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## Clinical application of reticulocyte counts in dogs and cats

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Reticulocytes are immature red blood cells (RBCs) that contain a reticulum network of RNA, mitochondria, and organelles, which are visible with supravital stains, such as new methylene blue (NMB) or brilliant cresyl blue (BCB) [1–4]. First recognized in 1865 by Erb using acetic acid, reticulocytes were originally named in 1891 by Ehrlich using NMB to stain reticulum. In 1932, Heilmeyer and Westhaeuser described the morphology and clinical utility of reticulocytes [3].

Two types of reticulocytes, aggregate and punctate, are observed with supravital stains. The aggregate reticulocyte is larger and more immature with coarsely clumped collections of reticulum. With Romanowsky stains, they appear grayish to bluish pink [1,2]. Aggregate reticulocytes are referred to as polychromatophils or polychromatophilic red cells, and the presence of these cells on a blood smear is called polychromasia. Because aggregate reticulocytes are larger and contain incomplete hemoglobin content, they appear macrocytic on a blood smear (and result in increased mean corpuscular volume [MCV] and decreased mean corpuscular hemoglobin concentration [MCHC]). Despite the decrease in rough endoplasmic reticulum (RER), ribosomes, and mitochondria from the more immature erythroid cells, aggregate reticulocytes are more metabolically active than mature erythrocytes and synthesize up to 20% of the final hemoglobin concentration. Punctate reticulocytes, which stain similar to mature red cells with Romanowsky stains, are smaller and more mature with small sparse granules of residual RNA [1–4].

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Reticulocytes are prematurely released from the bone marrow in response to elevated erythropoietin levels induced by tissue hypoxia and indicate enhanced bone marrow erythropoiesis. Reticulocytes are used to classify anemias into regenerative (reticulocytosis) and nonregenerative (reticulocytopenia) categories, to assess bone marrow integrity, and to monitor therapy for anemia [1,2,4]. Numerous methods for reticulocyte enumeration exist. Although the manual count by light microscopy remains the gold standard, newer automated methods using RNA-specific fluorochromes, flow cytometry, and dedicated reticulocyte analyzers are becoming more prevalent in veterinary medicine [3,4].

This article reviews the physiology and maturation of the reticulocyte, current laboratory techniques for reticulocyte enumeration (including newer methods that may become the standard of measurement in the future), clinical utility of reticulocyte counting, and species variation in the reticulocyte response.

### **Erythropoiesis and reticulocyte maturation**

Two types of erythroid progenitor cells, the burst-forming unit–erythroid (BFU-E) and the colony-forming unit–erythroid (CFU-E), have been identified by *in vitro* culture assays. Under the influence of interleukin-3 (IL-3), granulocyte macrophage colony-stimulating factor (GM-CSF), and other factors, BFU-E differentiates from the colony-forming unit–granulocyte, –erythrocyte, –monocyte, –megakaryocyte (CFU-GEMM). The latter cell develops from a primitive hematopoietic stem cell. CFU-E matures to BFU-E under the influence of erythropoietin, which further develops into recognizable erythroid precursors [5,6].

Erythropoietin is a glycoprotein and a hematopoietic growth factor made by the kidney. Reduced renal oxygen tension stimulates erythropoietin production. Erythropoietin potentiates the differentiation of BFU-E and enhances erythroid cell division at all stages of development. One of its major effects is stimulating committed resting (G0) erythroid stem cells (CFU-E) to enter the cell cycle and subsequently produce more erythroid precursors. Erythropoietin prevents apoptosis of progenitor cells and prorubricytes, allowing continued division and maturation. Additionally, erythropoietin stimulates increased hemoglobin synthesis and early release of young bone marrow reticulocytes into the peripheral blood [4–6].

The first morphologically recognizable erythroid precursor is the erythroblast. As the erythroblast matures to the reticulocyte, it undergoes a series of biochemical and structural changes. These changes include the following: loss of nucleoli, the Golgi apparatus, ribosomes, and mitochondria; synthesis of hemoglobin; reduction in cell volume and changes in membrane lipid and cholesterol levels with a shape change to the biconcave form; loss of cell-surface membrane receptor function; and phagocytosis

of the pyknotic condensed extruded nucleus by macrophages of the erythropoietic island. Removal of these organelles and nuclei from maturing erythrocytes is essential to produce a biconcave flexible cell that can readily pass through capillary beds. This progression from an erythroblast to a nonnucleated red blood cell, or reticulocyte, takes 3 to 5 days [4–6].

Maturation and differentiation of the reticulocyte into a mature red blood cell involves loss of cellular organelles, decreased hemoglobin synthesis, and loss of the transferrin receptor (necessary for entry of ferric iron into the cell). The reticulocyte contains remnants of Golgi bodies, ribosomes, and mitochondria and can be prematurely released from the bone marrow under the influence of erythropoietin. A peripheral reticulocytosis after a hemorrhagic or hemolytic event may not be seen until 2 to 5 days after the episode [1,2,4,5].

In human beings, reticulocytes are classified into four “age” groups (groups I–IV) based on density of RNA and organelles (reticulum) so as to obtain information about bone marrow function [1,2]. Similarly, reticulocytes are classified into aggregate and punctate groups in the cat, but only aggregate reticulocytes are counted in the dog. Aggregate reticulocytes are larger and more immature cells with coarsely clumped reticulum, whereas punctate reticulocytes are smaller and more mature with one to three or more small granules of residual RNA [7]. In cats and dogs, after a single episode (not ongoing) of blood loss or a hemolytic event, the aggregate reticulocytes usually appear in peripheral blood within 48 hours, peak at 4 to 7 days, and disappear in 10 to 14 days. Punctate reticulocytes in the cat are slower to increase, however, and remain in the circulation longer (2–3 weeks) [1].

### **Laboratory measurement of reticulin**

The standard method of reticulocyte enumeration is the supravital staining technique. A few drops of supravital stain are added to equal volumes of anticoagulated peripheral blood and incubated for 15 minutes at room temperature to allow the stain to enter the cell and precipitate the reticulum and polyribosomes before making the blood smear. The slide can be counterstained with a Romanowsky stain to identify mature red cells better or left without a counterstain [3,4,8]. Under oil immersion, an adequate number of erythrocytes (typically 1000 consecutive) are examined in a well-stained area of the monolayer, and the reticulocyte percent is determined [3,4]. To avoid inaccuracies, blood should not be visibly hemolyzed, the sample should be collected into EDTA, and counting should be performed within 6 hours to avoid time and temperature-dependent *in vitro* maturation [8].

