PRACTICAL HEMATOLOGIC TECHNIQUES

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Blood Smear Preparation

Veterinary nurses with an interest in clinical pathology are certainly capable of examining a stained blood smear. However, since smears can be sent to a clinical pathologist for an expert opinion, there is little need for nurses to become highly competent cytologists. There is, however, a need for all veterinary nurses to become adept at making good quality blood smears, since a freshly-made smear may preserve diagnostic features that will otherwise be lost during the transportation of anticoagulated blood. Manual estimates of platelet counts, and the microscopic visualization of RBC parasites, for example, are both far better performed on a smear made from a freshly collected blood sample.

Blood smears are prepared from well-mixed blood placed in an EDTA anticoagulant tube. A microhematocrit capillary tube is used to transfer a small drop of blood to one end of a clean microscope slide. A second angled slide is then slid backwards into the blood drop for an instant, and then smoothly slid forward along the first slide, dragging a thin smear of blood from the droplet along the slide. The freshly-made smear is rapidly air-dried, and may then be sent out unstained to an outside laboratory, or stained in-house. Although inexperienced veterinary nurses can have some trouble producing a high quality smear, with practice and repetition this is a simple technique that can be mastered by all nurses.

Buccal Mucosa Bleeding Time (BMBT)

The BMBT is the time until cessation of bleeding from a standardized incision made in the buccal mucosa. The BMBT detects abnormalities of primary hemostasis (platelet number/function, and vascular integrity). Theoretically, small vascular defects may be plugged by platelets alone, with no requirement for a functional clotting cascade. However, the BMBT may also be slightly prolonged in patients with defective secondary hemostasis (e.g. hemophilia A). Since vascular disorders causing hemostatic defects are rare, the BMBT is usually considered to be a specific test of platelet function if platelet number is adequate (over 100 x 10^9/litre) and secondary hemostasis is normal (normal ACT, PT and PTT).

Materials

Simplate (Organon Teknika), or Triplett (Helena):

- Spring-loaded disposable cutting device which makes one or two linear incisions of a standard length and depth. There are also pediatric versions available which are convenient for cats and small dogs. Although the best results are obtained with the specialized device, a scalpel blade may be used when a device is not available.
- 5 cm width gauze strip.
- Circular filter paper.
**Technique**

Position the patient in sternal or lateral recumbency. Sedation/anesthesia is usually not required for dogs. Lightly sedate cats with ketamine/acepromazine. Firmly tie a gauze muzzle around both mandible and maxilla, folding up the top lip on one side (although, in anesthetized cats, it is easier just to tie the gauze muzzle around the maxilla and across the hard palate, leaving the mandible untied). Place the Simplate device on the buccal mucosa of the upper lip, and push the trigger. Gently blot below the incision with filter paper until bleeding stops. Avoid blood running into the mouth, and avoid touching the incision with filter paper. Measure the time from incision until cessation of bleeding (paper no longer soaks up blood).

**Normal**

*Dogs:* Less than 5 minutes (but typically less than 3-4 minutes - 5 minutes is 'suspicious').

*Cats:* Less than 2-3 minutes.

**Prolonged BMBT**

If platelet number is normal, suspect defective platelet function:

- Hereditary thrombocytopenia
- Von Willebrand's disease
- Uremia
- Hepatopathy
- Drug-associated (esp. aspirin and other NSAIDs)

In some studies, a prolonged BMBT has been reported to be associated with excessive bleeding during subsequent surgery.

**Indications**

Since there are no other readily available tests of platelet function available to most practitioners, the BMBT should be part of the diagnostic work-up of any animal with a potential hemostatic defect and an adequate platelet number. The BMBT is not sensitive to subtle functional defects (such as mild vWD), and is not entirely reliable in terms of reproducibility, but it is still the best overall test that we have available for primary hemostasis. The BMBT is certainly sensitive enough to spot major hemostatic deficits, such as severe vWD.
Activated Clotting Time (ACT)

The activated clotting time is the time taken for whole blood to clot in a glass tube containing a contact activator such as diatomaceous earth. The ACT detects abnormalities in the intrinsic and common pathways of the clotting cascade (that is, secondary hemostasis), particularly clotting factor deficiencies, or the presence of a clotting inhibitor. Deficiencies affecting only the extrinsic pathway of secondary hemostasis (that is, congenital factor VII deficiencies or very early anticoagulant rodenticide poisoning) may have a normal ACT. However, since defects affecting only the extrinsic pathway are uncommon, the ACT is in reality a reasonable (albeit crude) test for most of the common disorders of secondary hemostasis. The ACT may also be mildly to moderately prolonged in the presence of very severe thrombocytopenia (platelet number < 10 x 10⁹/litre), as platelet factor 3 (PF 3) is essential for a normal ACT.

