Interpreting blood results: Facts and fallacies

Dawn Seddon
Gribbles Veterinary Pathology, 57 Sunshine Avenue, Hamilton

Abbreviations
ALP (alkaline phosphatase), CBC (complete blood count), GGT (gamma-glutamyl transferase), MCV (mean cell volume), nRBCs (nucleated red blood cells), PCV (packed cell volume), RBC (red blood cells), URL (upper reference limit), WCC (white cell count), PCR (polymerase chain reaction), ELISA (enzyme-linked immunosorbent assay)

General considerations that affect haematological and biochemical interpretation

There are a number of factors that affect test results, which include the effect of variables other than the disease on the test result itself.

These are divided into pre-analytical factors; analytical effects, and post analytical factors.

Pre analytical variables

These affect the composition of the body fluid before analysis and include:
1. Biological variables which affect the patient
2. Non-biological variables which include factors that affect sample collection and handling.

Analytical variables

Include factors that influence the analytical procedure such as haemolysis, lipaemia and icterus of the sample. Understanding the limitations of in house analysers especially haematology analysers is also essential when interpreting data.

Post analytical factors

Involve the presentation of data from the laboratory, data storage and the transferral of data to the clinician. Derivation of reference intervals, the effect of sensitivity and specificity including prevalence of the disease will impact on interpretation of data.

Pre analytical

Inherent patient or biological factors which include:
Species
Breed
Strenuous exercise
Age
Excitement
Stress
Sex/Pregnancy
Drugs
Fasting period pre bleeding

Pre analytical/biological variables which affect the patient

Species
There are numerous species-specific differences which must be taken into account. Examples include the use of different liver parameters for large and small animals, the comparison of reference interval for the same species e.g. donkeys versus horses. Splenic contraction in horses can increase PCV by 40%, but an increase in PCV in colic cases is reported to be a negative prognostic indicator. Equine RBCs have high intracellular potassium and have an energy dependent Na/K pump, and thus hyperkalaemia can occur before there is appreciable haemolysis of the sample.

Breed
Examples which demonstrate breed differences include:
• Athletic dogs such as greyhounds or working border collies have higher PCV & MCV's, and higher muscle mass (they tend to have higher levels of creatinine).
• Poodles have macrocytic RBC’s while Akitas and Shiba inus are microcytic.
• The potassium content of RBCs of these latter breeds (Akitas, and the Shar pei) are higher than other breeds which result in a pseudohyperkalaemia.
• Cavalier King Charles spaniels have large platelets (macrothrombocytosis) and have pseudothrombocytopenia.

Strenuous exercise
Racehorses and racing greyhounds have increases in RBC count, haemoglobin concentration and PCV.

Horses and dogs post racing demonstrate a leukocytosis with an absolute mature neutrophilia.

Age
Young animals have different age related findings. Most laboratory reference intervals used are for adults, and thus a number of perceived abnormalities are seen relative to the adult interval.

At 2-3 months of age, foetal RBC of puppies and kittens are replaced by adult RBC’s and a decrease in MCV and PCV are seen, but there should be evidence of regeneration.

Red cell indices reach adult values by 9-12 months (puppies) or 3-6 months (kittens) of age.

Normal neonatal puppies (<10 days) can have mild increases in band neutrophils and small numbers of metamyelocytes (earlier neutrophils), while kittens can have a mild leukocytosis at 2-3 months of age, which declines by 5-6 months to adult values.

Foals have higher ratios of neutrophils compared with lymphocytes at birth, but ratios are equal at 3-4 months.
In calves, neutrophils are the dominant cell until two weeks of age, but by three months, lymphocytes predominate and comprise 70-80% of the WCC.

Young animals also have a number of biochemical differences as compared with adult reference intervals.

They have decreased proteins which should reach adult levels by about 6-9 months of age, (can be breed dependent in dogs), and which can be up to 10-20g/l less than adult values.

The decreased muscle mass causes a decrease in creatinine.

Kittens and puppies can have ALP increases 20-30x URL post suckling (present in colostrum) which returns to 5-6 x URL by 10 days.

ALP stays increased for up to a year of age and is attributable to the bone isoenzyme due to growing bones.

Siberian Husky pups have a 6 x increase in ALP as compared to other breeds.

Adult Scottish terriers may have high ALP but with no overt liver malfunction.

Calves have a 3 fold increase in ALP 1-2 days after colostral intake which returns to pre-suckling levels by day three.

