QA AND QC IN ASSAY DEVELOPMENT

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Introduction

Over the past 10 -15 years there has been marked growth in the development of diagnostic tests. This is partly due to the rapid progress made in instrumentation development, bioinformatics and concerns about costs of, and access to, tests and their results. In both human and veterinary laboratory medicine this has raised issues of regulation of the development, implementation and continued quality monitoring of such tests. Major concern has been with rapid development of tests for infectious diseases, molecular biology based tests, genetic tests and test-algorithm relationships. In human medicine most commercially developed new tests are required to undergo rigorous approval testing This does not apply to much molecular diagnostics and they fall under the currently less well regulated category of laboratory developed tests LTDs. In veterinary medicine regulation is even less well defined, except for international guidelines for mostly infectious diseases of food animals. OIE guidelines which are reviewed and updated annually. In all testing fields Point of care (POC) tests are a concern because of their qualitative or semi-quantitative nature and rapid development. Genetic testing validation and standardization is a major concern in human medicine. Many "omics" genomics, proteomics, metabolomics which are multifaceted test platforms raise concerns in the clinical validation and interpretation at the point of use. Manufacturers of test kits received guidance for FDA approval applications which are mostly based on the guidelines and consensus standards developed by CLSI (clinical laboratory standards institute)

In veterinary diagnostic laboratories most tests fall into two main categories modification of manufacturer provided " approved " tests and LTDs. When developing assays it is wise to keep in mind the following

"Validation is a process that determines the fitness of an assay or test which has been properly developed, optimized and standardized, for an intended purpose"
Axel Collig et.al.OIE

"Is the new or adapted assay fit for purpose and will it remain so? "

This can be broken into three segments:

1. **Assay development**
   a. Select an assay./test that can be developed or modified and can likely be validated for the purpose it is intended.
   b. Define the intended purpose of the assay
   c. Test method: study design and protocol experimental studies
      This involves selection of appropriate reagents and controls
      Analysis of reference samples.

   d. Establish an operating range that the assay performs reproducibly and with precision over.
   e. Optimisation usually involves testing over low medium and high ranges
   f. Matrix considerations that this represents as closely as possible the sample to be tested (e.g. serum)
   g. Preparation of pooled reference samples for use during the validation of the assay.
   h. Standardization establishment of critical working ranges for reagents

i. Establishment of “interferences” inhibitors e. g. Haemolysis, intrinsic proteins that may interfere in biding in ELISA assays
j. Establishment of robustness of the assay: that the assay can be unaffected by minor changes in pH, temperature, reagent batch, surfaces used e.g. ELISA plates organic matrix factors
k. Preliminary testing of repeatability of the assay

2. Assay validation

This can be divided into four stages

1: **Analytical characteristics**

Analytical specificity:-the ability of the assay to distinguish the target analyte e g. antibody, organisms, gene sequence, from non target analytes e.g matrix cross reactions.

Analytical sensitivity:-includes LOD (limit of detection) LOQ

2: Diagnostic characteristics

Diagnostic specificity: Diagnostic sensitivity:
these use defined as Dsp. populations of animals / samples negative or healthy
Dse. populations of animals/samples positive or have the disease tested for and most important Animals or samples containing other “unknown“ conditions which may be intereferants or in context vaccinated animals.

Cut off determination: can be made using ROC curves and./or Byesian statistics.

3: Reproducibility

This may involve running of the test by other labs with other populations of animals/samples If this is successful then the assay can be declared validated for the original purpose it was designed.

4: Implementation

The use of the assay in practice allows collection of more information on its utility and validity. However this may not only involve scientific fitness for purpose but also its acceptance by the client community and its feasibility for use by other laboratories This may also include costs, ease of performance, turnaround times.
Before implementation of a test QC and QA systems for it should be in place and include preanalytical analytical and post analytical issues.

3. Validation status retention

This involves continuous monitoring of precision and accuracy, in house QC and proficiency testing QA

Conclusion:

For validation” of a test of either the LDT or modified approved test type the following is needed **PARR+AS+AS** (precision, accuracy, reportable range, reference range, analytical sensitivity, analytical specificity).Other considerations include sample stability, linearity of the assay, and possible carry-over /contamination. In addition good laboratory practice includes clinical validity, clinical sensitivity and clinical specificity and clinical utility. And possibly most important of all clinical acceptance and use.

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COMMUTABILITY OF COMMERCIAL QUALITY CONTROL MATERIALS WITH FELINE PLASMA

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Introduction:

Quality Control Materials (QCMs) or other reference materials are routinely used to monitor performance of analyzers and validate assay methods at human and veterinary clinical pathology laboratories and at point of care locations (1-2). Species-specific plasma or serum samples, pooled from multiple individuals, are considered the ideal standard for QCMs instead of commercially available QCMs (3), but may be problematic due to inability to consistently obtain sufficient volumes of suitable materials for routine use, degradation over time with freezing and/or refrigeration, and lack of representation of a range of analyte concentrations that may be desirable for monitoring of stable instrument performance (4).

Ideally, QCMs should be 'commutable' with patient plasma or serum; that is, the results from QCMs should be consistent with those from plasma or serum samples from healthy and diseased individuals (5). Commutability is defined as the equivalence of the mathematical relationships between the results of different measurement procedures for a QCM and for representative samples from healthy and diseased individuals.(5) Despite widespread recommendations (2, 6-7) and use of commercial QCMs, there are no peer-reviewed assessments of commutability with veterinary patient samples.

Essentially, commutability studies assess patient samples by two methods (instruments), perform linear regression on the results of the patient samples then determine if QCM samples tested by the same instruments fall within a specified range around the patient samples. The range around the patient samples can be determined by prediction limits or by assessment of residuals (the distance between any patient sample and the 'best fit' regression sample) (5, 8).

Commutability Study:

Pooled plasma was prepared from 12 healthy cats (PPN) and 12 cats known to have renal disease (PPR) over a 3 day period, divided into separate (daily) aliquots and frozen. After thawing aliquots on individual days, samples run on two analysers (IDEXX Catalyst Dx® Chemistry and Olympus® AU400 until ran out (10-12 samples).

Commercial QCMs: MAS® chemTRAK®-H (Thermo Fisher Scientific Inc, Waltham, MA USA), levels 1 and 3 (CT1 and CT3) was prepared, handled and stored according to the manufacturer's specifications. Run on same two analysers as pooled plasma on any days pooled plasma was run.

Patient samples from the routine clinical chemistry caseload ncluded clinically healthy as well as unhealthy. Age range: 3 months to 19 years (mean: 10.5 years). 38 male (4 entire), 36 female (3 entire). 33 DSH, 10 Burmese, 6 Brit SH, 5 DMH, 4 DLH, remainder from 10 other breeds. Tested (for 16 analytes) on same two analysers.

Statistical Design:

Patient samples:

- Compared analysers by assessing correlation and Passing-Bablok regression.
- Residuals (difference between each data point and regression line) were:
    - Determined.
    - Standardised (by dividing by SD of residual results).

Reference Materials (PPN, PPR, CT1, CT3)

- Determined mean, SD, CV for each analyte on each analyser.
- Residuals (fitted and standardised) determined using equation determined from regression (9).

Commutability of reference material with patient plasma samples if standardised residual within -3 to 3.

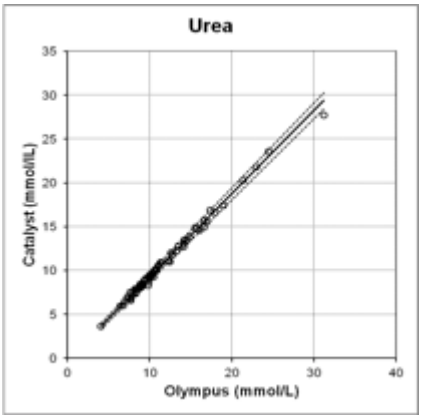

Figure 1: Passing-Bablok regression of urea for results on both analysers, R= 1.00, Slope= 0.95 (0.93-0.97), Intercept= -0.26 (-0.52-0.05).

