Diagnostic performances of manual and automated reticulocyte parameters in anemic cats

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Keywords:	anemia, diagnostic accuracy, feline, reticulocyte number, reticulocyte percentage, reticulocyte production index
Abstract:	Objectives: To evaluate the diagnostic performances of manual and instrumental measurement of reticulocyte percentage (Ret%), reticulocyte number (Ret#), and reticulocyte production index (RPI) to differentiate regenerative anemia (RA) from non regenerative anemia (NRA) in cats. Methods: Data from 106 blood samples from anemic cats with manual counts (n=74; 68 NRA, 6 RA) or instrumental counts of reticulocytes (n=32; 25 NRA, 7 RA) collected between 1995 and 2013 were retrospectively analyzed. Sensitivity, specificity, and positive likelihood ratio (LR+) were calculated using either cut-offs reported in literature or cut-offs determined from receiver operating characteristic (ROC) curves. Results: All the reticulocyte parameters were significantly higher in cats with RA than in cats with NRA. All the ROC curves were significantly different ($P<0.001$) from the line of no discrimination, without significant differences between the three parameters. Using the cut-offs published in literature, the Ret% (cut-off: 0.5%) was sensitive (100%) but not specific (<75%), the RPI (cut-off: 1.0) was specific (>92%) but not sensitive (<15%), and the Ret# (cut-off: 50 x 10^3/μL) had sensitivity and specificity >80% and the highest LR+ (manual count: 14; instrumental count: 6). For all the parameters, sensitivity and specificity approached 100% using the cut-offs determined by the ROC curves. These cut-offs were higher than those reported in the literature for Ret% (manual: 1.70%; instrumental: 3.06%), lower for RPI (manual: 0.39; instrumental: 0.59), and variably different, depending on the method (manual: 41 x 10^3/μL; instrumental: 57 x 10^3/μL) for Ret#. Using these cut-offs, the RPI had the highest LR+ (manual: 22.7; instrumental: 12.5). Conclusions and Relevance This study indicated that all the reticulocyte parameters may confirm regeneration when the pre-test probability is high, while when this probability is moderate, RA should be identified using the RPI providing that cut-offs lower than 1.0 are used.

Diagnostic performances of manual and automated reticulocyte parameters in anemic cats

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Anemia; Diagnostic accuracy; Feline; Reticulocyte number; Reticulocyte percentage; Reticulocyte production index;

Abstract

Objectives: To evaluate the diagnostic performances of manual and instrumental measurement of reticulocyte percentage (Ret%), reticulocyte number (Ret#), and reticulocyte production index (RPI) to differentiate regenerative anemia (RA) from non regenerative anemia (NRA) in cats.

Methods: Data from 106 blood samples from anemic cats with manual counts (n=74; 68 NRA, 6 RA) or instrumental counts of reticulocytes (n=32; 25 NRA, 7 RA) collected between 1995 and 2013 were retrospectively analyzed. Sensitivity, specificity, and positive likelihood ratio (LR+) were calculated using either cut-offs reported in literature or cut-offs determined from receiver operating characteristic (ROC) curves.

Results: All the reticulocyte parameters were significantly higher in cats with RA than in cats with NRA. All the ROC curves were significantly different ($P<0.001$) from the line of no discrimination, without significant differences between the three parameters. Using the cut-offs published in literature, the Ret% (cut-off: 0.5%) was sensitive (100%) but not specific (<75%), the RPI (cut-off: 1.0) was specific (>92%) but not sensitive (<15%), and the Ret# (cut-off: 50 x $10^3/\mu L$) had sensitivity and specificity >80% and the highest LR+ (manual count: 14; instrumental count: 6). For all the parameters, sensitivity and specificity approached 100% using the cut-offs determined by the ROC curves. These cut-offs were

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higher than those reported in the literature for Ret% (manual: 1.70%; instrumental: 3.06%), lower for RPI (manual: 0.39; instrumental: 0.59), and variably different, depending on the method (manual: 41 x 10^3/µL; instrumental: 57 x 10^3/µL) for Ret#. Using these cut-offs, the RPI had the highest LR+ (manual: 22.7; instrumental: 12.5).