In summary, the ACT is a simple, reasonably accurate test for significant abnormalities of the intrinsic and common pathways if platelet number is adequate.

Materials

Glass ACT vacutainer tube containing siliceous or diatomaceous earth as a contact activator (Becton Dickinson, Rutherford, New Jersey).

Optional: 38°C water bath or block heater.

Technique

- Using a clean venipuncture technique, collect and discard the first 1-2 mls of blood obtained (leave needle in vein). Collect a further 2 mls, and place in an ACT tube. Although this was the standard published technique, in all honesty many clinicians (myself included) simply collect the first 2 mls and put them directly into the ACT tube. In my opinion, I do not think that this significantly affects results.
- Gently invert the tube to mix, then leave undisturbed for 45 seconds. Thereafter, gently tilt the tube every 5-10 seconds, until an obvious clot begins to form. Complete clotting usually occurs within the next 15 seconds. The end of the ACT is timed from the point when clotting was first observed.
- Measure the time from addition of blood to the tube until clotting is first noted. There appears to be little practical extra benefit in the determining both the time that subtle clotting is first observed ('soft' clot) and the time at which a clot is obvious and solid ('firm' clot).
Normal

**Dogs:** 38°C: Less than 125 seconds.

*Room Temperature:* Less than 130 seconds (slightly less reproducible than at 38°C).

**Cats:** Less than 165 seconds. This is a new figure derived from a recent study, and is significantly longer than the previously quoted 65 seconds. Since I could rarely get an ACT in a cat of less than one minute, I think that the new figure is probably more accurate.

Prolonged ACT

A prolonged ACT suggests either a clotting factor deficiency or clotting inhibitor, if platelet number is adequate.

**Deficiency:** Hereditary (e.g. severe hemophilia A or B)  
Acquired lack of production (e.g. severe hepatopathy, or vitamin K antagonist)  
Excessive consumption (e.g. DIC or hemangiosarcoma)

**Inhibitors:** Heparin  
Fibrin degradation products (FDP) released by DIC

Indications:

The ACT tests the same clotting pathways (intrinsic and common) as the PTT, but is less sensitive and, in my opinion, a little less reproducible. Mild abnormalities of these pathways may therefore have a prolonged PTT, but a normal ACT. A prolonged ACT usually indicates a severe, clinically significant coagulopathy.

Since most veterinarians still don't have immediate access to PT and PTT results, the ACT is indicated in emergency situations where an answer is needed now. In these circumstances, I believe that the ACT does a reasonable job at identifying severe deficits, such as advanced rodenticide poisonings or severe hemophilia. The ACT is therefore part of the routine emergency work-up of suspected hemostatic defects if a PT (or PIVKA, as a reasonable PT substitute) or PTT are not available. However, I typically follow up with a PT and a PTT when available.

The classic tube ACT technique may become obsolete as new in-house analyzers (such as the SCA2000) become available that can measure PT, PTT and ACT. Currently, however, there is little data available comparing the utility and accuracy of the ACT tube technique with the newer in-house analyzer methods.
Evaluation of Platelet Numbers

Platelet numbers may be rapidly and relatively accurately estimated via examination of a stained fresh blood smear, or exactly quantitated by use of either a manual counting technique or an automated hematology analyzer. Blood for evaluation of platelet numbers should be collected into anticoagulant (usually either EDTA or sodium citrate) using a clean venipuncture technique, and preferably processed within a few hours of collection. In some animals, especially cats, spontaneous clumping of platelets can occur following the storage of anticoagulated blood for only a few hours, and may erroneously lower platelet counts (pseudothrombocytopenia).

