Mutational analysis and coagulation factor VIII sequence in a colt with hemophila A

by

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Abstract

Hemophilia A is an X-linked disorder caused by a deficiency in factor VIII (FVIII). Although foals have been diagnosed based on deficiency in FVIII activity, causative gene mutations have not been identified. The objectives of this project were to determine the sequence for the FVIII gene in the horse, identify the causative mutation in a colt with hemophilia A, and determine if the mutation was spontaneous or due to maternal inheritance. Genomic sequence analysis was compared from amplified PCR products from a colt diagnosed with hemophilia A, the colt's mare, and a normal horse. PCR reactions were also performed on hepatic cDNA and a western blot on hepatic lysate from the affected colt and normal horse. Based on the results, the mutation was isolated to the non-coding region of FVIII (specifically intron 1). Genomic sequencing of intron 1 suggested maternal inheritance. Transcription of the intron across the 5' splice site suggested activation of an aberrant splice site in intron 1. It is yet to be determined if this defect is responsible for hemophilia A in other horses.

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Abbreviations

ADP: adenosine diphosphate

aPTT: activated partial thromboplastin time

AT: antithrombin

BLAST: basic local alignment search tool

bp: base pairs

CDF5F8: combined deficiency of coagulation factor V and factor VIII

dNTP: deoxynucleotide triphosphate

FV: coagulation factor V

FVa: activated coagulation factor V

FVII: coagulation factor VII

FVIIa: activated coagulation factor VII

FVIII: coagulation factor VIII

FVIIIa: activated coagulation factor VIII

FVIII:C: factor VIII-coagulant

FIX: coagulation factor IX

FIXa: activated coagulation factor IX

FX: coagulation factor X

FXa: activated coagulation factor X

FXI: coagulation factor XI

FXII: coagulation factor XII

GT: Glanzmann thrombasthenia

hFVIII: human derived coagulation factor VIII

HMWK: high molecular weight kininogen

HSPG: heparin sulfated proteoglycans

kb: kilobases

LMAN-1: lectin mannose binding protein type 1

MCFD2: multiple coagulation factor deficiency 2

MPs: microparticles

mRNA: messenger RNA

PC: phosphatidylcholine

PCR: polymerase chain reaction

PE: phosphatidylethanolamine

PK: prekallikrein

PS: phosphatidylserine

PT: prothrombin time

PVDF: polyvinylidene fluoride

RI: reference interval

SNP: single nucleotide polymorphism

TaKaRa LA taq: TaKaRa long and accurate taq polymerase

TBS-T: tris-buffered saline with tween 20

TF: tissue factor

TFPI: tissue factor pathway inhibitor

TM: thrombomodulin

VWD: von Willebrand disease

vWF: von Willebrand factor

vWF:Ag: plasma vWF antigen levels

Chapter 1: Literature Review

Section 1: Coagulation

During hemostasis, blood flow is regulated by balances between coagulant and anti-coagulant factors. Precise control is required to prevent extremes of either branch, such as inappropriate clot formation leading to impedance of blood flow or excessive blood loss during minor injury. Activation of clot formation relies on a complex series of events that incorporate the role of both primary and secondary hemostasis. Primary hemostasis involves the adhesion and aggregation of activated platelets to subendothelial surfaces including collagen. Secondary hemostasis involves the formation of fibrin which serves to stabilize the initial platelet plug and includes the intrinsic (coagulation factors VIII, IX, XI and XII) and extrinsic (tissue factor and factor VII) arms of the coagulation system, as well as the common pathway (factors V, X, II, I, and XIII).[1]

The cascade model of coagulation was proposed in 1964 and described coagulation as a series of calcium dependent enzymatic cleavages of zymogen substrates with subsequent activation of enzymes.[2] The intrinsic pathway was described as being activated by exposure of coagulation factor XII (FXII) to a negatively charged surface, whereas the extrinsic pathway was activated via contact of coagulation factor FVII (FVII) with tissue factor.[1] This model focused on the independent roles of primary and secondary hemostasis, while suggesting that the intrinsic and extrinsic pathways had a redundant role in activation of coagulation factor X (FX), its cleavage of prothrombin to thrombin, and the formation of the stable fibrin clot.[3] The cascade model of coagulation became insufficient in explaining the variability noted in the *in vivo*

clinical signs associated with coagulation factor deficiencies.[1-3] For example, if the extrinsic and intrinsic pathways share a redundant role, then the extrinsic pathway should be able to compensate for a lack of coagulation factor VIII (FVIII) or IX (FIX), as seen with hemophilia, by independent activation of FX and allowing for sufficient clot formation.[2]

The cell based model, introduced by Hoffman and Monroe in 2001, described coagulation as three overlapping phases: initiation, amplification, and propagation.[2] The model rejected the previous concept of independent activation and focused on the synergistic and parallel role between each branch of coagulation while taking into account a dynamic vascular system.[4] Furthermore, the model offered a more precise explanation for localization of clot formation by incorporating the role of the cell and emphasizing the importance of tissue factor.[2]

Cellular Components of Coagulation

Cellular expression of tissue factor has been recognized as the primary initiator of coagulation. The majority of tissue factor remains extrinsic to the bloodstream with expression in the vasculature restricted to the blood vessel smooth muscle, pericytes, and adventitial fibroblasts.[5] Tissue factor (TF) is expressed on circulating neutrophils, lymphocytes, and monocytes but is thought to be quiescent with an inconsequential role in normal hemostasis.[5] Damage to the endothelium and basement membrane exposes the vasculature to the extracellular matrix and tissue factor expressing cells, activating the clotting cascade. It has been suggested that TF remains in an encrypted state with decryption transformation regulated by the binding of TF to activated coagulation factor VII (FVIIa) in conjunction with specific procoagulant factors such as elevations in cytosolic calcium, cell membrane expression of phosphatidylserine, and formation of microparticles.[6,7] Consequently, TF expression alone is not sufficient to fully propagate coagulation.[2]

All cells are composed of a phospholipid membrane which contains a large number of constitutively expressed membrane surface proteins. The neutral external leaflet is primarily composed of phosphatidylcholine (PC) and sphingomyelin, whereas the negatively charged internal leaflet is composed predominantly of phosphatidylethanolamine (PE) and phosphatidyldserine (PS). Under normal membrane conditions, the expression of PC on the outer surface does not support a procoagulant state, and any aberrant activation of coagulation enzymes occurs slowly with insufficient formation of thrombin.[1] Tightly regulated expression of these specific phospholipid molecules occurs via the expression of the enzyme flippase. During membrane injury, the enzymes floppase and scramblase (a calcium dependent bi-directional phospholipid transporter), predominate which initiates the movement of PS and PE to the outer membrane surface, effectively changing the membrane surface charge.[8] The presence of PS and PE on the cell surface creates a profoundly pro-coagulant state, with PS being largely responsible for increasing *in vivo* coagulation reaction times.[9] Increased expression of PS is also evident on the membrane of activated platelets, with an increase from negligible amounts of PS to approximately 12% surface expression.[10] The presence of PS contributes to assembly of coagulation factors on the platelet surface.[10] Thus, the ability of platelets to manipulate the composition of the phospholipid membrane during vascular injury while maintaining an anti-coagulant state during health, allows the activation of coagulation to occur on the surface of the cell effectively localizing and controlling clot formation.

