University of Veterinary Medicine Budapest

Department of Clinical Pathology and Oncology

Molecular Markers in Haematological and Haemostasis disorders of Animals

By

Elizabeth Clancy

Supervisor:

Professor Péter Vajdovich

Budapest, Hungary

2023

Molecular Markers in Haematological and Haemostasis disorders of Animals

Abstract

Molecular markers play a key role in the diagnosis, development, prognosis and subsequent management and therapeutic options of many diseases in the medical field. Their use in medicine have proven to have many benefits, not only in the veterinary field but also for further understanding those diseases associated with public health. Through their identification, the characterisation and treatment options available for haematological disorders of both companion and production animals have advanced in recent years. However, limitations still exist, and further investigations are required to define their pathognomonic significance and increase their potential for everyday clinical use.

In this review, I look at the advances and challenges currently surrounding molecular biomarkers and their role in managing some of the more commonly occurring haematological and haemostasis disorders in veterinary medicine.

Table of Contents

Abbreviations

ACT, Activated Clotting Time; AT, Antithrombin; aPL, anti-Phospholipid Antibodies; aPTT, Activated Partial Thromboplastin Time; DIC, Disseminated Intravascular Coagulation; FDP, Fibrin Degradation Products; INR, International Normalised Ratio; LA, Lupus Anticoagulants; PT, Prothrombin Time; PCR, Polymerase Chain Reaction; PIVKA, Proteins Induced by Vitamin K Absence or Antagonism; POC, Point of Care; RVVT, Russell Venom Viper Test; TT, Thrombin Time.

1. Introduction

In this thesis I will be using current literature to investigate molecular markers in the veterinary field and their use in the management of haematological disorders. I will particularly focus on those of dogs, cats, horses, cattle, swine, sheep and goats. The aim is to analyse the current information available surrounding this topic and discuss not only the benefits and advances surrounding biomarkers, but also the challenges and problems associated with their use in veterinary medicine today. Fundamentally this research is important as the identification of such markers and their implementation into clinical care are important steps towards disease characterization and management in veterinary medicine.

A biological marker, or biomarker in short, has been defined by the National Institute of Health Definitions Working Group (NIH DWG) as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" [1]. They consist of biological features such as gene variants, circulating proteins or metabolites that have been shown by genome sequencing and post-genomic technologies to correlate with the disease of interest. Large scale analyses at the DNA, RNA, mRNA, protein, lipid and metabolite level have been successful in identifying many different types of biomarkers [2] including those associated with patient susceptibility, diagnosis, safety and therapeutic response [3]. These markers can be used, individually or in combination, as disease indicators to improve and accelerate patient screening and detection [4] by having the ability to "differentiate between two or more biological states" [5].

The optimization of healthcare of companion animals as well as improving the health, welfare and production of livestock are all motivating factors for the development of molecular markers, [1]. Understanding the potential adverse effects these diseases, including zoonotic and vector borne infections, can pose on human health is also of huge importance [6], and can aid in the understanding of similar diseases associated with public health [7]. Their application has the ability to improve and develop precision medicine for both human and veterinary medicine [8].

The application of post-genomic technologies to research animal health and disease has been somewhat limited compared to human medicine [9]. Animal model studies of human disease and drug safety trials have significantly contributed to the initial recognition of diagnostic or

prognostic biomarkers [1]. While most proposed biomarkers hold great potential, many are yet to become widely available or routinely used, and their translation into clinical use in veterinary medicine has mainly proven successful in cancer, kidney and cardiovascular disease [2]. This can be due to a variety of reasons including complications in establishing diagnostic and monitoring cutoff values, lack of sensitivity or specificity and variations in available assays [1].

Haematological and haemostasis disorders can be inherited, such as haemophilia or von Willebrand disease, or acquired, (Schalm's Veterinary Haematology). They can present in a variety of ways and, if left untreated, can lead to catastrophic states in the affected individual. Routine Complete Blood Count tests and standard haematology and biochemistry profiles including typical biomarkers and have long been performed to indicate the presence of such disorders in a clinical setting [2]. Introducing molecular markers can increase the sensitivity and specificity associated with their diagnosis, ultimately leading to enhanced patient monitoring, care and improving clinical outcomes. However, in order to maximise the clinical potential of molecular markers surrounding these disorders, further research is needed to improve their diagnostic specificity as many are not yet pathognomonic [1].

2. Literature Review

2. 1 The Discovery and Importance of Molecular Markers

Developing reliable biomarkers is fundamental to improving medicine, with their application being made available to a wide range of fields including diagnostic, prognostic, monitoring or pharmacodynamics [11]. Technological advances in proteomics and metabolomics as well as Next Generation Sequencing (NGS) of animal genomes, including dogs, cats, pigs, horses and cows, has allowed for quicker developments in identifying the presence of a genetic background to the disease of interest, or a genetic basic resulting in an increased susceptibility or resistance to disease [12].

There are clear benefits in applying biomarkers to the veterinary field as they help to accelerate the confirmatory diagnoses that once relied solely on clinical signs and the limited range of tests available. Without this improvement, treatment was not only delayed which in the case of infectious diseases, resulted in mass culls and huge economic losses, but industry and public health also experienced widespread implications [2].

The process for producing an informative biomarker starts with first identifying the target population and defining species, age, sex, breed and disease status. A single, biological measurement to signify a disease, track it's progression and response to therapy should be identified. Understanding the pathogenesis of the disease in question as well as the pharmacodynamics and pharmacokinetics of treatment options available, is crucial to develop reliable biomarkers in diagnosing, staging, and monitoring the disease and its response to therapy. Collecting the appropriate data and samples, along with suitable analysis, assay development, validation and regulatory approval are all necessary steps in producing a biomarker, [13].

Predictive markers, such as positive and negative predictive values (PPV and NPV respectively), are essential in determining whether a particular marker will be of interest and produce reliable, useful results in a specific sample population. These markers are dependent on disease prevalence and can be used to evaluate the diagnostic performance of a biomarker [14]. Applying multiple markers as well as examining patterns generated can yield more powerful and reliable results than that of a single marker, therefore accelerating the staging of the disease or determining a prognosis.

Nucleic acid diagnostic techniques such as Polymerase Chain Reaction (PCR) and biochemical techniques such as Enzyme Linked ImmunoSorbent Assays (ELISA) and RadiommunoAssay (RIA) enable new diagnostic and susceptibility biomarkers to be developed for the early detection of disease for use in clinics [15].

2.2 Challenges in Producing a Reliable Biomarker

The level of complexity involved in biological systems can produce challenges when generating reliable biomarkers. Limiting the biologic variables originating from different genetic backgrounds, species, size, age, reproductive status and diet of a sample population can prove difficult when establishing cut-off values. Differences in sample handling, environments and stress responses of animals can result in varied data [6]. Using animal models and a standardised protocol is essential to avoid the introduction of unintentional bias into the study and allow a number of these variables be regulated wherever possible [16].

Large populations, including samples of both healthy and diseased animals, are required to validate the association as well as prognostic ability of potential biomarkers. This can prove difficult in terms of the financial support needed to obtain such sample sizes including both healthy and diseased controls, as well as the time needed for continuous biomarker investigations [2]. When translating technologies from experimental stages into a clinical environment, the practicalities should be considered to ensure it is a feasible and worthwhile endeavour, [6]. The cost-benefit ratio is an important consideration throughout the biomarker development stages to ensure the type of biomarker is suitable with the clinical setting it is being applied to, [2].

Ideally a biomarker for veterinary medicine should be appropriate for use in a primary care setting and enable animals to be directly monitored for specific diseases. In producing a reliable biomarker, confirmation that it is not just linked to the disease in question, but actually predicts or accurately correlates with the clinical outcome is crucial, [1]. Issues surrounding specificity and sensitivity are among those faced when attempting to translate a biomarker assay from the laboratory to it's use in clinic, resulting in many investigations failing to progress to larger scale investigations required for their validation [6].

2.3 Different Methods of Biomarker Application

Biomarkers can be classified in many ways, the most common of which being according to their function. In the veterinary field, developing diagnostic biomarkers that aid in the early detection of disease are prioritised, [2]. These markers can identify those affected by the disease of interest and can be applied to define a subset of the disease [6].

Susceptibility or risk biomarkers can identify animals of increased resistance or vulnerability to developing a disease or condition of interest are also of major importance, as their application can aid in limiting or eliminating the likelihood of the individual becoming affected. Markers for monitoring patients undergoing treatment, pharmacodynamic markers to analyse their response to it and safety markers to measure exposure levels in order to prevent drug toxicity offer support to clinicians and aid in their development of therapeutic plans [6]. Blood and body fluids such as urine or saliva are the samples most often required to develop these tests, [11].

The analysis of coagulation parameters can not only indicate the presence of haematological disorders but neoplastic and oncotic disease too, as excessive activation of coagulation and extravascular proteolysis has been shown to correspond with tumour growth, vascularization and metastasis, [17].

2. 4 Haemostasis and it's Physiological Regulation

Blood consists of both cellular and plasma components. It is essential for transporting substances such as nutrients, waste and oxygen around the body as well as being a crucial source of information for coagulation parameters, hypercoagulability, and alterations in fibrinolysis [18]. There are three cellular elements; red blood cells distribute oxygen throughout the body, white blood cells protect the individual from infection and platelets initiate clot formation. Plasma consists of majority water as well as proteins such as albumin and fibrinogen [19].

The term 'Haemostasis' originates from the Greek language and translates as "haeme" meaning blood, and "stasis" meaning to stop [20]. The concept dates back to the 1960's where Davie, Ratnoff and Macfarlane described a "waterfall sequence" or cascade of

proenzymes that subsequently activate their target substrates through proteolytic cleavage [21]. There are two independent mechanisms of platelet activation that can stimulate the initiation of this cascade, the first being dependent on tissue factor localised to the vessel wall and the second requiring endothelial disruption and subsequent exposure of the subendothelial matrix proteins, such as collagen [22].

The coagulation process begins with primary haemostasis, whereby vasoconstriction causes the blood vessels to contract to restrict blood flow, therefore preventing excessive blood loss through the site of damage. Platelets play a pivotal role by adhering, with the help of collagen and von Willebrand factor (vWF), to the endothelium of the damaged vessels, [23]. With the help of thrombin, platelets are then activated which ultimately leads to platelet aggregation, which are interconnected by fibrinogen. This provides a surface whereby fibrin strands can polymerize and form a temporary plug and matrix for secondary haemostasis to solidify, [24].

Secondary haemostasis consists of clotting factors acting in a cascade or sequence of enzymatic reactions in either an intrinsic or extrinsic coagulation pathway, which converge at factor X to form a common pathway. The intrinsic pathway includes the clotting factors I, II, IX, X, XI, and XII (fibrinogen, prothrombin, Christmas factor, Stuart-Prower factor, plasma thromboplastin, and Hageman factor respectively). Factors I, II, VII, and X make up the extrinsic pathway. The common pathway is initiated via activation of Factor Xa and consists of factors I, II, V, VIII, X, [24].