**Direct Smear Technique**

*Microscopic examination of an air-dried stained blood smear is a quick and reliable method of detecting significant thrombocytopenia. In normal animals, 10 to 30 platelets will be seen in each oil immersion (1,000 power) monolayer field. To obtain an estimated platelet count (x 10^9/litre), the number of platelets within 10 consecutive oil immersion fields is counted, divided by 10 (thereby obtaining an average number per field) and then multiplied by 20. The edges of the blood smear should be scanned (particularly if platelet numbers are estimated to be low) to ensure that thrombocytopenia is not simply an artifact due to excessive platelet clumping.*

*Examination of a direct smear also permits the identification of megathrombocytes ('shift' or 'stress' platelets), large platelets that are at the same size as or bigger than adjacent red blood cells. Megathrombocytes are (arguably) believed to be young platelets with increased hemostatic activity that are produced in response to thrombocytopenia. The presence of numerous megathrombocytes in a thrombocytopenic patient strongly suggests that the marrow is actively producing platelets to compensate for an increased rate of platelet loss.*
Diagnosis of Immune-Mediated Hemolytic Anemia

Hematology in patients with IMHA typically reveals a moderate to severe anemia, which is most commonly regenerative, with anisocytosis, polychromasia, a high corrected reticulocyte count and, sometimes, increased numbers of nucleated RBCs. Reticulocyte counts can however sometimes be inappropriately low, either because antibodies are also directed against RBC precursors, or because anemia is peracute (since it takes about 5 days for the marrow to mount a strong regenerative response). White cell and neutrophil counts are often moderately to markedly increased, probably in response to both non-specific marrow stimulation and the inflammatory process associated with RBC breakdown. Occasionally, white cell counts can be high enough to mimic myelogenous leukemia, a reaction sometimes called a ‘leukemoid response’. Platelet counts are usually normal unless the animal also has immune-mediated thrombocytopenia (IMT). Concurrent IMHA and IMT, a condition known as Evan’s syndrome, may affect up to approximately 10% of dogs with IMHA.

Hematology can often also reveal clues that suggest a specific etiological diagnosis:

1. **Spherocytosis:**

   Spherocytes are small spherical erythrocytes that, when present in high numbers, strongly suggest a diagnosis of either primary or secondary IMHA. The absence of spherocytes, however, does not absolutely exclude a diagnosis of IMHA. Spherocytes are formed when tissue macrophages remove a piece of RBC membrane without cell destruction or a significant loss of cytoplasm. Spherocytes can be difficult to recognize in cats, because normal feline RBCs tend to be smaller and less discoid than canine RBCs. Experienced veterinary clinical pathologists, however, may be able to recognize the presence of spherocytes in the cat.

2. **Agglutination:**

   Examination of blood smears may reveal microscopic autoagglutination (clumping) of RBCs. Such agglutination can form large rafts of RBC that, when a collection tube containing anticoagulated blood is closely inspected, are visible to the naked eye as multiple red speckles. Similar speckles can however be created by rouleaux formation, a phenomenon that can occur in normal animals, especially cats. Clinicians should therefore perform a saline dilution (one drop of RBCs to one drop of saline in dogs, one drop of RBCs to two drops of saline in cats) slide
agglutination test to differentiate rouleaux from genuine autoagglutination. True agglutination can be seen grossly as persistent speckles despite dilution with saline, and microscopically as non-linear clumps of RBCs.

**Mechanism of a Positive Slide Agglutination**

A  Antibody-Mediated Agglutination

![Diagram](image)

**Clearance of Rouleaux with Saline**

B  Rouleaux Formation

![Diagram](image)
A positive slide agglutination result is highly suggestive of a diagnosis of IMHA, and also suggests that the condition is likely to be acute and severe. A negative slide agglutination does not rule out IMHA, since in fact a negative result is reported to be the most common result in small animals with IMHA because most actually have non-agglutinating antibodies. Recent clinical studies of canine IMHA, however, report a much higher incidence of positive slide agglutination, perhaps reflecting a referral bias as a result of practitioners tending to refer only the more severe cases of IMHA. Automated hematology analysers sometimes register a clump of agglutinated RBCs as a single cell, often of a size too large to even be recorded as a RBC at all. Resultant erroneous results may include an artifactually high MCV or, if clumped cells are not recognised as erythrocytes, lowering of the calculated hematocrit. Since the hemoglobin within all RBCs is still measured by the analyser, this leads to an erroneously high estimation of mean corpuscular hemoglobin concentration (MCHC). When agglutination is suspected to be the cause of a lower than expected hematocrit, packed cell volume (PCV), which is not affected by RBC clumping, should be monitored using microhematocrit tube centrifugation rather than an automated analyser.

3. Other RBC Abnormalities:

Careful examination of RBC morphology may suggest an underlying cause of either immunological or non-immunological hemolysis. Diagnostically useful RBC abnormalities include detection of parasites such as *M. hemofelis* (which may cause secondary IMHA), Heinz bodies (suggesting hemolysis secondary to oxidative damage) and schistocytosis (suggesting a microangiopathic hemolytic process such as DIC).