Conclusions and Relevance This study indicated that all the reticulocyte parameters may confirm regeneration when the pre-test probability is high, while when this probability is moderate, RA should be identified using the RPI providing that cut-offs lower than 1.0 are used.

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Introduction

The differentiation between regenerative anemia (RA) and non regenerative anemia (NRA) may drive further diagnostic or therapeutic procedures.[1] The identification of RA relies on the quantification of reticulocyte responses. To this aim, the current literature recommends to use the absolute number of reticulocytes (Ret#) rather than the reticulocyte percentage (Ret%) or the reticulocyte production index (RPI).[2,3] However, the cut-offs reported in the literature for feline Ret# are variable (e.g. 40-60 x $10^3/\mu L$) and have been determined using different methods, including laser-based counters, that have a higher analytical sensitivity and provide higher Ret# compared with manual counts.[2,4-8] Moreover, the Ret# and the Ret% are higher in some feline breeds than in others (the Ret% may be as high as 0.8% in Norwegian Forest cats, 1.2% in Holy Birman cats, 1.9% in Siberian cats and 3.3% in Maine Coon cats, and the Ret# may be as high as 250.00 in Main Coon cats)[9,10] Additionally, the magnitude of reticulocytosis should inversely correlate with the severity of anemia.[2,11] In human medicine the RPI has been proposed as a tool to correct the Ret% for the severity of anemia.[3] The calculation of RPI is based on the maturation time of human circulating reticulocytes, that is higher in RA, when reticulocytes released in blood are younger than in healthy individuals.[3] The maturation time of feline reticulocyte is unknown but it is likely different from other species, since feline erythroid cells have some peculiarities such as a

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shorter erythrocyte lifespan, a prolonged maturation time of punctate reticulocytes and a weaker maximal response of aggregate reticulocytes.[12] Independent of the correctness of the formula we recently demonstrated that the Ret% and the RPI may be used to diagnose canine RA.[13] However, no studies on the utility of the three reticulocyte parameters in cats are available.

Hence, this study was aimed to assess the diagnostic performances of Ret%, Ret# and RPI counted manually or by a laser-based analyzer, for the diagnosis of RA in cats.

Materials and methods

Case selection criteria

The laboratory information system was analyzed to retrieve data recorded between January 1995 and January 2013 from anemic cats. The database included data generated using an impedance counter (SEAC Hemat 8) followed by reticulocyte counts on brilliant cresyl blue stained smears, or using a laser counter validated in cats (Sysmex XT2000-iV).[14,15] Only aggregate reticulocytes were counted manually, since punctate reticulocytes do not indicate active or recent regeneration in cats.[12] Moreover, a moderate to high correlation between aggregate reticulocyte counts and automated reticulocyte counts with Sysmex has previously been reported.[14,15]

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The inclusion criteria were the following: presence of anemia based on the comparison with the reference intervals in use at our institution for cats (RBC <5.0 x 10^3/µL, Ht <27%, Hb <10 g/dL); availability of manual or instrumental reticulocyte counts; availability of stored glass slides to review the original classification; diagnosis of RA or NRA based on history, diagnostic tests (necropsy, cytology; serum biochemistry, bone marrow cytology; serology/PCR for infectious diseases) or follow up (recovery within one month for RA; no improvement during one year follow up for NRA). Post-hemorrhagic acute anemia was classified in the RA group when sampling were done at least 5 days after the hemorrhage, or within the NRA group when sampling were done 1-2 days after the hemorrhage (pre-regenerative phase of acute anemia).

Samples from cats treated with drugs that influence bone marrow activity, or belonging to breeds known to have high reticulocyte counts[9,10] were excluded from the study.

Calculation of reticulocyte parameters

Based on RBC numbers and on the Ret% generated by manual or instrumental counts, the following parameters were calculated using the formula reported in literature:[3]

- Ret# = number of erythrocytes x (Ret%/100)
- Corrected Ret% = Ret% x (Hct/37)

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Comment [a21]: Removed: Independent of the time of recovery.