Microparticles (MPs) are derivatives of activated, apoptotic or damaged eukaryotic cell membranes and are formed from calcium dependent proteolytic cleavage of the cytoskeleton.[11] During coagulation, MPs are formed from the cellular activation of platelets, monocytes, and endothelial cells and contain procoagulant proteins similar to that of the parent cell (P-selectin,

tissue factor, and von Willebrand factor, respectively).[8] Platelets, which are thought to contribute up to 60-90% of circulating MPs, also release MPs with a PS predominant membrane.[12] MPs which express PS or tissue factor have the highest level of procoagulant activity due to the PS-facilitated assembly of clotting factors on the cell membrane and the interaction with FVII and tissue factor to activate coagulation FX and FIX.[12]

Initiation

The initiation phase involves injury of the blood vessel, formation of a platelet plug, and exposure of TF (see figure 1). Although each component is critical to clot formation, this phase emphasizes tissue factor as the key cellular surface for activation of coagulation. Vascular damage leads to disruption of the endothelium and basement membrane and contact of the blood to tissue factor expressing cells. Exposed collagen causes activation, adhesion and aggregation of platelets.

Activated FVII (FVIIa) accounts for approximately 1% of circulating FVII. [13] Uniquely, FVII circulates in the vasculature both as a zymogen and as a cleaved two-chain enzyme; however, FVIIa remains quiescent in a zymogen like conformation, requiring its obligatory cofactor, TF, to become fully activated.[13] Decrypted TF binds to FVIIa which forms the TF:FVIIa complex and promotes proteolysis and further activation of FVII, auto-activation of TF:FVII complexes,[14] and activation of small amounts of FIX and FX.[7] Activated FX (FXa) binding to negatively charged phospholipid cell membrane is mediated by ionized calcium, which along with the cofactor coagulation factor V (FV), forms the prothrombinase complex. The prothrombinase complex is responsible for the proteolytic cleavage of the substrate prothrombin to thrombin.[1] The generation of thrombin in the initiation phase is vital for further activation of platelets and thrombin formation, but alone is insufficient to form a stable fibrin

clot.[2] Activated FX is effectively restricted to the surface of the TF bearing cell due to immediate degradation by circulating tissue factor pathway inhibitor and anti-thrombin (AT); whereas, activated coagulation factor IX (FIXa) is able to circulate with minimal inhibition from AT and binds to the surface of circulating platelets.[1]

Amplification

The amplification phase focuses on the vital role of thrombin in ramping up coagulation via its interaction with platelets and the activation of coagulation factors V, VIII, and XI (see figure 2). The scant amount of thrombin produced from the initiation phase binds to the surface of platelets which have come in contact with the extra-vascular matrix.[1] Thrombin activation of platelets occurs by thrombin binding to potent protease-activated receptors on platelet surfaces,[2] and leads to shuffling of the cell membrane so that the anionic phospholipids (PS and PE) predominate, creating a highly pro-coagulant surface necessary for the assembly of coagulation factors during the propagation phase.[10] The negative cell membrane also causes formation of platelet microparticles which bud off the cytoskeleton and are highly pro-coagulant due to their PS predominant surface and expression of P-selectin.[12]

Activated platelets release platelet granule contents including von Willebrand factor (vWF), fibrinogen (coagulation factor I), adenosine diphosphate (ADP), serotonin, and FV.[8] Platelet intracytoplasmic mobilization of calcium contributes to further membrane concentration of PS, enhancing the pro-coagulant nature of the membrane surface.[1] Thrombin also activates FV and coagulation factor XI (FXI) on the platelet surface and cleaves the non-covalent bond between circulating FVIII and vWF. Once unbound, vWF is available to mediate platelet adhesion and aggregation while thrombin activates FVIII.[1-3,7]

Propagation

The propagation phase emphasizes the role of the intrinsic coagulation factors both on the platelet surface and within the vasculature (see figure 3). Binding of FIXa, generated from the initiation phase, and activated FVIII (FVIIIa), generated from amplification phase, on the platelet surface forms the intrinsic factor X activation complex, also known as the intrinsic tenase complex. Additional FIX is activated on the platelet surface by FXIa generated during the amplification phase, increasing the available FIXa to bind with FVIIIa.[1-3,7] The intrinsic tenase complex rapidly activates FX, generating 50 fold more FXa than produced during the initiation phase by TF-FVIIa complexes.[15] FXa directly binds activated coagulation FV (FVa) on the platelet surface, forming the prothrombinase complex which leads to massive cleavage of prothrombin to thrombin and a subsequent explosive burst in thrombin activity.[1-3,7] Thrombin cleaves fibrinopeptide A from fibrinogen, forming fibrin which spontaneously polymerizes into an insoluble fibrin matrix stabilized by amide or isopeptide covalent crosslinks generated by thrombin-activated coagulation factor XIII.[16]

Control of Coagulation

Although activation of coagulation is a rapid and efficient mechanism to prevent blood loss from damaged endothelium, unregulated coagulation could lead to widespread thrombus formation and disruption of blood flow to tissues. Multiple mechanisms are in place to ensure coagulation does not progress beyond the site of vascular injury.