Performing reticulocyte counts on a blood film can introduce inaccuracies, because reticulocyte distribution may not be uniform. Using ocular

devices, such as the Miller ocular reticle, reduces bias by standardizing the counting area (Fig. 1), but following “edge rules,” where cells touching only two of four edge lines (top and right edges) are counted, is essential to obtain a more accurate reticulocyte count. Discrepancies or errors in manual counts include the method of enumeration, lack of or improper use of the edge rule for the Miller reticle method, type and quality of blood film, and variation in the distribution of reticulocytes on the slide [3,4,8]. The major cause of imprecise reticulocyte counts involves variation between observers in how they define aggregate and punctate reticulocytes. The presence of a coarsely clumped reticulum, even a single clump, on an RBC

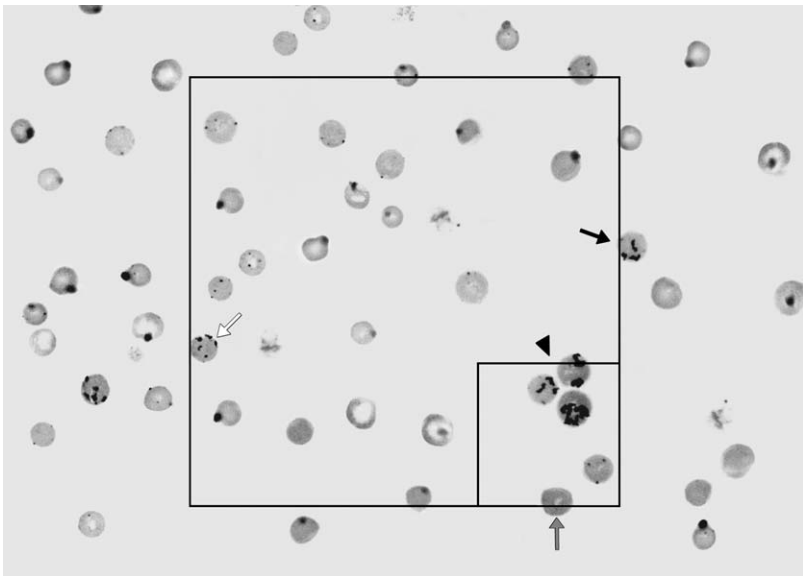


Fig. 1. Feline peripheral blood smear, new methylene blue stain. The superimposed illustration demonstrates the microscopic appearance of the smear when viewed with a Miller ocular reticle. All reticulocytes in the entire large square but only those erythrocytes present in the smaller square are counted. Reticulocyte counts and erythrocyte counts are recorded separately. A reticulocyte present in the smaller square is counted as both an erythrocyte and a reticulocyte. Cells touching the upper and right sides of the large and small squares are included in the counts, although cells touching the lower and left sides of the two squares are excluded (edge rule). Additional fields are examined in the same manner until at least 111 erythrocytes in the small square have been counted. The percent of reticulocytes is then calculated as:

$$\% \text{ Reticulocytes} = \frac{(\text{Total Reticulocytes in Larger Square} \times 100)}{(\text{Total Erythrocytes in Smaller Square} \times 9)}$$

In this figure, the aggregate reticulocyte indicated by the black arrow is included in the aggregate reticulocyte count, whereas the one indicated by the white arrow is excluded. The cell indicated by the gray arrow is excluded from the erythrocyte count, and the three aggregate reticulocytes indicated by the black arrowhead are included in both the aggregate reticulocyte and erythrocyte counts. A total of four erythrocytes in the small square and four aggregate reticulocytes in the entire large square are counted in this field.

in an appropriately stained preparation is the hallmark of an aggregate reticulocyte. Conversely, greater than three to five dots or granules representing residual RNA are criteria used to identify punctate reticulocytes. Another problem is erroneously counting red cells with cytoplasmic inclusions as reticulocytes. These “confounding” inclusions include Heinz bodies (Fig. 2), parasites like *Mycoplasma* sp (formerly *Haemobartonella* sp) (Fig. 3), Howell-Jolly bodies (Fig. 4), cellular debris or stain precipitate, and basophilic stippling (see Fig. 4). Basophilic stippling represents aggregates of ribosomes and is evident on Romanowsky-stained slides, whereas reticulum is evident only with the supravital stains [3,4]. Table 1 summarizes potential interferences with reticulocyte counts.

Flow cytometry, which uses a wide variety of fluorochromes, detects reticulocytes using both RNA/DNA fluorescence and forward and right angle light scatter for cell size and complexity. Flow cytometric methods for reticulocyte enumeration offer many advantages. With flow methods, reticulocytes are stained with RNA/DNA-specific fluorochromes, such as thiazole orange or auramine-O, and at least 10,000 cells are typically counted. This decreases the sampling error and increases precision of flow cytometry

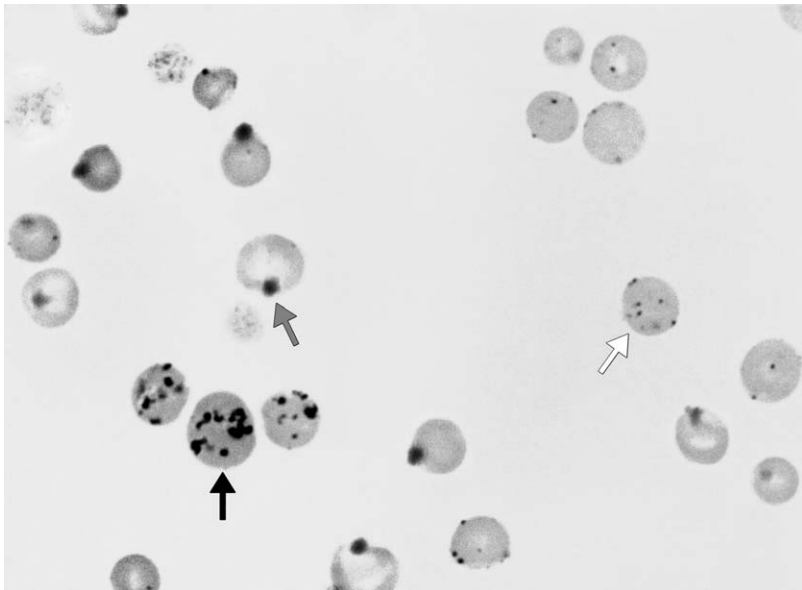


Fig. 2. Feline peripheral blood smear, aggregate reticulocytes, punctate reticulocytes, and Heinz bodies, new methylene blue (NMB) stain. Aggregate reticulocytes are larger with coarsely clumped collections of reticulum, whereas punctate reticulocytes are similar in size to mature erythrocytes and contain few small granules of residual RNA. Heinz bodies are also visible with NMB staining and can be misidentified as reticulocytes by inexperienced individuals and by many automated techniques. The black arrow indicates an aggregate reticulocyte, the white arrow indicates a punctate reticulocyte, and the gray arrow indicates a Heinz body.