These factors comprise of proteins or ‘zymogens’ which circulate in an inactive form until serine proteases activate them by proteolysis. The active enzyme binds it’s substrate and subsequently leads to it’s cleavage, acting as a catalyst to activate the next enzyme in the cascade, [25]. This is a rapid process activated either by exposed endothelial collagen, in the case of the internal pathway, or through tissue factor (FIII) released by damaged endothelial cells, in the case of the external pathway, The end result of secondary haemostasis sees prothrombin activated to thrombin which crosslinks with fibrin to form a stabilised clot, as seen in figure 1;

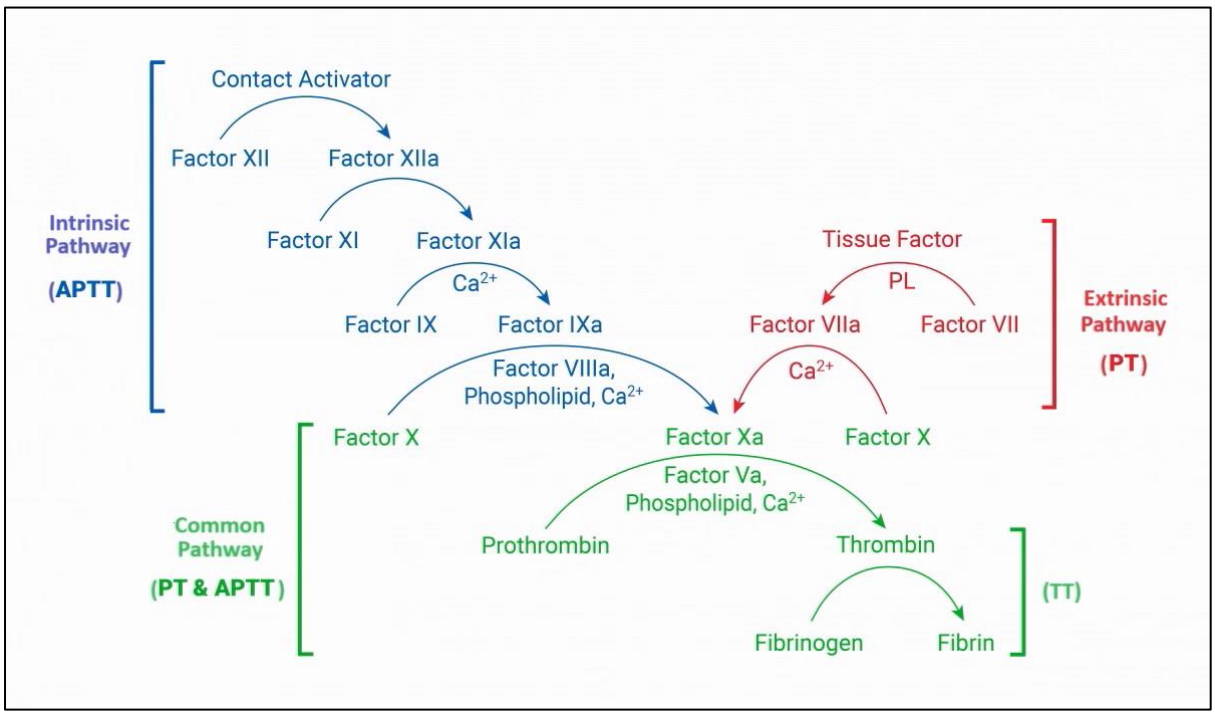

Figure 1. An overview of the blood clotting cascade showing the system and it's distinct pathways (adapted from [26])

Fibrinolysis is a highly regulated enzymatic process and essential component of haemostasis. It ensures thrombus dissolution and the removal of any unnecessary thrombi. Mediated by plasmin, it results in the cleavage of fibrin, fibrinogen and soluble fibrin polymers resulting in degradation products such as Fibrin Degradation Products (FDP) or D-dimer [27].

Many regulatory mechanisms surround the coagulation process to prevent any imbalance from occurring as this could lead to unwanted thrombosis or fibrinolysis and excess blood loss. Events such as trauma, infectious agents, surgery or anaesthesia can trigger the haemostatic system and disturb this delicate equilibrium. Physiological disturbances, disruptions in primary haemostasis or abnormalities in the blood, plasma or disorders such as disseminated intravascular coagulation (DIC), may also result in coagulopathies [28].

Physiological anticoagulants such as Antithrombin, Protein C and Tissue Factor plasminogen inhibitor exhibit regulatory roles over physiological coagulation. Coagulation enzymes are tightly monitored and inhibited through several mechanisms including enzyme cleavages, active site blockades, stable complex formations, and substrate modifications [29].

2.5 Disorders of Haemostasis

Coagulation or haemostasis disorders include any defect or abnormality in blood clot formation. As the aim of haemostasis is to prevent excess haemorrhaging upon endothelial injury, abnormalities in coagulation may result in either defective clot formation and therefore excess blood loss, or alternatively blood hypercoagulability in arteries or veins predisposing the affected to thrombosis [24].

These abnormalities can be congenital and therefore present at birth, arising from mutations of genes involved in the coagulation pathway. Von Willebrands Disease (vWD) is the most common inherited bleeding disorder of dogs which results in a defective or deficient von Willebrand factor (vWF) [30]. As vWF is essential for normal platelet adhesion, those affected with vWD can present with haematuria, epistaxis or prolonged bleeding during surgical interventions [31].

Acquired coagulopathies, resulting from liver disease or DIC, can appear at any stage in life and can present with similar coagulation abnormalities but varying background pathophysiology. Therefore, these cases would ideally be diagnosed early in clinic using coagulation screening tests to define and efficiently manage them [29]. Due to the majority of procoagulant factors and anticoagulant proteins C and S being produced in the liver, hepatic disease can also result in clotting factor defects. Vitamin K deficiencies are also prevalent among liver failure patients often due to malabsorption [32]. Recent evidence proposes that tissue factor (TF) acts not only as the primary activator of the coagulation cascade but is a critical mediator of coagulation in liver disease. Therefore, it can be beneficial to evaluate a serum biochemistry panel evaluating liver function if a clotting disorder is suspected, [33].

2.6 Clinical Significance & Coagulation tests

Clinical symptoms such as delayed coagulation or bruising deep in the tissues can suggest a blood clotting defect, while platelet defects tend to present as superficial small bruises, epistaxis, or prolonged bleeding at injection and surgery sites. Most often, young animals are diagnosed upon eruption of their adult teeth or during a surgical procedure [30]. Along with these clinical signs, functional screening tests such as buccal mucosal bleeding time (BMBT), Activated Partial Thromboplastin Time (aPTT), Activated Clotting Time (ACT), Prothrombin Time (PT) and Thrombin Time (TT) are routinely used in clinic to assess blood clotting function in patients by reflecting the intrinsic pathway, extrinsic pathway and

conversion of fibrinogen to fibrin [34]. Among others, these laboratory tests aid in exposing the true cause of any unexplained bleeding and identifying coagulopathies. Rapid diagnosis of these disorders is crucial as, if left untreated, they may lead to the development of anaemia or severe life-threatening haemorrhaging [31].

Blood coagulation monitoring has become crucial not only in diagnosing the cause of excess blood loss in a patient, but is also important throughout the process of developing anticoagulant drugs, determining the risk associated with surgical procedures, and examining the efficacy of haemostatic therapies [18]. While the functional tests indicate the presence of a coagulopathy in the individual, quantitative and structural analytical tests can be applied to specify the underlying cause of the disease [30].

2.7 Activated Clotting Time

2.7.1 Description

Activated clotting time is a rapid screening test for the coagulation factors of both the intrinsic and common clotting pathways and until recently was the only simple and widely available way to do so, [35].

2.7.2 Synthesis

The ACT test uses fresh whole blood added to a tube containing a negatively charged activator to cause the formation of a clot. Previously diatomaceous earth was used as the clot activator but has been replaced by celite, glass or kaolin activators that can result in variations in clotting times depending on which is selected. A combination of celine-kaolin-glass beads are incorporated in some versions of the test [36]. The inclusion of endogenous platelets as a source of phospholipid is required for the ACT test.

The tube was originally kept at 37°C using a heating block or water bath and tilted slowly until a clot has formed but is now more often used alongside a fully automated technique which detects and records the clot formation electronically (37).

Hand-held point of care (POC) devices containing kaolin or celite as activators for clot formation have been applied to detect canine ACT intervals. These devices provide rapid results, within 15 seconds, and require only a 40µl blood sample making them extremely appealing and easy to use in clinic [38].

2.7.3 Function

ACT measures the time taken, in seconds, for clot formation to occur in fresh non-coagulated whole blood in a test tube, after a clotting cascade activator such as kaolin or celite is added [38].

Due to the rapid result generated by the ACT test, it is often used to monitor acute changes during surgery when anti-coagulants, such as heparin, are used and has been successful in monitoring heparinised ponies during prolonged cardiopulmonary bypass [39]. However, it can be potentially misleading in this role due to it's ability to be influenced by external factors such as hypothermia or platelet dysfunction [40].

2.7.4 History

The ACT test is a 1966 modification of the original Lee-White whole blood clotting time assay which was originally developed to monitor patients suffering from haemophilia. This method left the tube undisturbed for 5 minutes, checking it every minute afterwards for clot formation, [41]. Unlike most other conventional clotting tests, it is simple, inexpensive, reproducible and therefore not limited to larger veterinary laboratories [35].

2.7.5 Analytical Method

Variability in test results can occur using the visual clot detection method which was the original method used, and which determined when a fibrin clot was first detected by vision alone [42].

Developments of end-point detection via an optical or electromagnetic sensor method, and an ACT monitor has improved both the accuracy and efficiency of the test. Automated coagulometers equipped with these sensors has improved the efficacy of the test, as the change in both viscosity and turbidity is measured during transformation of plasma into a clot, [38].

2.7.6 Diagnostic Importance

As aPTT can also be used to assess intrinsic and common coagulation pathways, measuring the ACT has been found to offer no additional information regarding secondary haemostasis and seemed to produce more variable data than the aPTT method according to Tseng et al., 2001 [35]. It is less sensitive than aPTT, with a functional or quantitative decrease in coagulation factors of at least 90% needed to result in a prolonged ACT [43].

However, a study in 2009 showed that ACT has a stronger correlation with inflammatory markers, such as CRP and could therefore lead to an earlier indication of systemic

dysfunction [44]. A study by Connell, JM et al., 2007 found that a steer which had a coagulation defect in factor XI, but showed no clinical haemorrhagic symptoms, would not have been diagnosed had it not been for the whole blood clotting time assay which was being done routinely on all calves when the steer was born, [45].

Although ACT alone is sufficient in the demonstration of severe inherited or acquired coagulopathies, such as haemophilia A or DIC respectively, using it alongside other screening methods of increased sensitivity is preferred.

2.7.7 Species Differences

Table 1. ACT times among different species (*whole blood clotting time at 42℃). (Adapted from [36], [45a], & [46])

Species	ACT (sec)
Canine	55 - 80
Feline	55 - 85
Bovine	120 - 180
Domestic Chicken	8-180 min*

In determining ACT for dogs, no substantial differences exist between male and female dogs and age and body weight seem to have no influence on ACT [38] and the mean was found to be 77.5 seconds, [48]. In clinically healthy cats, an ACT of < 165 seconds is considered normal with on average a range of 55-85 seconds being seen in clinic, as shown in Table 1 [49], [50]. Pigs ACT is on average 64 seconds [51] while sheep ACT range is approximately 195–215 seconds and 155–175 seconds in the goat [45]. Horses ACT has been shown to be 2 minutes 38 seconds [39]. Although there is a lack of key contact factors in avian species, tubes containing diatomaceous earth have been successful in obtaining ACT for some species [47].

2.8 Activated Partial Thromboplastin Time

2.8.1 Description

The Activated Partial Thromboplastin Test (aPTT) is an assay that can be used to indicate the function of the intrinsic and common coagulation pathways. It differs to the ACT in that it is of increased sensitivity to a deficiency in factors of these pathways. It also does not depend on platelets to support the reaction, [36].

2.8.2 Synthesis

Many different commercial kits have been produced for widespread clinical use. A variation of reagents and phospholipids exist, with those involving kaolin and human placenta

respectively found to be the most sensitive [52]. Each kit contains instructions matching the manufacturer's recommendations and reagents either ready for use or needing prior preparation. The test consists of first preincubating citrated plasma with a surface activator such as kaolin or ellagic acid to activate factors XII and XI. [53]. CaCl2 is then added, which triggers a calcium-dependent cascade resulting in a fibrin clot. With this step a stopwatch is started simultaneously. Coagulation activity (%) is calculated using reference curves generated as standards for each reagent [52]

Until recently viscoelastic tests were only available in larger clinical labs and referral centres. The development of a handheld monitor designed for POC use has made viscoelastic coagulation monitoring more widely accessible, [54]. This device uses 340 µl of fresh, whole untreated blood transferred immediately after venipuncture onto a disposable cassette and is taken up via capillary action. The loaded cassette is then placed in the device and analysis begins on the clotting kinetics. Information is transmitted via optical sensors which measure both the motor and free moving arm of the device once coagulation is initiated. A receiving device collects this information, and a graph and series of values are displayed on a monitor for clinical interpretation [54].

2.8.3 Function

aPTT is a global test, with a heightened sensitivity to low levels of coagulation factors than ACT. It detects discrepancies in the contact (prekallikrein, high molecular weight kininogen), intrinsic and common coagulation pathways, occurring due to either a deficiency in coagulation factors or the presence of inhibitors, [55].

Viscoelastic testing allows for the dynamics of fibrin formation, clot strength, platelet function and fibrinolysis to be analysed [54], previously overlooked by standard aPTT tests. This application has not only resulted in accelerated screening for hypercoagulability but also improved management of haemostasis during and post-surgery [56].