Regulatory control of coagulation occurs from circulating protease inhibitors, such as AT, heparin cofactor II, tissue factor pathway inhibitor (TFPI), and C1 inhibitor which actively patrol and cleave activated coagulation factors within the vasculature.[7] Approximately 80% of the circulating pool of TFPI is associated with lipoproteins while the remaining 20% remains

Phase 1: Initiation

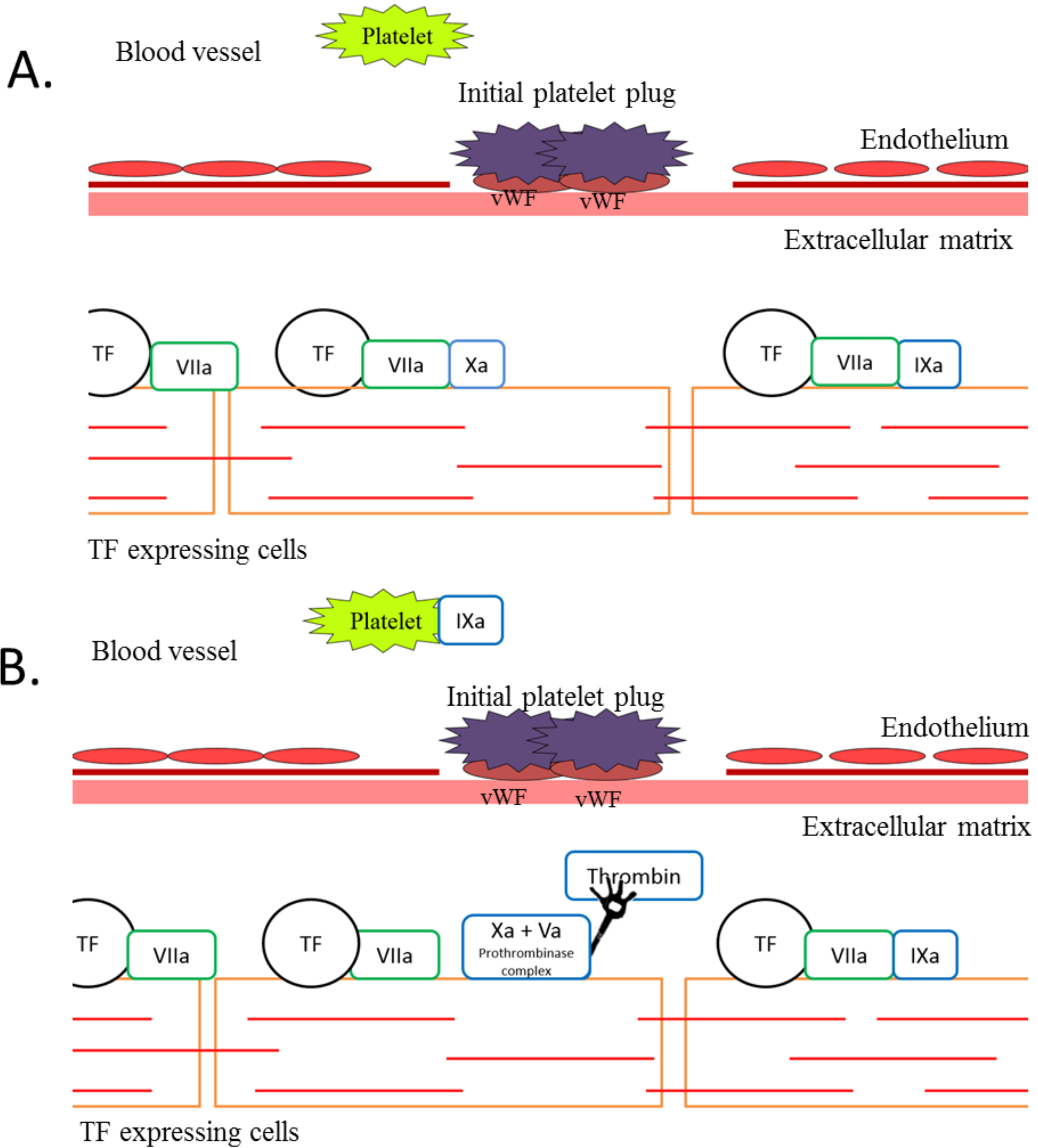


Figure 1: Initiation phase of coagulation begins immediately after injury to the blood vessel with (A) formation of the platelet plug and exposure of coagulation factor VII and X to TF expressing cells and subsequent activation of FVII. The FVII:TF complex activates coagulation factors X and IX. (B) Xa binds with coagulation FV to form the prothrombinase complex which is responsible for proteolytic cleavage of prothrombin to thrombin. FIXa is able to circulate and binds to the platelet cell membrane.
TF: Tissue factor, vWF: von Willebrand factor