Comment [a22]: Removed: hemorrhages occurred at least 5 days before sampling

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- RPI = corrected Ret% / [(-0.05) × Hct + 3.25]

Fact that the maturation time of feline reticulocytes is unknown, the RPI was calculated using the formula used in people,[3] assuming that also in cats the maturation time is longer if younger reticulocytes are released in blood. Moreover, As a further support to this assumption, the application of this formula in dogs, provided useful information for patient's management.[13]

Statistical analysis

Data from manual and instrumental counts were analyzed separately. The Ret%, Ret# and RPI for each group (RA, NRA) were compared with the Friedman test with the Bonferroni correction using a commercial software (Analyse-it Ltd).

For each parameter, the number of true positives and false negative (samples with RA with values higher and lower, respectively, than each operating point), true negative and false positive (samples with NRA with values lower and higher, respectively, than each operating point),were calculated.[16] Sensitivity, specificity and positive likelihood ratio (LR+) were calculated using standard formulae[17] either at the cut-offs determined with the receiver operating characteristic (ROC) curves, or using the upper reference limits reported in the literature (Ret% = 0.5%; Ret# = 50 x 10^3/µL; RPI = 1.0).[2] ROC curves were designed

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Comment [a27]: Removed: The rationale of this approach was that also in cats the maturation time should be longer if younger reticulocytes are released in blood.

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Comment [a30]: Removed: n dogs it has been demonstrated that this calculation may be useful also if the maturation time is different

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Comment [a32]: Removed: In routine practice, the gold standard test to confirm regeneration (bone marrow cytology) is not recommended if regeneration is suspected.[2] Hence, cats were classified as affected by RA or NRA based on history, clinical and laboratory findings suggestive of diseases associated with RA or NRA, and on the restoration (or not) of the RBC mass during the follow up.

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to determine the ability of each parameter to identify cats with RA and to identify which cut-off best differentiates RA from NRA.[18] The level of significance in all the statistics above was set at $P<0.05$

Results

Composition of groups

The retrospective search in the database according to the selection criteria (figure 1) allowed us to include 106 cases in the study (table 1). The RA group (table 1) included cats with hemoplasma infection or with acute hemorrhage occurred at least 5 days before sampling. The NRA group included either recent hemorrhage or infectious, metabolic or neoplastic conditions that depress the bone marrow activity.

Reticulocyte parameters

With both methods, results for all the reticulocyte parameters were significantly higher in cats with RA than in cats with NRA (figure 2). In cats with RA, the Ret%, Ret# and RPI were higher than the upper reference limit reported in literature[2] in 6/6, 4/6 and 0/6 cases after manual counts and in 6/6, 0/7 and 6/7 cases after instrumental count. In the NRA

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group, the Ret%, Ret# and RPI were abnormal in 13/68, 4/68 and 0/68 cats after manual counts and in 7/25, 4/25 cats and 2/68 cats after instrumental counts.

Hence, using both methods, the Ret% was more sensitive than specific, the RPI was very specific but not sensitive, and the Ret# had sensitivity and specificity >80.0% and the highest LR+ (table 2). However, for all the parameters, sensitivity and specificity approached 100.0% if cut-offs determined by the ROC curves were used. These cut-offs were higher than those reported in the literature for Ret%, lower for RPI, and variably different, depending on the method used to enumerate the reticulocytes, for Ret#. Using these cut-offs, the RPI had the highest LR+ using both methods. However, the discriminating power of all the parameters, as defined by the ROC curves (figure 3) was always close to 100% and significantly higher (P<0.001) than the line of no discrimination, without significant differences between the three parameters.

Discussion

This study indicated that all the reticulocyte parameters may identify cats with regenerative anemia. Moreover, the different counting methods provided similar results, contrary to what occurred in dogs.[13] This was likely due to the fact that instrumental counts mostly detect aggregate reticulocytes[15] (i.e the only cells that are counted manually in cats) and not