Pre suckling foals can be up to 20 x URL for adult horses which decreases to 5 x URL by 3 weeks of age. However, colostral increase in ALP is not seen in horses.

ALP is generally not widely used in large animals (GGT is the enzyme of choice to evaluate the cholestatic status).

Calves have a 20 x increase in GGT post suckling, and which can be used as an indicator of passive transfer.

Puppies demonstrate a GGT increase of up to 100x URL post suckling, which returns to reference levels within 10 days. GGT is also high in queen colostrum, but is not found in mare colostrum, although foals have GGT increases 1.5-3 x URL.

Young animals have increased calcium and phosphate levels as compared with adults (puppies and kittens can be double the quoted adult reference intervals).

A high calcium phosphate product can predispose adult animals to metastatic calcification of soft tissues but this does not seem to occur in young animals.

Increased bilirubin can also be seen in neonatal animals.

**Excitement**

Adrenalin release can cause a physiological leukocytosis with a mature neutrophilia and an absolute lymphocytosis (mainly seen in cats).

Lymphocytes are higher than neutrophils in cats for approximately 30 minutes post adrenalin response.
**Stress**
Stress is caused by release of either endogenous or use of exogenous corticosteroids. A typical stress response includes a mature neutrophilia (which is usually less than 2x top normal reference interval), monocytosis, lymphopenia (or low normal lymphocytes) with an eosinopenia.

Not all four components are generally seen together, but the typical findings are commonly manifested by a mature neutrophilia and a lymphopenia/low normal lymphocytes.

A single injection of exogenous corticosteroid such as dexamethasone can result in a stress leukogram within four hours, and which may persist for up to 24 hours.

Neutrophils shift from the marginating to the circulating pool and there is an increase in circulation time of neutrophils.

**Sex/Pregnancy**
Pregnancy reduces PCV, haemoglobin and red cell count, but these parameters return to normal during lactation.

A stress leukogram may occur during late pregnancy in the dog.

In birds, different reference intervals are often used for different sexes.

**Drugs**
A minimum database should be obtained before administering intravenous fluids or other drugs which may alter haematological and biochemical parameters.

Intravenous fluids, sedation or anaesthesia may decrease the PCV (haemodilution). PCV can decrease by up to 15% with the use of drugs such as ACP (acetylpromazine) or Ketamine due to splenic sequestration of RBC.

Interpretation of results should always be made with full knowledge of the animal’s medication history.

**Fasting period pre bleeding**
If at all possible, animals should be fasted for eight hours prior to bleeding.

This helps prevent lipaemic samples and post prandial increases in glucose.

Many birds can be fasted overnight (eight hours), however fasting should be done with caution in debilitated birds or smaller species.

**Pre-analytical – non-biological/sample variables**
Sample collection, handling and separation of sample and transport to the laboratory all affect the condition of the sample.

**Sample collection - venipuncture**
A clean venipuncture technique minimises artefactual changes (such as haemolysis), but is also imperative for coagulation testing.

The correct technique for filling tubes should be used to obtain a “clean” venipuncture with no tissue contamination. The sample should be taken from a large vein (generally jugular but
cephalic can be used in large dogs).

A full CBC and biochemical profile requires approximately 5ml blood.

Pediatric (small) tubes (1.3ml) are available for small animals such as cats and small dog breeds.

Tubes for sample collection are colour coded with different coloured stoppers:

**Serum tubes (red) do not contain anticoagulant**

Blood is expected to clot so that serum can be harvested for biochemical profile and many other tests. Serum tubes (red tops) cannot be used for haematology. Blood is allowed to clot before centrifugation which separates out the blood components. The serum is separated from cells and can be placed into a new tube (no anticoagulant). The tube should then be labelled “serum”.

**Serum separator tubes (red and black)**

Serum separator tubes (SST)/Gel tubes or Corvac tubes can be used which separates serum from clotted RBC’s by a layer of inert silicone, without transferring serum to a different tube.

Whole blood is allowed to clot, followed by centrifugation which forces the RBC below the silicon gel and the serum above the gel.

**Heparin (green)**

Heparin works by activating antithrombin III which blocks thrombin from clotting blood.

Heparinised plasma is used for specific tests e.g. Relaxin testing for pregnancy in dogs. Heparin can also be used in exotic animals for both chemistry and haematology, but has the disadvantage of causing morphologic changes in white blood cells.

Heparin is not recommended for collection of blood for mammalian haemogram interpretation, due to the morphological changes of leukocytes. It also fails to prevent platelet aggregation.