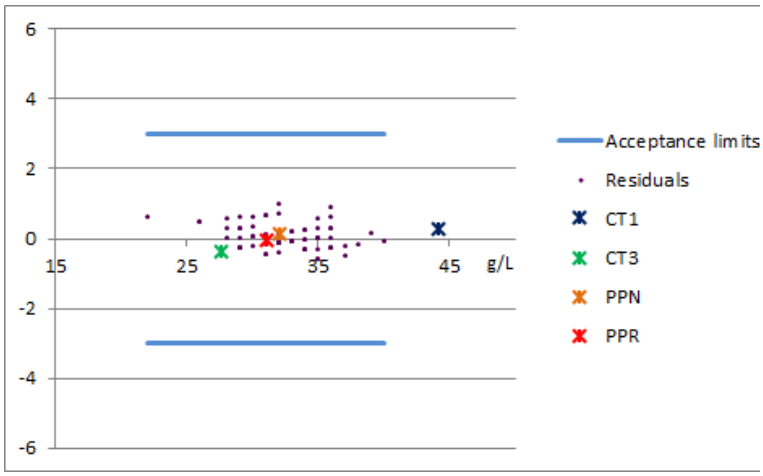

Figure 2: Standardised residuals plot for albumin. Residuals represent patient data results' distance from the regression line; acceptance limits are only shown over the range of the patient samples; reference materials are considered definitively commutable if within acceptance limits and within range of patient samples. Note that CT1 is outside the range of patient results so appears likely to be commutable but this cannot be definitively stated.

Results:

32 commercial QCM assessments (16 CT1, 16 CT3).
20/32 QCM's definitively commutable.
2/32 QCM's NOT commutable (CT3: ALP, K+).
4/32 QCM's unable to be assessed unable to regress (bilirubin and GGT for CT1 and CT3).
8/32 QCM's outside range of patient samples (including CT3 ALP and CT K+).
20/20 QCM's within patient range commutable.

Interchangeability Study:

Additionally compared CVs of commercial QCMs to pooled plasma by bootstrap analysis and assessed if CVs of all reference materials were within quality standards (optimal, desirable, minimal) determined by biological variation.

Bootstrap analyses:

No significant difference in precision for each reference material: Glucose, Sodium
No significant difference CT1 to PPN: Calcium, Chloride, Glucose, Potassium, Sodium, Total Protein, Urea (7/16)
No significant difference CT1 to PPR: Calcium, Cholesterol, Glucose, Potassium, Sodium, Total Protein, Urea (7/16)
No significant difference CT3 to PPN: ALP, Chloride, Glucose, Sodium, Urea (5/16)
No significant difference CT3 to PPR: ALP, Creatinine, Glucose, Sodium (4/16)

Quality standards:

Olympus:
PPN: Optimal (6/16), Desirable (5/16), Minimal (2/16), None (3/16)
PPR: Optimal (8/16), Desirable (4/16), Minimal (1/16), None (3/16)
CT1: Optimal (7/16), Desirable (0/16), Minimal (6/16), None (3/16)
CT3: Optimal (7/16), Desirable (3/16), Minimal (3/16), None (3/16)
Catalyst: (NB GGT = 0 U/L for all PP samples)

PPN: Optimal (6/15), Desirable (4/15), Minimal (1/15), None (4/15)
PPR: Optimal (7/15), Desirable (3/15), Minimal (1/15), None (4/15)
CT1: Optimal (8/16), Desirable (2/16), Minimal (0/16), None (616)
CT3: Optimal (8/16), Desirable (2/16), Minimal (1/16), None (5/16)

Conclusions:
Despite commutability, commercial QCM do not necessarily behave equivalently to pooled plasma when assessed for precision. “Equivalence of mathematical relationships” does not extend to precision! Commutability ensures results for clinical assays have numerically equivalent values. Equivalence of biochemical methods important for common/harmonised reference intervals (will this ever happen for veterinary clinical pathology?)
Non interchangeability affects daily QA method comparison. In most cases, differences between PP and CT were within quality standards (exceptions tightly regulated so limited by technology).

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STATISTICAL QC FOR VETERINARY HEMATOLOGY ANALYSER

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Quality requirements such as the total allowable error (TEa) are necessary for objective judgement of method validation studies and results of internal and external quality control. The TEa is the desired total error, whereby the total observed error (TE_{obs}) is the total error based on the analyzers´ performance. While there are recommendations for TEa veterinary clinical biochemistry measurands, similar guidelines are still missing for veterinary haematology. Moreover, it is largely unknown if veterinary haematology analyzers are meeting clinicians´ expectations. Thus, it was the aim of our study to evaluate TEa for veterinary haematology measurand by performing a prospective survey about experts´ opinions and to compare the median TEa derived from the survey to TE_{obs} obtained from meta-analysis of published method validation data.

Veterinary experts in clinical pathology and small animal internal medicine had to fill in the maximal allowable deviation from a given result for each measurand. The calculation of the percent TEa (%) was done with following formula: **TEa (%) = [allowable median deviation / clinical threshold] * 100.** Second, TE_{obs} for laser-based bench top haematology analysers and point of care analysers respectively were calculated based on method validation studies published between 2005 and 2013 (n=4 bench top analysers and n=9 point of care analysers): **TE_{obs} = 2*CV [%] + bias [%].** Laser-based bench top analysers included the ADVIA 2120 (Siemens), Sysmex XT2000iV (Sysmex) (1–4), and the CellDyn 3500 (Abbott) (5). Point of care analysers included laser based systems such as the ProCyte Dx (IDEXX) (6), the LaserCyte (IDEXX) (7, 8), as well as the impedance based systems Mythic 18 (Orphee´) (9), ForCyte (Oxford Science Inc) (8), Scil VetABC (Scil) (8), MS45 (Melet Schloesing) (8), Heska CBC (Boule, Medicalab) (8), VetScan HMT (Abraxis) (8), as well as Poch100iV (Sysmex) (10, 11). Inclusion criterion for the meta-analysis was that CV and regression equation was given by the authors. The bias was estimated from regression equation given in published studies as reported previously (12) to obtain comparable results for all included studies. Briefly, the upper limit of a published reference interval (13, 14) was entered in the regression equation. The bias (%) was consistent with the difference (%) between the calculated result and the original value entered in the formula. The coefficient of variation (CV) was also obtained from publications except for the ADVIA 2120 (own unpublished data). An ideal TE_{obs} should be < Tea (15). Overall, answers from 41 veterinary experts (19 diplomates, 8 residents, 10 post-graduate students, 4 anonymous specialists) were received. Interestingly, there was a wide range of proposed TEa of factor 5 to 20 for all variables, but for the majority of measurands the median postulated TEa had to be ≤ 20%. Regarding the bench top analyzers, TE_{obs} was generally < TE_{ad} for all variables and analysers. The only exceptions were high TEas for canine and feline haemoglobin (high bias, low CV) when the ADVIA 2120 was used and platelets (high bias in dogs and cats, high CV in cats). For the canine and feline monocyte count (high bias in dogs, also high CV in cats) as well as for the feline eosinophil count (high CV), TE_{obs} > $TE_{ad.}$ As for the large bench top analysers, TE_{obs} > TE_{ad} was observed for all studies using the ADVIA 2120 as reference method in both cats and dogs due to a high bias. TE_{obs} > TE_{ad} was also seen for the haematocrit value in dogs and cats (ProCyte Dx vs. ADVIA 2120, high bias) and in cats the comparison of LaserCyte and CellDyn (high bias). For all analysers, TE_{obs} > TEa_{d} was seen for the platelet count due to a high bias and/or CV in both cats and dogs. The only exceptions were comparisons between LaserCyte and CellDyn, LaserCyte and ADVIA 120 as well as the PocH100 iV and the CellDyn in dogs. In both dogs and cats, the comparison between MS45 and VetScan with the ADVIA120 revealed TE_{obs} > TEa_{d} for both dogs and cats due to a high bias. In only few studies evaluating point of care analysers, absolute numbers of cellular populations were compared for the differential count. Except for the