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punctate reticulocytes that are included in manual counts in dogs. However, all the reticulocyte parameters had better performances if cut-offs different from those reported in the literature are used. This is particularly true for the RPI, that corrects the magnitude of reticulocytosis for the severity of anemia.[2,3] The RPI is based on the maturation time of human reticulocytes, which is likely different than in cats, due to the peculiarities of feline erythroid cells.[12] However, it is very likely that also in cats the maturation times of circulating reticulocytes increases if these cells are released earlier than in normal conditions. Hence, we assessed whether RPI, as well as the other reticulocyte parameters, may provide useful diagnostic information at the cut-off reported in the literature or at a different cut-off, identified through a ROC curve analysis. This approach revealed that the cut-off reported in the literature for humans is very specific but not sensitive. This is not surprisingly, based on the peculiarities of feline reticulocytes described in the introduction.[12] Hence, further studies on the maturation time of feline reticulocytes are needed in order to increase the accuracy of the formula used to calculate the RPI in cats. However, using lower cut-offs, the RPI had the highest LR+. A similar finding was recorded in dogs,[13] which also have different maturation times compared with humans. Although the ROC curves did not detect significant differences between the discriminating power of the three parameters, the RPI, at cut-offs lower than those of humans, is preferred

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Comment [a51]: Removed: and confirms that the formula for calculating the feline RPI should be likely revised when precise information on the maturation time of these cells will be determined.

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175 if the pre-test probability of regeneration is unknown, since in this case a test with high
176 LR+ increases the post-test probability of disease.[16,18] Conversely, if the pre-test probability
177 of regeneration is high (evident blood loss or hemolysis) a test with high specificity, that
178 avoids false positive results, may be appropriate as a confirmatory test. Based on our
179 results, any reticulocyte parameter, except the Ret% at the cut-off reported in literature,
180 may play this confirmatory role.
181 The main limitation of this study is the low number of cats, especially in the RA group.
182 However, the performance of each parameter was expressed in terms of LR+, that,
183 differently from predictive values, is not affected the prevalence of the diseases,[18] thus
184 minimizing the effect of the low number of cats with RA. The prevalence of RA in our
185 caseload ranged from 8% (manual counts) to approximately 20% (instrumental counts).
186 This is in agreement with the opinion that hemolytic anemia is uncommon in cats,[19]
187 although recent reports suggest that hematologic patterns consistent with RA or pathogenic
188 mechanisms responsible for RA are present in more than 40% of anemic cats.[8,20] However,
189 the latter studies were based on cut-offs that, according to the current study are poorly
190 specific and may have overestimated RA. The low prevalence of RA in our study, and the
191 lack of cases of immune-mediated hemolytic anemia, were likely due to the application of
192 strict exclusion criteria, which excluded the cases with unknown etiology or biased by

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multiple pathogenic mechanisms or by inaccurate classification of RA. This is important since the bone marrow cytology, that is considered the most accurate marker to assess the presence of regeneration is not recommended in routine practice when blood loss or hemolysis are suspected based on clinical or hematological findings.[2] Hence, cats were classified based on the final diagnosis and on the restoration of the RBC mass during the follow up, as in a similar study in dogs.[13] To this aim, we established a long time limit to assess whether restoration of the RBC mass occurred or not (one year), to not misclassify cases of regenerative anemia in the NRA group. Independent of the time of recovery, acute post-hemorragic anemia was included in the NRA group when samples were obtained in the first days after the hemorrhage, since during pre-regenerative anemia reticulocytosis is not fully detectable in peripheral blood. Conversely, in the similar study in dogs,[13] the ability of reticulocyte parameters to detect regeneration was assessed either including pre-regenerative anemia in the NRA group, to detect full regeneration, or in the RA group, to detect early regeneration. However, in the current study this approach was hampered by the low number of cases with pre-regenerative anemia. Future studies including higher number of cases and especially a higher number of cats with of pre-regenerative anemia are needed to confirm that Ret#, Ret% or RPI may be early indicators of regeneration in cats.

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Comment [a61]: Removed: The only exception to this rule regarded post-hemorrhagic anemia, that was included in the NRA group when sampling were performed in the first days after the hemorrhagic event

Comment [a62]: Removed: It would be interesting, in the future, to extend this study to a higher number of cases of pre-regenerative anemia in order to assess if Ret#, Ret% or RPI may be early indicators of regeneration as it was the case in dogs.

In conclusion, this study indicated that all the reticulocyte parameters confirm regeneration when the pre-test probability is high, while when this probability is moderate, RA should be identified using the RPI at cut-offs lower than 1.0.

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Comment [a64]: Removed: However, further studies on a higher caseload should be encouraged to support the results of the current study.