2.8.4 History

The test to measure aPTT is derived from the test originally designed in 1953 to aid Brinkhous et al. [55] in purifying factor VIII from plasma. In this 'partial' form of the test instead of both the phospholipid and the surface activator being controlled, only the phospholipid concentration is, [57].

Initially the test involved adding a phospholipid, such as cephalin, as a platelet substitute, as well as calcium chloride to plasma but was later modified by Proctor and Rapaport who

added kaolin also [58]. This enhanced the activation of the contact phase, shortened the coagulation time and increased the reliability of the results, [55].

Viscoelastic monitoring using thromboelastography (TEG) or rotational thromboelastography (ROTEM) was first introduced in 1910 by Koffman [56] to measure the viscoelastic properties of clot formation and allow for a more global assessment of haemostasis in the individual, [59].

2.8.5 Analytical Method

Mathematic modelling can be used to analyse the aPTT test [53] but most labs apply an automated method using an optical density to indicate clot formation once a certain threshold is exceeded. It is often analysed in combination with PT, TT and fibrinogen concentration, [57].

2.8.6 Diagnostic Importance

As well as being involved in identifying any existing deficiency in coagulation factors or the presence of inhibitors to factors of the intrinsic or common coagulation pathways, a prolonged aPTT can indicate the presence of acquired deficiencies, such as those resulting from liver dysfunction or vitamin K deficiency. To be of highest reliability and accuracy, the interpretation of aPTT is ideally accompanied by prothrombin and fibrinogen concentrations [36].

More recently, an association between prolonged aPTT and the presence of circulating anticoagulants such as lupus in those affected by Systemic Lupus Erythematosus has been identified [60]. As coagulation factors XI, IX, VIII, II and fibrinogen have been recognised as being risk factors for VTE, a shortened aPTT has enabled the test to be used to categorise patients at increased risk of developing VTE [61] as well as being the test of choice for dose adjustment of unfractionated heparin for these patients [55]. aPTT is considered a more reliable indicator than alternative coagulative tests such as ACT for determining anti-coagulability, and so is also the preferred method of monitoring for sheep undergoing long-term treatment with heparin [62].

Liver failure, a vitamin K deficiency or an increased consumption of clotting factors during DIC can produce a prolonged aPTT along with PT. aPTT proved to be a significant marker of DIC in cats, as Estrin MA et al. [63] showed it to be prolonged in all cats suffering from this condition. As there is not one individual marker pathognomonic for DIC, several abnormalities in the coagulation profile are expected, although variation in synthesis rates of

clotting factors and their half-lives may result in only one abnormal clotting time (PT or APTT) showing. In a study of calves suffering from suspected septic shock, a prolonged APTT and PT, increased fibrin degradation products, thrombocytopaenia and schistocytes were found to be common abnormalities seen in the coagulation profiles [64].

2.8.7 Species Differences

Ranges can vary considerably between species, as shown in table 2, and between strains within species, which could be attributed to physiological differences or changes in testing methodology. It has also been reported that different reagents used can impact the test's sensitivity [52], with the type of phospholipid material in the reagent showing correlation to the sensitivity of the APTT test. As birds lack both factor XII and XI, aPTT endpoints can vary considerably and are often prolonged compared to those of mammals [46].

Table 2. The Activated Partial Thromboplastin Reference Intervals of Different Species (adapted from [36a], [47] & [46]).

Species	aPTT (sec)
Canine	11 – 17.5
Feline	15 - 21
Equine	35.8 – 48.6
Capri	16.2 – 24.3
Domestic Chicken	66 - 296

2.9 Prothrombin Time

2.9.1 Description

The Prothrombin test (PT) is used to analyse the coagulation factors of the extrinsic and common pathways, with the exception of tissue factor (FIII), [36].

2.9.2 Synthesis

The plasma is incubated at 37 °C with the addition of an activating agent such as thromboplastin. After plasma recalcification, the time required for fibrin filaments to form is measured. This can vary between species as shown in table 3. These intervals can also vary between labs based off the chosen reagent properties, instruments and method followed. Therefore, results are not directly comparable and reference values are established for each reagent or laboratory [65].

2.9.3 Function

The Prothrombin Test (PT) uses citrated plasma to identify any abnormality in the extrinsic and common pathways (coagulation factors VII, X, V, II, and fibrinogen), with the exception of Tissue Factor (FIII), by measuring the time necessary to generate fibrin upon the activation of factor VII.

The test is deemed to be more sensitive for deficient levels of factors than aPTT, as a prolonged result is seen with even a relatively small drop in levels of factor VII. It is considered one of the most important coagulation screening tests in dogs, [66].

2.9.4 History

Prothrombin was first hypothesised to exist as a precursor for thrombin by Alexander Schmidt in the late nineteenth century, upon realising that thrombin could not exist in it's true form in the circulation due to its ability to coagulate blood [67].

The PT test was first developed in 1935 by Professor Armand Quick and later altered by Professor Paul Owren, who changed the reagent used to a combined thromboplastin reagent [68].

The PT-International Normalised Ratio (INR) value was introduced as a standardisation system for PT in 1983 by the World Health Organisation (WHO). PT-INR is advantageous due to it being directly comparable between different laboratories using varying PT reagents. This enables the production of consistent dosage guidelines and a target anticoagulant intensity be attained. Target ranges for PT-INR for venous thrombotic syndromes have been identified as 2.0–3.0, and 2.0–3.5 for valve replacement. Values >4.0–4.5 suggest an increased risk of bleeding complications, [69]. PT-INR is the preferred method used for those patients taking anticoagulants such as vitamin K antagonists or warfarin [70].

2.9.5 Analytical Method

Interpretation of PT should ideally be accompanied with an aPTT test and as with a prolonged aPTT, a prolonged PT can suggest the presence of either a factor deficiency or a coagulation inhibitor circulating, [36].

The PT can be measured in plasma via activation of the coagulation cascade with tissue factor visually detecting clot formation. PT-INR can be used to account for inter-device variations and different sensitivities to the activator. This conventional method can be timely,

hindering it's reliability particularly for those conditions which can change or develop rapidly, [71].

In dogs, the application of a human POC test for PT is a reliable and cost-effective way of assessing haemostasis, providing results in minutes. However, when a prolonged PT is generated, confirmation using a standard method is still recommended (Kelmer, E et al., 2014). Although confirmational studies are required, when applied to horses this POC device also proved specific and sensitive to abnormal PT values however, when applied to those with low PCV (<0.25), the results are less reliable [72].

A Laser Speckle Rheology sensor, is a portable, battery-operated optical sensor, that can be applied to quantify a patient's PT/INR within seconds. It does so through the analysis of fluctuations in laser speckles resembling the alterations in the viscoelastic properties produced from a blood sample activated by the addition of a thromboplastin reagent, [71].

2.9.6 Diagnostic Importance

The inherited factor VII deficiency is a rare bleeding disorder characterized by a normal aPTT but prolonged PT. A modified PT using factor VII-deficient plasma is required in order to confirm a specific factor VII deficiency [36]. Acquired deficiencies are often related to hepatic synthesis disorder, warfarin therapy, DIC, severe bleeding, or massive transfusion. A prolonged PT and aPTT can appear in sheep infected with babesiosis, an extremely detrimental tick-borne disease to sheep and goats, which can indicate the presence of DIC [73]. Cats and horses suffering with DIC will have a prolonged PT and normal APTT while the PT of most dogs with DIC tends not to change [74]. As anticoagulant rodenticides result in a prolonged PT coinciding with a depleted factor VII, measuring PT can also be used for monitoring a deficiency in vitamin K1 and response to vitamin K therapy and will produce a prolonged PT result before aPTT [75].

Unfortunately, PT or aPTT do not screen for factor XIII deficiency which was found to be the most consistent alteration among cats suffering with liver disease. As this factor plays an important role in regulating fibrinolysis as well as playing a key role in the final stage of the clotting process, it's indicated that PT and APTT do not sufficiently represent the entire clotting process as it occurs in vivo [76].

2.9.7 Species Differences

Although PT has been standardized for domestic chickens, there remains a lack of validated coagulation tests for nondomestic avian species, limiting the investigation of acquired

haemostatic disorders in these species. In 2014, a PT reagent based off human placental thromboplastin showed promise for potential use in avian species, but further study is still required [78].

Table 3. Prothrombin Time Reference Intervals (Adapted from [36a], [79], [46], [47])

Species	PT (sec)
Canine	11.0 - 15.5
Feline	15.0 - 20.0
Equine	16.0 - 20.0
Bovine	20.1 - 30.1
Domestic Chicken	49 - 132
Capra	15.7 - 19.8

2.10 Thrombin Time

2.10.1 Description

The thrombin time (TT), or thrombin clot time, can be used to analyse the final reaction of the clotting cascade as it directly measures functional fibrinogen and the time required to drive the formation of fibrin in the presence of thrombin [27].

2.10.2 Synthesis

The thrombin time test uses citrated plasma which is incubated at 37°C. After the addition of thrombin, the time necessary for fibrin filaments to form is measured in seconds [66], with the reference range varying depending on the concentration of thrombin used. It does so by first directly acting on fibrinogen to produce a soluble fibrin polymer, then converting this to insoluble or cross-locked fibrin polymer by factor XIII [66].

2.10.3 Function

Thrombin time directly measures the time needed to convert fibrinogen to fibrin, in a thrombin solution, and form a blood clot [36]. Along with PT, aPTT, TT is one of the most useful tests when it comes to coagulopathies and anticoagulant drug monitoring in patients and can be applied to qualitatively test for unfractionated heparin in blood as it is sensitive to inhibitors in the plasma, [18].

As the coagulation factors FXI, FVIII, FX and FV are all activated by thrombin resulting in further thrombin generation, referred to as “thrombin burst”, [80].

2.10.4 History

Thrombin was first coined by Virchow in 1976 and in the early twentieth century it was understood that thrombin, once produced from prothrombin could convert fibrinogen to fibrin [81].

Thrombin Generation Assays (TGA) arrived in the early 1950s from Macfarland and Biggs which developed over the years to enable computer software to calculate parameters from the thrombin generation curve or 'thrombogram'. It was further modified to include a fluorogenic substrate which can be measured by a 405-nm wavelength upon thrombin splitting. TGA is also suitable to thrombin measurements in platelet poor plasma due to its ability to be unaffected by the turbidity of clotting plasma, [82].

2.10.5 Analytical Method

The time taken for a clot to form upon addition of thrombin to plasma is recorded in seconds by sampling the clotting mixture at regular intervals, [36].

Although used in conjunction with PT and aPTT, TT is considered to be a more sensitive indicator to low fibrinogen measuring the time taken in seconds for clot formation upon the addition of thrombin to plasma [36]. A Reptilase Time assay can be applied to exclude any disturbances to fibrinogen activity due to heparin, as this test is unaffected by it. Therefore, a prolonged TT result but normal Reptilase Time result indicates the presence of heparin in the patient's blood, [83].

TEG and Thrombin generation assays can be used to evaluated thrombin burst times by analysing the balance between thrombin generation and decay. TGA is ran in an automated fashion, requiring reagents such as Tissue Factor, Phospholipid, a fluorogenic substrate and calcium chloride to initiate coagulation. The fluorescent signal produced is recorded by computer software and to measure thrombin activity [82].

2.10.6 Diagnostic Importance

Usual blood concentrations of fibrinogen range from 1.3g/L – 4.8g/L in dogs, [84]. Abnormal TT results can indicate the presence of a qualitative fibrinogen abnormality known as "dysfibrinogenemia" or a fibrinogen deficiency, "hypofibrinogenemia" if the concentration is < 100 mg/dl. Alternatively, abnormalities may indicate the presence of an inhibitor to thrombin's activation [66]. These abnormalities can be caused by a variety of reasons, be it due to a congenital or acquired background.

A lack of fibrinogen, afibrinogenemia, or a decrease in concentration, hypofibrinogenemia, can be acquired, most often due to a consumptive coagulopathy such as DIC, or develop as a result of impaired hepatic synthesis or through massive haemorrhaging. Since hypofibrinogenemia is rare in large animals, a prolonged TT is often indicative of the presence of FDPs in these species [64].

Although rare, primary fibrinogen deficiency can occur, and although there is no specific treatment available, should be included in differential diagnoses for locked jaw syndrome or eye protrusions in dogs, especially when young [84]. Afibrinogenemia is a rare bleeding disorder with an autosomal recessive pattern of inheritance [30] and has been reported in Saanen goats. Specific factor assays and pedigree analysis are crucial in determining the definitive diagnosis of the hereditary deficiency, [85].