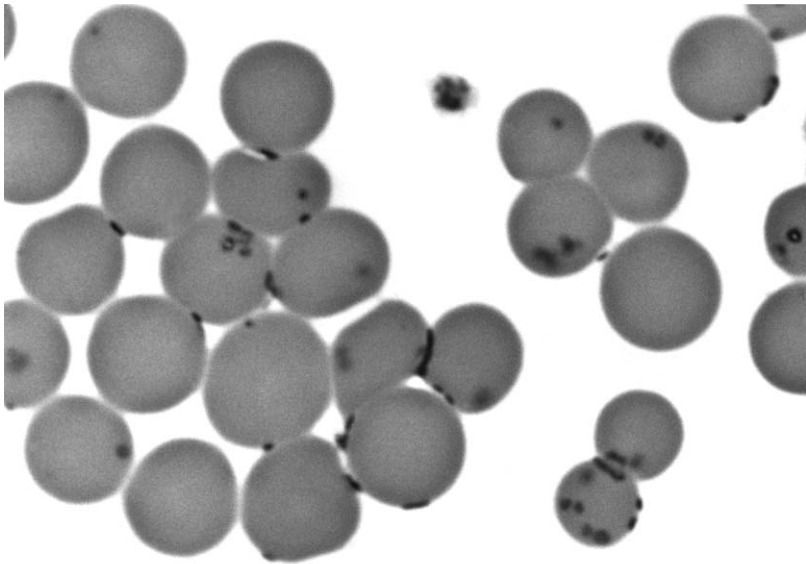


Fig. 3. Canine peripheral blood smear, Wright-Giemsa stain. Numerous *Mycoplasma* organisms are adhered to the erythrocyte membranes; these can result in inaccurate manual or automated reticulocyte counts.

over manual methods. In addition, the flow cytometers are fast and objective and require less technical labor [3,4]. Another advantage of these methods is that reticulocytes may be quantified and classified based on their RNA content or fluorescence intensity into two or three fractions. The immature reticulocyte fraction (IRF) in conjunction with the absolute reticulocyte count can be used to assess the response to anemia similar to the reticulocyte production index (RPI) [8]. The flow cytometer proved more reliable and sensitive than manual techniques for identification of feline reticulocytes and was able to detect both punctuate reticulocytes and low levels of aggregate reticulocytes [7–9]. Disadvantages of this technique include falsely elevated reticulocyte counts as a result of interfering erythrocyte inclusions, such as nucleated RBCs, Howell-Jolly bodies and parasites, and the expense and technical training for a complex instrument [4,9].

Hematology analyzers, such as the Cell Dyn 3500 and 3700 (Abbott Diagnostics, Santa Clara, CA) and the Bayer Advia 120 hematology system (Bayer/Miles Diagnostics Division, Tarrytown, NY), use helium laser light absorption or a combination of impedance and optical light scatter for reticulocyte enumeration. These instruments incubate whole blood with a supravital stain (NMB or oxazine 750) and analyze it at 0°, 10°, and 90° light scatter or at low and high angle light scatter. Less mature reticulocytes contain more residual RNA in a greater cell volume and consequently scatter more laser light than more mature reticulocytes, allowing identifi-

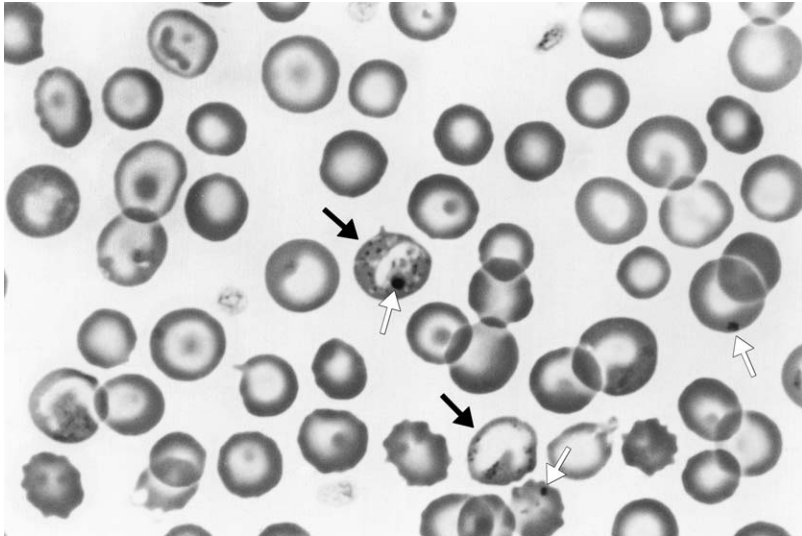


Fig. 4. Canine peripheral blood smear, Wright-Giemsa stain. Basophilic stippling (*black arrows*) and Howell-Jolly bodies (*white arrows*) are present. Both can result in inaccurate manual or automated reticulocyte counts.

cation of three distinct populations of reticulocytes (those of low, medium, and high RNA content). Typically, reticulocyte percent and absolute reticulocyte counts are reported, and reticulocyte indices (reticulocyte MCV, reticulocyte MCHC, reticulocyte cell hemoglobin content, and distribution widths) are sometimes calculated [4,10]. Like the flow cytometer

Table 1  
Potential Interferences with reticulocyte counts

Interfering factor	Counting method affected
<b>DNA/RNA</b>	
Howell-Jolly bodies	Most automated machines
Nucleated red blood cells	Most automated machines
Basophilic stippling	Manual, most automated machines
Parasites	Manual, most automated machines
<i>Mycoplasma haemofelis</i> and <i>M. haemocanis</i>	
<i>Babesia</i> sp	
Large immature platelets or platelet clumps	Manual, most automated machines
Leukocytes (uncommon) or leukocyte fragments	Most automated machines
<b>Miscellaneous</b>	
Heinz bodies	Manual, most automated machines
Pappenheimer bodies	Most automated machines
Autofluorescence (porphyrin, drugs)	Most automated machines
Paraproteins/cold agglutins	Most automated machines

Data from Refs. [3,4,9,10].

enumeration system, these hematology analyzers detect the DNA/RNA of parasites, nucleated RBCs, Howell-Jolly bodies, and other inclusions with their supravital dyes [10,11]. These reticulocyte methods are more expensive, require advanced training to perform and interpret, and may not be accurate in distinguishing punctate from aggregate reticulocytes in cats.

