Current Diagnostic Trends in Coagulation Disorders Among Dogs and Cats

Marjory B. Brooks, DVM*, James L. Catalfamo, MS, PhD

KEYWORDS

• Coagulopathy • Fibrinolysis • Hemorrhagic disorders • Hemostasis
• Platelet aggregometry • Platelet function • Thrombin(IIa) generation
• Thrombelastography

KEY POINTS

- Include hemostatic defects in the initial differential diagnosis of patients with signs of hemorrhage.
- Collect appropriate samples for platelet count and coagulation panel early in the diagnostic workup. Remember that sample quality is critical for valid results.
- Use results of initial screening tests and patient response to guide further testing.
- New techniques such as flow cytometry, thrombin-generation assays, thrombelastography, and anticoagulant drug monitoring are under investigation for veterinary patients; however, their ability to improve diagnosis or treatment requires further study in clinical trials.

INTRODUCTION

Hemostasis Overview

Hemostasis is a complex process involving temporospatially regulated interactions among the blood vessel wall and circulating platelets, membrane-associated tissue factor (TF), and procoagulant, anticoagulant, and fibrinolytic plasma proteins.[1] In healthy vessels, the balance favors anticoagulant reactions that maintain blood in a fluid state flowing at high pressure in a sealed compartment (**Fig. 1**). Blood vessel injury tips the balance to favor platelet and procoagulant factor activation that results in a burst of sustained thrombin (factor IIa) generation, which in turn leads to fibrin clot formation and cessation of blood loss. Thrombin-mediated feedback loops

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Comparative Coagulation Section, Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, 240 Farrier Road, Ithaca, NY 13501, USA
* Corresponding author.
E-mail address: mbb9@cornell.edu

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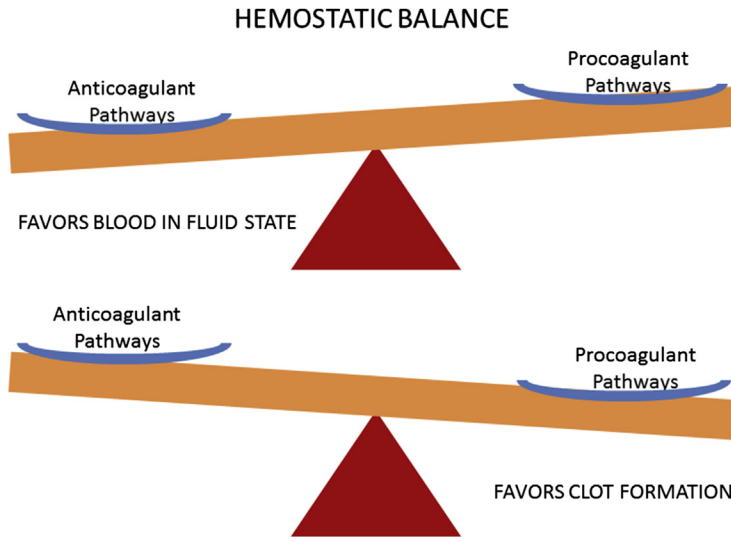

Fig. 1. Hemostatic balance. Blood circulates in a fluid state because anticoagulant reactions are slightly favored. Injury tips the balance toward procoagulant pathways and clot formation. An inadequate procoagulant response caused by deficiencies or dysfunction of platelets and clotting factors, or dysregulation of fibrinolysis, causes hemostatic imbalance and clinical signs of a bleeding diathesis.

simultaneously trigger activation of anticoagulant proteins that limit clot size, with the subsequent actions of the fibrinolytic pathway promoting clot dissolution and wound healing (**Fig. 2**).

Clinical Classification of Bleeding Disorders

The diagnostic workup to differentiate hemorrhage caused by vascular injury from a systemic hemostatic imbalance typically involves a combination of broad screening tests and specific assays. The characterization of 3 overlapping phases of primary hemostasis, secondary hemostasis, and fibrinolysis provides a simple diagnostic framework for evaluating patients with clinical signs of hemorrhage (**Fig. 3**).

Primary hemostasis

Platelets play a dominant role in primary hemostasis. At the site of injury they rapidly tether, change shape, spread, and firmly anchor to exposed subendothelial proteins.[2] Platelet adhesion requires the presence of von Willebrand factor (VWF), and the exposure of collagen and other adhesive proteins in the subendothelial matrix.[3] Agonists such as thrombin, collagen, adenosine diphosphate (ADP), thromboxane, and serotonin binding to their platelet membrane receptors initiate intracellular signaling pathways leading to platelet activation.[4] Activated platelets bind fibrinogen and VWF, aggregate to each other, and release granule contents that support the formation of a stable platelet plug sufficient to control hemorrhage from small vessels and capillaries. The process is regulated by inhibitors released from activated platelets and expressed on the surface of nearby endothelial cells.[5] Primary hemostatic defects include thrombocytopenia,[5,6] platelet dysfunction,[7] and von Willebrand disease (VWD).[6]

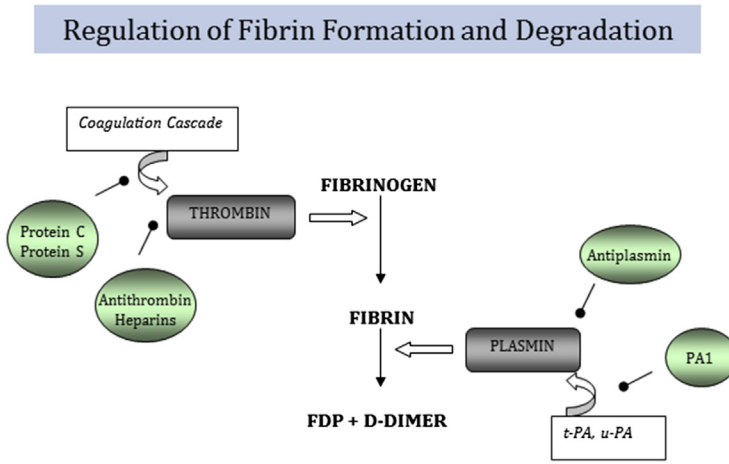

Fig. 2. Coagulation and fibrinolysis. Procoagulant and profibrinolytic reactions (*squares*) and their inhibitors (*circles*) modulate the generation and degradation of fibrin. The coagulation cascade culminates in the production of thrombin, which cleaves soluble fibrinogen to form insoluble fibrin. The actions of tissue plasminogen activator (t-PA) and urokinase (u-PA) generate plasmin, which lyses mature fibrin to form a series of degradation fragments (FDP) and the terminal lytic fragment, D-dimer. Thrombin generation is opposed by the anticoagulants Protein C and Protein S. These proteins act together to inhibit the cofactor activities of Factor V and Factor VIII. Antithrombin inhibits thrombin formation, and directly binds to and neutralizes free thrombin. Antithrombin's activity is enhanced by endogenous (or exogenous) heparin. Plasminogen activator inhibitor-1 (PA1) inhibits fibrinolysis by complexing with t-PA and u-PA to prevent their interaction with plasminogen. Antiplasmin neutralizes free plasmin.

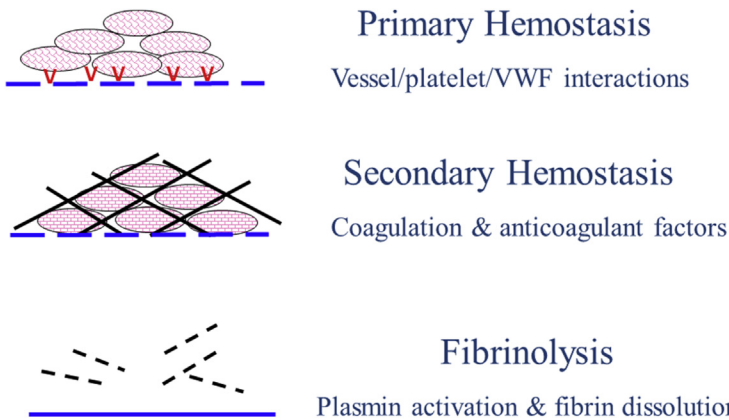

Fig. 3. Three phases of hemostasis. Primary hemostasis involves platelets and von Willebrand factor (VWF) at the site of vascular injury, and results in formation of a platelet aggregate. Secondary hemostasis refers to the reactions of the coagulation cascade that generate a fibrin/cellular meshwork and stable hemostatic plug. In the process of fibrinolysis, plasmin gradually degrades the mature clot to reestablish blood flow after vessel healing.