Phase 2: Amplification

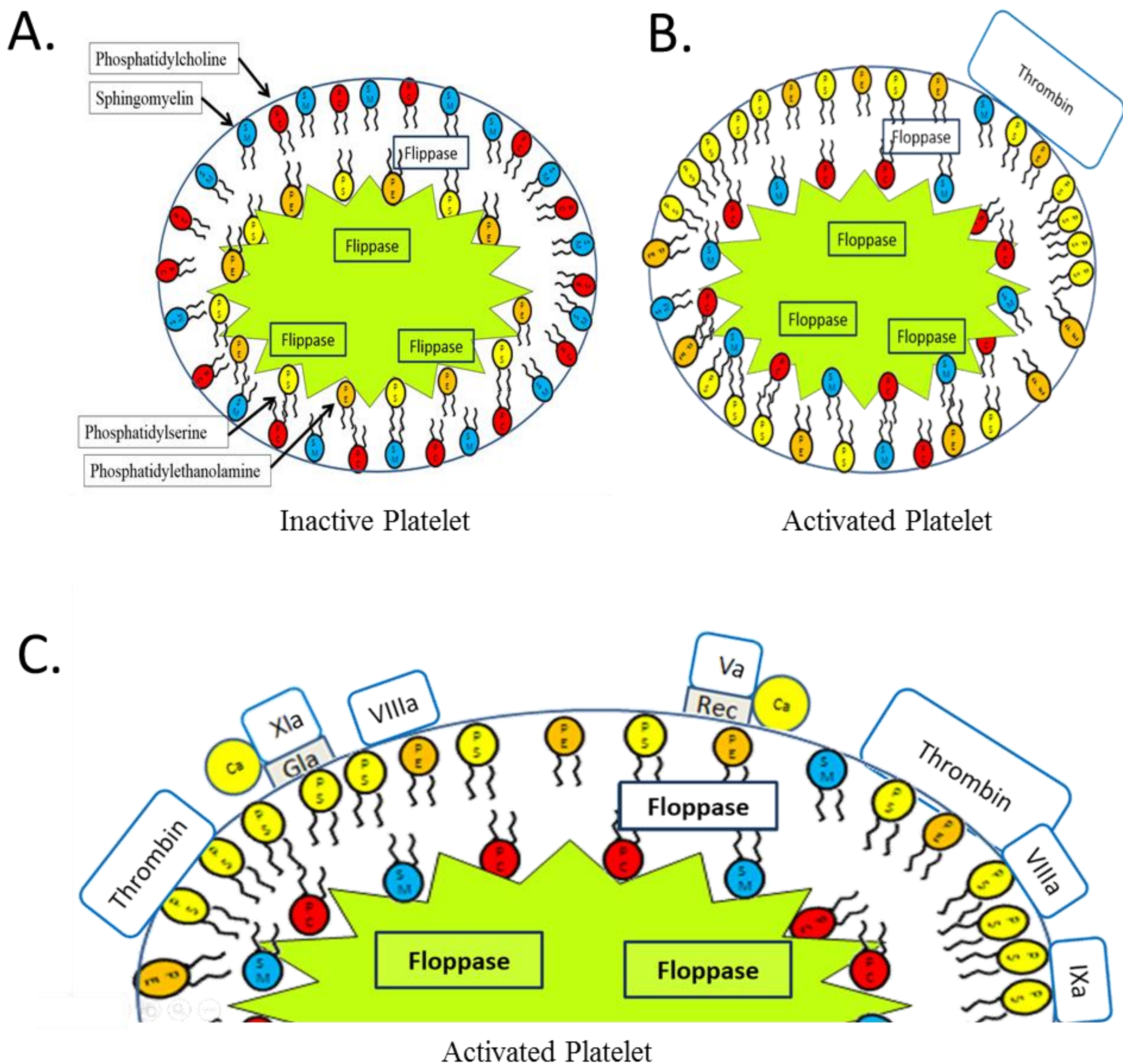


Figure 2: The propagation phase focuses on the role of thrombin and activated platelets. (A) Platelets are activated by thrombin which leads to a predominant expression of the enzyme floppase, shuffling PS and PE to the membrane surface and creating a procoagulant, anionic membrane surface with concurrent release of the platelet granules. (B) Thrombin cleaves coagulation FVIII from vWF and activates coagulation factors V, VIII, IX on the platelet surfaces.
Ca: calcium, Gla: glutamic acid

Phase 3: Propagation

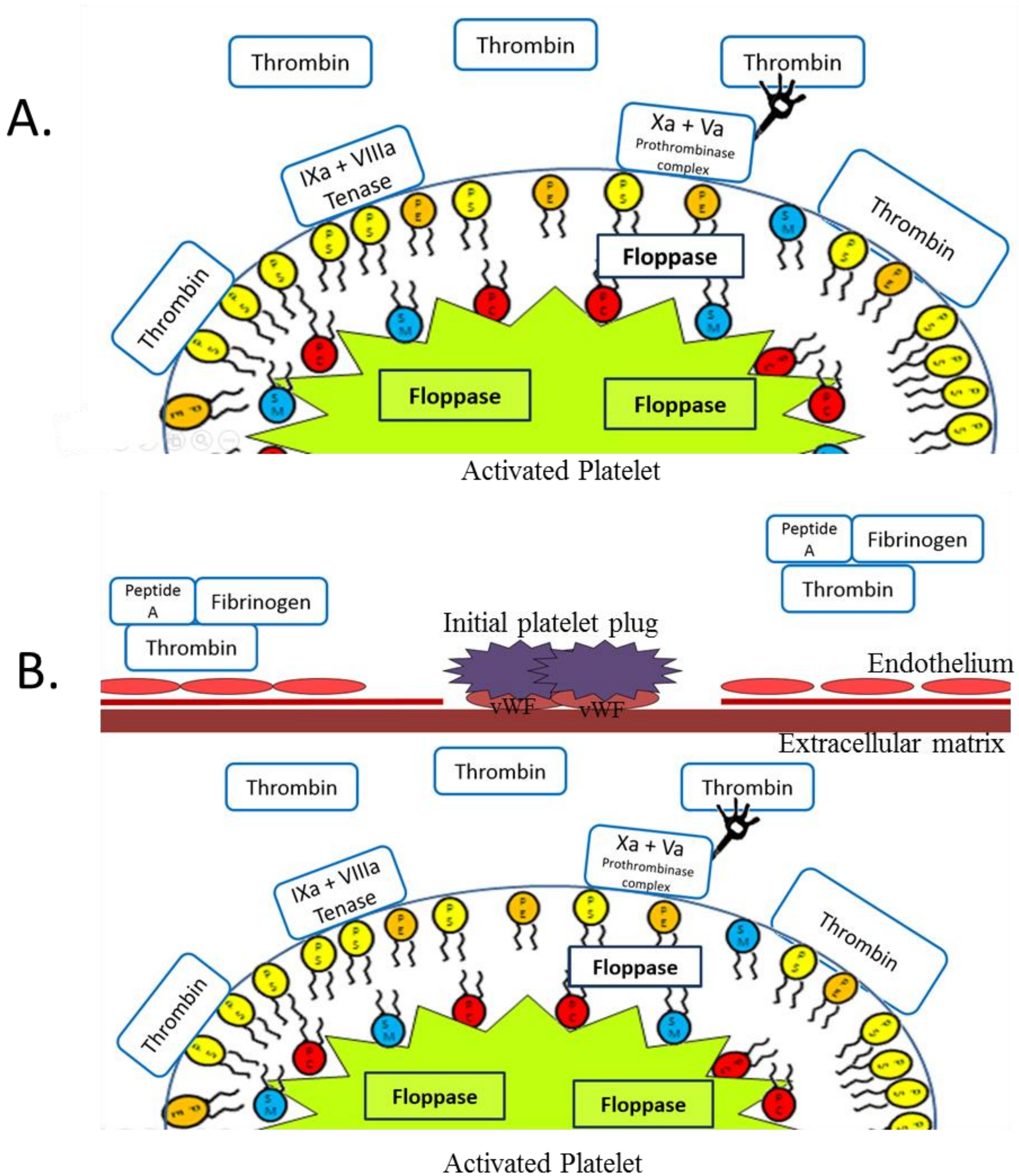


Figure 3: The propagation phase focuses on the role of the intrinsic coagulation factors. (A) Binding of FIXa and FVIIIa forms the intrinsic tenase complex which activates the substrate FX. FXa and FVa form the prothrombinase complex which converts large amounts of substrate prothrombin to thrombin. (B) Thrombin cleaves fibrinopeptide A from fibrinogen to form fibrin.