The Mascot Hemavet 3500 (Hemavet; CDC Technologies, Oxford, CT), another veterinary instrument, identifies reticulocytes with a “focused flow” system [12]. The automated reticulocyte count is determined by classifying cells by their size and intracellular complexity as reticulocytes. Although this machine has shown good correlation with manual counts, any artifacts or anomalies may result in inaccurate counts. For example, other causes of macrocytosis (eg, artifactual swelling of erythrocytes in EDTA, breed-associated macrocytosis) may result in false high reticulocyte readings [12].

More sophisticated instruments, such as the Cell-Dyn 4000 (Abbott Diagnostics) hematology analyzer, which has an argon laser light source and focused flow impedance, operate with a fluorescence dye (CD4K530) for detection of RNA similar to conventional flow cytometers. In human beings, this instrument can separate the most immature reticulocytes from the nucleated RBCs and white blood cells (WBCs), gives reticulocyte quantitative maturational data, and has shown great correlation with the manual counts. The staining is not specific for RNA, however, because cellular inclusions of DNA also stain [4,8,11].

Other techniques used in human medicine include flow cytometry with fluorochrome-labeled monoclonal antibodies specific for several different reticulocyte antigens, including CD71 (transferrin receptor) and CD36 (gpIV, gpIIIb), as well as density gradient fractionation [4]. In addition, image processing systems (automated counting devices for NMB-stained blood smears) are being developed but are currently unavailable in the United States [3,4].

Regardless of how reticulocyte percent is generated (manual or automated techniques), an absolute reticulocyte count must be calculated. The absolute aggregate reticulocyte count, a good indicator of regeneration in the dog and cat, is calculated by multiplying the RBC count in millions by the percent of reticulocytes generated manually or by the machine. The reticulocyte response should be greater as the hematocrit drops if the bone marrow is responding properly. Therefore, correcting the reticulocyte percent for packed cell volume (PCV) is also helpful for the clinical interpretation of results. The reticulocyte percent is corrected with the following formula:

$$\text{Corrected Reticulocyte \% (CRP)} = \text{Observed Reticulocyte \%} \times \frac{\text{PCV}_{\text{patient}}}{\text{PCV}_{\text{normal}}}$$

The mean normal PCV used for the dog is 45%, and that for the cat is 37%; however, if the patient’s own normal PCV is known or if the breed has a different mean PCV (ie, Greyhounds) [13], these values are preferable to the species mean values. A corrected reticulocyte percent above 1 in the dog

and 0.4 in the cat is a “bare minimum” to indicate active erythropoiesis. Conversely, an absolute count greater than 60,000 cells/ $\mu$ L in the dog and greater than 42,000 cells/ $\mu$ L in the cat suggests a regenerative response to the anemia. The greater the corrected percent or absolute number of reticulocytes, the better is the regenerative response. Only aggregate reticulocytes are used in the dog and cat to determine degree of regeneration [1,2,7,12]. Table 2 summarizes the percent of reticulocytes expected for the degree of anemia and subsequent bone marrow stimulation.

In the dog, the RPI may be calculated to correct for premature release of reticulocytes and prolonged maturation in peripheral blood. The RPI, which is based on human maturation of reticulocytes, is calculated as follows:

$$\text{RPI} = \text{CRP} / \text{Reticulocyte Maturation Time}$$

The following maturation times are used:

- 1 day for PCV of 45
- 1.5 days for PCV of 35
- 2 days for PCV of 25
- 2.5 days for PCV of 15

An RPI less than or equal to 1 indicates a nonresponsive anemia, an RPI between 1 and 2 indicates some response to anemia with an active marrow, an RPI greater than 2 suggests accelerated erythropoiesis, and an RPI greater than or equal to 3 reflects significant response like that seen with hemolysis. Because reticulocyte lifespans in blood with increasingly severe anemia have not been well determined in cats, the RPI is typically not used in this species [1,2,12].

### **Clinical utility of reticulocyte enumeration**

Reticulocytosis refers to an increase in the number of circulating reticulocytes and is a reflection of the erythropoietic activity of the bone marrow. It normally occurs in response to elevated erythropoietin levels in dogs and cats that have functional bone marrow. Listed in Table 3 are differentials for two broad categories that may be associated with reticulocytosis and regenerative anemia: hemolysis and blood loss [1,2,14,15]. As mentioned previously, this regenerative response may not become evident in circulation for 2 to 5 days. Before regeneration is seen peripherally, the anemia appears nonregenerative. Typically, a greater regenerative response occurs in hemolytic anemias than in external blood loss anemias, because the iron and protein of the destroyed RBCs are readily available for erythropoiesis. For the same reason, internal blood loss may stimulate a stronger regenerative response than external blood loss as breakdown products and erythrocytes are efficiently recycled [1,12].

Table 2  
Evaluation of reticulocyte response

Method	Canine	Feline
Percent reticulocytes		
Normal <sup>a</sup>	0–1.5	0–0.4
Slight	1.5–4	0.5–2
Moderate	5–20	3–4
Marked	21–50	5+
Absolute reticulocyte count (aggregate)		
Normal <sup>a</sup>	<60,000–80,000 (<1%)	<15,000–42,000 (0%–0.4%)
Slight	80,000–150,000	42,000–70,000
Moderate	150,000–300,000	70,000–100,000
Marked	> 500,000	> 200,000
Absolute reticulocyte count (punctate)		
Normal <sup>a</sup>	N/A	<200,000 (0%–10.8%)
Slight	N/A	200,000–500,000
Moderate	N/A	500,000–1,000,000
Marked	N/A	1,000,000–1,500,000
Corrected reticulocyte percent (CRP)		
Normal <sup>a</sup>	≤1	≤0.4
Regenerative	> 1	> 0.4
Reticulocyte production index (RPI)		
Normal <sup>a</sup>	<1	N/A
Slight	1–2	N/A
Moderate	> 2	N/A
Marked <sup>b</sup>	≥3	N/A

Abbreviation: N/A, not applicable.