Secondary hemostasis and anticoagulant proteins

Secondary hemostasis is triggered by the exposure of TF at sites of large-vessel injury, and sustained by the procoagulant properties of platelets that promote fibrin formation. These properties include release of polyphosphates,[8] outer membrane externalization of aminophospholipids,[9] and shedding of membrane vesicles referred to as platelet microparticles (PMP).[10] Procoagulant platelets and PMP provide physiologic anchoring sites for assembly of calcium-dependent, coagulation-factor complexes that generate a large burst of thrombin, which cleaves soluble plasma fibrinogen to form a stable cross-linked fibrin clot. A cell-based model of coagulation (**Fig. 4**) describes the physiologic interactions among endothelial cells, TF-bearing cells, platelets, and the subendothelial matrix that promote fibrin formation.[11] This model illustrates the key role for the TF–Factor VIIa complex as the in vivo initiator of coagulation, amplification of thrombin generation by platelets, and the opposing actions of thrombin in promoting and inhibiting coagulation.

The classic coagulation cascade model[12] depicts the intrinsic and extrinsic pathways as 2 distinct series of activation reactions that coalesce into common terminal

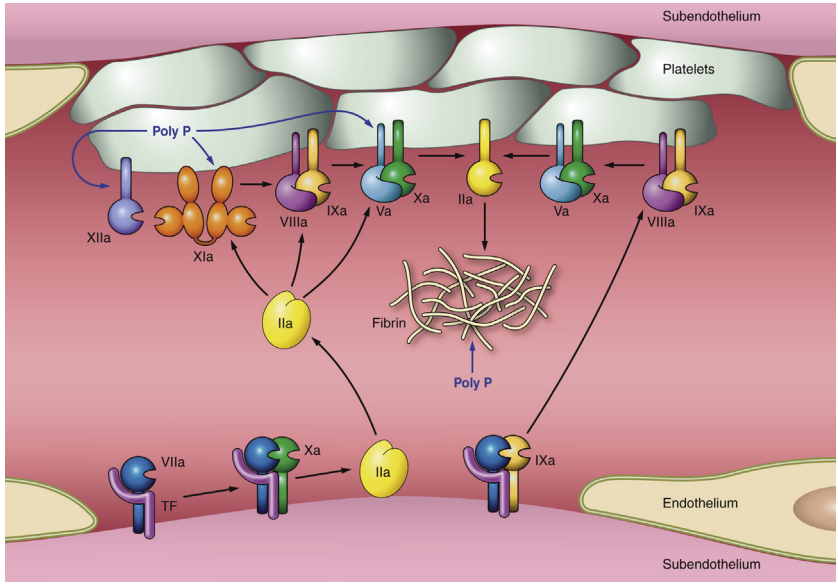

Fig. 4. Cell-based model of coagulation. Injury exposes flowing blood to subendothelial collagen and von Willebrand factor, and surrounding cells bearing tissue factor (TF). Trace amounts of circulating Factor VIIa bind TF and generate small amounts of Factor IXa and Factor Xa, which in turn activate prothrombin to thrombin (IIa). Thrombin plays a central role in amplifying and propagating coagulation through a series of reactions taking place on the surface of procoagulant platelets, formed in response to combined collagen and thrombin stimulation. These platelets also release polyphosphates (poly P) that may activate Factors XII, XI, and V, and inhibit clot lysis. Procoagulant platelets provide a phospholipid surface for assembly of the tenase complex (Factors VIIIa and IXa) and prothrombinase complex (Factors Va and Xa) that rapidly generate Factor Xa and thrombin, respectively. The resultant burst of high concentration thrombin cleaves plasma fibrinogen to form a fibrin clot. Recent murine models of coagulation suggest that collagen and neutrophil-elaborated fibrillar material (neutrophil extracellular traps, or NETs) have procoagulant actions similar to those of polyphosphates. (*From* Versteeg HH, Heemskerk JW, Levi M, et al. New fundamentals in hemostasis. Physiol Rev 2013;93:328; with permission.)

reactions, ultimately producing a fibrin clot (**Fig. 5**). These pathways represent the in vitro, fluid-phase reactions of the traditional coagulation screening tests, the activated partial thromboplastin time (aPTT) and prothrombin time (PT), as discussed later in this article.

Plasma anticoagulant proteins are critical negative regulators of coagulation (**Fig. 6**). Antithrombin (AT) and tissue factor pathway inhibitor (TFPI) are direct protease inhibitors that neutralize circulating active factors, thereby restricting fibrin formation to the site of vessel injury. The targets of AT include activated Factors IX, X, and thrombin (Factors IXa, Xa, and IIa). Protein C, with its cofactor, Protein S, binds to endothelial cell receptors and inhibits coagulation by degrading the coagulation cofactors Va and VIIIa at the endothelial cell surface.[13] Defects of secondary hemostasis that cause signs of hemorrhage include acquired and hereditary coagulation factor deficiencies and physiologic and pharmacologic coagulation inhibitors, whereas relative deficiencies of anticoagulant proteins are associated with thrombotic syndromes.

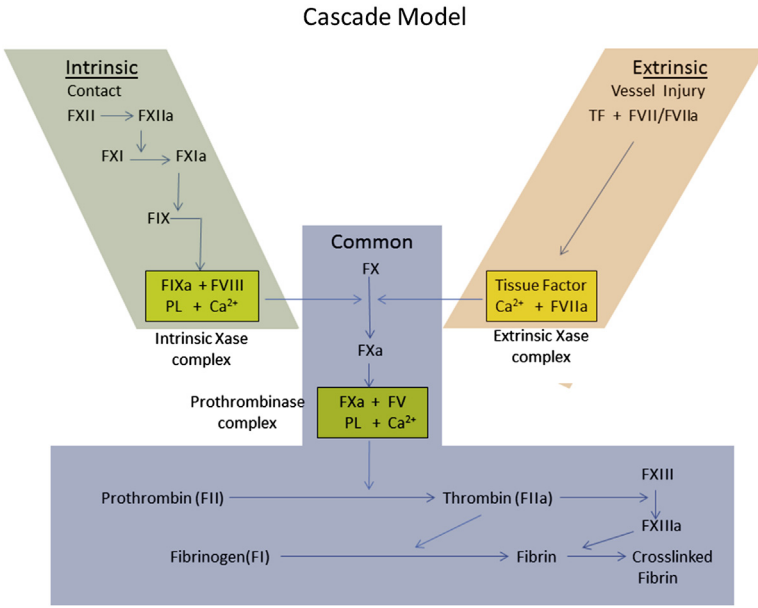

Fig. 5. Cascade model of coagulation. Coagulation factors circulate in plasma in zymogen form and are activated by a cascading series of calcium-dependent proteolytic reactions, referred to as pathways. The extrinsic pathway is initiated by the introduction of tissue factor (TF), which binds to trace levels of Factor VIIa. Factor VIIa–TF and Ca^{2+} form the extrinsic tenase (Xase) complex, which activates Factor X to Factor Xa. The intrinsic coagulation pathway is initiated by interactions among the contact group factors, prekallikrein, kininogen, and Factor XII, with a negatively charged surface. Factor XIIa activates Factor XI to Factor XIa, which in turn generates Factor IXa. Factor IXa assembles with Factor VIIIa, phospholipid (PL), and calcium to form the intrinsic Xase complex and generate Factor Xa. In the common pathway, Factor Xa generated by the extrinsic and intrinsic pathways interacts with Factor Va, PL, and calcium to form the prothrombinase complex that converts prothrombin (FII) to thrombin (FIIa). Thrombin then converts fibrinogen to fibrin, which polymerizes to form the insoluble fibrin clot. Factor XIIIa cross-links fibrin to further strengthen the clot.

