, by considering the patient's haematocrit	✗ This index originated in human medicine, without having been completely standardised in veterinary medicine. Difficult to personalise depending on the patient's data.	■ Any value above 1 is considered a sign of regeneration.

In this formula, the value for 'reticulocyte maturation days' is obtained depending on the patient's HCT:

Patient's HCT	Reticulocyte maturation days
36 – 45 %	1
26 – 35 %	1.5
16 – 25 %	2
≤ 15 %	2.5

Reticulocyte maturity: meaning and relevance

Aside from counting the absolute number of reticulocytes, the Sysmex XN-V analyser can use its fluorescence flow cytometry to determine the degree of maturity of circulating reticulocytes [5], classifying them according to their RNA content in the following categories:

- LFR (low-fluorescence reticulocytes) or 'mature' reticulocytes.
- MFR (medium-fluorescence reticulocytes) or 'semi-mature' reticulocytes.
- HFR (high-fluorescence reticulocytes) or 'immature' reticulocytes, with a high RNA content.

In addition to that, the XN-V analyser provides the immature reticulocyte fraction (IRF), obtained as the sum of the MFR and HFR fractions. The distribution of these reticulocytes may also be studied using the corresponding scattergram of the reticulocyte channel (see Figure 1).

These parameters are already used in human medicine to obtain an initial suspected diagnosis and classify anaemic conditions according to their aetiology, among other applications [6].

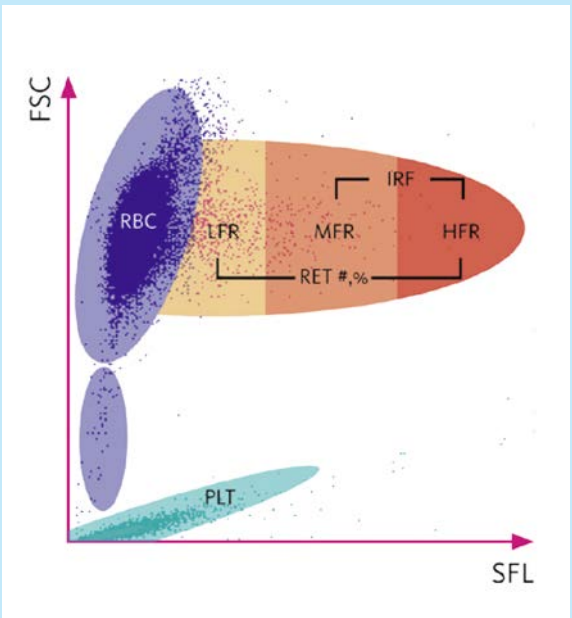

Fig. 1 *Representation of the different reticulocyte maturity fractions in the reticulocyte channel scattergram of the Sysmex XN-V haematology analyser*

Reference ranges for reticulocyte maturity in canines

In order to assess the usefulness of the reticulocyte maturity parameters obtained with the Sysmex XN-V analyser, it is first necessary to establish the reference range for these values in healthy canines. Although there are previous studies [5], these used a different analyser system, a limited number of patients and focused only on the Beagle breed. The research team at the University of Córdoba is analysing these parameters among the animals admitted to the Hospital Clínico Veterinario Francisco Santisteban (ongoing project). Following the initial assessment of 489 non-anaemic canines (n = 489), the reference ranges for these parameters are included in Table 4 and a typical scattergram for a non-anaemic dog is depicted in Figure 2.

Table 4 *Mean value and reference range (95 % confidence interval) of the reticulocyte maturity parameters in non-anaemic dogs (n = 489)*

	LFR	MFR	HFR	IRF
Mean (%)	75.4	9.8	14.5	24.6
Reference range (%)	60–88	4–16	5–26	11–39

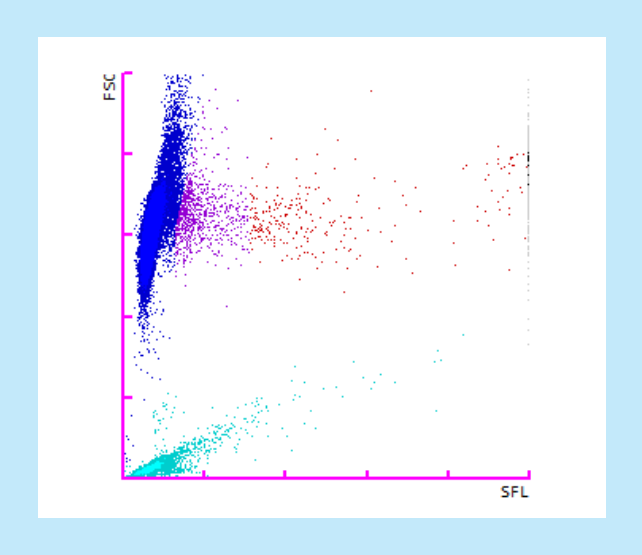

Fig. 2 *Scattergram of the RET channel in a non-anaemic canine patient*

This study population included animals of both sexes from 45 different breeds with an age ranging from 7 days to 15 years. Following a preliminary statistical analysis, no significant effects on these values have been detected depending on sex, age, or breed of the patient, while the data is still being collected and analysed.

Diagnostic interpretation of reticulocyte maturity in anaemic canines: the Écija-Mendoza diagram

With the objective of assessing the capacity of the reticulocyte maturity parameters to distinguish among the various types of canine anaemia, the research team at the University of Córdoba studied these parameters in a total of 153 anaemic canines (n = 153) with a confirmed aetiological diagnosis. Following the statistical analysis of the data, we have created the specific Écija-Mendoza diagnostic diagram for canine anaemia (see Figure 3).