Lithium heparin or EDTA samples can be used for haematology in snakes and lizards, but chelonian haematological samples should be collected in lithium heparin only as EDTA lyses RBCs.

Citrate should be avoided in all of these species as it may also result in increased cell lysis.

**Fluoride oxalate (grey)**

Most mammalian RBC’s use glucose for energy (pigs use inositol, and birds use amino acids).

Fluoride oxalate anticoagulant (sodium salt) inhibits glucose metabolism by RBC, WBC and platelets.

Glucose values decrease by 10% per hour in un-separated serum and the decrease in glucose is enhanced if there are increased leukocyte or platelet counts.

Prompt separation is recommended as sodium fluoride is hypertonic and causes lysis of RBCs which in turn causes release of intra-erythrocyte water which dilutes the glucose concentration. Glucose concentrations from sodium fluoride samples are lower than in promptly separated serum samples by approximately 7-12%.
Lipaemia and haemolysis may interfere with methodology.

**Sodium citrate (blue)**
Sodium citrate is in liquid form in the tube and is used for coagulation studies and for blood transfusion bags.

It can also be used in the form of ACD (acid citrate dextrose), and its mechanism of action is to bind calcium (which is a cofactor used in many steps of coagulation).

The correct proportion of citrate to blood is crucial because of the dilution – i.e. tubes should be filled to labeled capacity.

Whole blood is collected with minimum trauma into 3.2% sodium citrate in a ratio of 9 parts of blood to 1 part citrate.

Citrate can be used for CBC (although not routinely recommended) but a calculation is required to compensate for the dilution.

Citrate and EDTA anticoagulants should not be used for chemistry.

Short or under filled citrate tubes may falsely prolong coagulation times by more than 5 fold due to incorrect blood to anticoagulant ratio.

Under filled and/or haemolysed samples cannot be evaluated as they have often been activated during collection.

Activation of samples may cause reduction or prolongation of clotting times.

**EDTA (lavender/purple top tubes)**
EDTA is a potassium salt of ethylene-diamine-tetra-acetic acid which preserves cell morphology.

Adequately filled tubes are required for CBC.

Fresh smears should be made at the time of taking blood sample and which should be submitted with the full blood.

Blood samples can be refrigerated at 4°C if there is a known delay in testing, but the smears should not be refrigerated as condensation ruins the smears.

EDTA or citrate samples for haematology should not be frozen, but citrated plasma may be frozen for coagulation studies.

A short (under) filled tube has excessive EDTA relative to the blood volume which causes artefactual changes in RBC parameters such as false decreases in PCV and RBC count. It also causes shrinkage of RBC's which artefactually reduces the MCV.

Under filling also causes a false increase in total plasma proteins.

It is important when taking blood samples, that EDTA contamination of serum tubes does not occur - this markedly increases the potassium and decreases the calcium.
EDTA (best) or heparin anticoagulants can be used for bird haematology but there may be different lab preferences.

Ostrich and emu samples are best collected into acid citrate dextrose (ACD – yellow top) to the full mark.

A note about cats…and small breed dogs:
• Slow or difficult bleeds may cause minute clots to form, platelet clumping and thus WBC counts and platelets may be artefactually low.
• It is impossible to get an accurate CBC from a clotted sample as cells will be in the clot, and thus a fresh EDTA is required.

Reticulocyte count
If animals are anaemic, reticulocytes counts should be performed. Reticulocytes must be stained with a supravital stain e.g. new methylene blue which has to be added to the full blood (EDTA sample) before making the smear.

Reticulocytes (“retics”) are young, anucleate RBC, and are the hallmark of regenerative anaemia, and represent a bone marrow response (not seen in peripheral blood of horses).

Reticulocytes in normal dogs make up 1-2% of the RBC’s and in cats 0.5-1%.

After acute blood loss or destruction it takes 72 hours before reticulocytes are seen in peripheral circulation. Maximal production is seen after seven days.

Absolute reticulocyte counts should be used which corrects for the variation in RBC number i.e. the degree of anaemia:

Other pre analytical - non biological sample variables

Storage and transport: courier services should offer speedy sample delivery.

If there is a known 24-hour delay, prompt separation of serum/plasma from cells is recommended for chemistry samples.

Samples should be kept cool with an icepack in the summer months. This is essential to maximise enzyme stability and prevent artefactual changes.

If a long (>24 hour) delay is anticipated, then samples should be separated and frozen for chemistry, and sent to the laboratory frozen, on dry ice if possible.