comparison of the canine neutrophil count using the LaserCyte and CellDyn, TE_{obs} > TE_{ad} for all analysers and species due to either a high CV and/or bias.
As far as we are aware of, this was the first introduction of TEa derived from experts´ opinion for veterinary hematology. Most striking, however, was the extremely large variation in experts´ opinion and thus desired TEas. Possible reasons for the huge variety of desired TEas might be the different needs of experts based on their specialization as well as different knowledge about analyser capability. The results, however, are limited by the fact that data are derived from meta-analysis and thus different methodology and statistical methods (i.e., regression equation). Moreover, it has to be noted that the bias obtained from method validation studies is highly dependent on the used reference method. The high bias of approximately 20% for haemoglobin in all studies using the cyanide-free method of the ADVIA 2120 as reference is well known (16) and can be easily compensated with software adaptation. Interestingly, there were relatively high experts´ expectations for the haematocrit value which could not be fulfilled by some point of care analysers due to a high bias, i.e., the VetScan (dog), the ProCyte (cat), and the LaserCyte (cat). A possible reason for the discrepancy between TEa and TE_{obs} is the fact that the haematocrit value is not measured but calculated from the MCV and RBC values so that errors are easily adding up. The measurement of PLTs is also problematic due to well-known analytical and prae-analytical difficulties i.e., the presence of PLT aggregates especially in cats and impedance analysers. TE_{obs} was > TE_{ad} for the WBC count of two impedance analysers due to a bias, so that adaption of the threshold might be considered. “Problematic” cellular populations of the differential count included monocytes due to their low number and pleomorphism and feline eosinophils. For point of care analysers, nearly all cell populations of the differential count are problematic, however, only data of few analysers have been included due to the lack of reported absolute values for cell populations. Overall, veterinary analyzers fulfilled experts´ requirements apart from haemoglobin due to method related bias and platelet counts due to known pre-analytical/analytical difficulties. “Rare” and pleomorphic cell populations such as monocytes are problematic for large bench-top analyser, whereas for point of care analysers non statistical QC is necessary for the differential count.

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NON STATISTICAL QC FOR VETERINARY HEMATOLOGY ANALYSER

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In both human and veterinary medicine there has been a large technical evolution of automated blood cell counts resulting in a greater precision than microscopic 100-cell differential count. Besides its imprecision especially in rare cell populations, the manual differential count is time consuming (1). Thus, in human laboratories, attempts have been made to reduce the costs and turnaround time by reducing the rate of blood smear reviews (1).
In 2002, 20 experts (the international consensus group for haematology review) were meeting and established 83 criteria and rules for review of CBC and automated differential count derived from haematology analysers. The criteria were then evaluated in 15 laboratories with overall 13,298 samples. Compared to the blood smear as reference method, there were 11.2% true positives, 18.6% false positives, 67.3% true negatives, and 2.9% false negatives, whereby the comparatively high rate of false positives was mainly due to instrument flags. According the experts´ opinion, the false negative rate should be as low as possible but generally below 5%. Based on the results, rules were redefined and consolidated to 41. Overall, the rules included cut-off values for abnormalities of the erythron, leukon and thrombon requiring blood smear review as well as instrument flags such as flags indicating the presence of immature granulocytes or atypical lymphocytes (1). Prior to defining the rules, the rate of blood smear review was highly variable in the laboratories ranging from 5 to 95%. So far no “official” guidelines are published for veterinary laboratories. Moreover, the rate of blood smear review to perform manual differentials is unknown.

First part - ADVIA 120:
Our aim was to evaluate the rate and causes of performing manual leukocyte differential counts in a university clinical pathology laboratory using the laser-based haematology analyser ADVIA 120 (Siemens Medical Solution Diagnostics, Eschborn, Germany) (2). Between August 2004 and December 2006, 14,953 complete blood cell counts from dogs (n=10,139), cats (n=2,494) and horses (n=2,320) were reviewed by veterinary clinical pathologists (1 diplomate and residents of the European college of Veterinary Clinical Pathology). Manual leukocyte differentials were requested by clinical pathologists based on assessment of the peroxidase and baso cytograms of the ADVIA 120. All samples in which a manual differential count was requested by clinical pathologists where reviewed by one of the authors (MS) and causes for its request were classified by applying 4 specific criteria which were based on the authors´ long experience of reviewing ADVIA cytograms. The criteria were as follows:

1) Suspicion of left shift (e.g, “worm with swollen neck” or “short worm” in baso cytogram)
2) Inappropriate separation of cell populations
3) Suspicion of atypical lymphocytes
4) Suspicion of abnormal cells (e.g., blasts)

Moreover, the rate of false positive alerts i.e., an abnormal ADVIA 120 cytogram but normal blood smear was determined in a subgroup of canine (n=436), feline (n=153), and equine (n=76) samples. At the time of performing the study, slides were reviewed by the clinical pathologists in case of abnormalities of the erythron and thrombon. Overall, a manual differential was requested in 21% of canine, 32% of feline, and 20% of equine samples respectively. Depending on the species, indistinct separation of the cell population was present in 10% to 15% of the cases, atypical lymphocytes were suspected in 2% to 12%, left shift in 13% to 25%, and blasts were rarely suspected in less than 0.4% of the cases. The relatively high rate of requested manual differential counts in cats was mainly based on the suspicion of left shift.

Sensitivity and specificity for detecting a left shift based on ADVIA cytograms were 80% and 49% in dogs, 94% and 24% in cats and 97% and 18% in horses, respectively. The rate of false positive alerts was 13% in dogs and 30% in cats and horses respectively. Overall, our results were comparable to human studies reporting a slide review rate of approximately 25% (3, 4).
Our results are limited by the retrospective nature of the study and the fact that the rate of false negative results was not evaluated. Moreover it has to be considered that rubricytes and parasites are not detected. The findings are further dependent on the type of the samples i.e, they might be different in laboratories receiving a large number of specimens for health check. Sample aging as it is commonly seen in commercial laboratories might lead to the presence of more cells in the lyse resistant and mononuclear area of the baso cytogram (5, 6). Recently, laboratory-specific cut-off values have been established based on the criteria recommended by the international consensus group for haematology review (1) resulting in an increase of diagnostic efficiency from 84% to 87% (7). Including historical patient data was followed by a marked decline of the rate of blood smear review from 25% to 6% (4). Moreover, it has been discussed if instrument flags might be too sensitive. Although future research is needed even in human medicine, it can be concluded that the rate of manual differentiation could possibly be further reduced in veterinary laboratories, if the microscopic examination would be used as a validation procedure, rather than as a reflexive substitute for automated differentiation.

Second part – ProCyte Dx:
Aim of this study was the prospective evaluation of diagnostic utility of the flag "band neutrophils suspected" (Flag LS) and "WBC abnormal distribution" (Flag WBC_{abn}) of the laser based haematology analyzer ProCyte Dx (IDEXX Laboratories Inc, USA) to detect a left shift. Moreover, the diagnostic utility of the ProCyte Dx WBC scattergrams (dotplots) were evaluated as well as diagrams not routinely available for the user such as the density plot (1. presence of enlarged neutrophil “cloud”, and 2. presence of not separated “clouds”) and the histogram of WBC fluorescence distribution. Consecutive blood samples of dogs (n=143) and cats (n=103) submitted to our laboratory between May 2014 to June 2014 were included. Samples were measured with the ProCyte Dx and compared with the results of a 100-cell manual differential count performed by two experienced examiners (MB, JF).
A low (bands > $0.5x10^9$/L) and a high cut-off value (bands > $1.0x10^9$/L) were used to define a left shift. Results for dogs and cats are shown in table 1. In dogs, the Flag LS showed a high sensitivity at a cut-off > $1.0x10^9$/L bands to detect a left shift but a low specificity. At the same cut-off value, the Flag WBC_{abn} had a comparatively low sensitivity but very high specificity. In the cat, the Flag LS had a markedly lower sensitivity but much higher specificity than in the dog. In contrast, the Flag WBC_{abn} was both less sensitive and less specific than in the dog.
In both species, the review of the dotplot was the best parameter routinely available for the user. Overall, the enlargement of the neutrophil “cloud” in the density plot was the best indicator for presence of a left shift in cats and dogs, however, this feature is actually not available for the user.