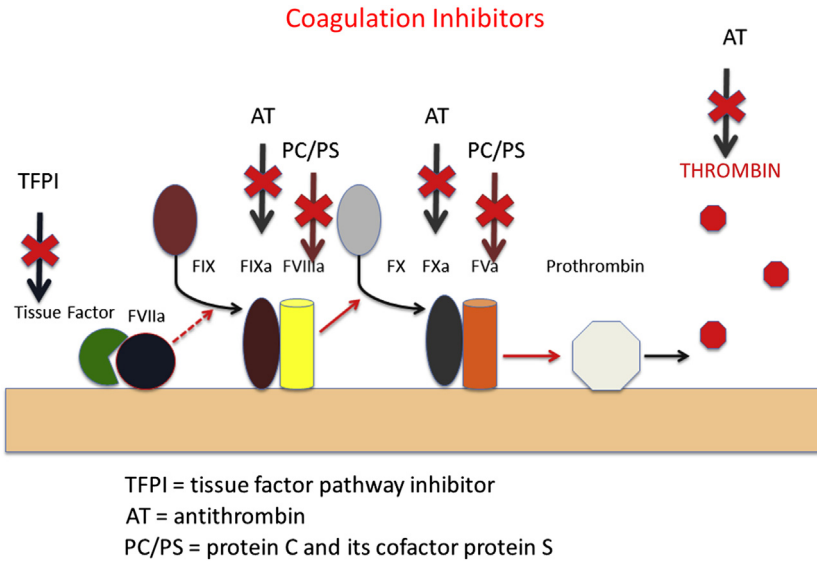

Fig. 6. Coagulation inhibitors. Tissue factor pathway inhibitor (TFPI), antithrombin (AT), and Protein C (PC) are key anticoagulant plasma proteins that neutralize active clotting factors, thereby limiting the extension of a fibrin clot. TFPI binds to Factor Xa and the Factor VIIa–TF complex, and acts to rapidly neutralize Factor Xa. Protein C becomes activated at cell surfaces to form activated protein C (aPC) which, in combination with Protein S, proteolytically degrades coagulation Factor VIIIa and Factor Va. In the absence of coagulation cofactors VIIIa and Va, the tenase and prothrombinase complexes do not form, resulting in a marked decrease in thrombin formation. Antithrombin binds to and neutralizes free plasma Factor IXa, Factor Xa and thrombin. Red crosses denote inhibition.

Fibrinolytic pathway

Tissue and vascular injury initiate coagulation and fibrinolysis (see **Fig. 2**). Thrombin is the terminal enzyme of the coagulation cascade, whereas plasmin is the enzyme responsible for fibrinolysis. Tissue plasminogen activator (tPA) is the major initiator of intravascular fibrinolysis. When bound to fibrin, tPA is highly efficient in activating the proenzyme plasminogen to form plasmin, thereby localizing fibrinolysis to the mature fibrin clot. The circulating levels of tPA reflect secretion by the vascular endothelium, clearance by the liver, and its inhibition by plasminogen activator inhibitor type 1 (PAI-1).[14] Plasmin degrades cross-linked fibrin in a series of cleavage steps that generate intermediary fibrin degradation products (FDP) and terminal D-dimer fragments. Circulating free plasmin is rapidly degraded by the protease inhibitor antiplasmin. Pathologic states associated with dysregulated fibrinolysis and signs of hemorrhage include severe trauma, hepatic cirrhosis, and disseminated intravascular coagulation.

TESTING HEMOSTASIS

Primary Hemostasis Testing

Thrombocytopenia is the most common acquired hemostatic defect; therefore, platelet count is the first diagnostic test in the evaluation of primary hemostatic disorders (**Fig. 7**). Normal platelet counts for dogs and cats range from approximately 150 to 450 $\times$ 10^3 platelets/μL. Spurious low automated platelet counts are common laboratory artifacts and should be confirmed by examination of a blood smear to rule out

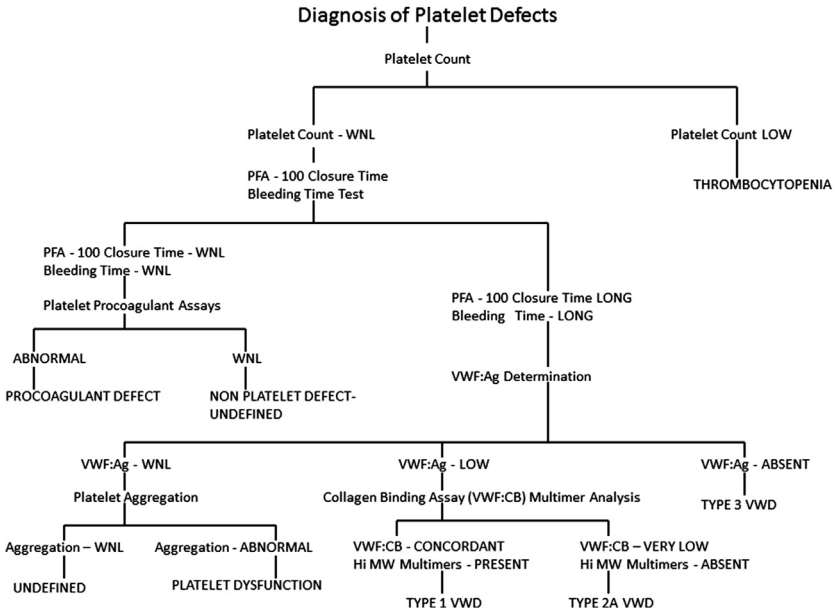

Fig. 7. Diagnostic algorithm of platelet defects. Thrombocytopenia should first be ruled out in the diagnostic workup of suspect platelet defects, followed by functional screening tests, von Willebrand factor assays, and specific tests of platelet activation response. MW, molecular weight; PFA-100, platelet function analyzer 100; WNL, within normal limits; VWD, von Willebrand disease.

platelet clumping. Platelet counts (per microliter) can be estimated from the blood smear by multiplying the average number of platelets counted per 10 oil-immersion fields by 15,000.[15] Thrombocytopenia is rarely the sole cause of hemorrhage at platelets counts above 35,000/μL; however, low platelet count influences end points in functional screening tests such as in vivo bleeding time and whole blood assays.

Platelet function and VWF screening tests: bleeding time and closure time

The buccal mucosal bleeding time (BMBT) using the template system (Simplate II; Organon-Teknika, Durham, NC) and the PFA-100 closure time using the Platelet Function Analyzer (PFA-100; Siemens Diagnostics, Marburg, Germany) have been described as screening tests of primary hemostasis in dogs and cats (**Fig. 8**).[16,17] The BMBT is an in vivo assay that measures the time for cessation of blood flow from a standard incision made in the oral mucosa. Prolonged BMBT (>4 minutes) occurs in patients with platelet aggregation defects and VWD; however, the test may be prolonged in patients with anemia, thrombocytopenia, and hyperproteinemia, and is subject to interoperator variability. Bleeding-time tests are now rarely performed in medical practice because of this nonspecificity, variability, and lack of predictive value for surgical bleeding.[18,19]

The PFA-100 assay evaluates platelet function in citrated whole blood samples that are aspirated at high shear rates (5000–6000 per second) through disposable plastic cartridge assemblies. The cartridges contain a membrane coated with both collagen and epinephrine (CEPI) or collagen and ADP (CADP). As blood flows through a small aperture in the membrane, the agonists trigger platelet adhesion, activation, and aggregation, leading to occlusion of the membrane. The assay end point is reported as

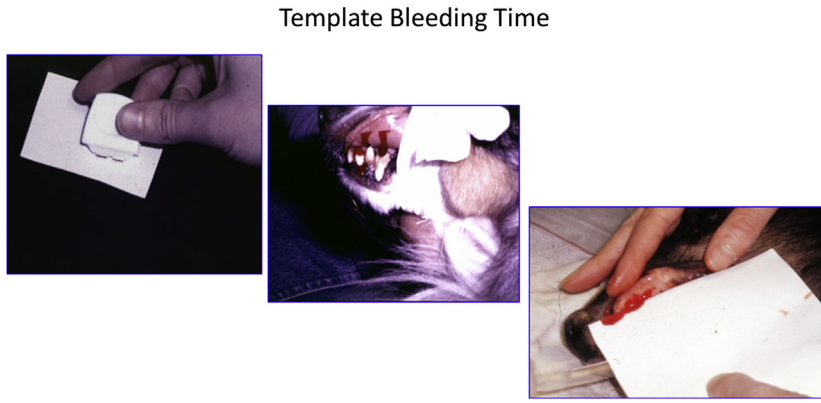

Fig. 8. Buccal mucosal bleeding time. (*Top*) A template device is used to produce a standard depth and length incision. (*Center*) The upper lip is everted and secured with gauze wrapped snugly around the muzzle. The test is initiated by triggering the template device and simultaneously starting a timer. (*Bottom*) Blood is gently blotted from below the incision. The time from incision to the cessation of blood flow is the bleeding time. After completion of the test, the gauze is removed and direct pressure applied to the wound. Tissue glue should be applied to the wound if bleeding persists beyond a 12-minute observation time or rebleeding occurs.