<sup>a</sup> Nonregenerative if anemic.

<sup>b</sup> Suggests hemolytic disease.

Data from Refs. [7,12,19].

In healthy dogs with an acute moderate to marked anemia and functional bone marrow, a strong regenerative response with aggregate reticulocytes is expected. Although punctate reticulocytes may be observed while performing a canine reticulocyte count, they are present in small insignificant numbers and are disregarded. In dogs, an absolute reticulocyte count of greater than 60,000 to 80,000 cells/ $\mu$ L or greater than 1% to 1.5% typically indicates regeneration [12,14,16]. The aggregate reticulocyte response peaks at days 4 to 7 and may return to normal by day 15 even though the anemia has not completely resolved. Consequently, it is possible for the anemia to appear nonregenerative, because the peak in reticulocytosis and peripheral regenerative response has already occurred. Once the PCV reaches 30% to 35%, the bone marrow may simply respond to this relatively mild anemia by releasing increased numbers of mature erythrocytes instead of reticulocytes. The hypoxia is too mild at this point to stimulate further erythropoietin synthesis [12]. Caution must be used when interpreting regenerative responses in puppies

Table 3  
Causes of regenerative anemia in veterinary medicine

Hemolysis	Blood loss
Chemical	Trauma
Oxidants (onions, acetaminophin, propylene glycol, vitamin K, garlic, moth balls, kale)	Hit by car
Drugs (cephalosporins)	Penetrating wounds
Zinc	Burns
Ricin	
Snake venom	
Copper toxicity	
Osmotic	Surgery
Water intoxication or hypotonic fluids	
Hypophosphatemia (hyperalimentionation, diabetes mellitus)	
Metabolic	Coagulopathies
Pyruvate kinase deficiency (Basenji, Beagle, West Highland White and Cairn Terriers, Miniature Poodle, Dachshund, Pug, Chihuahua, Abyssinian, Somali, DSH cats)	Factor deficiencies
Phosphofructokinase deficiency (English Springer and Cocker Spaniels, mixed breed)	Warfarin toxicity
Stomatocytosis (Alaskan Malamute)	Vitamin K deficiency
Osmotic fragility (Abyssinian and Somali cats)	Disseminated intravascular coagulation
Parasitic	Severe thrombocytopenia
RBC parasites ( <i>Babesia</i> sp, <i>Mycoplasma haemofelis</i> or <i>M haemocanis</i> , <i>Cytauxzoon</i> sp)	von Willebrand's disease
<i>Trypanosoma</i> sp	
Bacteria ( <i>Leptospira</i> sp, <i>Clostridium</i> sp)	
Immune mediated	Gastrointestinal ulceration
Primary	Parasitism
Autoimmune hemolytic anemia	Ancylostomiasis
Secondary	Extreme flea or tick infestation
Neonatal isoerythrolysis (rare)	
Transfusion reaction	
Infectious (RBC parasites, <i>Ehrlichia</i> sp)	
Drugs (penicillin, cephalosporins)	
Microvascular Disease	Tumors
Vena caval syndrome	Gastrointestinal
Disseminated intravascular coagulation	Hemangiosarcoma
Vasculitis	
Hemangiosarcoma	

Abbreviations: DSH, domestic shorthair; RBC, red blood cell.

Data from Refs. [2,12,14,15].

and probably in other young animals, because reticulocyte counts are high at birth (3%–4% in 6- to 8-week-old puppies) and decrease with age (up to 1% in adult dogs). Young animals may be less capable of responding to blood loss than adults because of physiologic iron deficiency [12,13,16].

Unlike canine reticulocytes, feline reticulocytes exhibit a continuum as they mature from the aggregate reticulocyte with a clumped reticulum network to the punctate reticulocyte with a faint stippling of reticulum [16]. As previously stated, the aggregate reticulocytes appear in circulation within 48 hours after blood loss and return to normal by 9 to 13 days [1,7,9]. Consequently, the aggregate reticulocyte count indicates a recent event and a current bone marrow stimulation or response. Conversely, punctate reticulocytes do not increase above the reference interval for 1 to 3 weeks; when increased, they indicate that the inciting event for anemia occurred 2 to 4 weeks earlier [1,7,9]. Table 4 depicts this response to anemia and the delayed appearance of punctate reticulocytes in peripheral blood. Because a morphologic continuum exists between aggregate and punctate reticulocytes, it is sometimes difficult to classify the cells manually as one or the other, which may lead to interobserver variation and inaccuracies in the reported reticulocyte count. When evaluating a Romanowsky-stained blood smear in either the dog or cat, it also is important to remember that polychromasia only reflects the aggregate reticulocyte numbers [12].

Although reticulocytosis is seen in many causes of anemia with functional bone marrow, reticulocytopenia occurs in dogs and cats with reduced or defective erythropoiesis [12]. Table 5 lists general causes of nonregenerative anemia. If the reticulocyte response is absent or inadequate for the degree of anemia, a bone marrow examination is warranted to determine the cause and to assess the degree of erythropoietic response. This procedure may be repeated to assess the response to therapy; however, evaluation of serial PCVs may provide the clinician with similar information. Aside from assessing the response of the animal to anemia and classifying the anemia, reticulocyte enumeration has other important applications. A reticulocyte count may be valuable when evaluating a patient receiving cytotoxic chemotherapeutic drugs, following a patient given iron for previously diagnosed iron-deficient anemia, or monitoring a response to recombinant human erythropoietin (rhEPO) given for chronic renal failure [4].

Table 4  
Reticulocyte response to experimental blood loss<sup>a</sup>

Response	Aggregate reticulocytes (dog and cat) <sup>b</sup>	Punctate reticulocytes (cat) <sup>b</sup>
Initial	2–5 days	4–11 days
Maximal	4–7 days	11–20 days
End <sup>c</sup>	10–14 days	25–30 days

<sup>a</sup> 30 mL/kg of blood removed.

<sup>b</sup> Days after blood loss.

<sup>c</sup> Packed cell volume (PCV) may still be below baseline; PCV should return to baseline in 2–4 weeks.