unbound in the vasculature. Free TFPI, in conjunction with protein S, is responsible for immediate inactivation of circulating FXa.[17] TFPI is known to be functionally dependent on protein S which acts as cofactor for TFPI and enhances the stability of free TFPI within the plasma.[17] AT is a serine protease inhibitor and considered one of the most important anticoagulants due to its high affinity for binding and inhibiting thrombin, FIXa, and FXa; its activity is amplified when the protein is bound to heparin. In fact, heterozygous deficiency in AT leads to a ten-fold increased risk of venous thrombosis; homozygous deficiency in AT is a congenitally lethal trait.[7]

Healthy, intact endothelium also acts as a potent anticoagulant, in contrast to the pro-coagulant properties of the activated cell membrane and tissue factor bearing extra-vascular cells, effectively limiting coagulation to the site of injury. Heparin sulfated proteoglycans (HSPGs), thrombomodulin (TM), and TFPI are all proteins expressed on the endothelial surface.[1] HSPGs acts as a binding site for AT which rapidly inactivates thrombin within the circulation. Thrombin also binds to TM at its growth factor like domains (GF1-6), altering the substrate specificity of thrombin from pro-coagulant to anti-thrombotic by initiating the activation of the protein C anticoagulant pathway.[18] This activation is increased by 20 fold when protein C is bound to endothelial protein C receptor on the endothelial surface.[19] Activated protein C, in conjunction with its cofactor protein S, prevents FVa and FVIIIa from further participating in thrombin production by irreversible cleavage and inactivation.[7] The protein C and protein S complex also inactivates plasminogen activator inhibitor 1, an important inhibitor of fibrinolysis, upregulating the breakdown of the fibrin clot.[19] TFPI forms a quaternary complex with FVIIa, FXa, and TF; terminating further contribution of these factors in coagulation.[7]

Fibrinolysis

Breakdown of the fibrin clot is essential for continued tissue repair and restoration of normal blood flow. Fibrinolysis is kept quiescent by a plethora of circulating inhibitors and requires fibrin as a cofactor for optimal function to prevent activation of fibrinolysis in the absence of clot formation.[3] Plasminogen, synthesized by the liver, is transformed to plasmin by plasminogen activators, tissue plasminogen activator and urokinase plasminogen activator. The activity of plasminogen activators is enhanced in the presence of fibrin. Plasmin is responsible for degradation of polymerized fibrin into smaller fragments referred to as fibrin degradation products.

The cell based model of coagulation allows for a more accurate description of a finely regulated event which incorporates both the prevention of coagulation during normal blood flow, formation of localized fibrin matrix over the site of injury, and eventual breakdown of the clot. Acquired or inherited disorders of hemostasis can occur at any phase, altering the equilibrium and shifting toward hypercoagulable or hemorrhagic diatheses. An improved understanding of the parallel activity and cellular contribution of coagulation has led to a better recognition of the clinical manifestation and management of many hemostatic derangements.

Section 2: Inherited hemostatic disorders in horses

The occurrence of acquired hemostatic disorders in horses is well described with hemorrhagic diatheses such as disseminated intravascular coagulopathy being extensively investigated in septic foals and horses suffering ischemic or inflammatory gastrointestinal disease.[20-22] Conversely, inherited coagulopathies are relatively rare with sparse information in the literature making identification, diagnosis, and management more challenging. A hereditary

hemostatic defect implies that a mutation has occurred within the gene encoding a specific hemostatic protein, leading to impaired synthesis or function of that protein and altering normal coagulation. Identification of the causative mutation is imperative for acquiring a confirmatory diagnosis and obtaining appropriate evidence to distinguish between mutations which are inherited or due to a *de novo* stochastic event.[23,24] However, only a handful of mutations have been identified for equine inherited hemostatic derangements making diagnosis reliant on clinical presumption, coagulation testing results, coagulation factor activity, and functional assays.[25]

Both inherited quantitative and functional defects of von Willebrand factor (vWF) have been detected in horses.[26-28] vWF is a high molecular weight glycoprotein which is synthesized by megakaryocytes and endothelial cells; it is found in platelets and the endothelium and circulates within the plasma bound to coagulation FVIII. vWF functions to stabilize and protect circulating coagulation FVIII from immediate degradation by protease inhibitors, while also providing a scaffold for platelet adherence and formation of the platelet plug after endothelial damage occurs.[29] Patients with von Willebrand disease (VWD) typically present with spontaneous bleeding from mucosal surfaces or impaired hemostasis after trauma or surgery; clinical variability in phenotype is dependent on the amount of functional vWF present.[30] Diagnosis is based on assessment of circulating vWF antigen concentrations (vWF:Ag), vWF function (based on ristocetin cofactor activity or collagen-binding capacity), evaluation of multimeric forms of vWF, and comparison of vWF:Ag to activity ratio.[30]

Although three distinct types of (VWD) have been described in people and dogs, only two types have been reported in horses.[25-28] Type 1 VWD is defined as a partial quantitative protein deficiency with diagnosis based on normal vWF multimeric structure and low levels of

circulating vWF:Ag with a concomitant reduction in vWF function.[29] In horses, type 1 VWD has been reported in an Arabian filly and a Quarter horse colt.[25,27] The Quarter horse colt was evaluated at 8 days of age due to multiple hematomas and hemarthrosis. Diagnosis of type 1 VWD was based on a prolonged activated partial thromboplastin time (aPTT), decreased vWF:Ag activity (8%), reduced vWF function, and low-normal FVIII:C activity. Although the dam was clinically asymptomatic and genetic analysis was not performed, maternal inheritance was suspected based on a decreased vWF:Ag activity (30%) in the mare.[27] Type 2 VWD is defined as a qualitative defect in vWF and can be further broken down into subtypes 2A, 2B, 2M, and 2N.[29] Two equine case reports of type 2 VWD have been described in the literature and include a Quarter horse filly and Thoroughbred mare and foal. In all three clinical cases, a diagnosis of VWD type 2A, consistent with a loss of platelet -dependent function due to abnormal multimers, was suspected.[31] Diagnoses were made based on prolonged mucosal bleeding time, low plasma vWF:Ag with disproportionately severe loss of vWF function, and decreased concentrations of high molecular weight vWF multimers.[26,28] Interestingly, after diagnosis of the Quarter horse filly, the dam was also evaluated and noted to have fragmentation of multimers on agarose gel electrophoresis, categorizing her as a VWD type 2C (or type 2A subtype II).[26] Type 3 VWD is an autosomal recessive disease and represents a severe quantitative defect of vWF characterized by immeasurable or extremely low levels of vWF protein.[29] Type 3 VWD is considered rare in animals and in some instances may indicate a lethal trait; however, cases in Shetland sheep dogs, Scottish terriers, Chesapeake Bay retrievers, and Dutch Kooiker dogs have been reported.[32,33] No clinical cases of type 3 VWD have been documented in horses.