closure time (CT) (seconds) representing the time for cessation of blood flow. Although more readily standardized than in vivo bleeding time tests, the PFA-100 assay system is subject to preanalytical artifacts caused by improper anticoagulant, platelet activation during blood collection and transport, or prolonged (>4 hour) delays in analysis. In addition, the CT is subject to the same nonspecific influences as BMBT. Species differences in platelet epinephrine response limit the utility of the CEPI cartridge for evaluating platelet function in animals. A failure of CEPI membrane occlusion, reported as CT greater than 300 seconds, has been reported for healthy dogs (and horses) with normal platelet function.[17]

Reference ranges have been reported for canine CADP CT (approximately 60–120 seconds)[17] and feline CADP CT (60–180 seconds)[16]; however, in-house reference ranges should be established by each testing site to account for preanalytical variables. The finding of prolonged CADP CT is compatible with either VWD or intrinsic platelet aggregation defects (see **Fig. 7**). In human studies, CT has a sensitivity of greater than 98% for diagnosis of severe (types 2 and 3) VWD, and an overall sensitivity of 85% to 90% for all VWD subtypes.[20] A significant shortening of CADP CT was found in dogs with type 1 VWD treated with desmopressin[21]; however, clinical studies relating normalization of CT to positive clinical outcomes have not been reported. The PFA-100 assay system is not sensitive to defects of platelet procoagulant activity, and in human studies has not proved useful in detecting platelet-secretion defects.[19,22]

Specific Platelet Function Tests

Platelet function testing is logistically and technically challenging. Fresh blood samples are required because of platelets' short ex vivo viability, and quality assurance requires assay of paired control samples to confirm that reagent systems perform within accepted limits.[23] Preanalytical variables have a major impact on assay results. Nontraumatic venipuncture and blood-collection techniques are critical determinants for

all subsequent procedures. Blood samples for platelet function testing should be withdrawn gently into premeasured anticoagulant, with no turbulent flow, and maintained at room temperature until analysis. It is of the utmost importance to confirm appropriate sample quality before any platelet function testing.

Light-transmission aggregometry

Light-transmission aggregometry (LTA) is the traditional gold-standard platelet function test, and remains a critical tool for the diagnosis of hereditary and acquired platelet function defects and the monitoring of antiplatelet drugs (**Tables 1** and **4**).[20,23] The assay end point is the amount of light transmitted through a suspension of platelets in plasma or buffer. Changes in light signal are registered electronically by the aggregometer (**Fig. 9**, inset) and displayed dynamically over time after the addition of platelet agonists. After agonist addition, responsive platelets change shape from discoid to spiny spheres, an event that causes a transient decrease in light transmission, followed by progressive increase in transmitted light that parallels ongoing platelet aggregate formation. As the size of the platelet clumps grow, more light is transmitted until a sustained plateau is reached representing maximal, irreversible platelet aggregation (see **Fig. 9**). Reversible aggregation refers to a qualitatively different profile that develops if platelets disaggregate and light transmittance decreases toward baseline after an initial increase. Low dose or weak agonist stimuli may induce biphasic aggregation, appearing as an early plateau (primary aggregation) followed by a subsequent increase to a plateau of light transmission, representing a secondary wave of irreversible aggregate formation.

Analytical variables such as platelet number, choice of agonists and agonist concentration, and reaction stir speeds all influence LTA. Traditionally, platelet numbers are adjusted to 200 to 300 $\times$ 10^3 per microliter of platelet-rich plasma (PRP) using

Table 1
Hereditary platelet disorders in dogs and cats

Disorder	Affected Breeds	Platelet Abnormalities
Glanzmann thrombasthenia	Great Pyrenees, otterhounds	GpIIb–IIIa receptor complex absent or reduced, profound aggregation and retraction defect
Macrothrombocytosis	Cavalier King Charles spaniels, Norfolk terriers	Low platelet count, large platelets, no functional defect
Procoagulant deficiency	German shepherds	Normal aggregation and retraction, failure of stimulated phosphatidylserine externalization, and microparticle release
Storage pool disorder (Chediak-Higashi)	Persian cat	Abnormal aggregation, abnormal dense granule contents and secretion
Storage pool disorder	American Cocker spaniel	Abnormal aggregation, abnormal dense granule adenosine diphosphate (ADP) content
Storage pool and thrombocytopenia	Collie	Cyclic hematopoiesis defect, abnormal aggregation to some agonists, abnormal dense granule serotonin
Thrombopathia	Basset hound, Landseer, Spitz	CalDagGEF signal transduction defect, poor aggregation to ADP, collagen, normal clot retraction

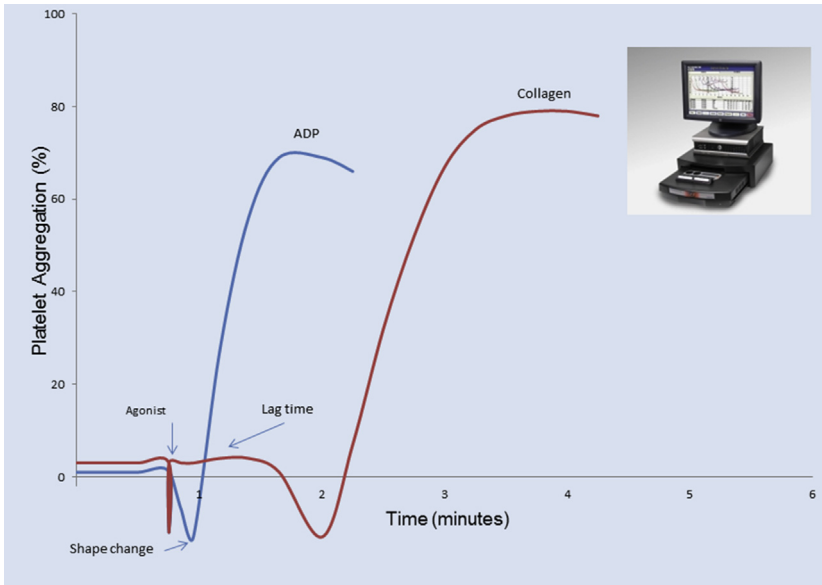

Fig. 9. Light-transmission aggregometry. Representative response profiles of canine platelet-rich plasma (PRP) activated with adenosine diphosphate (ADP; *blue tracing*) or collagen (*red tracing*). A baseline of 0% aggregation is set before agonist addition based on light transmission through turbid PRP, whereas 100% aggregation represents light transmission through platelet-poor plasma. After agonist addition, platelets change shape from flat discs to spiny spheres that block light more efficiently, resulting in a negative deflection from baseline. When platelet aggregation begins, there is a steady increase in light transmission until maxima are reached. Data collection and analyses can be semiautomated using modern instrumentation. An 8-channel platelet aggregometer, Model PAP-8E (Bio Data Corporation, Horsham, PA, USA), is shown (*inset*). Parameters of aggregometry include lag time to onset of aggregation, slope of the reaction, time to maximal platelet aggregation, maximal percent platelet aggregation, reversible aggregation, and the presence of primary and secondary waves of aggregation.

platelet-poor plasma or suspension buffer. This practice has recently been questioned as unnecessary,[24] and may actually result in a decrease in platelet function caused by inhibitory effects of substances in platelet-poor plasma.[23] The LTA reaction mixtures must be stirred at a constant speed to assure the platelets are maintained in an even suspension and to facilitate the platelet-to-platelet contact critical for aggregation. Stir speeds of 1000 rpm are often used; however, stir speeds ranging from 700 to 1200 rpm have been reported. High stir speed risks damaging platelets and inducing spontaneous secretion and aggregation.

Lumi-aggregometry

Routine LTA is an insensitive test in identifying platelet-secretion defects. However, the LTA technique has been modified for this purpose through the use of an instrument (Lumi-Aggregometer; Chrono-Log, Havertown, PA) that is capable of detecting luminescence resulting from the release of adenosine triphosphate (ATP) in the aggregation reaction mixture. In this modification, ATP released from aggregating platelets is quantified through its reaction with a chemiluminescent reagent (Chrono-lume).[25] This technique is generally restricted to specialized veterinary hemostasis laboratories

because the agonists and the chemiluminescent reagents used must be optimized to account for species differences in platelet response.[26] Complete characterization of secretion defects requires additional analyses of the platelet content of adenine nucleotides and serotonin.

Whole blood aggregometry

In contrast to LTA, impedance-based whole blood aggregometry requires minimal specimen preparation, and has been adapted for antiplatelet drug monitoring and the assessment of platelet-granule secretion in human studies. In this method, spaced-platinum electrodes are placed in whole blood warmed to 37°C and as the blood is stirred, platelets coat the electrodes with a monolayer. When an agonist is added to the reaction mixture, activated platelets, leukocytes, and red cells form an aggregate on the monolayer and impede the current between the 2 spaced electrodes.[20] The change in electrical impedance is recorded and expressed in ohms. The method has also been modified to monitor ATP release by the addition of chemiluminescent reagents. Although relatively simple, whole blood aggregometry is subject to artifacts caused by hemolysis and improper sample handling, and nonspecific influences of high or low platelet count and hematocrit.