2.10.7 Species Differences

A qualitative deficiency in fibrinogen (dysfibrinogenemia) is also quite a rare condition in all species and can be acquired or inherited in an autosomal dominant pattern. In cats, fibrinogen is an acute phase protein with elevated levels being seen in response to inflammation and infection. It is less affected by consumption than FXIII in cats with liver disease, [76].

Siller-Matula et al in 2008 showed sheep to need a 100-fold lower dose of thrombin to decrease the clotting time compared to rats, pigs and rabbits [86].

Table 4. Reference intervals for Thrombin Time Coagulation Test for different species, [36], [79].

Species	TT (sec)
Canine	5.0 - 9.0
Feline	5.0 - 8.0
Equine	5.0 - 9.0
Bovine	12.4 - 17.2

2.11 D-Dimer

2.11.1 Definition

D- Dimers are produced during plasmin mediated fibrinolysis, or plasmin-mediated degradation of cross-linked fibrin and therefore are useful biomarkers of fibrinolysis. They consist of the D-domains from two adjacent fibrin monomers, cross-linked by their γ chains. [76].

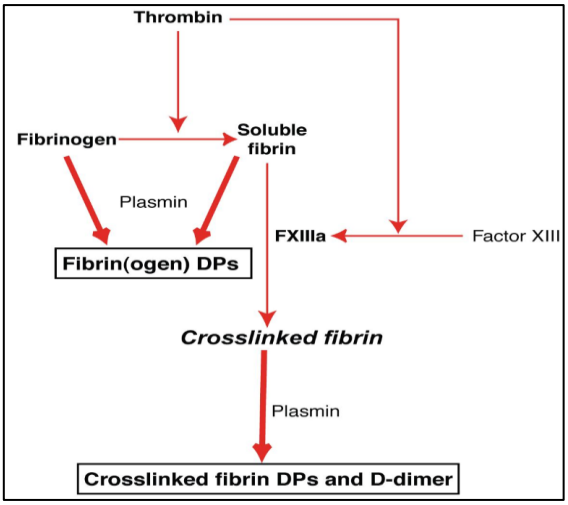

Figure 2. Pathway illustrating the synthesis of FDPs and D-dimer [36].

2.11.2 Synthesis

D-dimer is a specific product of crosslinked fibrin, generated through it's plasmin mediated breakdown, as shown in Figure 2. The exposure of D-dimer allows it to be detectable by immunological-based methods [36].

2.11.3 Function

D-dimer concentrations are specific for fibrinolysis, with their presence being an indication of thrombin and plasmin generation. This makes D-dimer concentration measurements particularly useful in the diagnosis of systemic thrombosis including PTE and DIC, as D-dimer is more specific for fibrinolysis than FDPs [36].

2.11.4 History

In 1973, Gaffney reported the identification of a unique fragment produced through the hydrolytic action of plasmin on human and bovine fibrin subsequently known as D-dimer, and predicted it's use and detection in assays would be a useful addition to diagnostic tools [87].

2.11.5 Analytical Method

D-dimer concentrations can be measured using a citrated plasma sample and a variety of assays, including latex agglutination assays, whole blood agglutination assays or ELISA techniques, using monoclonal antibodies bound to latex beads against the D-dimer epitope [27].

The immunoturbidimetric assay is performed using an automated chemistry analyser, which measures the change in turbidity once antigen-antibody complexes are formed from adding monoclonal antibody-coated latex beads to blood samples, [88]. Results are reported as concentration of D-dimer (ng/ml) compared to the species standard.

A precise value, rather than a concentration range, offers more accurate monitoring for continuous assessments of patient status. Combining this result with clinical criteria early in the diagnostic work-up can be useful for ruling out thrombosis and PTE. ELISA assays have been identified as being the most sensitive method, with the development of a rapid fluorescence quantitative ELISA assay resulting in a quicker turnaround of results in less than one hour [89].

An immunometric POC device exists which requires no specialised equipment and is therefore a very attractive option for clinical use as a screening test for those with DIC or thromboembolic disease, [90].

2.11.6 Diagnostic Importance

An increased D-dimer concentration has been associated with DIC; an acquired disorder of haemostasis secondary to systemic inflammatory disease or infection, characterized by the activation of both the coagulation and fibrinolytic pathways, [63]. DIC is a considerable risk factor for mortality in all species and therefore requires rapid diagnosis which, along with the presence of D-dimers, is based off a prolonged aPTT, PT, TT, thrombocytopaenia and hypofibrinogenemia among others. [63].

An increased D-dimer value is also associated with venous thromboembolic disease, internal haemorrhage, variceal bleeding, cardiac disease, renal disease, liver disease as well as a strong correlation existing between D-dimer values and pulmonary emboli [91]. Monitoring D-dimer concentrations for colic horses at admission and throughout hospitalisation can function as a prognostic marker and aid in recognising DIC in those with severe gastrointestinal disease [92]. In a study performed by Dirck et al. [76] an increased D-dimer concentration was shown to be present in about half of the feline sample population suffering from liver disease, and was among the most consistent abnormalities of all cats with liver diseases. A significantly higher D-dimer concentration was also observed in those with neoplastic, inflammatory liver diseases, and hepatic lipidosis, compared to the control group.

Although difficult to confirm, as cut-off values were previously lacking for cattle, analysis carried out by Gunes et al., showed D-Dimer to be a sensitive and specific test in cattle

suffering from theileriosis. It also has the potential to be an accurate marker of BVD-MD, peritonitis and for identifying cows with Left Abomasal Displacement [91]. In swine, D-dimer levels were accurately measured using saliva samples and an automated immunoturbidimetric assay, and were seen to show a positive correlation with an autonomic nervous system response to stress [93].

D-dimer values have also been shown to be of interest for use as a tumour biomarker and prognostic marker. A positive correlation has been identified, with higher D-dimer concentrations in dogs suffering from malignant tumours compared to those with benign tumours, as well as elevated levels in those with distant metastases. A recent study has shown that increased D-dimer concentrations subsequently decreased after surgical removal of the benign or malignant tumour was performed in affected dogs [94] showing a strong correlation to tumour burden in patients. Boye et al, 2021 showed that D-dimer levels have the potential to be applied as a prognostic indicator in dogs suffering from non-Hodgkin Lymphoma [95].

D-Dimer measurement is extremely sensitive but not specific for patients with DIC and therefore, has been found to be most beneficial when used in tandem with other tests, such as FDP to maximise both sensitivity and specificity [96]. As it is sensitive to a number of clinical conditions, including any physiological process leading to increased fibrin breakdown, it has been found to be of limited use as a lone indicator in diagnosing hypercoaguable states in dogs [90].

2.11.7 Species Differences

Normally dogs and cats have D-dimer values of < 250 ng/ml, whereas healthy horses lie < 500ng/ml but can be as high as 1000 ng/ml as seen in Table 5.

An elevated D-dimer level in dogs has been found to be a sensitive marker of fibrinolysis in those with DIC, as well as high values being indicative of neoplasia, inflammatory disease or haemorrhagic effusions [36]. In horses D-dimer concentrations are increased in severe colic cases and can be applied as a prognostic indicator in these cases [97]. In cats, D-dimer concentration as a useful tool for detecting DIC or thrombosis is less apparent [36].

Table 5. Reference Intervals of D-dimer values according to each species, (adapted from [90], [36], [91], [47]).

Species	D-Dimer (ng/ml)
Canine	0 - 575
Feline	0 - 250
Equine	0 - 1000
Bovine	230 - 650
Capra	0 - 680

2.12 Fibrin Degradation Products (FDP)

2.12.1 Description

Assays for Fibrin Degradation Products (FDP) represent the final degradation products resulting from plasmin acting on fibrinogen or soluble non-crosslinked fibrin, [17] as demonstrated in Figure 2.

2.12.2 Synthesis

Assays for FDP exist using either serum or plasma. The serum test uses polyclonal antibodies that cross react with intact fibrinogen and therefore require its removal via Reptilase tubes or application of an inhibitor [36].

The plasma test is more advantageous as it uses a citrated plasma sample and a latex agglutination kit consisting of monoclonal antibodies against human fibrin(ogen) degradation products, that, unlike the serum test, do not cross-react with intact fibrinogen [98].

A plasma FDP kit been successfully used in dogs as it is deemed more sensitive than serum FDP assays for diagnosing disorders such as DIC or thromboembolism [99].

2.12.3 Function

FDP assays indicate the activation of plasmin as the fibrinolytic system relies on it's proteolytic activity [17]. Plasmin acts on the circulating fibrinogen and soluble (non-crosslinked) fibrin, cleaving them and ultimately producing terminal fragments E and D [36].

2.12.4 History

The products of fibrinolysis were first introduced in 1960 by Nussenzweig and Seligmann using immunoelectrophoresis and immunodiffusion assays to demonstrate the split products that present with fibrinogen digestion in vitro [100].

2.12.5 Analytical Method

Standard plasma FDP kits provide reagents for the detection and quantification of FDP using latex particles coated with monoclonal antibodies. The concentration of FDP can be determined through the macroscopic agglutination that occurs between the antibodies and particles, and comparing this to a positive control, with <5µg/ml plasma being deemed as a negative result [101].

Plasma FDP assays can also use the immune complexes formed via the antibody-FDP relationship to produce light scattering, which is proportional to the FDP concentration in the sample. Turbidity is read at 500- 600nm to measure the amount of light scattering, [102].

2.12.6 Diagnostic Importance

As with D-dimer assays, FDP assays are most beneficial when used alongside other coagulation tests to fully characterise any disorders present. As any pathologic intravascular coagulation, thrombosis, or severe internal haemorrhage or liver disease, can result in an increased concentration of FDP, their detection is not pathognomonic to any one specific disorder [36].

Most properties of fibrinogen, fibrin and their fragments are of particular interest in tumour development as they actively contribute to cancer progression. They can stimulate endothelium indirectly to secrete von Willebrand factor, enabling them to have proinflammatory activity leading to platelet activation which accompanies neoplastic disorders, [17]. In dogs with sepsis, both FDP and D-Dimer have been recognised as important markers as elevated concentrations were identified compared to controls, indicating fibrinolysis activity [103].

As mentioned previously, the diagnosis of DIC is often based off a number of markers including FDPs, prolonged aPTT, PT, thrombocytopaenia, phenotypic abnormalities in the RBCs, decreased levels of coagulation factors and reduced antithrombin activity, [64]. Therefore, an array of these tests is considered most effective when diagnosing this condition.

2.12.7 Species Differences

The canine latex agglutination kits based on monoclonal antibodies used on citrated plasma samples have been validated, with results < 5 µl/mL being recognised as abnormal. However, horses with colic as a result of gastrointestinal disorders have increased levels of FDP in blood and peritoneal fluid than healthy control horses. The plasma and serum FDP assay has been associated with a higher frequency of false positive results when used in horses and

therefore are not considered valid indicators of abnormal or pathologic fibrinolysis in horses with colic, [104].

2.13 Proteins Induced by Vitamin K Absence or Antagonism (PIVKA)

2.13.1 Description

Proteins Induced by Vitamin K Absence or Antagonism (PIVKA) are inactive circulating precursors; FII, FVII, FIX and FX, that rely on vitamin K for their activation, [105].

2.13.2 Synthesis

The PIVKA test is a modified version of the PT test and uses diluted plasma with a specific thromboplastin reagent less sensitive to coagulation factors II, VII and X than that of PT, [105].

2.13.3 Function

As approximately 4% of animals die due to rodenticide poisoning, whether through malicious intent or animals such as swine, wild mammals or birds ingesting poisoned rodents [106], the PIVKA test is considered to be a crucial diagnostic test in veterinary medicine for diagnosing those suffering from anticoagulant rodenticide poisoning.

2.13.4 History

The PIVKA test was first introduced into veterinary medical literature in 1986, [105] and has since been demonstrated to be a sensitive and diagnostic marker in distinguishing anticoagulant rodenticide intoxication from other coagulopathies [106].

However, some controversy surrounds the PIVKA test as to whether it is sensitive to an increased concentration of these nonfunctional precursors or rather just a decrease in concentration of the specified coagulation factors, [105].

2.13.5 Analytical Method

The PIVKA test has been shown to be most beneficial when used concurrently with other coagulation tests such as PT in detecting prolonged clotting times, with a threefold increase indicative for diagnosing rodenticide poisoning [106].