Table 1: Sensitivity and specificity and area under the receiver operating characteristic curve (ROC) for 143 canine and 103 feline samples respectively for a cut-off value of bands > $1.0x10^9$/L.

Species	Cat			Dog		
Variable/result	Sens	Spec	ROC	Sens	Spec	ROC
Flag LS (left shift)	50	91	0.70	97	37	**0.67**
Flag WBC abn. distribution	**38**	**88**	**0.64**	**61**	**99**	0.80
DotPlot abnormal	89	78	0.83	82	94	0.88
Density plot- Neutrophil „cloud“ enlarged	**100**	**74**	**0.87**	**97**	**90**	**0.93**

Density plot– No seperation between „clouds“	67	93	0.80	70	99	0.84
Histogramm abnormal	94	73	0.84	97	87	0.92

Overall, the seize of the neutrophil cloud was the best parameter for detection of a left shift, however, when not available, the dotpot should be reviewed to decide whether a manual differential count is necessary. Future research in veterinary clinical pathology is needed to establish guidelines for the cut-off of numerical data and the optimization of flagging options.

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HEMATOLOGY CASE STUDIES – AN INTERACTIVE CHALLENGE

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During this session, a series of unknown hematology case studies will be presented for interactive discussion and demonstration. As an example of the case material, two cases are presented below. Additional cases will be available online at (http://bit.ly/1g46euB).

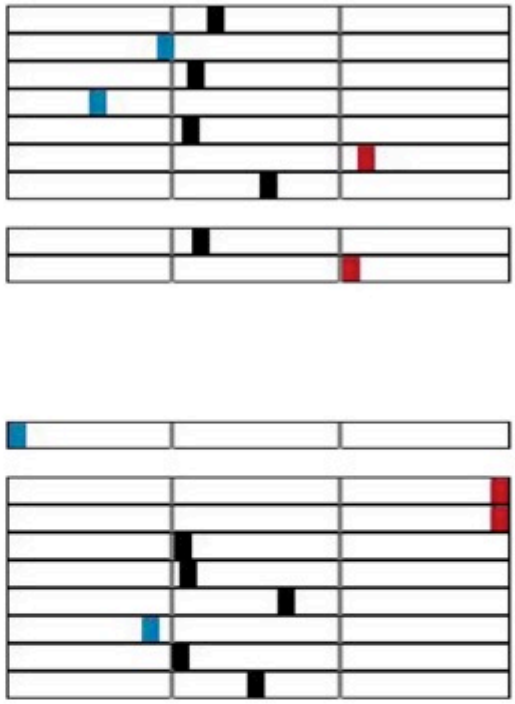
Case 1: 8-year-old, intact male Springer Spaniel in good body condition, presented with acute onset decreased exercise tolerance, extreme weakness, fever and intermittent vomiting.

Patient Name: Blizzard
Species: Canine

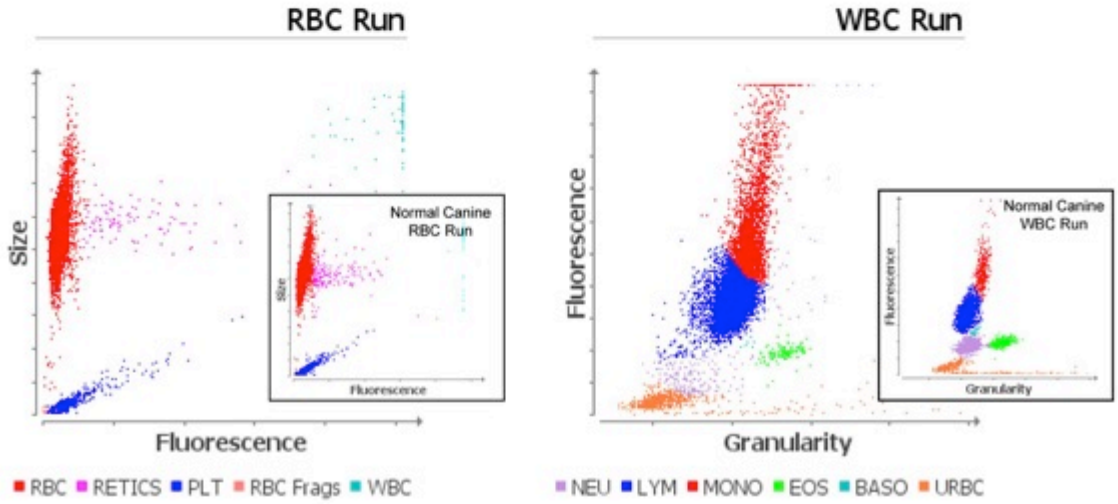
Gender: Male
Age: 8 Years

ProCyte Dx (February 10, 2013 11:54 AM)

Test	Results	Reference Interval	LOW / NORMAL / HIGH
RBC	6.50 M/µL	5.65 - 8.87	
HCT	36.8 %	37.3 - 61.7	LOW
HGB	14.1 g/dL	13.1 - 20.5	
MCV	56.6 fL	61.6 - 73.5	LOW
MCH	21.7 pg	21.2 - 25.9	
MCHC	38.3 g/dL	32.0 - 37.9	HIGH
RDW	18.3 %	13.6 - 21.7	
%RETIC	0.4 %		
RETIC	27.3 K/µL	10.0 - 110.0	
WBC	16.94 K/µL	5.05 - 16.76	HIGH
%NEU	* 1.3 %		
%LYM	* 71.2 %		
%MONO	* 26.6 %		
%EOS	0.8 %		
%BASO	0.1 %		
NEU	* 0.22 K/µL	2.95 - 11.64	LOW
BAND	* Suspect presence		
LYM	* 12.06 K/µL	1.05 - 5.10	HIGH
MONO	* 4.51 K/µL	0.16 - 1.12	HIGH
EOS	0.14 K/µL	0.06 - 1.23	
BASO	0.01 K/µL	0.00 - 0.10	
PLT	378 K/µL	148 - 484	
MPV	7.9 fL	8.7 - 13.2	LOW
PDW	9.5 fL	9.1 - 19.4	
PCT	0.30 %	0.14 - 0.46	



WBC Abnormal Distribution
Band neutrophils suspected



Case 2: 7-year-old, neutered male, DSH cat presented with a 10 month history of diabetes and PU/PD. The cat has been more lethargic and has had greater weight loss in the past few weeks.

Patient Name: Tike
Species: Feline
Breed:

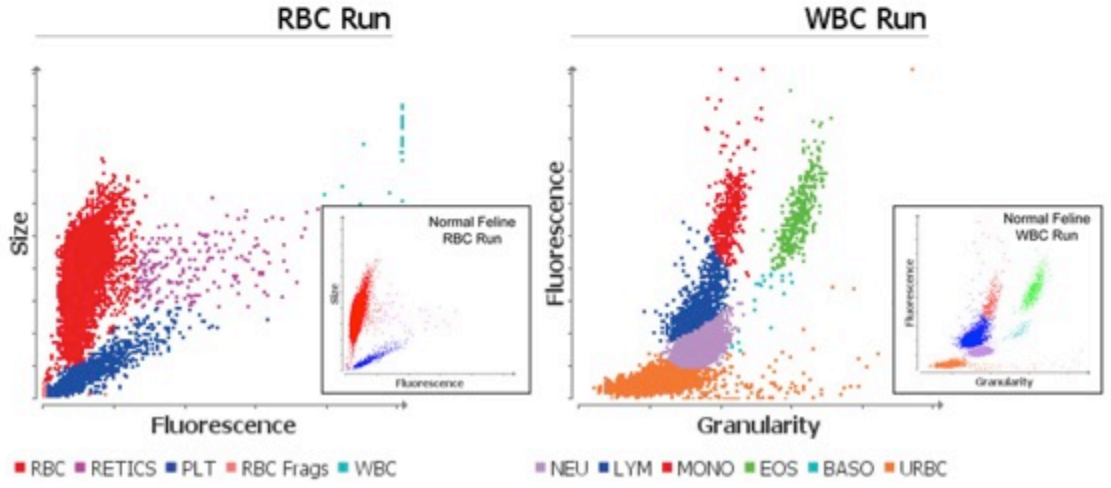
Gender: Male/Castrated
Weight: 6.48 lbs
Age: 7 Years