Flow cytometry

Whereas aggregation studies assess the overall function of an entire platelet population, flow cytometry is a technique that allows examination of platelet activation on a single-cell basis.[27] Cytometric assays detect platelet activation based on changes in light scatter and labeling with fluorescent probes. Platelet studies can be performed on suspensions of washed platelets, PRP, or dilute whole blood, and assays can be configured to assess constitutive membrane receptors, basal activation status, or activation response to different agonists.[27,28] Careful attention to blood collection and processing is as important for valid cytometry assays as for other platelet function assays. The numerous parameters of platelet activation that can be evaluated with cytometry include density of outer membrane surface glycoproteins and ligands, the expression of granule proteins and neoantigens induced by receptor activation, changes in ion flux, protein phosphorylation status, the permeability of platelet cytoplasmic and mitochondrial membranes, outer membrane lipid composition, and PMP release (**Table 2**). One of the most commonly used parameters of platelet activation in animal and human studies is detection of the α-granule protein P selectin (CD62P) on the platelet outer membrane surface (**Fig. 10**, lower left). Labeling with Annexin V, a protein that binds externalized phosphatidylserine, is often used to detect procoagulant platelets and PMP (see **Fig. 10**, lower right) and is also used as a marker of platelet-storage lesion. In addition to the instrumentation and expertise required for cytometric analyses, the application of cytometric assays in veterinary medicine depends on the availability of species-specific and/or cross-reactive antibodies to identify canine and feline platelet antigens.

Von Willebrand Factor Assays

The diagnosis and subtype classification of VWD, the most common hereditary bleeding disorder in dogs and people,[6,29] is based on quantitative and functional assays of VWF protein (see **Fig. 7**; **Table 3**). Type 1 VWD is a partial quantitative VWF deficiency with concordant levels of protein concentration and function. Type 2 VWD is characterized by qualitative defects in VWF structure and function, often combined with protein deficiency. In human medicine, subtype 2A VWD refers to the specific lack of high molecular weight VWF multimers. To date, type 2A is the only type-2

Table 2
Flow cytometric measures of platelet activation

Activation Response	Markers	Process or Receptor Detected
Adhesion	CD42 (a, b, c)	VWF receptor complex (Gp Ib-V-IX)
	CD49b/CD29	Collagen receptor
	CD49e/CD29	Fibronectin receptor
	CD41/61	Fibrinogen receptor
Aggregation	CD41/61	Fibrinogen receptor
	PAC-1	Ligand induced binding site-active fibrinogen receptor
	Fibrinogen	Platelet membrane-bound fibrinogen
Degranulation	CD62P	Alpha granule release
	CD63	Lysosomal integral membrane protein
	Mepacrine	Released from dense granule after ex vivo loading
Signaling	Fluo-3	Cytosolic free calcium
	JC-1	Mitochondrial membrane potential
	TMRE	Mitochondrial membrane potential
Procoagulant activity	Annexin V	Phosphatidylserine externalization
	Lactadherin	Phosphatidylserine externalization
	Factor V	Membrane-bound Factor V
	Fibrinogen	Derivatized membrane-bound fibrinogen
Platelet microvesiculation	CD42b, CD41/61	High-density constitutive platelet membrane antigens

variant identified in dogs. Subtype 2B describes increased binding affinity of VWF to platelet GpIb, and subtype 2N is associated with impaired VWF–Factor VIII binding. Type 3 VWD, the most clinically severe form, is characterized by the virtual absence of plasma VWF (<1% VWF).

Quantitative VWF assays

Measurement of VWF protein concentration, referred to as von Willebrand factor antigen (VWF:Ag), is the first step in the diagnosis of VWD (see **Fig. 7**). Species differences in VWF antigenic structure require the use of species-specific or cross-reactive antibodies in enzyme-linked immunosorbent assays or latex-immunoassay platforms. Test results are conventionally reported in comparison with a normal, same-species standard having 100% or 100 U/mL VWF:Ag. In general, plasma VWF:Ag values of less than 50% (<50 U/mL) indicate VWF deficiency.

Functional VWF assays

Functional VWF assays measure the ability of VWF to interact with platelets, collagen, or Factor VIII. The ristocetin cofactor assay (VWF:RCo) is based on the ability of the antibiotic ristocetin to induce conformational changes in VWF that enhance its binding to platelet GpIb.[30] The application of VWF:RCo assays for canine or feline plasmas is complicated by the tendency of ristocetin to cause protein-precipitate formation that interferes with the assay end point. A reagent purified from snake venom, botrocetin, has been used as a surrogate for ristocetin in animal plasmas.[31]

Functional assays that measure VWF's collagen-binding activity (VWF:CB) have been developed and validated for use with human[32] and canine plasma.[33] In human and canine VWF:CB assays, purified bovine collagen is immobilized to the polystyrene surface of microtiter plate wells; plasma is added to allow for VWF binding; the wells are washed free of unbound VWF; and collagen-bound VWF is detected

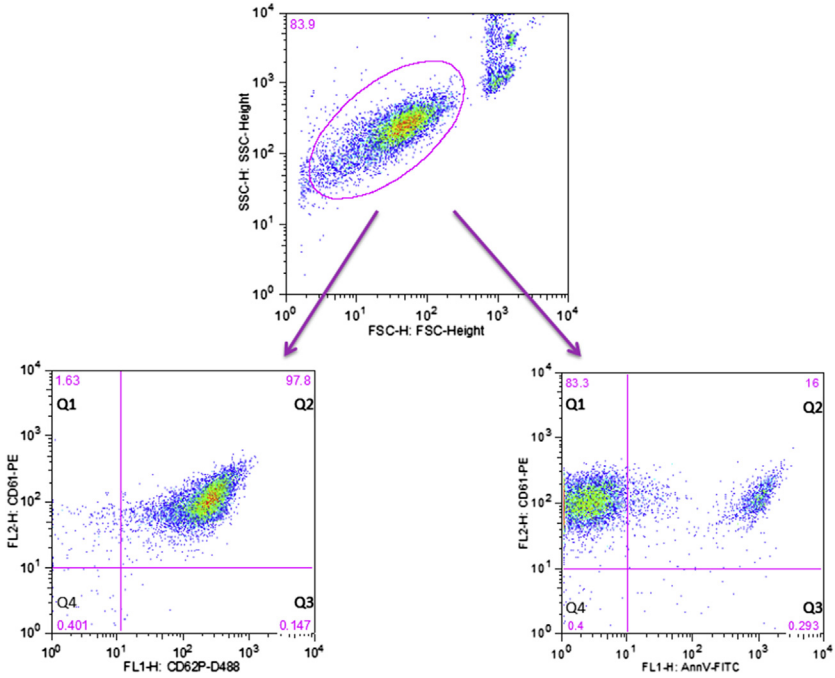

Fig. 10. Flow-cytometric analyses of platelet activation. Dot plots depicting thrombin stimulation of canine platelet-rich plasma (PRP). (*Upper panel*) Platelets are gated (*ellipse*) for analysis based on size and light-scattering properties (forward scatter [FSC-Height] and side scatter [SSC-Height]). (*Lower panels*) Fluorescence intensity of the gated population is shown on the y-axis for a constitutive platelet membrane antigen, CD61 (GPIIIa), and on the x-axis at left for a marker of P-selectin expression (CD62P) or at right, a marker of phosphatidylserine externalization (Annexin V). In each lower panel, Q1 represents nonactivated platelets and Q2 represents platelets expressing the activation marker. Fluorochrome abbreviations: D488, DyLight green; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Table 3
Subtype classification of von Willebrand disease