Acknowledgment

Part of this study was presented at the 15th Conference of the European Society of Veterinary Clinical Pathology and the European College of Veterinary Clinical Pathology, Berlin, Germany, October 2013.

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Conflict of interest

The authors do not have any potential conflicts of interest to declare

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279 **Figure legends**

280 **Figure 1**. Flow diagram illustrating the selection procedure employed in this study.

281

282 **Figure 2**. Distribution of Ret%, Ret# and RPI in cats with regenerative anemia (RA) and
283 with non-regenerative anemia (NRA), after manual (row A) or automated counts (row B).
284 The boxes indicate the I-III interquartile range (IQR), the horizontal line the median value,
285 and the whiskers extend to further observation within quartile 1 minus 1.5 x IQR or to
286 further observation within quartile 3 plus 1.5 x IQR. The grey area represents the reference
287 interval of the laboratory. For each parameter and method, results of the RA group were
288 significantly higher ($P < 0.001$) than results of the NRA group.

289

290 **Figure 3**. Comparison of ROC curves of the Ret% (gray circle), Ret# (black circle), and
291 RPI (open circle) obtained after manual count (A) or instrumental count (B). The gray line
292 indicates the line of no discrimination.

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293 Table 1: final diagnosis in the 106 cats with regenerative anemia (RA) or non regenerative
294 anemia (NRA) included in this study

		Number	Breed		Condition	
Manual counts	RA	6	Domestic Shorthair	3	Hemorrhage	3
			Persian	2	*Mycoplasma hemofelis*	3
			Exotic Shorthair	1		
	NRA	68	Domestic Shorthair	56	Tumors	20
			Persian	8	FIP	19
			Abyssinian	2	CKD	14
			Siamese	2	FIV/FeLV	9
					Inflammation	4
					Recent hemorrhage	2
Instrumental counts	RA	7	Domestic Shorthair	4	Hemorrhage	3
			Persian	2	*Mycoplasma*	4

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					hemofelis	
			Scottish Fold	1		
	NRA	25	Domestic Shorthair	14	Tumors	9
			Abyssinian	3	FIP	6
			Persian	3	CKD	5
			Siamese	2	FIV/FeLV	3
			Chartreux	1	Inflammation	1
			Devon Rex	1	Recent hemorrhage	1
			Exotic shorthair	1		

295 Table 2. Sensitivity (Sens), specificity (Spec), and positive likelihood ratio (LR+) of Ret% and Ret#
296 using pre-determined cut-offs (i.e. the cut-off corresponding to the upper reference limit reported in
297 literature for humans.[2]) or using the cut-offs determined by the ROC curve
298

		Manual counting (n=74)				Automated counting (n=32)			
		cut-off	Sens %	Spec %	LR+	cut-off	Sens %	Spec %	LR+
Pre-defined cut-off	Ret%	0.5	100.0 (54.1/100.0)	73.5 (61.4/83.5)	3.78	0.5	100.0 (59.0/100.0)	72.0 (50.6/87.9)	3.57
	Ret#	50.0	83.3 (36.9/99.6)	94.1 (85.6/98.4)	14.17	50.0	100.0 (59.0/100.0)	84.0 (63.9/85.5)	6.25
	RPI	1.00	0.0 (0.0/45.9)	98.5 (92.1/100.0)	0.00	1.00	14.3 (0.04-57.9)	92.0 (74.0/99.0)	1.79
Cut-off determined	Ret%	1.7	100.0 (54.1/100.0)	94.1 (85.6/98.4)	17.00	3.06	100.0 (59.0/100.0)	88.8 (68.8/97.5)	8.33

by the ROC curve	Ret#	41.4	100.0 (54.1/100.0)	92.6 (83.7/97.6)	13.60	57.7	100.0 (59.0/100.0)	88.8 (68.8/97.5)	8.33
	RPI	0.35	100.0 (54.1/100.0)	95.6 (87.6/99.1)	22.67	0.59	100.0 (59.0/100.0)	92.0 (74.0/99.0)	12.50

AUC = area under the ROC curve. Values in parentheses are 95% confidence intervals.