*Data from Refs. [7,19,20,21].*

Table 5  
Causes of nonregenerative anemia in veterinary medicine

Reduced erythropoiesis	Defective erythropoiesis
Erythropoietin deficiency	Disorders of nucleic acid synthesis
Chronic renal disease	Vitamin B <sub>12</sub> folic acid deficiency (Giant Schnauzers)
Endocrine	
Hypoadrenocorticism	
Hypothyroidism	
Hypopituitarism	
Hypoandrogenism	
Anemia of chronic disease	Disorders of globin synthesis (rare)
Chronic inflammation	
Neoplasia	
Liver disease	
Toxic microenvironment	Disorders of heme synthesis
Phenylbutazone	Iron deficiency
Trimethoprim-sulfa	Pyridoxine deficiency
Estrogen	Copper deficiency
Radiation	Lead poisoning
Cytotoxic cancer drugs	
Griseofulvin	
Chloramphenicol	
Bone marrow necrosis	
Immune mediated (early precursors)	
Myelophthisis	Abnormal maturation
Myeloproliferative disease	Leukemias
Lymphoproliferative disease	Myelodysplastic disorders
Metastatic neoplasia	Dyserythropoiesis
Myelofibrosis	
Infectious or granulomatous disease	
Infections	
Feline leukemia virus	
Feline panleukopenia virus	
Canine parvovirus	
<i>Ehrlichia</i> sp	

*Data from Refs. [2,12,14].*

Remember when interpreting reticulocyte responses to consider the patient's health status as well as the cause, degree, and duration of anemia. In clinical practice, anemias are more likely to be complex than straightforward, and animals may not show the classic response anticipated with one-time blood loss anemia in healthy animals. Therefore, when evaluating an animal's response to anemia, consider that multiple etiologies may be responsible for the anemia and that other disease conditions or therapies (eg, blood transfusions, oxyglobin therapy, chemotherapy) may alter the response. For example, chronic hemorrhage in an animal with neoplasia may initially present as a regenerative anemia. Over time, an iron-deficient and chronic disease component can develop, resulting in a nonregenerative state [17]. Sequential monitoring of PCV and reticulocytes is essential in

these patients, and bone marrow analysis is indicated when an appropriate response is not observed.

### **Clinical approach to diagnostic dilemmas**

Discrepancies between the red cell morphology (polychromasia) seen in a Romanowsky-stained slide and the manual or machine-generated reticulocyte count can occur. This is apparent if the reticulocyte percent does not closely agree with the semiquantitative value or percent of polychromasia. Appropriate steps to resolve this disagreement or “laboratory error” include the following:

1. Verify that the same and correct sample was used for the blood smear and reticulocyte count, and obtain a new sample if necessary.
2. Recheck the procedure for the reticulocyte count, and ensure that it was done properly.
3. Determine the method of the reticulocyte count (manual or automated). Rule out known interferences with the automated counts (see Table 1). For manual counts, especially samples having low PCVs, reticulocytes are often drawn to the feathered edge rather than being evenly distributed in the monolayer counting area.
4. Re-evaluate the Romanowsky-stained smear. Confirm that the smear is well-stained. If the stain quality was poor on the first slide, stain another slide using fresh reagents. Alternatively, use a different stain, such as a Diff-Quik–like stain or a quick Wright’s stain. Although a poorly stained slide could result in all red cells staining slightly bluish, the polychromatophilic cells that correspond to aggregate reticulocytes are larger bluish-red cells. If the red cells are larger but fully hemoglobinized, consider macrocytosis of poodles, B<sub>12</sub>/folic acid deficiency, or a sodium-potassium pump disorder.

In the absence of reticulocytosis or increased polychromatophils, moderate to marked anisocytosis, macrocytosis, Howell-Jolly bodies, nucleated RBCs (normoblastemia), and basophilic stippling cannot be interpreted as a regenerative response [1,2]. Splenic disease, bone marrow injury, lead poisoning, or myeloproliferative disease should be considered in these cases. Basophilic stippling without reticulocytosis may indicate dyserythropoiesis or lead toxicity in dogs and cats, and macrocytosis without polychromasia may result from feline leukemia virus (FeLV), an EDTA-induced artifact, or a breed-associated inheritance that occurs in Miniature Schnauzers and Poodles [1,2].

Sample timing can also cause some misinterpretations of an animal’s response or ability to respond to anemia. As previously discussed, the absolute number or corrected percent of reticulocytes is not increased immediately after blood loss or a hemolytic event. It may take several days

before a response is apparent in the peripheral blood. Further, the stimulus (hypoxia) may not be strong enough to promote continued synthesis of erythropoietin once the PCV has begun to normalize. A bone marrow evaluation in these instances would typically indicate erythroid hyperplasia [1,2].

### **Conclusion and future directions**

The number and characteristics of circulating aggregate reticulocytes indicate the regenerative capacity of the bone marrow and the animal's ability to respond to anemia. Enumeration is extremely important in assessing anemic patients. Differences of enumeration exist in dogs and cats, because only the aggregate reticulocytes are counted in dogs, whereas both the aggregate and punctate reticulocytes are reported in cats. In both species, however, the degree of anemia must be extreme enough to stimulate erythropoietin and the early release of reticulocytes. When anemia is acute (<2–5 days after onset), reticulocyte numbers will be within the reference range despite bone marrow erythroid hyperplasia. Similarly, anemia may appear nonregenerative late in the course of disease (>10–14 days after onset) even though the bone marrow is releasing increased numbers of mature erythrocytes. Therefore, knowing the duration of anemia is important when interpreting the animal's reticulocyte parameters. The response to anemia is also influenced by therapy and concurrent disease.

Veterinary diagnostic laboratories and hospitals are beginning to use automated techniques more commonly to evaluate anemia, although light microscopy remains the standard for reticulocyte counting. With both methods of enumeration, errors (including improper classification of reticulocytes and poor quality of the blood film) may result in false reticulocyte counts.

### **Summary**

Reticulocytes are anucleate immature red blood cells that contain a network of RNA, organelles, and mitochondria, which stain with supravital dyes. Both aggregate and punctate reticulocytes are present in domestic cats; only aggregate reticulocytes are used to assess the degree of regeneration in anemic dogs and cats. Multiple factors influence the degree of regenerative response to anemia. These factors include time of reticulocyte measurement, concurrent diseases, species, and ongoing therapy. Although many automated systems for reticulocyte enumeration exist, manual counts remain the gold standard in veterinary medicine.