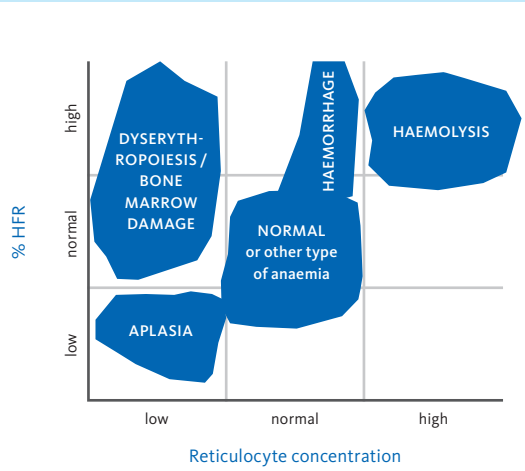

Fig. 3 *Écija-Mendoza diagram displaying the classification of different anaemia aetiologies, based on the percentage of HFR and reticulocyte concentration*

In the Écija-Mendoza diagram both the HFR (high if above 26 % and low if lower than 5 %) and the reticulocyte concentration (high if above 150,000 and low if below 20,000 reticulocytes/µL) are displayed. In this diagram various diagnostic grids are obtained, which allow suspicion of and differentiation between the presence of haemolysis, haemorrhage and bone marrow damage.

Table 5 presents the data on sensitivity, specificity and diagnostic predictive values for each of the cited causes of anaemia using preliminary cut-off values established by the research team at the University of Córdoba.

It must be emphasised that the data presented in this document is preliminary and that the research team at the University of Córdoba continues to collect results and perform statistical analysis to improve this diagnostic tool. Also, this diagram does not replace other aetiological diagnostic confirmatory tests, and its objective is rather to assist with an easy and early differentiation between haemorrhagic, haemolytic and bone marrow damage disorders.

Table 5 *Diagnostic potential of the Écija-Mendoza diagram, using statistical data available to date (Aug 2020)*

Aetiology and number of cases	HFR	Number of reticulocytes	Diagnostic specificity*	Diagnostic sensitivity**	Disorders present
Haemolysis (n = 50)	High (≥ 26 %)	High (≥ 150,000/µL)	92 %	76 %	Immunohaemolytic anaemia (idiopathic and secondary), babesiosis, etc.
Acute haemorrhage (n = 25)	Normal-high (≥ 21 %)	Normal-high (100,000 – 150,000/µL)	97 %	72 %	Trauma, intoxication due to coumaric agents, other coagulation disorders
Bone marrow aplasia (n = 15)	Low (≤ 5 %)	Low (≤ 20,000/µL)	99 %	87 %	Viral damage (parvovirus, etc.), toxic (chemotherapy, etc.)
Other types of dyserythropoiesis including bone marrow damage (n = 15)	Normal-high (> 5 %)	Low (≤ 20,000/µL)	99 %	80 %	Leukaemia (myeloid, lymphoid), terminal renal disease, etc.

* The diagnostic specificity indicates the algorithm's capacity to rule out the aetiological agent if the patient presents different values of HFR and reticulocytes [7]. For example, 92 % of the patients not classified as haemolytic will indeed not have haemolysis.

** The diagnostic sensitivity represents the algorithm's capacity to recognise the anaemia's underlying aetiology considering the HFR and circulating reticulocytes [7]. For example, 76 % of the patients who fulfil the 'haemolysis' classification criteria, do indeed have haemolysis.

Conclusions

The early differentiation of the underlying cause of canine anaemia is one of the biggest diagnostic challenges that veterinary clinicians currently face. An accurate, reliable total reticulocyte count, together with the study of the different circulating degrees of maturity may allow a much more thorough and detailed assessment of each patient in the veterinary lab. In this preliminary study, reference ranges have been established for the different reticulocyte maturity fractions (LFR, MFR, HFR, IRF) determined with the Sysmex XN-V analyser. Following the assessment of more than 150 real cases of anaemia, the Écija-Mendoza diagram was created. This is a diagnostic tool that allows differentiation between conditions caused by haemolysis, acute haemorrhage and anaemia due to dyserythropoiesis. While this algorithm does not replace the definitive test to confirm the aetiological diagnosis, the diagnostic sensitivity and specificity of the proposed categories allow the clinician to quickly form a suspected diagnosis.

References

[1] ***Thrall MA et al. (2012):*** *Veterinary hematology and clinical chemistry. 2nd ed. Wiley-Blackwell. Section II. Chapter 6.*

[2] ***Latimer KS (2011):*** *Duncan and Prasse's veterinary laboratory medicine clinical pathology. 5th ed. Wiley-Blackwell. Chapter 1.*

[3] ***Cowgirl ES et al. (2003):*** *Clinical application of reticulocyte counts in dogs and cats. Vet Clin North Am Small Anim Pract 33(6):1223–1244.*

[4] ***Sysmex Europe GmbH (2016):*** *SEED haematology: the importance of reticulocyte detection. Online educational material.*

[5] ***Mathers A et al. (2012):*** *Reticulocyte measurements in rat, dog and mouse whole blood samples using the Sysmex XT-2000iV. Comp Clin Pathol 21:631–637.*

[6] ***D'Onofrio G et al. (1996):*** *Indicators of haematopoietic recovery after bone marrow transplantation: the role of reticulocyte measurements. Clin Lab Haem 18(Suppl.1):45–53.*

[7] ***Paltrinieri S et al. (2012):*** *Sensitivity and specificity of manual and automated measurements of reticulocyte parameters for classification of anemia in dogs: 174 cases (1993–2013). J Am Vet Med Assoc 249(7):776–786.*