The term inherited thrombopathies encompasses a broad range of diseases including abnormalities in platelet granule secretions, signal transduction pathways, membrane receptors, and membrane phospholipids.[25] In horses, clinical cases of Glanzmann thrombasthenia (GT) have been described in a Peruvian Paso, Quarter horse, Thoroughbred cross, and an Oldenburg; in all four cases the genetic mutations were documented.[34-38] GT is an inherited, intrinsic quantitative or qualitative defect in the heterodimer platelet membrane receptor $\alpha_{IIb}\beta_3$, which acts as the fibrinogen receptor on the platelet membrane and is essential for normal platelet aggregation.[39] With vascular damage, platelets adhere to the exposed subendothelial matrix via vWF, resulting in activation of the platelets and a conformational change of the $\alpha_{IIb}\beta_3$ integrin. Once activated, the $\alpha_{IIb}\beta_3$ receptors strongly and more readily bind fibrinogen, allowing for aggregation of platelets and formation of the primary platelet plug.[35,40] Diagnosis of GT is based on normal platelet numbers and morphology in the face of prolonged bleeding time, decreased to absent clot retraction, and absent platelet aggregation in response to all agonists. In human beings, the most common clinical signs of GT are associated with exaggerated bleeding manifesting as purpura, epistaxis, gingival bleeding, and prolonged hemorrhage after trauma or surgery.[41] Similar findings have been documented in horses. In three of the four equine cases, epistaxis was the chief complaint; a coagulopathy was suspected in the Oldenburg filly due to hematoma formation and excessive bleeding after arthroscopy and venipuncture.[35-37]

The $\alpha_{IIb}\beta_3$ integrin is encoded by the ITGA2B and ITGB3 genes. In people diagnosed with GT, nonsense, missense, deletions, insertions and splice mutations have been identified in either gene with a notable variation in phenotype.[39,42] Mutational analysis performed in the Thoroughbred cross and Oldenburg identified both horses to be homozygous for a missense mutation (leading to a predicted amino acid change from arginine to proline) in exon 2 of the

gene encoding αIIb.[34,38] Mutational analysis in the Peruvian Paso identified a homozygous ten base pair deletion encompassing the last three base pairs of exon 11 and the first 7 base pairs of intron 11 of the gene encoding the glycoprotein αIIb. The mutation was predicted to affect normal splicing of intron 11.[37] The Quarter horse was determined to be a compound heterozygote possessing both of the above described mutations.[43]

In 2005, a novel, heritable bleeding diathesis associated with decreased thrombin generation by activated platelets was described in a two year old Thoroughbred mare. The mare presented for epistaxis and significant bleeding after injection and was noted to have prolonged platelet aggregation in response to thrombin and collagen.[44] Further analysis concluded that the mare's platelets were able to bind thrombin but insufficiently propagated thrombin production via a reduction in prothrombinase activity, indicating a defect in platelet secretion. Similar results were noted in two of the filly's offspring suggesting an inherited defect.[45]

Inherited defects caused by coagulation factor deficiencies identified in horses include deficiencies in coagulation FVIII (hemophilia A), FIX (hemophilia B), and combined factor deficiencies.[25,46-50] The genes encoding coagulation FVIII and FIX are located on the X chromosome; clinical disease primarily affects males with mutations occurring either as *de novo* stochastic events or as an X-linked mode of inheritance from phenotypically normal females. Both syndromes can manifest as mild, moderate, or severe clinical bleeding based on the percent of residual coagulation factor activity. Due to the size and athletic nature of horses, even mild cases of hemophilia carry a poor prognosis due to bleeding tendencies after minor injury and recurrent hemarthrosis.[51,52] As reported in other species, hemophilia A is the more prevalent form of hemophilia and is also considered the most common inherited coagulopathy in horses being reported in Thoroughbreds, Standardbreds, Quarter horses, and a Shetland pony.[46,49,50,53]

Other inherited coagulopathies associated with coagulation factor deficiencies have not been well characterized in horses.[25] In 1977, Hinton, et al described a two month old Arabian colt with clinical signs of epistaxis, hematoma formation, and excessive bleeding from injection sites. Blood work revealed a prolonged aPTT and decreased coagulation factor activity for factors VIII, IX, and XI with normal coagulation factors II, V, VII, and X. The colt was diagnosed with a multiple coagulation factor deficiency of the intrinsic pathway; although a single factor deficiency with secondary consumption of the remaining factors could not be ruled out. Alterations in coagulation panels and coagulation factor activity were not found in the dam, sire, half sister or brother of the affected colt.[47]

Additional inherited intrinsic pathway defects include deficiencies in the contact activator prekallikrein (PK), or Fletcher factor deficiency, and have been described in specific families of miniature and Belgian horses.[54,55] PK is a glycoprotein which functions in conjunction with high molecular weight kininogen (HMWK) and coagulation FXII to form the intrinsic contact system.[56] PK circulates in plasma bound to HMWK; activation of coagulation FXII occurs due to contact of the negatively charged surface of damaged subendothelium with the prekallikrein-HMWK complex.[57] Small amounts of activated FXII cleave PK to kallikrein and thus acts as a source of auto-activation for additional coagulation FXII. Kallikrein also augments the cleavage of HMWK to form bradykinin, an inflammatory mediator, and the activation of the plasminogen activator pro-urokinase.[56] Deficiencies in PK have an autosomal recessive mode of inheritance and are diagnosed via a prolonged aPTT and decreased level of serum PK (10-35% activity).[57] Interestingly, in people, both mild coagulopathies and thrombotic events have been described. However, most affected individuals are asymptomatic due to the alternative activation of coagulation FXII by unbound HMWK, and the primary initiation of the intrinsic pathway via

formation of the intrinsic tenase complex as previously described (see coagulation section).[57] It has been suggested that patients with PK deficiencies and active coagulopathies may represent individuals with multiple coagulation defects or a concurrent acquired coagulopathy.[57] In the two equine cases, clinical suspicion was roused by *in vitro* failure of normal clot formation in a miniature horse and excessive bleeding after castration in a Belgian stallion. Presumed diagnosis was based on a prolonged aPTT and decreased serum PK concentrations with normal HMWK and coagulation factor activity. Similar discrepancies were detected in several related horses for both index cases.[54,55]