2.13.6 Diagnostic Importance

Rodenticide poisoning inhibits vitamin K epoxide reductase; an enzyme required for the regeneration of vitamin K, necessary for the synthesis of factors II, VII, IX and X. Therefore, poisoning ultimately results in a decreased concentration of the functional hepatic coagulation factors, and an increase in nonfunctional factors or PIVKAs [107]. An acute deficiency in vitamin K, characteristically recognised by disturbed coagulation, can also be

induced by therapeutic oral anticoagulants, such as warfarin, which act as vitamin K antagonists [106].

In differentiating the presence of alternate haemostatic disorders, such as DIC, the addition of PIVKA to the coagulation tests performed has proven to be of value as, unlike aPTT, PIVKA values do not increase and remain below critical value, allowing clinicians to exclude rodenticide poisoning in the differential diagnoses [105].

The PIVKA test has also been identified to be a potentially useful marker of mast cell tumours in dogs and cats, as heparin, a bioactive substance released from the cytoplasmic granule of mast cells, results in an increased PIVKA value, [105]. Mast cell tumours are ideally identified and surgically removed before any regional or distant metastases has occurred, as they have the potential to be malignant and threaten the life of the affected [108].

2.13.7 Species Differences

A hereditary deficiency in factors II, VII, IX and X occurring in Rambouillet sheep was identified though applying the PIVKA test to plasma. This deficiency resulted in increased lamb mortality due to excess blood loss through the umbilicus or into subcutaneous tissue, [109].

The use of PIVKA tests has, to my knowledge, not yet been fully explored in horses but could be of use in exploring coagulopathies induced by vitamin K antagonists, as discrepancies in PT results among species has been noted [69].

2.14 Russell Venom Test

2.14.1 Description

The Russell Venom Viper Test (RVVT) is a widely used laboratory test for identifying the presence of acquired autoantibodies, known as Lupus Anticoagulants (LA's) or anti-phospholipid (aPL) antibodies. These are IgG or IgM immunoglobulins known to inhibit procoagulant phospholipid complexes or anticoagulant phospholipid dependent reactions [110].

2.14.2 Synthesis

Russell's viper venom of the *Daboia* species has a serine protease, which activates factor V and a metalloprotease, which activates factor X. The dilute test (dRVVT) uses diluted venom and a limiting concentration of the phospholipid reagent, [111].

The dRVVT is performed by incubating a small volume of plasma (0.1ml) with equal volume of dilute venom and phospholipid at 37°C for 30 seconds. Clotting time is recorded after the addition of calcium chloride, [111].

2.14.3 Function

RVVT is used to directly activate factor X, which in the presence of factor V and phospholipid, converts prothrombin to thrombin and forms a fibrin clot. As the dilute test results in a prolonged clotting time, excess plasma or phospholipid can be added to differentiate whether anti-phospholipid antibody concentrations or a deficiency in coagulation factors is the cause [36].

2.14.4 History

Recognised in 1948 in patients suffering with Systemic Lupus Erythematosus [112], LAs result in a prolonged clotting time and, contrary to what their name indicates, are highly associated with thrombotic events [113].

The Russel Venom viper was originally recognised by Dr. Patrick Russell in India in 1796, while Dr. Robert McFarlane later applied it to people suffering with haemophilia [114]. The test dates back to 1975, with the dilute test being later applied in 1985 for LA diagnosis in a large sample population [111].

2.14.5 Analytical Method

The Dilute RVVT (dRVVT) is not affected by inhibitors of factor VIII or IX and therefore is more sensitive for detecting LA than other coagulation tests due to it's phospholipid reagent being rate limited, [115]. Other LA assays such as the kaolin clot time or thrombin thromboplastin inhibition time are alternative methods to dRVVT in identifying LA patients [116]. It has been shown that a combination of assays or different test methods followed by a confirmational functional clotting test yields the highest accuracy in excluding or detecting LA [117], with RVVT and aPTT being the highest recommended tests to be used in combination. Compared to aPTT, RVVT is a shorter coagulation pathway test as it is directly initiated by the effect of the venom on factor X and is recognised to have increased sensitivity to LA [113].

2.14.6 Diagnostic Importance

As concomitant anticoagulant therapy, such as the vitamin K antagonists heparin and warfarin, or direct thrombin inhibitors, can interfere with RVVT, applying the test for LA can yield false positive or negative results in their presence,-Although this suggests a role for RVVT in identifying the presence of such anticoagulants, this sensitivity can reduce the test's

specificity to LA and therefore an exclusion test such as TT can be performed prior to RVVT [116].

A study in 2011 used dRVVT to detect LA in dogs suffering from immune mediated haemolytic anaemia, spontaneous thrombosis and hyperadrenocorticism. However, although rare in healthy dogs, those found those with aPL present had no increased risk of thrombosis [118]. Unfortunately, the conventional RVVT cannot be used in birds due to phospholipids in the rabbit-brain cephalin inhibiting coagulation when RVV is present. However, using purified factor X activating enzyme can reduce this disruptioin, [119]. In deer infected with epizootic haemorrhagic disease, abnormal RVVT results reflected reduced numbers and functioning platelets while prothrombin time showed no significant change [120].

2.14.7 Species Differences

The dRVVT test results can vary between species and depending on the concentration of phospholipids involved in the test. Canine dRVVT results were found to average at 21.2 seconds [118] while an interval of 9–16.1 seconds was identified for domestic turkeys and 11-17 seconds being that for vultures [46].

2.15 Antithrombin

2.15.1 Description

Antithrombin is a single-chain glycoprotein belonging to a family of serine protease inhibitors (serpins). Produced primarily in the liver and to a lesser extent in the endothelia of blood vessels, it is an extremely important coagulation inhibitor as it inactivates thrombin as well as factors Xa, XIa XIIa, IXa, and when in the presence of heparin, Factor VIIa [121]. This class of inhibitors employ a “suicide-substrate” mechanism, luring their target enzyme by mimicking their substrates, then form a stable complex through binding and inducing a conformational change [122].

2.15.2 Synthesis

Citrated plasma is collected and added to a reagent containing heparin, excess thrombin and a chromogen-labelled thrombin specific substrate. Chromogen released by thrombin-mediated cleavage of the substrate is measured, with the amount being proportional to the residual thrombin activity, which in turn is inversely proportional to the level of antithrombin activity [36].

Measurements can also be obtained through the inhibition of FXa. In this assay, excess FXa is added that is inhibited in the presence of AT, resulting in a lack of chromogenic substrate

cleavage. As AT activity is inversely proportional to the amount of substrate produced, low concentration of AT results in more substrate cleavage [36].

2.15.3 Function

Assays for antithrombin levels mainly measure either functional activity or protein quantity to determine whether any qualitative or quantitative defects are present, to identify those at increased risk of unwanted coagulability [123].

2.15.4 History

The discovery of heparin by McLean in 1916 and the later identification of pro-antithrombin led to better understanding of these circulating anticoagulants, [124]. A deficiency in antithrombin was originally recognised in 1965 by Egeberg to be related to an increased likelihood of thrombotic events, [125], with new methods to measure its concentration and the natural inhibitor of FX being devised in 1970 [126].

2.15.5 Analytical Method

The functional activity assay is based on the ability of the sample plasma to inhibit an excess of either thrombin or factor Xa, with results being expressed as percent activity compared to reference plasma, [36]. Upon the substrate being cleaved by thrombin, a chromogenic compound is released. A spectrophotometer detects the change in absorbance, usually at 405 nm, which is inversely proportional to antithrombin activity in the patient plasma, [123].

Immunological methods such as ELISA, radial immunodiffusion and immunoturbidimetric methods are available for measuring antithrombin antigen levels. Latex agglutination methods also are available however are less reliable. The type of assay and incubation time can influence both the sensitivity and specificity of the activity assays, [123].

2.15.6 Diagnostic Importance

Decreased concentration of antithrombin has been shown to predispose patients to thrombotic events, organ failure and may ultimately result in death, by causing a shift in haemostasis towards hypercoagulability. In dogs, hypo-antithrombinemia has been reported in hepatobiliary disease, porto-systemic shunt, sepsis, DIC, Protein Losing Nephropathy, congestive heart failure, trauma, neoplasia, and immune-mediated haemolytic anaemia (IMHA), and after surgery. Increased consumption through inflammatory disease or surgery, reduced synthesis due to liver disease, or loss through haemorrhage, increased capillary permeability or proteinuria are the main mechanisms leading to an AT deficiency [121].

Elevated plasma levels of antithrombin III have been identified in diabetic dogs [127], as well as in cats with Feline Infectious Peritonitis, cardiac disease and hyperthyroidism [36] toxic neutrophils and hypoalbuminaemia were also less common in cats with higher AT concentrations [128]. It is possible that high antithrombin activity play a protective role against excessive coagulation activation [127].

In human studies AT has been identified as a marker with potential use in prognostics as well as in therapeutic planning, which could also be true for animals. Although alone found to be unsuccessful in diagnosing sepsis in neonatal foals [129], it has been shown in humans that a critical and immediate thrombotic risk exists when AT Activity is 30%, a high thrombotic risk at 60%, and potential hypercoagulability when activity is above 60%, [121]. Therefore, further research into it's prognostic ability in animals is warranted as hypo-antithrombinaemia is increasingly associated with a high risk of mortality.

2.15.7 Species Differences

Antithrombin levels can differ between animal species, as shown in Table 3. The half-life in dogs is quite short, being 1.7days on average, [128].

Table 3. Antithrombin values as % for each species. Adapted from [36a] & [47].

Species	Anti-Thrombin (%)
Canine	65 - 145 %
Feline	75 - 110 %
Equine	85 - 130 %
Bovine	80 - 153 %
Capra	108.6 - 156.5 %

2.16 Problems Associated with Coagulation Tests

The current lack of cut off values available in veterinary literature creates a huge challenge in producing reliable results, as a lack of comparative data limits the reliability and therefore credibility of the data generated in research studies. For example, Gunes et al 2017 established the D-dimer cut-off value for the first time, as well as it's specificity and sensitivity in cattle affected with theileriosis, and therefore were unable to compare their values to others of the same animal species [91].

Investigations into acquired haemostatic disorders in avian species is also limited as, although the PT has been standardised for domestic chickens, validated coagulation tests are lacking in

non-domestic species [77]. This may prove problematic in terms of misinterpreting and therefore misdiagnosing cases. Viscoelastic assays used for parrots revealed inaccurate clot formation times and clot strengths as the conditions of the test may not represent those optimised for birds, [46]. Unique challenges are faced in biomarker development in veterinary medicine from the need to qualify each biomarker multiple times, for each applicable species, [2].

Variations may be seen among coagulation tests results for several reasons and cause potential discrepancies to develop among studies. Considering TP, venipuncture technique, sample handling and storage methods, type and lot number of thromboplastin used, and the test method, including that of endpoint determination can all contribute to the production of variable results. The widespread use of similar tools, reagents and methods may enable significant comparisons of coagulation test results take place between clinics and facilitate multi-centre coagulation studies [35].

2.17 Future of Biomarkers & Their Use in Veterinary Medicine

Monitoring blood coagulation with a high degree of both accuracy and reliability is of huge importance in all aspects of medicine today. Advancements in artificial intelligence and machine learning have the potential to improve how we diagnose and manage disease in veterinary medicine. The development of algorithms to measure multiple parameters simultaneously throughout a diagnosis or treatment plan and discover patient specific patterns, would hugely benefit patient care and be potentially one of most noteworthy achievements in coagulation measurement technologies [18].

Recent advances in post-genomic research are mostly due to public repositories, such as GenBank and MSDB, for genomic and proteomic information. Whilst databases for metabolomic studies remain less developed, appropriate bioinformatic resources are increasingly becoming available [6]. Similarly, databases specific for veterinary medicine have been limited compared to that of human medicine, as the genome sequences of many animal species have proven difficult to structurally and functionally annotate [6]. Genome projects focused on those species and diseases of highest biological and economical importance will help to develop species specific assays, and provide an extremely useful tool for those involved in veterinary research.

Although viscoelastic assays, such as TEG, provide additional insight into haemostatic function compared with traditional plasma-based tests such as PT and aPTT [130], the production of a method to evaluate the entire coagulation process could provide further insight and better reflect bleeding and thrombotic tendencies. This method would ideally reflect all components and interactions involved during coagulation, including the endothelium, platelets, coagulation factors and their inhibitors, as well as fibrinolysis and blood flow [131].