Type	VWF Defect	Affected Breeds
1	Low VWF concentration; residual protein has normal structure and function	Airedale, Akita, Bernese mountain dog, Dachshund, Doberman pinscher, German shepherd, Golden retriever, Greyhound, Kerry blue terrier, Manchester terrier, Mini pinscher, Papillon, Pembroke Corgi, Poodle, others Himalayan cat
2A	Low VWF concentration, selective loss of largest multimers, abnormal collagen binding	German shorthaired and wirehaired pointers
2B	Increased platelet-VWF binding	Not identified in dogs/cats
2M	Decreased platelet-VWF binding	Not identified in dogs/cats
2N	Decreased Factor VIII-VWF binding	Not identified in dogs/cats
3	Complete lack of VWF	Dutch kooiker, Scottish terrier, Shetland sheepdog, Border collie, Chesapeake retriever, Cocker spaniel, Labrador retriever, Maltese, Pomeranian Domestic short-haired cat

using VWF-specific monoclonal antibodies in a manner similar to that of VWF:Ag. Paired determinations of VWF:Ag and VWF:CB can be used to discriminate between type 1 and type 2A VWD. The ratio of VWF:Ag to VWF:CB is close to 1 for patients with type 1 VWD, whereas the disproportionate lack of VWF:CB in patients with type 2A VWD results in a VWF:Ag to VWF:CB ratio of more than 2. The VWF:CB is considered an indicator of VWF multimer structure, because VWF collagen-binding activity is a property of high molecular weight forms of VWF.[29]

Structural VWF assays

Characterization of VWF multimeric structure is technically demanding, time consuming, and difficult to standardize; however, VWF protein structure is relevant for understanding protein activity and stability, and is used to further define abnormal results obtained in functional and quantitative VWF assays.[34] To determine VWF multimeric structure, test samples are subjected to sodium dodecyl sulfate–agarose gel electrophoresis to achieve separation of VWF forms according to their molecular mass. The separated protein bands are then electrotransferred to nitrocellulose or polyvinylidene difluoride membranes and visualized using anti-VWF detector antibodies (**Fig. 11**). The full complement of VWF multimers ranges in size from 500 kDa (VWF dimer) to more than 20 million kDa for the high molecular weight forms. Multimer analyses can also discern subtle qualitative changes in VWF, including abnormal protein migration or reduced intensity, and changes in the VWF triplet structure within individual multimer bands. These changes can provide insight into the biochemical basis for abnormal VWF processing, function, or susceptibility to proteolysis.[29,34]

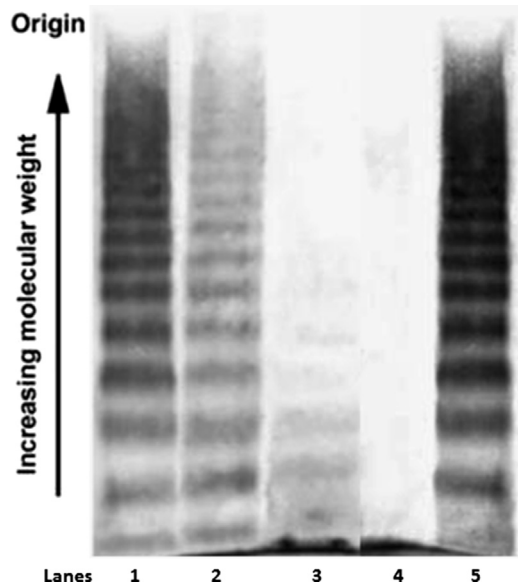

Fig. 11. Von Willebrand factor (VWF) multimer analysis. Western blot of VWF multimers from plasma samples from control dogs (lanes 1 and 5) and dogs with type 1 von Willebrand disease (VWD) (lane 2), type 2 VWD (lane 3), and type 3 VWD (lane 4). The samples were electrophoresed, blotted to nitrocellulose membranes, and immunostained to detect VWF. Type 1 VWD is characterized by a proportional reduction in all multimers, whereas type 2 VWD plasma shows specific reduction in the highest molecular weight forms, and type 3 VWD plasma lacks any detectable VWF protein.

Secondary Hemostasis Testing

Most coagulation assays are functional tests, measuring the enzyme, coenzyme, or inhibitory activity of various hemostatic proteins in samples of citrate anticoagulated whole blood or plasma. The same procedures used to collect blood samples for platelet function testing are therefore applicable to retaining hemostatic protein function.[35] The kinetics of fibrin formation and the relative activities of coagulation factors and inhibitors vary among species. Assay systems optimized for human coagulation testing may not provide accurate values for samples from other species, and different reagent and instrumentation combinations yield different clotting time values within a species.[36–38] Coagulation laboratories thus should provide species-specific reference ranges and controls to facilitate interpretation of patient values.

Coagulation screening tests

Coagulation screening tests are configured with specific reagents to differentially initiate the coagulation cascade via the extrinsic or intrinsic pathway (see **Fig. 5**).[39] Though traditionally performed on plasma samples in a testing laboratory, point-of-care instruments for use with anticoagulated whole blood are now available for veterinary practice. The PT screening test is triggered by the addition of a TF reagent and calcium to the test sample. Patients deficient in any single (or >1) factor in the extrinsic (Factor VII) or common pathways (Factors I [fibrinogen], II [prothrombin], V, and X) demonstrate prolonged clotting time in the PT. The aPTT reagent is composed of phospholipid and negatively charged contact particles. Coagulation complexes assemble during preincubation of the reaction mixture; the addition of calcium then triggers clot formation. This test is sensitive to factor deficiencies in the intrinsic (Factors VIII, IX, XI and XII) and common pathways. The activated clotting time (ACT) is a simple point-of-care test of the intrinsic and common pathway.[40] The ACT, however, is more susceptible to nonspecific prolongation because of thrombocytopenia, platelet dysfunction, hematocrit, or plasma protease activity. The thrombin clotting time (TCT) and measurement of fibrinogen concentration are performed by adding an excess of thrombin (Factor IIa) to the test plasma. These tests measure the conversion of fibrinogen to fibrin and are sensitive only to deficiency, dysfunction, or inhibition of fibrinogen. The pattern of abnormalities in coagulation screening tests thus depends on which coagulation factor or factors the patient lacks (**Fig. 12**).

Coagulation factor assays

The specific procoagulant activity of individual coagulation factors and cofactors can be measured in modified aPTT and PT screening tests, which are configured with a series of single factor–deficient plasmas.[39,41] Factor activity of the test sample should be compared with same-species standards because clotting times vary among species, and human standards generally overestimate the factor content of animal plasmas. Values are reported as percentages, U/mL, or U/dL, depending on the laboratory. In general, factor activities greater than 50% are sufficient to support in vivo fibrin formation. The clinical relevance of factor activities lower than this value depends on the identity and severity of the factor deficiency, and whether a single factor or multiple factors are involved.[41,42] In addition to modified clotting time tests, the procoagulant activities of some coagulation factors can be measured in colorimetric assays based on cleavage of chromogenic substrates. In human medicine these assays are generally reserved for pharmacodynamic studies and evaluation of factor concentrates.[43]

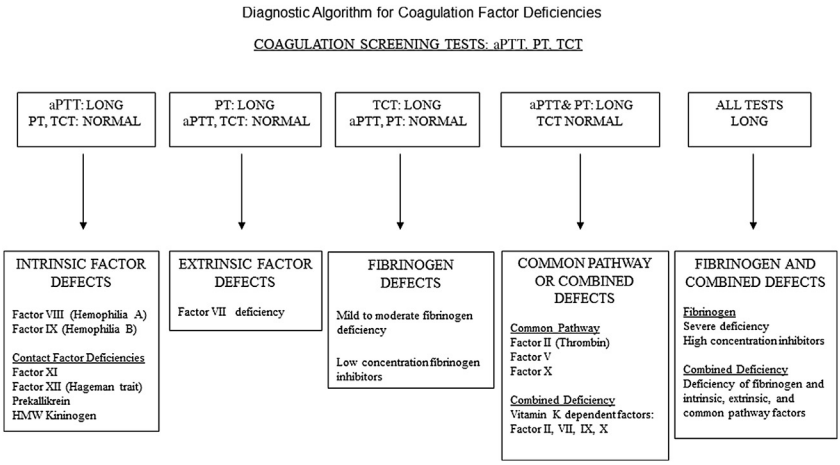

Fig. 12. Diagnostic algorithm of coagulation defects. Coagulation factor deficiencies cause distinct patterns of abnormalities in coagulation screening tests. aPTT, activated partial thromboplastin time; HMW, high molecular weight; PT, prothrombin time; TCT, thrombin clotting time.