Inherited intrinsic platelet function disorders, deficiencies in coagulation factors, alterations in the intrinsic contact system, and qualitative and quantitative defects in vWF have all been identified in horses. Although not considered common, an inherited hemostatic defect should be considered in any horse with an unexplained bleeding diathesis. Coagulation profile and coagulation factor activity assays were utilized in a case of a 2 day old colt that presented with hemophilia-like clinical signs and was diagnosed with a deficiency in coagulation FVIII. Examination of the genetic sequence of FVIII in this colt is the basis of this thesis project

Section 3: Coagulopathies associated with coagulation factor VIII deficiency

The coagulation FVIII gene contains 186 kilobases (kb) and is located on the most distal region of the long arm of the X-chromosome (Xq28 in human beings).[51] The gene consists of 26 exons, predominately containing 69-262 base pairs (bp) per exon with the exception of exons 14 and 26, containing 3106 bp and 1938 bp, respectively, with the majority of exon 14 consisting of a 3' untranslated region. The spliced messenger RNA (mRNA) is 9.6 kb with the final translated protein containing 2332 amino acids (see figure 4).[58]

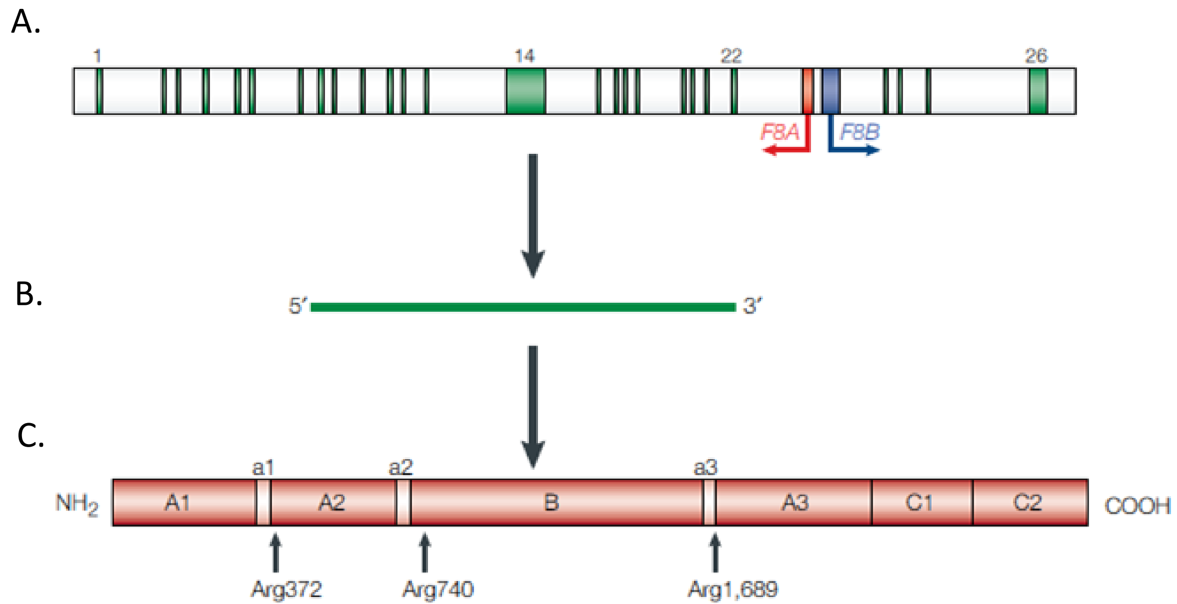


Figure 4: (A) The FVIII gene contains 186 kilobases (kb) and is located on the most distal region of the long arm of the X-chromosome (Xq28 in humans). The gene consists of 26 exons, predominately containing 69-262 base pairs (bp) per exon with the exception of exons 14 and 26, containing 3106 bp and 1938 bp, respectively. Intron 22 contains two further genes (F8A and F8B) with the arrows indicated the orientation of transcription. (B) The spliced mRNA is 9.6 kb with (C) the final translated protein containing 2332 amino acids. Cleavage at Arg 372, 740 and 1689 produces the final product
Figure adapted from: Graw J, Brackmann HH, Oldenburg J, et al. Haemophilia A: from mutation analysis to new therapies. Nat Rev Genet 2005; 6:488-501.

Coagulation FVIII is a vital component of the intrinsic pathway in secondary hemostasis. During coagulation, activated FVIII acts as a cofactor for factor IX leading to the formation of the intrinsic tenase complex and activation of coagulation FX. Activated FXa is responsible for cleavage of prothrombin to thrombin; thrombin cleaves fibrinogen to fibrin allowing for the formation of an insoluble fibrin clot.[1,59] Therefore, deficiencies in coagulation FVIII result in coagulation type bleeding including hematoma formation and hemorrhage into joints, deeper tissues, and body cavities. Several inherited coagulopathies have been identified associated with deficiencies in coagulation FVIII.

Hemophilia A

Coagulation FVIII deficiency, also known as hemophilia A, is the most common inherited coagulation disorder. As a sex-linked disease, hemophilia A primarily affects males with a frequency in people as high as 1/5000 male births.[51] The disease is most often transmitted by heterozygous females, who are usually phenotypically normal due to random X-chromosome inactivation, allowing the individual to produce enough FVIII to remain clinically normal. A carrier female has a 50% chance of producing an affected male and a 50% chance of producing a carrier female. Although rare, cases of females with severe hemophilia A have been documented; these cases are usually associated with monosomy X or skewed (non random) X inactivation.[60,61]

In people with hemophilia A, mutations occur within the FVIII gene which can affect either the protein structure and / or function of FVIII. FVIII stochastic mutations occur 3.6 times more often in male germ cells, and have been associated with gene rearrangements (intron 1 and intron 22 inversions), single base substitutions, deletions, insertions, and compound mutations.[51] Identification of FVIII mutations has become increasingly important due to the direct relationship of particular mutations with severity of clinical signs, prognosis, and

development of anti-factor VIII antibodies.[51,62-64] As of May 2013, the international Haemophilia A Mutation, Structure, Test and Resource site (HAMSTeRS database) listed 2158 unique mutations associated with hemophilia A.[65] The high mutation rate of the FVIII gene has been attributed both to the size and location of the gene.