3.0 Conclusion

The identification and development of molecular markers have resulted in significant advances in veterinary medicine, not only the early diagnostics of animal disease, but in their management and treatment too. As mentioned previously, monitoring coagulation and the factors involved contributes enormously not only in determining the underlying cause of excess haemorrhaging or blood loss in a patient, but also to the pharmaceutical industry as it can aid in both the developmental stages of medications as well as in determining their efficacy during treatment.

Although there have been many developments and advances over the years, many challenges still surround that of biomarkers, their development and application to the field of veterinary medicine. Unfortunately, as discussed, of the many biomarkers identified few have made their way into routine clinical use compared to that of human medicine. Problems associated with the developmental stages of these markers, including their application and reliability in different species as well as in achieving a high level of specificity and sensitivity, delay or prevent their progression to the large-scale studies required for their proper validation.

Further research into the development of molecular markers and their application to a wide range of domestic species will prove invaluable in accelerating and enhancing our knowledge surrounding disease in veterinary medicine, and guide how we diagnose, manage and prevent disease going forward.

4.0 Acknowledgements

I would like to thank my supervisor Péter Vajdovich for his invaluable guidance, advice and support throughout writing this literature review. I would also like to thank the University of Veterinary Medicine for an unforgettable 4 years and my friends and family, particularly my mum and dad; Agatha and Gerard Clancy without whom the pursuit of this career would not have been possible.

5.0 References

[1]. Myers, M.J., Smith, E.R. and Turfle, P.G., (2017). Biomarkers in veterinary medicine. *Annual Review of Animal Biosciences*, *5*, pp.65-87.

[2]. Perera, T.R., Skerrett-Byrne, D.A., Gibb, Z., Nixon, B., and Swegen, A. (2022). The Future of Biomarkers in Veterinary Medicine: Emerging Approaches and Associated Challenges. Animals 12, 2194. https://doi.org/10.3390/ani12172194

[3]. FDA-NIH Biomarker Working Group. BEST (Biomarkers, EndpointS, and other Tools) Resource [Internet]. Silver Spring (MD): Food and Drug Administration (US); 2016-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK326791/ Co-published by National Institutes of Health (US), Bethesda (MD).

[4]. Vékey, K., Telekes, A. and Vertes, A. (2008). *Medical Applications of Mass Spectrometry*, Amsterdam: Elsevier. Chapter 22 - Biomarker discovery, Thatcher, B J., Caputo, E. Pages 505-532, ISBN 9780444519801, https://doi.org/10.1016/B978-044451980-1.50024-0.

[5]. LaBaer J. (2005). So, you want to look for biomarkers (introduction to the special biomarkers issue). J Proteome Res. Jul-Aug;4(4):1053-9. doi: 10.1021/pr0501259. PMID: 16083254.

[6]. Moore, R. E., Kirwan J., Doherty, M. K., and Whitfield, P. D., (2007). Biomarker discovery in animal health and disease: the application of post-genomic technologies. Biomark Insights. Jul 10;2:185-96. PMID: 19662203; PMCID: PMC2717813.

[7]. Simpson, S., Edwards, J., Ferguson-Mignan, T. F., Cobb, M., Mongan, N. P., and Rutland, C. S. (2015). Genetics of Human and Canine Dilated Cardiomyopathy. *International journal of genomics*, *2015*, 204823. https://doi.org/10.1155/2015/204823

[8]. Mobasheri, A., and Cassidy, J. P. (2010). Biomarkers in veterinary medicine: Towards targeted, individualised therapies for companion animals. Veterinary journal (London, England: 1997), 185(1), 1–3. https://doi.org/10.1016/j.tvjl.2010.04.003

[9]. Witkamp RF. (2005). Genomics and systems biology–how relevant are the developments to veterinary pharmacology, toxicology and therapeutics? *J Vet Pharmacol Ther*;28:235–45

[10]. Brooks, M. B., Erb, H. N., Foureman, P. A., and Ray, K. (2001). von Willebrand disease phenotype and von Willebrand factor marker genotype in Doberman Pinschers. American Journal of Veterinary Research 62, 3, 364-369, available from: < https://doi.org/10.2460/ajvr.2001.62.364> [Accessed 01 November 2023]

[11]. Califf R. M. (2018). Biomarker definitions and their applications. *Experimental biology and medicine (Maywood, N.J.)*, *243*(3), 213–221. https://doi.org/10.1177/1535370217750088

[12]. Womack J. E. (2005). Advances in livestock genomics: opening the barn door. *Genome research*, *15*(12), 1699–1705. https://doi.org/10.1101/gr.3809105

[13]. Wilson, J. L., and Altman, R. B. (2018). Biomarkers: Delivering on the expectation of molecularly driven, quantitative health. *Experimental biology and medicine (Maywood, N.J.)*, *243*(3), 313–322. https://doi.org/10.1177/1535370217744775

[14]. Florkowski, C. M. (2008). Sensitivity, specificity, receiver-operating characteristic (ROC) curves and likelihood ratios: communicating the performance of diagnostic tests. *The Clinical biochemist. Reviews*, *29 Suppl 1*(Suppl 1), S83–S87.

[15]. Schmitt, B., and Henderson, L. (2005). Diagnostic tools for animal diseases. *Revue scientifique et technique (International Office of Epizootics)*, *24*(1), 243–250.

[16]. Ransohoff D. F. (2005). Bias as a threat to the validity of cancer molecular-marker research. *Nature reviews. Cancer*, *5*(2), 142–149. https://doi.org/10.1038/nrc1550

[17]. Kołodziejczyk, J., and Ponczek, M. (2013). The role of fibrinogen, fibrin and fibrin(ogen) degradation products (FDPs) in tumor progression. Contemporary Oncology/Współczesna Onkologia, 17(2), pp.113-119. https://doi.org/10.5114/wo.2013.34611

[18]. Mohammadi Aria, M., Erten, A., and Yalcin, O. (2019). Technology Advancements in Blood Coagulation Measurements for Point-of-Care Diagnostic Testing. *Frontiers in bioengineering and biotechnology*, *7*, 395. https://doi.org/10.3389/fbioe.2019.00395

[19]. Farley, A., Hendry, C. and McLafferty, E., (2012). Blood components. *Nursing Standard (through 2013)*, *27*(13), p.35.

[20]. Thornton, P., and Douglas, J. (2010). Coagulation in pregnancy. *Best practice & research. Clinical obstetrics & gynaecology*, *24*(3), 339–352. https://doi.org/10.1016/j.bpobgyn.2009.11.010

[21]. Davie, E. W., and Ratnoff, O. D. (1964). Waterfall Sequence For Intrinsic Blood Clotting *Science (New York, N.Y.)*, *145*(3638), 1310–1312. https://doi.org/10.1126/science.145.3638.1310

[22]. Achneck, H. E., Sileshi, B., Parikh, A., Milano, C. A., Welsby, I. J., and Lawson, J. H. (2010). Pathophysiology of bleeding and clotting in the cardiac surgery patient: from vascular endothelium to circulatory assist device surface. *Circulation*, *122*(20), 2068–2077. https://doi.org/10.1161/CIRCULATIONAHA.110.936773

[23]. Andrews, R. K., and Berndt, M. C. (2004). Platelet physiology and thrombosis. *Thrombosis research*, *114*(5-6), 447–453. https://doi.org/10.1016/j.thromres.2004.07.020

[24]. Garmo, C., Bajwa, T., and Burns, B. (2022). Physiology, Clotting Mechanism. In: StatPearls. StatPearls Publishing, Treasure Island (FL). PMID: 29939572.

[25]. Chaudhry, R., Usama, S. M., and Babiker, H. M. (2022). Physiology, Coagulation Pathways. In StatPearls. StatPearls Publishing.

[26]. Ying, J. W., Lin, C. Y and Anne, C. M. (2020) The revised coagulation Cascade - LearnHaem: Haematology Made Simple, LearnHaem. Available at:

https://www.learnhaem.com/courses/coagulation/lessons/normal-haemostasis/topic/the-revised-coagulation-cascade (Accessed: 01 November 2023).

[27]. Stokol T. (2003). Plasma D-dimer for the diagnosis of thromboembolic disorders in dogs. *The Veterinary clinics of North America. Small animal practice*, *33*(6), 1419–1435. https://doi.org/10.1016/s0195-5616(03)00096-2

[28]. Palta, S., Saroa, R., and Palta, A. (2014). Overview of the coagulation system. *Indian journal of anaesthesia*, *58*(5), 515–523. https://doi.org/10.4103/0019-5049.144643

[29]. Brooks, M.B. and De Laforcade, A., 2022. Acquired coagulopathies. *Schalm's Veterinary Hematology*, pp.804-811.

[30]. Barr, J. W., and McMichael, M. (2012). Inherited disorders of hemostasis in dogs and cats. *Topics in companion animal medicine*, *27*(2), 53–58. https://doi.org/10.1053/j.tcam.2012.07.006

[31]. Boudreaux M. K. (2012). Inherited platelet disorders. *Journal of veterinary emergency and critical care (San Antonio, Tex. : 2001)*, *22*(1), 30–41. https://doi.org/10.1111/j.1476-4431.2011.00702.x

[32]. Muciño-Bermejo, J., Carrillo-Esper, R., Uribe, M., and Méndez-Sánchez, N. (2013). Coagulation abnormalities in the cirrhotic patient. *Annals of hepatology*, *12*(5), 713–724.

[33]. Kopec, A. K., and Luyendyk, J. P. (2014). Coagulation in liver toxicity and disease: role of hepatocyte tissue factor. *Thrombosis research*, *133 Suppl 1*(0 1), S57–S59. https://doi.org/10.1016/j.thromres.2014.03.023

[34]. Brooks M. (1999). A review of canine inherited bleeding disorders: biochemical and molecular strategies for disease characterization and carrier detection. *The Journal of heredity*, *90*(1), 112–118. https://doi.org/10.1093/jhered/90.1.112

[35]. Tseng, L. W., Hughes, D., and Giger, U. (2001). Evaluation of a point-of-care coagulation analyzer for measurement of prothrombin time, activated partial thromboplastin time, and activated clotting time in dogs. American Journal of Veterinary Research, 62(9), 1455-1460. Retrieved Oct 1, 2023, from https://doi.org/10.2460/ajvr.2001.62.1455

[36]. Coagulation assays (2019) eClinpath. Available at: https://eclinpath.com/hemostasis/tests/screening-coagulation-assays/ (Accessed: 01 November 2023).

[36a]. Assay reference intervals (2022) Cornell University College of Veterinary Medicine. Available at: https://www.vet.cornell.edu/animal-health-diagnostic-center/laboratories/comparative-coagulation/AssayIntervals (Accessed: 02 November 2023).

[37]. Practical-haemostasis.com (2022a) Activated Clotting Time [ACT]. Available at: https://practical-haemostasis.com/Miscellaneous/act.html (Accessed: 01 November 2023).

[38]. Hellemans, A., Devriendt, N., De Somer, F., Marynissen, S., Daminet, S., Paepe, D., and Smets, P. (2023). Reference interval, longitudinal variability and reliability of activated clotting time in healthy dogs using a

point-of-care analyser. Veterinary medicine and science, 9(4), 1534–1540. https://doi.org/10.1002/vms3.1148

[39]. Rawlings, C.A., Byars, T.D., Van Noy, M.K. and Bisgard, G.E., (1975). Activated coagulation test in normal and heparinized ponies and horses. American Journal of Veterinary Research, 36(5), pp.711-713.

[40]. Van Poucke, S., Stevens, K., Marcus, A.E. and Lancé, M., (2014). Hypothermia: effects on platelet function and hemostasis. Thrombosis journal, 12(1), pp.1-5.

[41]. de Brito Sousa, J. D., Sachett, J. A. G., de Oliveira, S. S., Mendonça-da-Silva, I., Marques, H. O., de Lacerda, M. V. G., Fan, H. W., and Monteiro, W. M. (2018). Accuracy of the Lee-White Clotting Time Performed in the Hospital Routine to Detect Coagulopathy in Bothrops atrox Envenomation. The American journal of tropical medicine and hygiene, 98(5), 1547–1551. https://doi.org/10.4269/ajtmh.17-0992

[42]. Song J. (2022). Advances in laboratory assessment of thrombosis and hemostasis. Blood research, 57(S1), 93–100. https://doi.org/10.5045/br.2022.2022048

[43]. Themes, U. (2020) 9: Coagulation, Veterian Key. Available at: https://veteriankey.com/9-coagulation/ (Accessed: 26 October 2023).