ProCyte Dx (December 8, 2014 9:15 AM)

Test	Results	Reference Interval	LOW / NORMAL / HIGH	12/7/14 11:30 AM
RBC	* 4.11 M/µL	6.54 - 12.20	LOW	* 4.74 M/µL
HCT	* 15.7 %	30.3 - 52.3	LOW	* 18.2 %
HGB	6.7 g/dL	9.8 - 16.2	LOW	7.9 g/dL
MCV	* 38.2 fL	35.9 - 53.1		* 38.4 fL
MCH	* 16.3 pg	11.8 - 17.3		* 16.7 pg
MCHC	* 42.7 g/dL	28.1 - 35.8	HIGH	* 43.4 g/dL
RDW	* 27.3 %	15.0 - 27.0	HIGH	* 26.3 %
%RETIC	0.8 %			0.3 %
RETIC	* 34.1 K/µL	3.0 - 50.0		* 16.1 K/µL
WBC	6.72 K/µL	2.87 - 17.02		9.40 K/µL
%NEU	69.2 %			* 75.2 %
%LYM	18.3 %			* 14.1 %
%MONO	6.7 %			* 6.8 %
%EOS	5.5 %			3.3 %
%BASO	0.3 %			0.6 %
NEU	4.65 K/µL	1.48 - 10.29		* 7.06 K/µL
LYM	1.23 K/µL	0.92 - 6.88		* 1.33 K/µL
MONO	0.45 K/µL	0.05 - 0.67		* 0.64 K/µL
EOS	0.37 K/µL	0.17 - 1.57		0.31 K/µL
BASO	0.02 K/µL	0.01 - 0.26		0.06 K/µL
PLT	478 K/µL	151 - 600		373 K/µL

RBC Abnormal Distribution

RBC Abnormal Distribution
Band neutrophils suspected



IMMATURE AND TOXIC NEUTROPHIL IDENTIFICATION – CAN TECHNOLOGY IMPROVE OUR ACCURACY?

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The presence of immature and toxic neutrophils in circulation for most of our domestic animals is the hallmark of inflammatory disease and historically, the microscopic identification of these neutrophil forms on a blood film has been our primary tool to identify the presence of systemic inflammatory disease. Clearly the introduction of c-reactive protein in the dog as well as measurement of other acute phase inflammatory proteins have added an additional dimension to our characterization of inflammatory disease, but the microscopic evaluation of the blood film is still the primary method for detecting inflammatory disease in most veterinary practices. The problem with the microscopic identification of inflammatory disease is that this is not performed in the vast majority of in-clinic CBCs. Many veterinarians indicate that they only perform a blood film microscopic evaluation when there are abnormalities. In the majority of cases regarding suspected inflammatory disease, this happens when the veterinarian records either an increased total WBC count or an increased total neutrophil count. In a recent review of 1,320 canine CBCs with confirmed inflammatory disease with the presence of immature and/or toxic neutrophils, approximately 50% of these cases have normal total WBC or immature/toxic neutrophil counts (figure 1).

Figure 1: Frequency of increased WBC and neutrophil counts with canine inflammatory leukograms

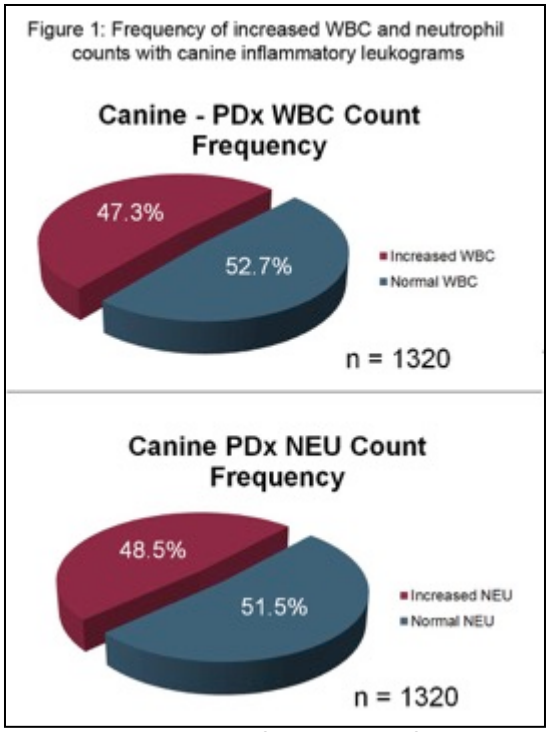
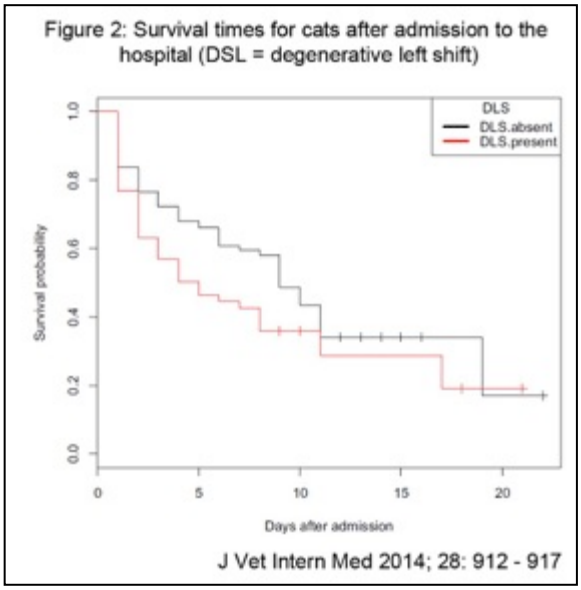


Figure 2: Survival times for cats after admission to the hospital (DSL = degenerative left shift)



In addition to the value of simply identifying the presence or absence of immature and/or toxic neutrophils on the peripheral blood film, identifying the relative and absolute numbers of these neutrophil forms assists in grading the severity of the inflammatory disease. This is especially of value when a degenerative left shift is seen. There are multiple definitions for a degenerative left shift; however, most accept the term degenerative left shift when the immature neutrophil forms outnumber the mature neutrophil forms. In a recent study of hospitalized cats with inflammatory disease, a significantly higher rate of animals being euthanized or dying while hospitalized were seen in cats with degenerative left shifts (figure 2).

Identification of immature and toxic neutrophils: Historically this process of identification and enumeration (relative and absolute counts) has been accomplished only with the microscopic review of

the peripheral blood film. However, there are multiple problems with this process. First, it is extremely difficult to obtain a well-prepared blood film with even distribution of leukocytes to assure an accurate leukocyte differential. This is especially true of blood films prepared in the average veterinary practice. An additional barrier that the average veterinary faces is the staining of peripheral blood films. Rapid stains such as Diff Quik are commonly used and if not deliberate in the staining process, specimens are stained too "blue", which interferes with one of the significant morphologic criteria for identifying immature or toxic neutrophil forms. Immature and toxic neutrophils have greater amounts of cytoplasmic RNA, which stains blue with a Romanowsky stain. If the amount of stain is only slight in amount, accurate identification is not possible.

Even if the perfectly prepared slide with perfect leukocyte distribution and a perfect stain was applied, both determination of what is to be labeled an immature and/or toxic neutrophil is challenging and the process of enumeration with a manual leukocyte differential is limiting in precision. Because of the nature of how neutrophils will be seen on the slide, there are often-times a challenge in accurate assessment of the shape of the nucleus, which is a commonly used feature to distinguish between mature and immature forms. One definition of a band neutrophil is when the nucleus is horseshoe shaped with parallel sides (and potential slight indentations) and another definition is when the thinnest portion of the nucleus is more than one-third the thickest portion of the nucleus. Applying these definitions are both subjective or too labor consuming to be feasible in accurate identification of immature neutrophil forms. Even if identification was simple to accomplish, the limitation in precision on a 100 or 200 leukocyte differential, especially for low cellular events that are low in frequency, is poor (figure 3). If there it were known that 10% of the leukocytes in a sample were immature and/or toxic neutrophils and only a 100 leukocyte differential was performed, the 95% confidence limits for this value are 4 – 18 %. Rather significant interpretations would be given to an animal based on these extreme limit values.