Correct labelling of the sample is important with the patient name or identification, the type of specimen (if not in the original tube), and if a body fluid should be labelled appropriately.

Fluid samples for cytology should be sent in both serum tubes for culture and EDTA for cytology.

Analytical variables

These include features inherent to the sample e.g. haemolysis, lipaemia, icterus and the analyser.

This is the degree to which endogenous substances interfere with the assay, and depend on the
type of analyser, methods used to detect the analytes, and the amount of interfering substances, all of which may be laboratory-specific. Understanding the limitations of in house bench top analysers are also of prime importance in assessing haematological results.

Causes of artefacts that affect sample quality:

**Haemolysis**
Haemolysis is defined by damage to the red blood cell membranes which causes liberation of haemoglobin.

RBC’s of small animals are more susceptible to lysis than other species.

Evidence of haemolysis can be seen on separated plasma or serum, but is not always evident on whole blood samples.

**Causes of Haemolysis**
*In vivo* - haemolytic anaemias.

*In vitro* - most cases are attributable to poor venipuncture technique, either due to excessive suction or expelation, too narrow a needle, vacutainer too large, or excessive shaking.

Other causes include freezing of whole blood samples, delayed sample submission (ageing), high environmental temperatures, and osmotic change - contact with hypotonic solutions from water or condensation, chemicals (e.g. alcohol, ether, acids), lipaemia, or incorrect volume of blood from the anticoagulant.

Haemolysis may falsely increase or decrease certain analytes (depending on methodology).

Certain breeds have higher potassium levels in their RBCs such as Akitas, Shiba inu, Shar pei, but this is also seen in horses.

**Lipaemia**
Hyperlipaemia/hyperlipidaemia is the visible turbidity (milkiness) due to the presence of triglycerides, and gives the sample the appearance of “cream of tomato soup”.

Lipaemia or large numbers of Heinz bodies can increase haemoglobin artefactually, as the lipaemia affects turbidity readings on photometric measurements.

Total plasma protein by refractometry will also be falsely elevated.

Triglycerides are usually in the form of lipoproteins, which are the largest fat particles absorbed from the small intestine and which are transported in the blood as chylomicrons or VLDLs (very-low-density lipoproteins).

Chylomicrons appear 30 minutes - six hours (average 1-3 hours) after feeding and can remain for 6-12 hours.

Miniature Schnauzers may require longer fasting for lipaemia to disappear.

Many parameters are affected by lipaemia – the worst affected are bile acids which are markedly increased with lipaemia, while electrolytes tend to be decreased due to displacement effect.
Lipaemia of the sample actually causes in vitro haemolysis.

**Icterus**

Bilirubin interference arises from its spectral properties and its ability to react chemically with other reagents.

Other parameters that affect analytical variables include hyperproteinaemia and certain drugs.

High concentrations of monoclonal immunoglobulins interfere with chemistry analysis e.g. hyperkalaemia is reported in dogs with lymphocytic leukaemia and an IgM monoclonal gammopathy.

High protein concentrations (usually >100g/l) produces similar volume displacing effects on electrolyte concentrations as does lipaemia.

There are certain methodology related drug interferences e.g. artefactual elevation of chloride values in dogs on bromide therapy.

**Haematology analysers**

A number of different technologies are used for in house haematology analysers which include impedance, laser, flow cytometery, and the QBC (IDEXX – old technology).

Most of the bench top analysers used in practice are based on impedance technology which is dependent on the principle that electrically neutral blood cells pass through an electrically charged aperture. Each cell causes a change in electrical resistance which impedes the current flow as the cell passes through the aperture, and a pulse is detected which is then amplified by the instrument. The differentiation of cell size is made on the magnitude of electrical current interference, and the basic cell types (red, white cells, and platelets) are determined by the size variation and susceptibility to various lysing agents.

Most impedance counters are designed for human cells and thus special modifications of software are required for veterinary species.

RBC of some species such as goats, sheep and some horses are too small to be reliably detected. e.g.

<table>
<thead>
<tr>
<th>Species</th>
<th>MCV - Erythrocyte Size (fl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog</td>
<td>60-72 fl</td>
</tr>
<tr>
<td>Cat, horse, cow</td>
<td>39-52 fl</td>
</tr>
<tr>
<td>Sheep</td>
<td>25-35 fl</td>
</tr>
<tr>
<td>Llama</td>
<td>21-29 fl</td>
</tr>
<tr>
<td>Goat</td>
<td>15-25 fl</td>
</tr>
<tr>
<td>Human</td>
<td>80-100 fl</td>
</tr>
</tbody>
</table>

The disadvantages of impedance technology is the poor differentiation between WBC and nRBC’s, aggregates of platelets may be counted as WBC (cats), but large platelets (cats some dog breeds) are often counted as RBC.