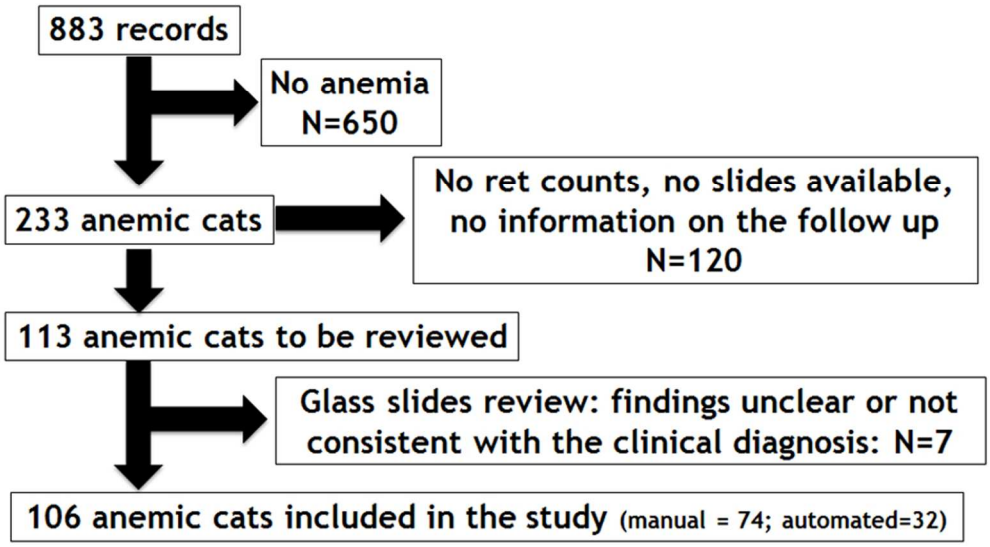

Figure 1. Flow diagram illustrating the selection procedure employed in this study.

80x44mm (300 x 300 DPI)