Figure 3: The 95% confidence limits for various percentages of leukocytes of a given type as determined by differntial counts on stained blood films.

a	n = 100	n = 200	n = 500	n = 1,000
0	0–4	0–2	0–1	0–1
1	0–6	0–4	0–3	0–2
2	0–8	0–6	0–4	1–4
3	0–9	1–7	1–5	2–5
4	1–10	1–8	2–7	2–6
5	1–12	2–10	3–8	3–7
6	2–13	3–11	4–9	4–8
7	2–14	3–12	4–10	5–9
8	3–16	4–13	5–11	6–10
9	4–17	5–14	6–12	7–11
10	4–18	6–16	7–13	8–13

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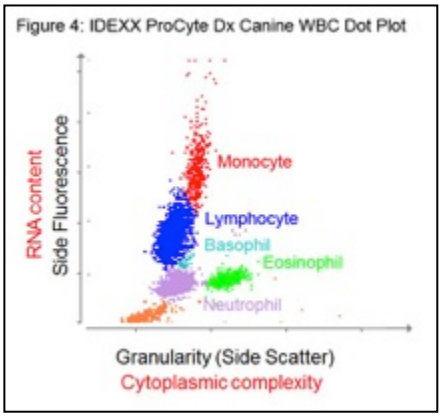

Newer hematology technology can dramatically assist in both the recognition of immature and/or toxic neutrophil forms and suggest the degree of their presence, which indirectly expresses the severity of the and disease state. The Siemens 120/2120, inflammatory process the Sysmex XT-v and the IDEXX ProCyte Dx all provide graphic and message indicators that there are immature and toxic neutrophils suspected in a sample. For the veterinarian in practice, the in-clinic IDEXX ProCyte Dx actually includes both the graphic representation of this suspect as well as a direct inclusion of a message in the final report that indicates the suspect presence of these neutrophil forms. With the Sysmex and IDEXX platforms, the immature and/or toxic neutrophils are identified based on their cytoplasmic content of RNA. The y-axis on their WBC cytogram is a measure of nucleic acid content and since the immature and toxic neutrophil has greater amounts of RNA, the digital signal for these cells is higher on the y-axis. For demonstration purposes, figure 4 demonstrates the WBC dot plot from the IDEXX ProCyte Dx on a normal canine sample. Figure 5 is an example of a ProCyte Dx WBC dot plot from a canine case of severe inflammatory disease with a prominent left shift that was flagged on the final report. Note the continuum of digitized events extending upward on the y-axis (optical fluorescence for nucleic acid); the degree of increase of digitized events on the y-axis is directly reported to the degree of left shift and/or toxic neutrophils in circulation.

Summary: The identification of immature and/or toxic neutrophils is a rapid means for determining both the presence and severity of inflammatory disease. This identification is critical for accurate assessment of patients as well as for monitoring the progression or regression of inflammatory disease.

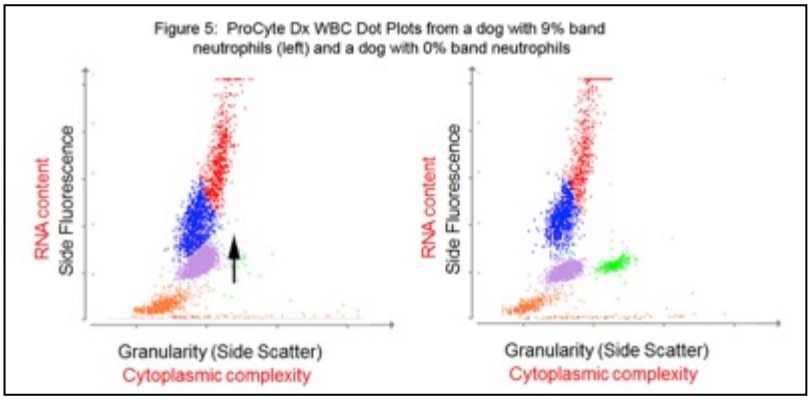

Figure 5: ProCyte Dx WBC Dot Plots from a dog with 9% band neutrophils (left) and a dog with 0% band neutrophils

There are limitations on the manual microscopic identification of these neutrophil forms; however, newer automated technologies provide an accurate and sensitive means for the identification of these cells.

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RETICULOCYTE COUNTS AND RBC INDICES IN REGENERATIVE ANEMIA

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Both laboratory data generated by current hematology analyzers and blood film microscopic evaluation are critical in characterizing an anemia as regenerative or non-regenerative and to assist in identifying the underlying mechanism for the anemia itself. Over the years, we have learned to associate various erythrocyte microscopic morphologic changes with different causes of anemia, particularly hemolytic anemias, but the process of microscopic evaluation with the light microscope has not changed for routine hematology evaluations. However, the generation of reticulocyte counts and the various erythrocyte indices has significantly improved over the years with the advancement of hematology instrumentation and our use of these values has evolved.

Reticulocyte counts: Although microscopic morphologic features or erythrocytes such as the presence of polychromasia associated with anisocytosis clearly support an active bone marrow response, the reticulocyte count is widely accepted as the most objective measure of bone marrow responsiveness. Historically we have evaluated reticulocyte responses in a variety of ways in an attempt to maximize our interpretive capabilities. These include 1) relative reticulocyte count, 2) corrected reticulocyte count, 3) reticulocyte production index and 4) absolute reticulocyte count. The determination of these is shown in figure 1. The absolute reticulocyte count ($x10^3/\mu L$) is the most direct and simplest parameter to follow for accurate assessment; it eliminates the need for any mental gymnastics. Caution should be made to not use the relative reticulocyte count by itself; a correction using a relatively normal species-specific hematocrit (canine = 45%, feline = 37%) avoids over-interpretation. In addition, calculating the reticulocyte production index (RPI) should also be used cautiously. The principle behind this calculation is sound; however, the details are not well founded. The principle is that with increasing severity of anemia, if the bone marrow can respond, reticulocytes are released from the hematopoietic tissue earlier than they would be when the animal is not anemic. The end result is that these cells will last in circulation longer since there is a greater amount of hemoglobin to produce and reticulum to be removed. The various maturation times suggested in the literature have never been well-documented; these were only suggested comparisons in early pharmaceutical studies based on human comparative studies and many of the known similarities between dog and human erythrocyte physiology. If the RPI is used, only use this as a crude guideline. The absolute reticulocyte count is the best parameter to characterize the patient's bone marrow response; it requires no mental gymnastics to correctly interpret the response.

Figure 1: Reticulocyte Reporting in Dogs

Relative reticulocyte count (RRC) = percent of non-nucleated erythrocytes that are reticulocytes (RETIC)

% Corrected reticulocyte count (CRC) =
RRC x (Patient PCV / Average Normal PCV of 45%)
PCV = Packed Cell Volume (%)

% Reticulocyte production index (RPI) =
CRC / Patient RETIC MT

MT = maturation time (in days)

PCV	MT
45	1.0
35	1.5
25	2.0
15	2.5

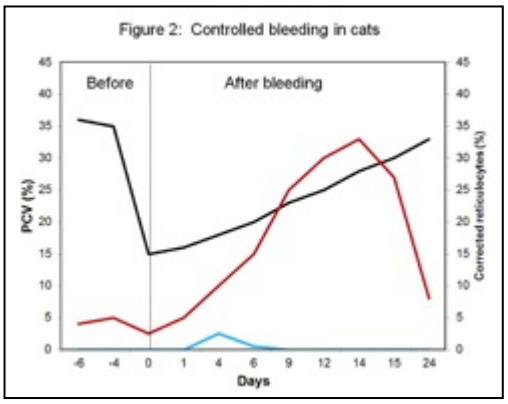
Absolute reticulocyte count (ARC) = RRC (%) x RBC ($x10^6/\mu L$)
RBC = red blood cell count ($x10^6/\mu L$)

Methodologies for the actual reticulocyte count determination have significantly evolved over the years. Prior to the availability of automated hematology analyzers with the capability of measuring reticulocyte numbers, labor-intensive and imprecise manual methods were used. The use of supravital stains such as new methylene blue cause a precipitation of the reticulum in the immature erythrocyte and also causes the presence of easily identified blue inclusions in the cells. These inclusions allow easy distinction between mature and immature erythrocytes and the amount of precipitated reticulum in the cell is inversely related to the degree of maturation stage of the reticulocytes. The greater the amount of precipitated reticulum, the less mature the reticulocyte.