Thrombin-generation assays

Coagulation screening tests and factor assays based on the PT and aPTT have a fibrin end point that forms from the action of approximately 10 nM thrombin in the assay system.[44] This value, however, represents only a small portion of the total thrombin potential within the plasma sample. Thrombin-generation assays (TGA) are kinetic assays that monitor thrombin formation over time based on the cleavage of a fluorogenic thrombin substrate. The assay can be performed on citrated PRP, plasma fractions free of platelets, or plasma centrifuged at high g-force to become free of cell-derived microparticles. The TGA reaction is triggered by the addition of a TF and phospholipid reagent, and proceeds through activation of the coagulation cascade and assembly of tenase and prothrombinase complexes that ultimately generate thrombin. The rate of increase and decrease in thrombin concentration over time is calculated from a thrombin calibrator and displayed as a thrombin-generation curve or thrombogram (**Fig. 13**). The resultant profile depicts different phases of coagulation representing net procoagulant and anticoagulant forces. The area under the thrombogram profile is a summary of the overall capacity for thrombin production and is referred to as endogenous thrombin potential. Additional derived parameters include lag time, slope, peak thrombin concentration, time to peak, and the total amount of thrombin generated (see **Fig. 13**).

The test principle of TGA is broadly applicable across species; however, commercially available assay reagents rely on recombinant human tissue factor (rhTF) to initiate coagulation. Species variability in response to rhTF and species differences in the contact pathway activation of Factor XII will influence the thrombogram parameters and may require adaptation of human TGA for measuring thrombin generation in animals. The potential clinical utility of TGA is still under investigation for people with hemorrhagic and thrombotic disorders, including hemophilia, sepsis, preeclampsia, and heart disease.[45]

Fibrinolytic Pathway Assays

Fibrinolysis involves the tPA-initiated formation of plasmin, which degrades fibrin to generate FDP. PAI is a major regulator of this process, and the protease inhibitor,

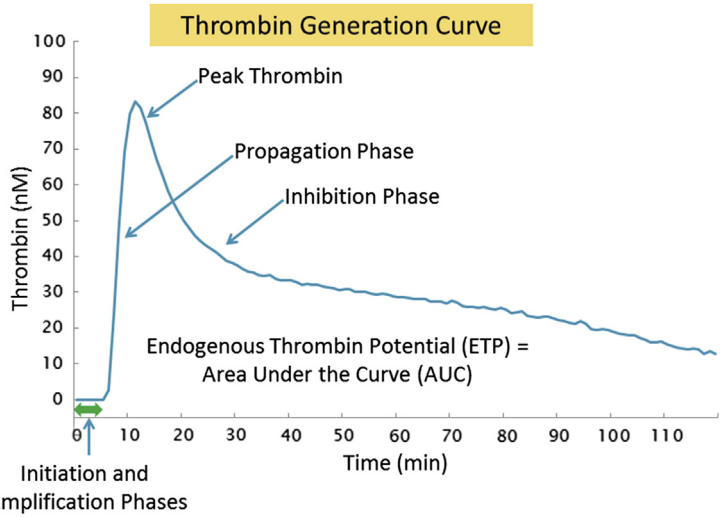

Fig. 13. Thrombin-generation curve. Thrombin generation in canine plasma activated with a tissue factor, calcium, and phospholipid reagent. The initiation and amplification phases of coagulation are measured as the parameter lag time; the propagation phase of coagulation is reflected in the parameter peak thrombin; and the net forces of thrombin generation and inhibition are summarized by the area under the thrombogram curve, also referred to as endogenous thrombin potential.

antiplasmin, further modulates fibrinolysis by neutralizing circulating free plasmin (see **Fig. 2**). The proenzyme plasminogen and its regulators can be measured in specific functional or quantitative assays; however, detection of circulating levels of FDP and the terminal fragment, D-dimer, are the most commonly used clinical tests of fibrinolysis. The D-dimer fragment consists of 2 cross-linked D domains of fibrin, and its presence is a specific indicator of plasmin's action on mature fibrin, rather than fibrinogen. The finding of high circulating FDP and D-dimer concentration indicates excessive or dysregulated fibrinolysis, and is included in the diagnostic criteria of disseminated intravascular coagulation.[46] Quantitative D-dimer assays are considered sensitive, but nonspecific, tests of pulmonary thromboembolism in people and animals.[47,48]

Viscoelastic Coagulation Assays

Thrombelastography (TEG) and rotational thrombelastometry are coagulation monitors with associated software designed to measure and display the kinetics and tensile properties of clot formation in whole blood. These tests reflect the contribution of cellular elements to clot formation, and characterize changes in clot strength and stability that occur beyond the time of initial fibrin formation.[49] In addition to a qualitative tracing, the instruments' software performs direct measurements and derived calculations that describe various parameters of clot formation and subsequent clot lysis. The routinely reported parameters using the TEG instrumentation are reaction time (R), clotting time (K), angle (α), maximal amplitude (MA), and lysis index (LY60). The R parameter is the interval from initiation of the assay until the first deviations of the tracing from baseline denoting initial fibrin formation; K is arbitrarily assigned as the time for deflection from 2 mm to 20 mm from baseline; α is the slope of a line drawn

from R to K; MA is the widest vertical amplitude of the TEG tracing, reflecting maximum clot strength; and LY60 is the TEG amplitude at 60 minutes after the time of MA, denoting the extent of fibrinolysis.

The assays are influenced by preanalytical and analytical variables, such as blood-storage time and temperature, and trigger-reagent composition (**Fig. 14**). Test interpretation also requires knowledge of the patient's platelet count, hematocrit, and fibrinogen concentration. Thrombocytopenia (platelet count <50,000), high hematocrit, and low fibrinogen produce relatively poor-quality clots, manifest as prolonged time to clot formation and low clot strength (ie, hypocoagulability). By contrast, low hematocrit and hyperfibrinogenemia typically generate tracings with short clot-formation times and clots of high tensile strength, characterized as hypercoagulable. Rather than a diagnostic test, viscoelastic assays are primarily used in human practice to guide transfusion therapy in patients undergoing cardiac bypass, and patients with complex coagulopathies undergoing surgery such as orthotopic liver transplantation. Most veterinary studies have been descriptive, and the clinical utility of viscoelastic monitors for disease diagnosis, prognosis, or as guides to therapy that improve clinical outcome requires further study.

Modified thrombelastography: clot life-span analysis

After in vivo fibrin formation, the nascent clot undergoes subsequent maturation, remodeling, and gradual dissolution. These late phases of hemostasis are important for patients in perioperative settings at risk for rebleeding and/or thrombosis. A clot life-span model has been developed to characterize imbalance of the fibrinolytic pathway that might induce hyperfibrinolytic or hypofibrinolytic states.[50] Examination of clot life span uses TEG with TF-activated or contact pathway–activated coagulation, coupled with the addition of tPA to initiate fibrinolysis. In addition to amplitude measurements of clot strength, the life-span data incorporate parametric resistance units to capture the velocity of changes in clot structure as the thrombus matures and then lyses. This technique provides a system to screen for fibrinolysis defects, and to visualize the effects of pharmacologic fibrinolysis inhibitors such as ε-aminocaproic acid and tranexamic acid.

Anticoagulant Assays and Drug Monitoring

Physiologic and pathologic coagulation inhibitors

Antithrombin and Protein C are major circulating anticoagulant proteins that regulate the generation and action of thrombin (see **Fig. 6**). Functional chromogenic assays of antithrombin and Protein C, modified by the use of same-species standards, have been used in clinical veterinary studies of patients at risk for thrombosis and as biomarkers of hepatic function. Acquired antithrombin deficiency is associated with thrombosis in patients with disseminated intravascular coagulation and protein-losing disorders. Protein C deficiency has been associated with poor prognosis in dogs with septic peritonitis and liver failure,[51,52] and aids in the diagnosis of portacaval vascular shunting.[53]

Pathologic coagulation inhibitors include antibodies that bind to and neutralize clotting factors to cause signs of hemorrhage, and antibodies that bind to protein-phospholipid membrane antigens that typically cause thrombosis. Coagulation inhibitors are detected in clotting time tests configured with mixtures of patient plasma and same-species control plasma. In contrast to factor-deficient plasmas that demonstrate correction of clotting time, the presence of coagulation inhibitors results in persistent prolongation of clotting time in a mixing study.[54]