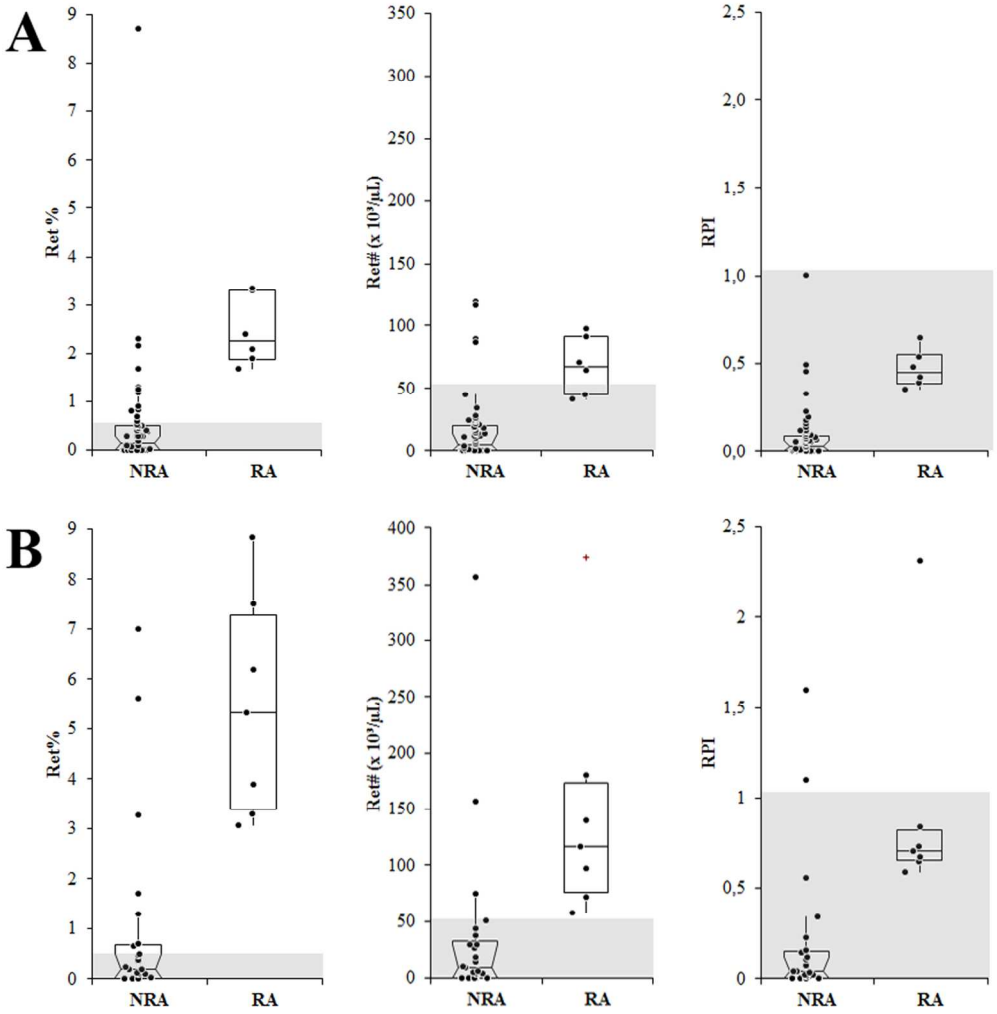

Figure 2. Distribution of Ret%, Ret# and RPI in cats with regenerative anemia (RA) and with non-regenerative anemia (NRA), after manual (row A) or automated counts (row B). The boxes indicate the I-III interquartile range (IQR), the horizontal line the median value, and the whiskers extend to further observation within quartile 1 minus 1.5 x IQR or to further observation within quartile 3 plus 1.5 x IQR. The grey area represents the reference interval of the laboratory. For each parameter and method, results of the RA group were significantly higher ($P < 0.001$) than results of the NRA group.

119x124mm (300 x 300 DPI)

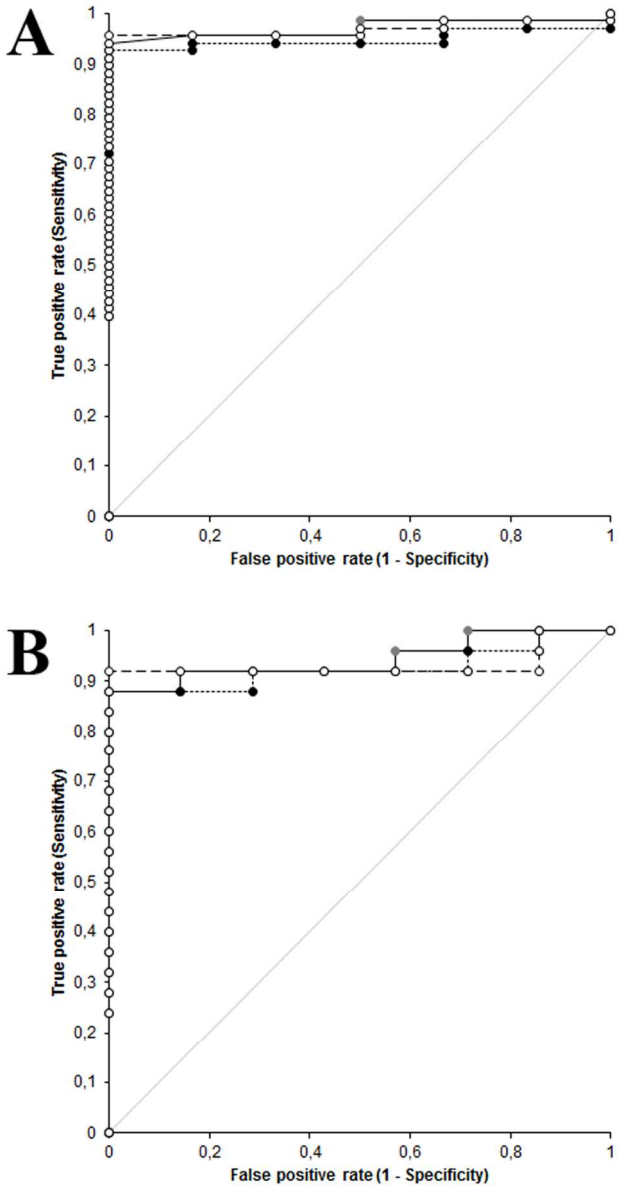

Figure 3. Comparison of ROC curves of the Ret% (gray circle), Ret# (black circle), and RPI (open circle) obtained after manual count (A) or instrumental count (B). The gray line indicates the line of no discrimination.

80x145mm (300 x 300 DPI)