[44]. Cheng, T., Mathews, K. A., Abrams-Ogg, A. C., and Wood, R. D. (2009). Relationship between assays of inflammation and coagulation: a novel interpretation of the canine activated clotting time. Canadian journal of veterinary research = Revue canadienne de recherche veterinaire, 73(2), 97–102.

[45]. Connell, J. M., Khalapyan, T., Al-Mondhiry, H. A., Wilson, R. P., Rosenberg, G., and Weiss, W. J. (2007). Anticoagulation of juvenile sheep and goats with heparin, warfarin, and clopidogrel. ASAIO journal (American Society for Artificial Internal Organs : 1992), 53(2), 229–237. https://doi.org/10.1097/MAT.0b013e31802e192b

[45a]. Riley, J. H., and Lassen, E. D. (1979). Activated coagulation times of normal cows. Veterinary clinical pathology, 8(1), 31–33. https://doi.org/10.1111/j.1939-165x.1979.tb00879.x

[46]. Brooks, M.B. and De Laforcade, A., (2022). Avian Haemostasis. Schalm's Veterinary Hematology, pp.867-870

[47]. Zersen, K. M., Russell Moore, A., Olver, C. S., and Mathis, J. C. (2018). Reference intervals for coagulation variables in healthy adult domestic goats (Capra aegagrus hircus). Veterinary clinical pathology, 47(3), 396–399. https://doi.org/10.1111/vcp.12621

[48]. Byars, T. D., Ling, G. V., Ferris, N. A., and Keeton, K. S. (1976). Activated coagulation time (ACT) of whole blood in normal dogs. American journal of veterinary research, 37(11), 1359–1361.

[49]. Bay, J. D., Scott, M. A., and Hans, J. E. (2000). Reference values for activated coagulation time in cats. American journal of veterinary research, 61(7), 750–753. https://doi.org/10.2460/ajvr.2000.61.750

[50]. See, A. M., Swindells, K. L., Sharman, M. J., Haack, K. L., Goodman, D., Delaporta, A., Robertson, I., and Foster, S. F. (2009). Activated coagulation times in normal cats and dogs using MAX-ACT tubes. Australian veterinary journal, 87(7), 292–295. https://doi.org/10.1111/j.1751-0813.2009.00450.x

[51]. Kessler, U., Grau, T., Gronchi, F., Berger, S., Brandt, S., Bracht, H., Marcucci, C., Zachariou, Z. and Jakob, S.M., (2011). Comparison of porcine and human coagulation by thrombelastometry. Thrombosis research, 128(5), pp.477-482.

[52]. Mischke R. (2000). Activated partial thromboplastin time as a screening test of minor or moderate coagulation factor deficiencies for canine plasma: sensitivity of different commercial reagents. Journal of veterinary diagnostic investigation : official publication of the American Association of Veterinary Laboratory Diagnosticians, Inc, 12(5), 433–437. https://doi.org/10.1177/104063870001200507

[53]. Kogan, A.E., Kardakov, D.V. and Khanin, M.A., (2001). Analysis of the activated partial thromboplastin time test using mathematical modeling. *Thrombosis research*, *101*(4), pp.299-310.

[54]. Buriko, Y., Drobatz, K. and Silverstein, D.C., (2020). Establishment of normal reference intervals in dogs using a viscoelastic point-of-care coagulation monitor and its comparison with thromboelastography. *Veterinary Clinical Pathology*, *49*(4), pp.567-573.

[55]. Tripodi, A., and Mannucci, P. M. (2006). Activated partial thromboplastin time (APTT). New indications for an old test?. *Journal of thrombosis and haemostasis : JTH*, *4*(4), 750–751. https://doi.org/10.1111/j.1538-7836.2006.01857.x

[56]. McMichael, M.A. and Smith, S.A., (2011). Viscoelastic coagulation testing: technology, applications, and limitations. *Veterinary clinical pathology*, *40*(2), pp.140-153.

[57]. Practical-haemostasis.com (2022a) Activated Partial Thromboplastin Time [APTT]. Available at: https://practical-haemostasis.com/Screening%20Tests/aptt.html (Accessed: 01 November 2023).

[58]. Proctor, R. R., and Rapaport, S. I. (1961). The partial thromboplastin time with kaolin. A simple screening test for first stage plasma clotting factor deficiencies. American journal of clinical pathology, 36, 212–219. https://doi.org/10.1093/ajcp/36.3.212

[59]. Bucknoff, M. C., Hanel, R. M., Marks, S. L., Motsinger-Reif, A. A., and Suter, S. E. (2014). Evaluation of thromboelastography for prediction of clinical bleeding in thrombocytopenic dogs after total body irradiation and hematopoietic cell transplantation. American journal of veterinary research, 75(5), 425–432. https://doi.org/10.2460/ajvr.75.5.425

[60]. Arnout J. (2001). Antiphospholipid syndrome: diagnostic aspects of lupus anticoagulants. Thrombosis and haemostasis, 86(1), 83–91.

[61]. Tripodi, A., Chantarangkul, V., Martinelli, I., Bucciarelli, P., and Mannucci, P. M. (2004). A shortened activated partial thromboplastin time is associated with the risk of venous thromboembolism. Blood, 104(12), 3631–3634. https://doi.org/10.1182/blood-2004-03-1042

[62]. Berk, Z. B. K., Shah, A., Sun, W., Griffith, B. P., and Wu, Z. J. (2022). An ex vivo comparison of partial thromboplastin time and activated clotting time for heparin anticoagulation in an ovine model. Artificial organs, 46(3), 501–505. https://doi.org/10.1111/aor.14094

[63]. Estrin, M.A., Wehausen, C.E., Lessen, C.R. and Lee, J.A., (2006). Disseminated intravascular coagulation in cats. Journal of veterinary internal medicine, 20(6), pp.1334-1339.

[64]. Sobiech, P., Radwińska, J., Krystkiewicz, W., Snarska, A., and Stopyra, A. (2008). Changes in the coagulation profile of cattle with left abomasal displacement. Polish journal of veterinary sciences, 11(4), 301–306.

[65]. Mischke R. (2011). Prothrombin time standardisation in canine samples with regard to inter-batch and inter-reagent variability. Veterinary journal (London, England : 1997), 188(3), 301–306. https://doi.org/10.1016/j.tvjl.2010.06.005

[66]. Walker, H. K., Hall, W. D., and Hurst, J. W. (Eds.). (1990). Clinical Methods: The History, Physical, and Laboratory Examinations. (3rd ed.). Butterworths.

[67]. Douglas, S., (1999). Coagulation history, Oxford 1951–53. British journal of haematology, 107(1), pp.22-32.

[68]. Horsti J. (2009). The progress of prothrombin time measurement. *Hematology Reviews*, *1*(2), e19. https://doi.org/10.4081/hr.2009.e19

[69]. Romano, M.C., Francis, K.A., Janes, J.G., Poppenga, R.H., Filigenzi, M.S., Stefanovski, D. and Gaskill, C.L., (2022). Determining an approximate minimum toxic dosage of diphacinone in horses and corresponding serum, blood, and liver diphacinone concentrations: a pilot study. Journal of Veterinary Diagnostic Investigation, 34(3), pp.489-495.

[70]. Shikdar, S., Vashisht, R., and Bhattacharya, P. T. (2023). International Normalized Ratio (INR). In StatPearls. StatPearls Publishing.

[71]. Tripathi, M. M., Egawa, S., Wirth, A. G., Tshikudi, D. M., Van Cott, E. M., and Nadkarni, S. K. (2017). Clinical evaluation of whole blood prothrombin time (PT) and international normalized ratio (INR) using a Laser Speckle Rheology sensor. *Scientific reports*, *7*(1), 9169. https://doi.org/10.1038/s41598-017-08693-5

[72]. Berlin, N., Kelmer, E., Segev, G., Aroch, I. and Kelmer, G., (2019). Assessment of the CoaguChek-XS portable prothrombin time point-of-care analyzer for horses. *Journal of Veterinary Diagnostic Investigation*, *31*(3), pp.448-452.

[73]. Kilinc, O.O., Ozdal, N., Bicek, K., Deger, M.S., Yuksek, N., Yilmaz, A.B. and Oguz, B., (2018). Relationship between cardiac injury, selected biochemical parameters, DIC, and hemogram levels in cattle with theileriosis. *Med. Weter*, *74*(6), pp.383-386.

[74]. Honse, C. O., Figueiredo, F. B., de Alencar, N. X., Madeira, M.deF., Gremião, I. D., and Schubach, T. M. (2013). Disseminated intravascular coagulation in a dog naturally infected by Leishmania (Leishmania) chagasi from Rio de Janeiro - Brazil. *BMC veterinary research*, *9*, 43. https://doi.org/10.1186/1746-6148-9-43

[75]. Mooney, E. T., Agostini, G., Griebsch, C., and Hickey, M. (2020). Intravenous vitamin K_1 normalises prothrombin time in 1 hour in dogs with anticoagulant rodenticide toxicosis. *Australian veterinary journal*, *98*(6), 225–231. https://doi.org/10.1111/avj.12931

[76]. Dircks, B., Nolte, I., and Mischke, R. (2012). Haemostatic abnormalities in cats with naturally occurring liver diseases. *Veterinary journal (London, England : 1997)*, *193*(1), 103–108. https://doi.org/10.1016/j.tvjl.2011.09.026

[77]. Morrisey, J.K., Paul-Murphy, J., Fialkowski, J.P., Hart, A. and Darien, B.J., (2003). Estimation of prothrombin times of Hispaniolan Amazon parrots (Amazona ventralis) and umbrella cockatoos (Cacatua alba). *Journal of Avian Medicine and Surgery*, *17*(2), pp.72-77.

[78]. Guddorf, V., Kummerfeld, N., and Mischke, R. (2014). Methodical aspects of blood coagulation measurements in birds applying commercial reagents--a pilot study. *Berliner und Munchener tierarztliche Wochenschrift*, *127*(7-8), 322–327.

[79]. Heuwieser, W., Biesel, M., and Grunert, E. (1989). Physiological coagulation profile of dairy cattle. *Zentralblatt fur Veterinarmedizin. Reihe A*, *36*(1), 24–31. https://doi.org/10.1111/j.1439-0442.1989.tb00698.x

[80]. Wada, H., Ichikawa, Y., Ezaki, M., Matsumoto, T., Yamashita, Y., Shiraki, K., Shimaoka, M., and Shimpo, H. (2021). The Reevaluation of Thrombin Time Using a Clot Waveform Analysis. *Journal of clinical medicine*, *10*(21), 4840. https://doi.org/10.3390/jcm10214840

[81]. Douglas, S., (1999). Coagulation history, Oxford 1951–53. *British journal of haematology*, *107*(1), pp.22-32.

[82]. Tripodi A. (2016). Thrombin Generation Assay and Its Application in the Clinical Laboratory. *Clinical chemistry*, *62*(5), 699–707. https://doi.org/10.1373/clinchem.2015.248625

[83]. Karapetian H. (2013). Reptilase time (RT). *Methods in molecular biology (Clifton, N.J.)*, *992*, 273–277. https://doi.org/10.1007/978-1-62703-339-8_20

[84]. Jolivet, F., Diquélou, A., Trumel, C., Privat, S., and Dossin, O. (2017). Fibrinogen deficiency in a dog - a case report. *BMC veterinary research*, *13*(1), 183. https://doi.org/10.1186/s12917-017-1110-8

[85]. Fecteau, G., Zinkl, J.G., Smith, B.P., O'Neil, S., Smith, S. and Klopfer, S., (1997). Dysfibrinogenemia or afibrinogenemia in a Border Leicester lamb. *The Canadian Veterinary Journal*, *38*(7), p.443.

[86]. Siller-Matula, J. M., Plasenzotti, R., Spiel, A., Quehenberger, P., and Jilma, B. (2008). Interspecies differences in coagulation profile. *Thrombosis and haemostasis*, *100*(3), 397–404.

[87]. Gaffney, P.J., (1993). D-dimer. History of the discovery, characterisation and utility of this and other fibrin fragments. *Fibrinolysis*, *7*, pp.2-8.

[88]. Stokol, T., Brooks, M. B., Erb, H. N., and Mauldin, G. E. (2000). D-dimer concentrations in healthy dogs and dogs with disseminated intravascular coagulation. *American journal of veterinary research*, *61*(4), 393–398. https://doi.org/10.2460/ajvr.2000.61.393

[89]. Hart, D.J., Hutchman, G. and Cuthbert, R.J.G., (2002). Evaluation of an automated latex d-dimer immunoassay in the clinical assessment of suspected venous thromboembolism. *Clinical & Laboratory Haematology*, *24*(3), pp.171-174.