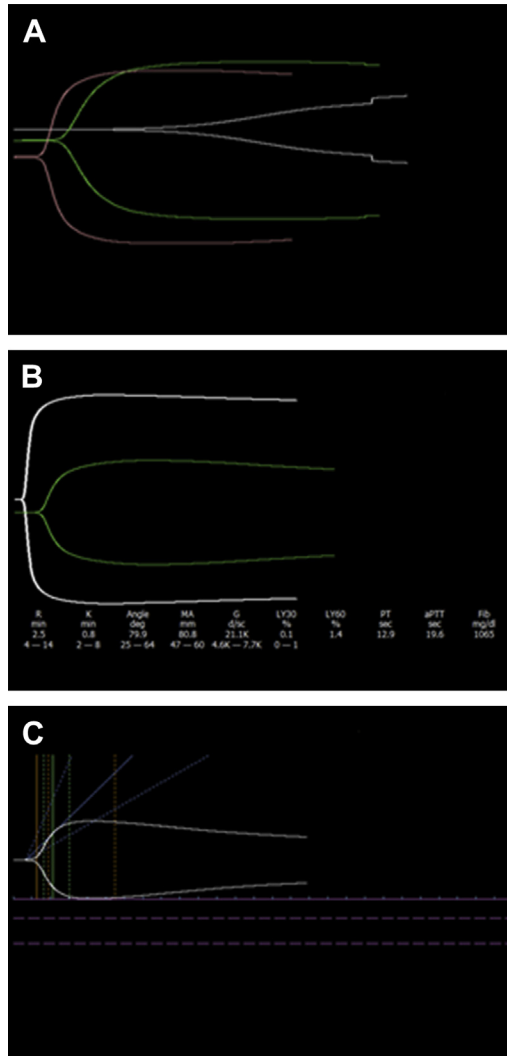

Fig. 14. Thrombelastography profiles. (*A*) Differential effects of trigger reagent composition. The 3 superimposed thrombelastograph tracings were generated from a citrated whole blood sample from a dog with hemophilia B (Factor IX coagulant activity = 3%; reference range >50%). The white tracing denotes activation with calcium alone, the green tracing activation with tissue factor and calcium, and the pink tracing activation with kaolin and calcium. Note the marked abnormality in fibrin formation in the calcium-activated sample, representing an almost complete failure of coupling between the sample cup and pin. (*B*) Thrombelastographic features of hypercoagulability. The superimposed tracings were generated from tissue factor and calcium-activated citrated whole blood samples from a control dog (green tracing) and a dog with pancreatitis and marked hyperfibrinogenemia (*white tracing*). Reference intervals for each thrombelastographic parameter are displayed below the tracing; reference interval for prothrombin time = 12.0 to17.5 seconds; activated partial thromboplastin time = 10.0 to 17.0 seconds; fibrinogen = 150 to 490 mg/dL. Angle, angle of tangent; G, clot strength; K, clot formation time; LY30, lysis at 30 minutes from MA; MA, maximal amplitude; R, reaction time. (*C*) Tissue plasminogen activator (tPA) activation to assess fibrinolysis. The tracing was generated from tissue factor and calcium-activated citrated whole blood from a control dog with the addition of tPA to the reaction mixture. The tPA activates endogenous plasminogen resulting in a relatively weak clot, with small amplitude and a rapid rate of lysis (compare with green tracing in panel *B* generated from same control dog sample with no tPA).

Antiplatelet and anticoagulant drug monitoring

Evidence-based dosage guidelines for antiplatelet and anticoagulant therapy have been established for defined clinical conditions in people, such as coronary syndromes, deep vein thrombosis, and perioperative thromboprophylaxis. Drug monitoring has been useful in establishing these guidelines (see **Table 4**). Thrombosis is also recognized as a severe complication of many common disease syndromes in animals. Pending clinical trials, drug monitoring may provide some guidance in the empirical use of these drugs to provide the benefits of appropriate antithrombotic intensity while minimizing the risks of iatrogenic hemorrhage.

Aspirin and clopidogrel

Aspirin remains the standard antiplatelet drug against which newer agents are compared.[55] Aspirin irreversibly inactivates platelet cyclo-oxygenase, thereby inhibiting the metabolism of arachidonic acid and the subsequent generation of thromboxane A_2. Thromboxane is a potent platelet agonist and acts as an amplification signal in platelet activation. Inhibition of PFA-100 CEPI CT has been advocated as a simple test to identify aspirin resistance in people; however, the CEPI cartridge is not a useful test for the monitoring of platelet function in animals, and CT has not been consistently correlated with positive clinical outcomes in people. Clinical end points such as thrombosis and hemorrhage, rather than laboratory monitoring, remain the typical means of assessing the efficacy and safety of aspirin therapy in human and veterinary practice.

Clopidogrel is a recently off-patent oral antiplatelet agent. The drug is a thienopyridine derivative that is metabolized in the liver to an active drug that binds to the platelet P2Y12 ADP receptor, thereby inhibiting the platelet release reaction and ADP-mediated activation of the platelet fibrinogen receptor.[55] The antiplatelet effects of clopidogrel vary among patients, and do not directly correlate with its plasma concentration because of differences in hepatic metabolism. Platelet ADP-induced

Table 4
Drug monitoring

Drug	Biologic Action	Monitoring Tests	TX. Target
Antiplatelet drugs			
Aspirin	Inhibit platelet COX → block TXA formation	Platelet aggregation PFA100	Variable
Clopidogrel	Inhibit platelet ADP P2Y12 receptor	Platelet ADP-induced aggregation	>50% inhibition from baseline, Residual aggregation <70%
Anticoagulants			
Heparin (unfractionated)	Enhance ATactivity → block thrombin formation and activity	ACT, aPTT Anti-Xa activity	1.5–2.5× control value 0.3–0.7 U/mL
LMW heparins	Enhance AT activity → block thrombin formation	Anti-Xa activity	0.5–1.0 U/mL

Abbreviations: ACT, activated clotting time; aPTT, activated partial thromboplastin time; AT, antithrombin, COX, cyclo-oxygenase; LMW, low molecular weight; TXA, thromboxane A_2; TX. Target, range of assay values associated with improved clinical outcome in human studies.

aggregation allows pharmacodynamic profiling of clopidogrel response; however, its routine clinical use in people is still based on fixed-dose administration and clinical end points for monitoring.[56]

Unfractionated and low molecular weight heparins

Unfractionated heparin (UFH) is a mixture of polysaccharide chains of highly variable length. Heparin exerts its anticoagulant effect indirectly, by enhancing AT's affinity for its target serine protease coagulation factors, particularly thrombin (Factor IIa) and Factor Xa.[57] A specific pentasaccharide sequence on the heparin molecule binds to AT; however, longer chain lengths mediate UFH's interactions with numerous plasma proteins (including thrombin) and intravascular cells. This extensive binding of protein and cells produces a complex pharmacokinetic profile and a wide variation in UFH anticoagulant effect among individuals. Individual patient monitoring and dosage adjustments are required to attain a desired anticoagulant intensity without causing hemorrhage.

Therapeutic targets for UFH in human medicine were first established based on prolongation of the aPTT screening test, with more recent studies defining target ranges based on in vitro inhibition of Factor Xa (anti-Xa activity).[57] Target prolongation of aPTT to 1.5 to 2.5 times the assay control (or patient baseline) or target range for anti-Xa activity of 0.3 to 0.7 U/mL have been associated with favorable clinical outcomes. Attainment of a target range of anti-Xa activity was associated with improved survival in a canine trial of immune hemolytic anemia.[58] Measurements of aPTT or anti-Xa activity are indicated for monitoring UFH therapy in animals to detect peak effect (approximately 4 hours after subcutaneous administration) to prevent iatrogenic hemorrhage in patients treated with high-dose UFH.

Low molecular weight heparins (LMWH) are produced by depolymerization of UFH and consist of short, approximately 15 monosaccharide-length chains.[57] The LMWH act via the same mechanism as UFH in enhancing AT activity; however, they demonstrate improved bioavailability and a more predictable pharmacokinetic profile. The shorter chain lengths of LMWH also reduce the formation of a ternary heparin-AT-thrombin complex, so that LMWH primarily inactivate Factor Xa. In clinical trials of human thrombotic syndromes, LMWH have proved as effective as UFH, and are generally associated with fewer bleeding complications.

Unlike UFH, therapeutic doses of LMWH do not prolong clotting times of the aPTT screening test. Pharmacokinetic studies and assessment of LMWH anticoagulant intensity are based on anti-Xa assays. A target therapeutic range of 0.5 to 1.0 U/mL anti-Xa activity is considered appropriate for human patients at risk for venous thromboembolism. Pharmacokinetic studies of LMWH in healthy dogs and cats also reveal improved bioavailability and predictability in comparison with UFH.[59,60] Species differences exist, however, in LMWH absorption and elimination. Cats demonstrate peak anti-Xa activity as early as 2 to 3 hours after subcutaneous LMWH injection, with trough values falling to baseline by 8 hours. Measurements of peak anti-Xa activity (2–3 hours after treatment for cats; 3–4 hours after treatment for dogs) can be used to gauge anticoagulant intensity for individual patients.

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