## Data interpretation

### *Case 1: immune-mediated hemolytic anemia and thrombocytopenia in a cat*

An adult, castrated, male American domestic shorthair cat was presented for progressive lethargy and weight loss. The cat had previously been diagnosed with immune-mediated hemolytic anemia (IMHA) and thrombocytopenia. Treatment with prednisone and doxycycline was successful, but treatment was discontinued 4 months ago. Laboratory data are given in Table 6.

### *Interpretation*

Severe slightly regenerative anemia resolving within 6 weeks with prednisone therapy supports the previous diagnosis of IMHA. The anemia was slightly regenerative based on the percent and corrected aggregate reticulocyte percents. The absolute and percent aggregate reticulocytes on day 1 and the percent and absolute punctate reticulocytes on day 2 were slightly increased, indicating a response, but values suggested a poor or inadequate response for the degree of anemia. Sequential PCVs and reticulocyte counts were necessary to determine the adequacy of the response. Notice that the erythrogram was normal by day 46.

The polychromasia, anisocytosis, Howell-Jolly bodies, and nucleated RBCs are consistent with regenerative anemia. The MCV and macrocytosis, however, are disproportionately elevated for the degree of reticulocytosis. Although the RBC agglutination and large platelets may artifactually increase the MCV, they cannot account for the macrocytosis observed on the blood smear. The cause of the marked macrocytosis was not definitively determined, but it resolved by day 46. Considerations for the macrocytosis included FeLV (multiple FeLV tests were negative and the macrocytosis resolved), B<sub>12</sub>/folic acid deficiency (rare in animals and not tested for in this cat), and artifact of fluid shifts (no delay in sample processing and no serum hypo-osmolality; in fact, the glucose was slightly elevated at 170 mg/dL, and the blood sample on day 1 was overanticoagulated, both of which have a tendency to crenate the red cells). RBC membrane damage was the most likely possibility and was supported by evidence of slight hemoglobinemia and hemoglobinuria.

### *Case 2: immune-mediated hemolytic anemia in a dog*

A 7-year-old, castrated, male, terrier mix dog was presented to the local veterinarian with lethargy, depression, inappetence, and pale and slightly icteric mucous membranes. Three days after clinical signs started, the dog was transfused (10% pretransfusion PCV and 7.8 g/dL total solids, 24% posttransfusion PCV) and sent to a referral veterinary hospital. The dog responded slowly to treatment with prednisone, azathioprine, antibiotics,

famotidine, and heparin in addition to repeated oxyglobin therapy and a blood transfusion (day 6 at referral hospital). Laboratory data are given in Table 6.

### *Interpretation*

A developing regenerative anemia with a persistently low PCV, RBC agglutination, spherocytes, and icterus suggests ongoing hemolysis in this dog with IMHA. The two blood transfusions and oxyglobin therapy may have delayed the reticulocyte response. Peak reticulocyte response (percent and absolute) occurred on day 10. Ongoing hemolysis was suspected, because with this regenerative response, the PCV should have returned to the reference range by day 35. Notice that the reticulocyte response is more marked in this dog than in the cat in the first case. Dogs normally have a more pronounced reticulocyte response than do cats. The polychromasia and nucleated RBCs corresponded well with the reticulocyte response. Red cell agglutination, large platelets, and reticulocytes all contributed to the increased MCV. The macrocytes were disproportionately increased compared with the reticulocytes on days 23 and 35. The differentials for this macrocytosis are similar to those for the cat (ie, artifacts of fluid shifts, B<sub>12</sub>/folic acid deficiency). Oxyglobin therapy (days 3 and 7) invalidated the MCHC and the total plasma protein. Hypochromic indices are an inconsistent finding in regenerative anemias and were not associated with the reticulocytosis in either case 1 or case 2. Hemolysis, oxyglobin therapy, and a WBC count greater than 50,000/ $\mu$ L can erroneously elevate the MCHC and may have masked a decreased MCHC in this dog. Transfusions can result in a dimorphic population of red cells. The transfused red cells can become spherocytes as they age or are attacked by antibodies. The appearance of spherocytes on day 10 is likely the result of the transfusions. The WBC counts and differentials (not shown) indicated inflammation and stress, a not unexpected response to hemolytic anemia in the dog.

### *Case 3: iron deficiency anemia in a dog*

A 12-year-old, spayed, female Collie was presented for bilateral carpal arthrodesis of hyperextended carpi. Historically, the dog had been treated for a diffuse pruritic dermatitis. Laboratory data are listed in Table 6.

### *Interpretation*

The nonregenerative microcytic and hypochromic anemia with thrombocytosis was consistent with iron deficiency. An iron panel, including total iron of 27  $\mu$ g/dL (reference range: 33–147  $\mu$ g/dL), iron saturation of 8.9% (reference range: 20%–60%, with an average of 33%) [18], and total iron-binding capacity (TIBC) of 305  $\mu$ g/dL (reference range: 282–386  $\mu$ g/dL) also supported iron deficiency. A normal lead level eliminated lead as a cause of the basophilic stippling seen in this dog. The dog responded to iron therapy with an increased MCV and decreased platelets; however, the MCHC was

Table 6  
Data from interpretative cases 1, 2, and 3

Days from initial hospitalization	Case 1: cat with immune-mediated hemolytic anemia					Case 2: dog with immune-mediated hemolytic anemia					Case 3: dog with iron deficiency		
	Day 1	Day 2	Day 46	Cat reference range	Day 1	Day 3	Day 7	Day 10	Day 16	Day 23	Day 35	Day 79	Dog reference range
Percent aggregate reticulocytes (%)	1.93	1.77	ND	0–0.4	1.3	0.4	14.2	19.4	4.9	3.2	5.9	0.8	0–1.5
Absolute aggregate reticulocytes (/ $\mu$ L)	44,969	34,161	ND	$\leq$ 42,000	32,500	4880	214,420	292,940	97,510	61,760	182,310	30,400	ND
Percent punctate reticulocytes	6.56	17.83	ND	2–17	NA	NA	NA	NA	NA	NA	NA	NA	NA
Absolute punctate reticulocytes (/ $\mu$ L)	152,848	344,119	ND	<200,000	NA	NA	NA	NA	NA	NA	NA	NA	NA
Corrected reticulocyte percent (%)	0.68	0.57	ND	$\leq$ 0.4	0.5	0.07	4.1	6.0	2.2	1.3	3.4	0.4	<1
Reticulocyte production index (%)	NA	NA	NA	NA	0.25	0.04	1.6	2.4	1.1	0.65	1.7	0.2	<1
RBC count ( $10^6$ / $\mu$ L)	2.33	1.93	9.28	7.1–11.1	2.50	1.22	1.51	1.51	1.99	1.93	3.09	3.80	4.78–8.23
Packed cell volume (%)	13	12	38	28–45	17	8	13	14	20	18	26	23	33–58
Mean corpuscular volume (fL)	61.2	60.4	48.0	38.9–48.9	73.5	69.6	77.6	84.9	86.6	87.6	80.9	58.4	63.9–72.8