Automated reticulocyte counting on analyzers typically found in the commercial and academic reference laboratories has almost eliminated the need for manual determination of reticulocyte counts. These analyzers have evolved to a new "reference method" in both human and veterinary medicine. Stains specific for RNA/DNA (Siemens 120/2120 – Oxazine 750; Sysmex XT-2000iV and IDEXX ProCyte Dx – polymethine dye) and flow cytometric analysis is used for enumerating reticulocytes. These newer automated systems are both more sensitive and much more precise compared to manual enumeration methods. A good example of their increased sensitivity is seen in how reference intervals for canine reticulocyte counts have changed. Most previous manual method reference intervals suggested an upper reference limit of 60 $x10^3$/µL; however, various laboratories using the Siemens or Sysmex methods suggest the upper reference interval limit for canine reticulocytes is between 110 and 125 $x10^3$/µL. Observing reticulocyte counts of greater than 800 to 900 $x10^3$/µL in a strongly regenerative response in the dog with current automated methods are occasionally seen where with manual methods, observing a reticulocyte count of greater than 600 $x10^3$/µL were rarely seen.

There is still a strong need for manual reticulocyte counting and that is with the cat. Cat reticulocyte responses are quite different in the cat compared to the dog. Both the dog and the cat have two types of reticulocytes, punctate and aggregate. In the dog, the punctate reticulocyte containing only extremely small amounts of RNA is the final stage of maturation to a mature erythrocyte and it is extremely short-lived. In the cat, the punctate reticulocyte is also the last stage in the reticulocyte maturation process, but it lasts in circulation for 7-21 days. The aggregate reticulocyte is the best parameter to characterize recent bone marrow responsiveness. Current hematology analyzers can be calibrated to accurately enumerate aggregate reticulocytes but cannot accurately count all punctate reticulocytes.

There are situations where punctate reticulocytes are of value; however, only examining a new methylene blue stained sample and microscopic recognition of significant numbers; actual manual enumeration is difficult because of their potential high prevalence in the blood. In the 1970's there were several experiments highlighting the need for evaluating a punctate reticulocyte response. Aggregate and punctate reticulocyte counts were followed over a course of several weeks following controlled bleeding of 40% of the blood volume in cats. The findings are summarized in Figure 2. The punctate reticulocyte count response is much greater than the aggregate response and as is seen in naturally occurring anemias in the cat, once the hematocrit is greater than approximately 20%, only a punctate response is typically seen in the peripheral blood. If only aggregate reticulocytes are evaluated, mild anemias in the cat will be incorrectly characterized as non-regenerative.



Erythrocyte Indices: The various erythrocyte indices generated by current hematology analyzers provide various measurements providing indirect morphologic information regarding the general erythrocyte population. Since these are "average" values related to cell size, hemoglobin content, etc., significant numbers of "abnormal" cells must be present in circulation before the values are reported outside of their reference intervals; therefore, they are relatively insensitive for detecting early and potential pre-clinical abnormalities. Trended values, particularly for mean cell volume (MCV), prove effective in improving sensitivity since during health, these values remain relatively stable.

Mean Cell Volume (MCV) and Mean Cell Hemoglobin Concentration (MCHC) – MCV is reported in fL and MCHC is reported in g/dL. MCV is most commonly measured with either impedance or optical fluorescence methods; it is the average cell volume for the population. The MCHC is calculated by dividing the hemoglobin value (g/dL) by the hematocrit (%) and then multiplied by 100. Historically, changes in MCV and MCHC were utilized to potentially identify a regenerative response. Normal reticulocytes are larger than mature erythrocytes and they have less hemoglobin content; therefore, the

classic profile for regenerative anemia would be increased MCV and decreased MCHC. In reality, this profile is seen in less than 10% of cases of regenerative anemia cases in the dog.

Red Cell Distribution Width (RDW) – The RDW is an electronic measure of anisocytosis and if there are a significant number of different sized erythrocytes, the RDW is increased. The two most common situations when this occurs are during regenerative anemia and iron deficiency anemia.

Reticulocyte hemoglobin content (Advia – CHr; XT-V and ProCyte Dx – RET-He) – These are sensitive indicators of recent production abnormalities of erythrocytes. Veterinary application is primarily with early identification of developing iron deficiency.

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VALUE OF RETICULOCYTE COUNTS IN NON-ANEMIC DOGS

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Reticulocyte counts have historically only been routinely requested when an anemia is present as an aid to distinguish between regenerative and non-regenerative anemia. However, two in-clinic hematology analyzers, IDEXX LaserCyte (2002) and IDEXX ProCyte Dx (2010), provided reticulocyte counts in all dog and cat CBCs. In 2012, the IDEXX Reference Laboratories, Inc. initiated a similar policy in reporting reticulocyte counts in all dog and cat CBCs. The driving force behind these policies was the fact that significant numbers of dogs without anemia have been identified in the general population and this finding can prove helpful in identifying significant erythrocyte pathologic changes or underlying metabolic disturbances before there is clinical evidence of disease. Early investigations (figure 1) into hematologic data from the field documented almost 10% of non-anemic canine samples demonstrated a significant reticulocytosis. Almost 300,000 CBCs were evaluated in this field investigation.

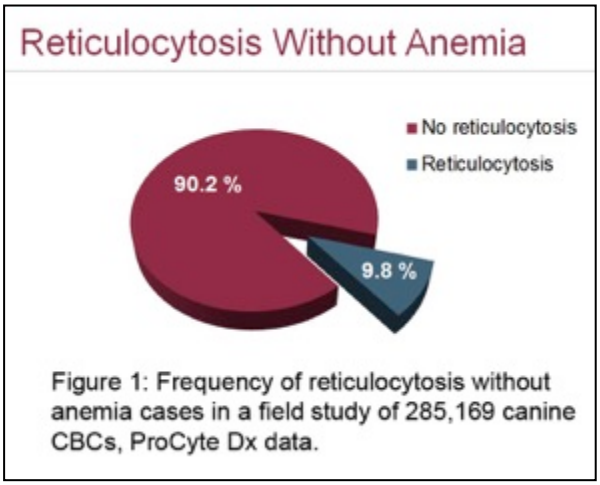
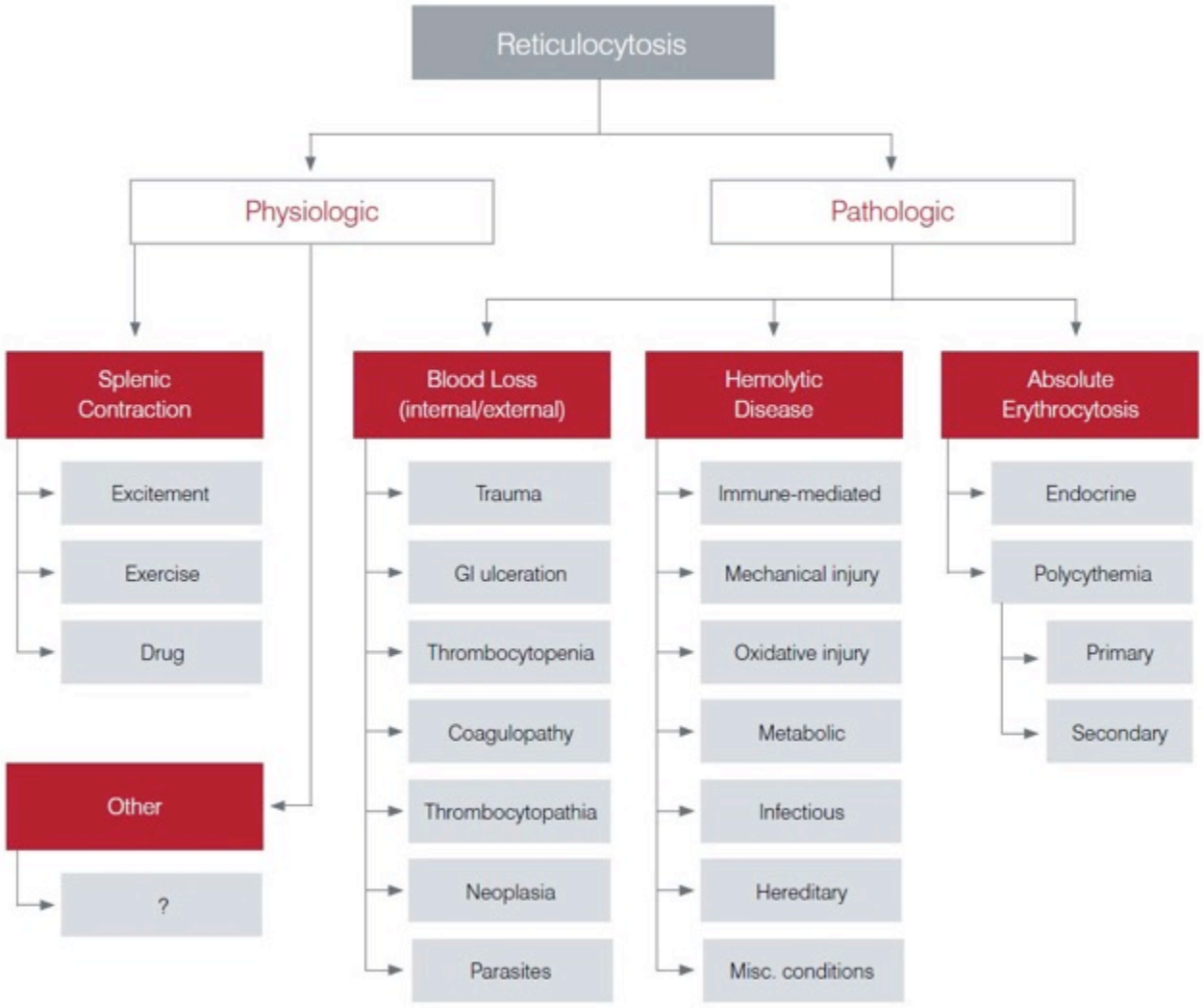