[90]. Dewhurst, E., Cue, S., Crawford, E. and Papasouliotis, K., (2008). A retrospective study of canine D-dimer concentrations measured using an immunometric "Point-of-Care" test. Journal of Small Animal Practice, 49(7), pp.344-348.

[91]. Gunes, V., Onmaz, A. C., Keles, I., Varol, K., and Ekinci, G. (2017). The diagnostic importance of coagulation parameters in cattle having natural theileriosis. *Polish journal of veterinary sciences*, *20*(2), 369–376. https://doi.org/10.1515/pjvs-2017-0045

[92]. Honoré, M.L., Pihl, T.H., Busk-Anderson, T.M., Flintrup, L.L. and Nielsen, L.N., (2022). Investigation of two different human d-dimer assays in the horse. *BMC Veterinary Research*, *18*(1), p.227.

[93]. López-Martínez, M. J., Escribano, D., Contreras-Aguilar, M. D., García-Martínez, J. D., Martínez-Subiela, S., and Cerón, J. J. (2020). Salivary D-dimer in pigs: Validation of an automated assay and changes after acute stress. *Veterinary journal (London, England : 1997)*, *259-260*, 105472. https://doi.org/10.1016/j.tvjl.2020.105472

[94]. Ke, C. H., Liu, C. C., Wang, S. L., and Lin, C. S. (2023). Paired Analysis of D-Dimer and Its Correlated Hemostatic Parameters in 30 Dogs with Neoplasms after Tumorectomy. *Animals : an open access journal from MDPI*, *13*(6), 969. https://doi.org/10.3390/ani13060969

[95]. Boyé, P., Serres, F., Floch, F., Fournel-Fleury, C. and Tierny, D. (2021). Prognostic value of pretreatment plasma D-dimer level in dogs with intermediate to high-grade non-Hodgkin lymphoma. *Veterinary and Comparative Oncology*, *19*(1), pp.44-52.

[96]. Han, H. J., and Kim, J. H. (2022). Correlation Between D-Dimer Concentrations and Thromboelastography in Dogs With Critical Illness: A Retrospective, Cross-Sectional Study. *Frontiers in veterinary science*, *9*, 844022. https://doi.org/10.3389/fvets.2022.844022

[97]. Nikvand, A. A., Jalali, S. M., Ghadrdan Mashhadi, A., Razi Jalali, M., and Hassanpour Amirabadi, S. (2019). Clinical, hematologic, hemostatic, and serum biochemical findings related to survival in Arabian horses with colic. *Veterinary clinical pathology*, *48*(3), 441–448. https://doi.org/10.1111/vcp.12779

[98]. Stokol, T., Brooks, M., Erb, H. and Mauldin, G.E., (1999). Evaluation of kits for the detection of fibrin (ogen) degradation products in dogs. *Journal of Veterinary Internal Medicine*, *13*(5), pp.478-484.

[99]. Boisvert, A.M., Swenson, C.L. and Haines, C.J., (2001). Serum and Plasma Latex Agglutination Tests for Detection of Fibrin (ogen) Degradation Products in Clinically III Dogs. *Veterinary clinical pathology*, *30*(3), pp.133-136.

[100]. Ferreira, H.C. and Murat, L.G., (1963). An immunological method for demonstrating fibrin degradation products in serum and its use in the diagnosis of fibrinolytic states. *British Journal of Haematology*, *9*(3), pp.299-310.

[101]. Fibrinogen degradation products (FDP), plasma (2023) Labcorp. Available at: https://www.labcorp.com/tests/115402/fibrinogen-degradation-products-fdp-plasma). (Accessed: 01 November 2023).

[102]. Kamiya biomedical company automated analyzer application K assay plasma FDP (2023) Kamiya Biomedical Company. Available at: https://www.kamiyabiomedical.com/pdf/KAI-111.pdf (Accessed: 01 November 2023).

[103]. de Laforcade, A.M., Freeman, L.M., Shaw, S.P., Brooks, M.B., Rozanski, E.A. and Rush, J.E., (2003). Hemostatic changes in dogs with naturally occurring sepsis. *Journal of veterinary internal medicine*, *17*(5), pp.674-679.

[104]. Stokol, T., Erb, H. N., De Wilde, L., Tornquist, S. J., and Brooks, M. (2005). Evaluation of latex agglutination kits for detection of fibrin(ogen) degradation products and D-dimer in healthy horses and horses with severe colic. *Veterinary clinical pathology*, *34*(4), 375–382. https://doi.org/10.1111/j.1939-165x.2005.tb00064.x

[105]. Mount, M. E., Kim, B. U., and Kass, P. H. (2003). Use of a test for proteins induced by vitamin K absence or antagonism in diagnosis of anticoagulant poisoning in dogs: 325 cases (1987-1997). *Journal of the American Veterinary Medical Association*, *222*(2), 194–198. https://doi.org/10.2460/javma.2003.222.194

[106]. Maniscalco, L., Varello, K., Zoppi, S., Abbamonte, G., Ferrero, M., Torres, E., Ostorero, F., Rossi, F., and Bozzetta, E. (2021). Abnormal Prothrombin (PIVKA-II) Expression in Canine Tissues as an Indicator of Anticoagulant Poisoning. *Animals : an open access journal from MDPI*, *11*(9), 2612. https://doi.org/10.3390/ani11092612

[107]. Long, J., Peng, X., Luo, Y., Sun, Y., Lin, G., Wang, Y., and Qiu, Z. (2016). Treatment of a long-acting anticoagulant rodenticide poisoning cohort with vitamin K1 during the maintenance period. *Medicine*, *95*(51), e5461. https://doi.org/10.1097/MD.0000000000005461

[108]. Misdorp W. (2004). Mast cells and canine mast cell tumours. A review. *The veterinary quarterly*, *26*(4), 156–169. https://doi.org/10.1080/01652176.2004.9695178

[109]. Baker, D. C., Robbe, S. L., Jacobson, L., Manco-Johnson, M. J., Holler, L., and Lefkowitz, J. (1999). Hereditary deficiency of vitamin-K-dependent coagulation factors in Rambouillet sheep. *Blood coagulation & fibrinolysis : an international journal in haemostasis and thrombosis*, *10*(2), 75–80. https://doi.org/10.1097/00001721-199903000-00003

[110]. McMahon, M. and Kalunian, K. (2005) *Measuring Immunity*, Academic Press. Chapter 17 - SLE-Associated Tests, Editor(s): Michael T. Lotze, Angus W. Thomson, Pages 210-220, ISBN 9780124559004, https://doi.org/10.1016/B978-012455900-4/50279-8.

(https://www.sciencedirect.com/science/article/pii/B9780124559004502798

[111]. Thiagarajan, P., Pengo, V. and Shapiro, S.S., (1986). The use of the dilute Russell viper venom time for the diagnosis of lupus anticoagulants. *Blood*, *68*(4), pp.869-874.

[112]. Conley, C. L., and Rathbun, H. K. (1948). Circulating anticoagulant as a cause of hemorrhagic diathesis in man. *Bulletin of the Johns Hopkins Hospital*, *83*(4), 288–296.

[113]. Favaloro, E.J., (2019). The Russell viper venom time (RVVT) test for investigation of lupus anticoagulant (LA). *American Journal of Hematology*, *94*(11), pp.1290-1296.

[114]. Thachil, J., (2023). Russell viper venom: a journey from the bedside to the bench and back to the bedside. In *Seminars in Thrombosis and Hemostasis*. 333 Seventh Avenue, 18th Floor, New York, NY 10001, USA: Thieme Medical Publishers, Inc..

[115]. Pengo, V., Bison, E., Banzato, A., Zoppellaro, G., Jose, S.P. and Denas, G., (2017). Lupus anticoagulant testing: diluted Russell viper venom time (dRVVT). *Hemostasis and Thrombosis: Methods and Protocols*, pp.169-176.

[116]. Rand, J.H. and Wolgast, L.R., (2013). Antiphospholipid syndrome: pathogenesis, clinical presentation, diagnosis, and patient management. *Consultative Hemostasis and Thrombosis. 3rd ed. Elsevier*, pp.324-341.

[117]. L. Dana DeWitt, (2003). Anti-Phospholipid Antibodies, Editor(s): Michael J. Aminoff, Robert B. Daroff, Encyclopedia of the Neurological Sciences, Academic Press, Pages 234-237, ISBN 9780122268700, https://doi.org/10.1016/B0-12-226870-9/01491-X.

[118]. Miller, A. G., Dow, S., Long, L., and Olver, C. S. (2012). Antiphospholipid antibodies in dogs with immune mediated hemolytic anemia, spontaneous thrombosis, and hyperadrenocorticism. *Journal of veterinary internal medicine*, *26*(3), 614–623. https://doi.org/10.1111/j.1939-1676.2012.00922.x

[119]. Kaneko, J.J., Harvey, J.W. and Bruss, M.L. eds., (2008). *Clinical biochemistry of domestic animals*. Academic press.

[120]. Debbie, J.G. and Abelseth, M.K., (1971). Pathogenesis of epizootic hemorrhagic disease. I. Blood coagulation during viral infection. *Journal of Infectious Diseases*, *124*(2), pp.217-222.

[121]. Kuzi, S., Segev, G., Haruvi, E. and Aroch, I., (2010). Plasma antithrombin activity as a diagnostic and prognostic indicator in dogs: a retrospective study of 149 dogs. *Journal of Veterinary Internal Medicine*, *24*(3), pp.587-596.

[122]. Brooks, M.B. and De Laforcade, A., (2022). *Schalm's Veterinary Hematology*, pp.768-769

[123]. Van Cott, E. M., Orlando, C., Moore, G. W., Cooper, P. C., Meijer, P., Marlar, R., and Subcommittee on Plasma Coagulation Inhibitors (2020). Recommendations for clinical laboratory testing for antithrombin

deficiency; Communication from the SSC of the ISTH. *Journal of thrombosis and haemostasis: JTH*, *18*(1), 17–22. https://doi.org/10.1111/jth.14648

[124]. Wilson, S.J., (1942). Quantitative studies on antithrombin. *Archives of Internal Medicine*, *69*(4), pp.647-661.

[125]. Egeberg, O., (1965). Inherited antithrombin deficiency causing thrombophilia. *Thrombosis and Haemostasis*, *13*(02), pp.516-530.

[126]. Biggs, R., Denson, K.W.E., Akman, N., Borrett, R. and Hadden, M., (1970). Antithrombin III, antifactor Xa and heparin. *British Journal of Haematology*, *19*(3), pp.283-305.

[127]. Milczak, A., Winiarczyk, D., Winiarczyk, S., Bochyńska, D., Adaszek, Ł., Winiarczyk, M., and Lechowski, R. (2022). Procoagulant and anticoagulant plasma indicators in diabetic dogs showing increased antithrombin III levels in canine diabetes mellitus. *BMC veterinary research*, *18*(1), 108. https://doi.org/10.1186/s12917-022-03179-7

[128]. Sun, P., and Jeffery, U. (2021). Decreased antithrombin activity and inflammation in cats. Journal of feline medicine and surgery, 23(6), 498–506. https://doi.org/10.1177/1098612X20960400

[129]. Panzani, S., Castagnetti, C., Vitiello, T., Pirrone, A., Scarpa, P. and Veronesi, M.C., (2014). Antithrombin: Could It be a Diagnostic and Prognostic Marker in Septic Neonatal Foals?. *Journal of Equine Veterinary Science*, *34*(10), pp.1211-1217.

[130]. Wang, W. H., Lynch, A. M., Balko, J. A., Duffy, D. J., Robertson, J. B., and Posner, L. P. (2022). Point-of-care viscoelastic coagulation assessment in healthy dogs during the perianesthetic period. *BMC veterinary research*, *18*(1), 346. https://doi.org/10.1186/s12917-022-03442-x

[131]. Duarte, R. C. F., Ferreira, C. N., Rios, D. R. A., Reis, H. J. D., and Carvalho, M. D. G. (2017). Thrombin generation assays for global evaluation of the hemostatic system: perspectives and limitations. *Revista brasileira de hematologia e hemoterapia*, *39*(3), 259–265. https://doi.org/10.1016/j.bjhh.2017.03.009