Mean	30.1	30.8	27.2	29.5–31.5	33.7	NA <sup>d</sup>	NA <sup>d</sup>	37.5	35.5	34.3	33.6	NA	32.3	33.6–36.4
corpuscular hemoglobin concentration (g/dL)														
Polychromasia	Mild	Mild	—	—	Mild	—	Moderate	Moderate	Mild	—	Mild	—	—	—
Anisocytosis <sup>a</sup>	Moderate	Moderate	Mild	—	Marked	—	Marked	Moderate	Moderate	Moderate	Mild	Mild	Mild	—
Howell-Jolly bodies	Rare	Rare	—	Rare	—	Few <sup>e</sup>	Few <sup>e</sup>	Rare	Occasional	Few <sup>e</sup>	—	Rare <sup>e</sup>	—	—
Macrocytes	Marked	Marked	—	—	Moderate	—	Moderate	Moderate	Moderate	Many	Many	Occasional	—	—
Microcytes	—	—	—	—	—	—	—	—	Few	—	—	—	Rare	—
Hypochromia	—	—	—	—	—	—	—	—	Mild	—	—	Moderate	Mild	—
Nucleated RBC/100 WBC's	12	10	0	Rare	10	20	20	23	4	5	3	—	—	—
Spherocytes	Present	Present	—	—	Present	Present	Present	Moderate	Few	Occasional	—	—	—	—
RBC agglutination	—	—	—	—	Present	Present	Present	Present	Present	Present	Present	—	—	—
Platelet count (10 <sup>3</sup> /μL)	69	57	Appears adequate	300–800	223	NA <sup>f,g</sup>	NA <sup>f,g</sup>	196	259	NA <sup>f,h</sup>	1054	743	392	181–350
Platelet Clumps <sup>b</sup>	—	—	Occasional	—	—	—	—	—	—	—	Occasional	Many	—	—
Large platelets	Many	Many	Moderate	Rare	Many	Moderate	Many	Many	Few	Many	Many	—	Occasional	—
WBC count (10 <sup>3</sup> /μL) <sup>c</sup>	13.8	10.0	14.2	6.4–15.8	26.2 <sup>i</sup>	45.9 <sup>i</sup>	77.8 <sup>i</sup>	72.9 <sup>i</sup>	38.1 <sup>i</sup>	27.5 <sup>i</sup>	36.5 <sup>i</sup>	7.4	13.6	6.4–15.8
Plasma protein	7.8	6.9	7.9	6.5–8.4	8.4	NA <sup>d</sup>	NA <sup>d</sup>	7.0	6.5 <sup>j</sup>	7.4 <sup>j</sup>	7.8	NA	6.2	6.1–7.5

Abbreviations: NA, not applicable; ND, not done; RBC, red blood cell; WBC, white blood cell.

<sup>a</sup> Above normal for the cat.

<sup>b</sup> Frequently observed in cats especially with difficult venipunctures.

<sup>c</sup> WBC's corrected for nucleated RBCs.

<sup>d</sup> Invalid because of presence of oxyglobin.

<sup>e</sup> Rare basophilic stippling seen.

<sup>f</sup> Platelet count cannot be determined by automated method because of large platelets.

<sup>g</sup> Increased estimated platelet numbers.

<sup>h</sup> Normal estimated platelet numbers.

<sup>i</sup> Leukogram revealed inflammation and stress characterized by neutrophilia, left shift, lymphopenia and monocytosis (Data not shown).

<sup>j</sup> Icteric plasma.

low and microcytic hypochromic red cells were still observed on the smear on day 79. The cause of the anemia was not determined, but chronic blood loss was suspected. Anemia of chronic inflammatory disease may have been a contributing factor, but the response to iron therapy and the low total protein are not expected with chronic inflammatory disease. Ferritin levels would also be helpful in differentiating iron deficiency (low ferritin levels, low serum iron, low iron saturation, and normal to increased TIBC) from chronic disease (normal ferritin levels, low serum iron, normal to increased saturation, and normal to decreased TIBC).

*Case 4: discrepancy between polychromasia and reticulocyte percent in a severely anemic dog*

An adult dog collapsed and was presented on an emergency basis. The mucous membranes were pale, and the spleen was palpably enlarged. The PCV was 8%, marked polychromasia was observed on the blood smear, and the reticulocyte count was 0%.

*Interpretation and resolution*

The reticulocyte percent and the degree (or percent) of polychromasia should always closely agree. Disagreement indicates an error. Laboratory samples, results, and procedures should be verified and reanalyzed if necessary. The blood samples for the complete blood cell count (CBC) and manual reticulocyte count were verified to be the correct samples. Polychromasia was confirmed on the Wright's Giemsa blood smear, but the NMB smear (PCV of 4%) had an uneven cellular (reticulocyte) distribution. All the reticulocytes were at the feathered edge rather than in the monolayer area. Unfortunately, the clinician saw the preliminary laboratory data and performed a bone marrow aspiration biopsy before the official laboratory report with the correct data was released. The bone marrow biopsy did confirm a marked erythroid hyperplasia consistent with the polychromasia and the reticulocytes at the feathered edge. This case reinforces the importance of scanning the whole blood smear at low power before proceeding to the monolayer area to perform the reticulocyte enumeration.

*Case 5: nonregenerative anemia in a dog with blood loss*

A young adult dog (approximate weight of 50 lb) donated a unit of blood (450 mL). The dog developed a mild transient anemia without a reticulocyte response.

*Interpretation*

The blood loss was insufficient to induce a reticulocyte response. A regenerative response would be expected if the dog had lost sufficient blood

to become hypoxic. Similarly, low-grade chronic blood loss (eg, gastrointestinal bleeding, tumor bleeding, hematuria) often does not stimulate a regenerative response. In experimental studies, about one third or more of the healthy animal's blood volume is removed to induce a regenerative reticulocyte response [7,19–21].

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