Figure 1: Frequency of reticulocytosis without anemia cases in a field study of 285,169 canine CBCs, ProCyte Dx data.

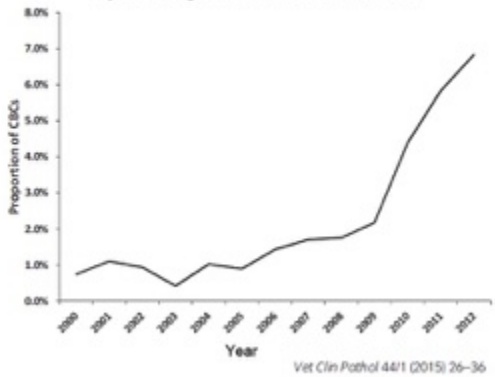
Pathologic Causes for Reticulocytosis in Non-Anemic Dogs: The bone marrow has tremendous capacity for responding with increased erythrocyte production when there is a demand. We typically only consider this when there is anemia; however, there are two primary situations when reticulocytosis is seen in the non-anemic dog. First consider the classic bone marrow response to either blood loss or hemolytic causes of anemia. During the recovery phase, the bone marrow continues to have an increased rate of production and release of reticulocytes until the animal's baseline erythrocyte mass (HCT, RBC or HGB). The reticulocytosis will gradually decrease until the return to normal oxygen carrying capacity is reached. The second situation is when there is a partially or complete compensated ongoing occult blood loss or underlying hemolytic disease, or an underlying disorder causing an absolute erythrocytosis. If there is low-grade blood loss or hemolytic disease, the bone marrow has the ability to adequately respond to maintain the erythrocyte mass within "normal" values. Essentially all blood loss and hemolytic diseases that cause anemia can be seen in the partially or fully compensated state. Figure 2 has a list of the general categories for both pathologic and physiologic reticulocytosis without anemia.

Figure 2
IDEXX Laboratories Expands the Information Included with Every Complee Blood Count
Diagnostic Update, IDEXX Laboratories, October 2012



Either there has been an increased prevalence of pathologic conditions resulting in these findings or we have not been recognizing the condition simply because we historically only have used the reticulocyte count to characterize the anemic patient. Both theories are viable. In fact, a recent publication documents an increased prevalence of reticulocytosis in non-anemic dogs (figure 3) between the years of 2000 and 2012. In this particular study, increased used of anti-inflammatory medications and nutraceuticals, associated with increased diagnosis of osteoarthritis were considered as contributing factors.

Figure 3: Prevalence of dogs greater than or equal to 1 year-of-age between 2000 and 2012



Physiologic Causes for Reticulocytosis in Non-Anemic Dogs: During normal erythropoiesis, relatively immature reticulocytes are often released from the marrow and these young reticulocytes often home to the spleen to

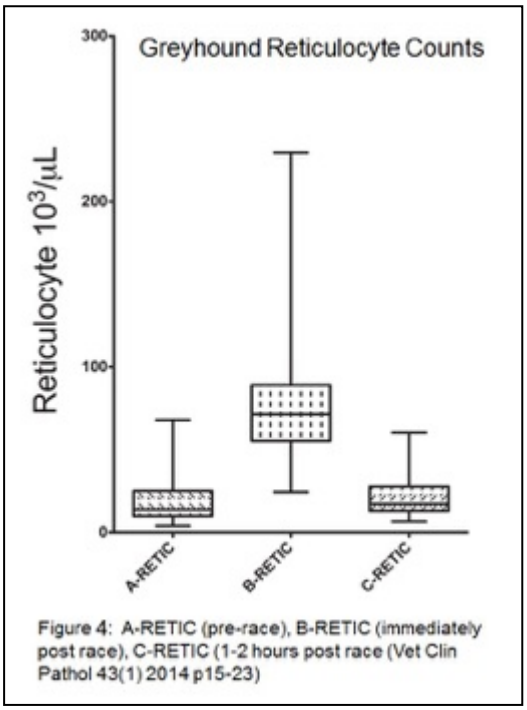


Figure 4: A-RETIC (pre-race), B-RETIC (immediately post race), C-RETIC (1-2 hours post race (Vet Clin Pathol 43(1) 2014 p15-23)

complete their maturation process. Surface markers including fibronectin and transferrin have been implicated in this homing process. If there is sudden splenic contraction, transient release of these young reticulocytes into the circulation is possible. After the cause for the splenic contraction is removed, the reticulocytes can re-home to the spleen to continue their maturation process. Although unknown how frequently physiologic reticulocytosis occurs in routine veterinary practices, splenic contraction and associated transient release of maturing reticulocytes can occur associated with excitement or exercise. Various laboratories have had anecdotal reports of mild to moderate reticulocytosis in dogs without anemia; these typically have been associated with excitement and therefore, a catecholamine-induced splenic contraction mechanism has been proposed. Similar catecholamine release associated with exercise is known and in a recent report, a post-race associated reticulocytosis has been documented in racing greyhounds. Blood samples were collected from greyhounds pre-race, immediately following a race and then one to two hours after the race. There was a significant increase in reticulocyte counts in the immediate post-race samples (Figure 4).

Summary: Our consideration of when reticulocyte counts should be performed must be dramatically broadened. Clear value in documentation of both pathologic and physiologic conditions resulting in reticulocytosis without anemia is evident. In the pathologic causes, the reticulocytosis could serve as a signal to the clinician that an underlying subclinical disease process is possible; this can lead to more detailed diagnostics and early disease detection with subsequent early intervention. Some of the types of cases detected primarily as a result of finding an unexplained reticulocytosis include: immune mediated hemolytic disease, Heinz body hemolytic disease, neoplastic disease involving the liver and/or spleen, cardio-pulmonary disease and others.

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