With impedance technology, leukocyte clumping leads to artefactual leukopenia.

Automated machines do not replace a manual differential however sophisticated the machine,
and thus manual differentials should be performed on all blood smears.

When submitting blood for a CBC, submission of a labeled blood smear, made at the time of sampling along with an EDTA tube is recommended.

The smear should be on correct side of slide, (and labelled).

EDTA can cause parasites such as *Mycoplasma* (*Haemobartonella*) to fall off the cells, and thus a fresh blood smear examination is required.

**Disadvantages of blood machines**

Leukocyte differentials from all instruments are less accurate as compared with the Advia or manual methods.

There are a number of cells and changes that cannot be detected without examination of the blood smear.

Instruments cannot differentiate band neutrophils from mature segmented neutrophils, and cannot detect RBC morphological changes, blood parasites (including microfilaria), reactive lymphocytes, blast cells, and mast cells.

**Data interpretation**

All relevant information must be used such as signalment, history, physical examination, and laboratory data. It is thus very important to submit samples with full signalment and history.

Results may exclude some differential diagnoses, may suggest additional differential diagnoses, or may be diagnostic for a specific disease.

Appropriate reference intervals should be used to interpret results, and a working knowledge of sensitivity and specificity of the test is important in for analysing results.

Results that are most outside of the reference interval are likely the most significant (possibly with the exception of enzymes).

Pattern Recognition of abnormal results is critical for making a diagnosis. e.g. an increased creatinine and urea (azotaemia) and a urine specific gravity indicating that animal is not concentrating urine is very suggestive of renal disease, but an increased creatinine and urea with a concentrated urine, indicates that animal is dehydrated or had blood loss into the gastrointestinal tract or had a very high protein meal.

**Reference Intervals**

Reference intervals should be established by each laboratory, and are usually determined from a population of healthy adult animals. Data should be distributed symmetrically around the mean (Gaussian Distribution). The reference interval is calculated as the mean + 2 SD, which includes 95% of normal samples regardless of the method.
A few (5%) healthy animals will have results slightly outside of the reference interval (2.5% either side).

If the result is far outside the reference interval, it probably indicates an abnormality.

One laboratory’s results should not be compared with another laboratory’s reference intervals as the intervals vary within laboratories depending on the instrumentation or reagent. Intervals are methodology dependent (wet versus dry chemistry) and serum enzymatic methods vary in pH, temperature, and specific cofactors or substrates.

Slight abnormalities in some test results, such as electrolytes, are more significant than slight abnormalities in others, such as serum enzyme activities.

Sensitivity is the positivity in disease. e.g. If the sensitivity of PCR for lymphoma is 91%, then 91% of animals with lymphoma will have a positive result and 9% will have a false negative result.

The specificity of a test is the negativity in health. If the specificity of a test is 95%, then 95% of unaffected (“normal”) animals will have normal results, but 5% will have false positive results.

**Prevalence**

This is the number of cases present at any time in a population, and false positive results are more likely to occur at a low disease prevalence.

If a test gives an occasional false positive, it is highly significant if there are only a few cases that are truly positive in the population.

An example of how predictive value impacts on clinical cases can be demonstrated using FeLV (Feline leukaemia virus). E.g. FeLV has a low disease prevalence in New Zealand – and may be as low as approximately 1%.

At 1% disease prevalence, using an ELISA test kit with a 95% sensitivity and specificity, statistically only 16.1% of cats testing positive for FeLV are truly infected with the disease.

This is of concern with diseases of low prevalence such as FeLV, in that most of the cats tested for FeLV in New Zealand that give a positive result that will be false positive.

Even if the sensitivity of the test is increased to 98%, the positive predictive value only rises to 33% which still means that two thirds of positive tests are false positives.

**References**

Barton M *et al.* Hemostatic indices in healthy foals from birth to one month of age. *Journal of Veterinary Diagnostic Investigation* 7, 380-385, 1995


Harper E *et al.* Age-related variations in hematologic and plasma biochemical test results in Beagles and Labrador Retrievers. *Journal of the American Veterinary Medical Association* 223 (10), 2003