Intron 22 inversions are estimated to be the cause of 40% of severe cases of hemophilia A, and occur at a 10 fold higher rate in male germ cells. Intron 22 contains 32 kilobases (kb) and is composed of two nested genes, F8A and F8B. Furthermore, intron 22 contains a 9.5kb region which is also present outside of the FVIII gene, near two extragenic copies located 400kb 5' to the FVIII gene. Intron 22 inversions result from flipping of the tip of the X-chromosome, with intrachromasomal recombination occurring in the F8A gene due to the similarity and opposite directions of the extragenic versus the homologous intragenic region (see figure 4). Depending on which portion of the F8A gene is involved, two main types of inversions have been identified with type 1 incorporating the distal portion of the gene and type 2 incorporating the proximal portion of the gene. Both type 1 and type 2 inversions result in the translocation of exons 1-22 away from exons 23-26, rendering the gene nonfunctional.[51,63,66] Approximately 40% of point mutations are at one of the seventy cytosine-phosphate-guanine rich sites, and 25% of small deletions occur in one of two adenine series in exon 14, which contain eight to nine consecutive adenine nucleotides.[51,63,67]

Additionally, hemophilia A has been identified in cats, pigs, horses, sheep and cattle.[46,68-71] Dogs exhibit clinical signs similar to people and have been described as an ideal animal model for hemophilia.[72] Colonies of hemophilia A dogs have been bred and maintained, offering invaluable knowledge on the safety and efficacy of novel hemostatic agents and therapeutic modalities. FVIII gene mutations identified in both canine hemophilia A colonies in

Chapel Hill, North Carolina and Queen's University, Canada were suggestive of a large genomic rearrangement, with notable similarities to intron 22 inversions in humans.[70,73] At the Queen's colony aberrant splicing was identified that led to premature termination of the transcription of the FVIII gene, resulting in disruption of the transcript distal to exon 22 and ending with a novel sequence element. A copy of the novel sequence element with hybridized banding patterns was identified in the canine intron 22 further suggestive of a large gene rearrangement.[73]

Although the two established dog colonies have become an important animal model, the high cost of production and maintenance, requirement for scale-up in comparison to human beings, and the robust immune response to initial injections of human FVIII (hFVIII) are notable limitations.[52] It has been suggested that sheep, which have historically been used as models for other human diseases, may provide an alternative large animal model for hemophilia.[74] Recently, a hemophiliac sheep colony was established utilizing frozen semen from a line of White Swiss Alpine sheep diagnosed with hemophilia A in the 1980s. Originally, all affected lambs died shortly after birth due to uncontrolled umbilical bleeding.[71] During establishment of the sheep colony, lambs were treated with two to seven doses of hFVIII which ceased umbilical bleeding; no lambs had adverse reactions to hFVIII. The sheep continued to be treated prophylactically (prior to tail docking or ear tagging) and symptomatically with hFVIII. Four of the six sheep developed inhibitor antibodies to FVIII at varying levels, establishing the sheep colony as a viable animal model for studying protein or gene based hFVIII therapeutics. Furthermore, mutational analysis revealed that the inciting mutation included an insertion leading to a frameshift and premature stop codon in exon 14, providing the first animal model with that particular mutation.[74]

Other causative mutations leading to hemophilia A have been identified in animals. In dogs, distinct missense mutations have been identified in boxers and German shepherds.[75] A German shepherd and another male dog of unspecified breed were found to have single base substitutions leading to nonsense mutations in exon 1 and exon 12, respectively.[76,77] Two related Havanese dogs were determined to have short interspersed element insertions of 220 bp in exon 14.[78] In cattle, two related Japanese Brown bulls were discovered to have a missense mutation (T>A), leading to a predicted amino acid substitution from leucine to histidine, in the highly conserved C1 portion of FVIII.[69]

In horses, hemophilia A is the most common inherited coagulopathy and has been identified in Thoroughbreds, Standardbreds, Quarter horses, Shetland ponies, and Arabians.[46,48-50,53,72] Diagnosis is based on clinical signs, coagulation panel findings (prolonged aPTT with a normal platelet count and prothrombin time), and decreased FVIII coagulant activity (FVIII:C). Although the incidence of hemophilia A in horses is unknown, according to Dr. Marjory Brooks from Cornell University Veterinary Diagnostic lab, approximately one case of hemophilia A is identified per year from that lab.[79] The incidence may be higher, because many of these foals are severely affected and are euthanized prior to a diagnosis being made. As with people, the severity of disease in horses varies inversely with the percent of FVIII:C activity. In people, severe disease has been associated with a FVIII:C activity of <2%; whereas in horses, severe disease can be associated with FVIII:C activity of <10-15%.[63,80] Due to the size and athletic nature of horses, severe cases of equine hemophilia A carry a poor prognosis both for athletic function and quality of life. Currently there is no cure for hemophilia A and treatment is often medically and financially limiting. Most hemophiliac foals are euthanized, with the loss having both an emotional and economic impact on the owner.[46,80]

Distinguishing between maternal inheritance and a *de novo* stochastic event is essential for making accurate recommendations on both future breeding of the mare and her female offspring. With no previous identification of mutations associated with hemophilia A in horses, practitioners have attempted to identify carrier mares based on FVIII:C activities. Unfortunately, carrier mares often have FVIII:C activities within the reference range, particularly immediately post-partum, decreasing the chance of identification of carrier mares.[46]

Type 2N von Willebrand Disease

Type 2N VWD is characterized as a qualitative defect in the FVIII binding site to vWF. In normal conditions, FVIII is tightly bound non-covalently to vWf in circulation.[1,4] FVIII is activated by two thrombin proteolytic cleavages at R372 and R1689. Cleavage at R1689 allows for dissociation of FVIII from vWF.[81,82] Premature dissociation of FVIII from vWF leads to immediate degradation of FVIII by phospholipid-binding proteases to prevent alterations in coagulation. Therefore, a qualitative defect in the FVIII binding site to vWF leads to secondary FVIII deficiency and hemophilia-like phenotype (see figure 5).

In people, VWD is the most common inherited bleeding disorder; however, type 2N VWD is considered a relatively rare form and only accounts for 1-2% of identified cases of VWD and 18% of type 2 VWD.[83] The predominant clinical signs in people mimic those of mild hemophilia such as hemarthrosis and excessive bleeding after trauma or during surgery.[31] The level of disease severity has been found to be dependent on the location of the mutation in the vWF binding site, indicating that genotype directly correlates with the expressed phenotype.[31] In human beings, exons 18-25 of the vWF gene, encode the FVIII binding site. Approximately 20 different type 2N mutations have been identified accounting for three patterns of inheritance in people: homozygotes for FVIII binding missense mutations, compound heterozygotes for two

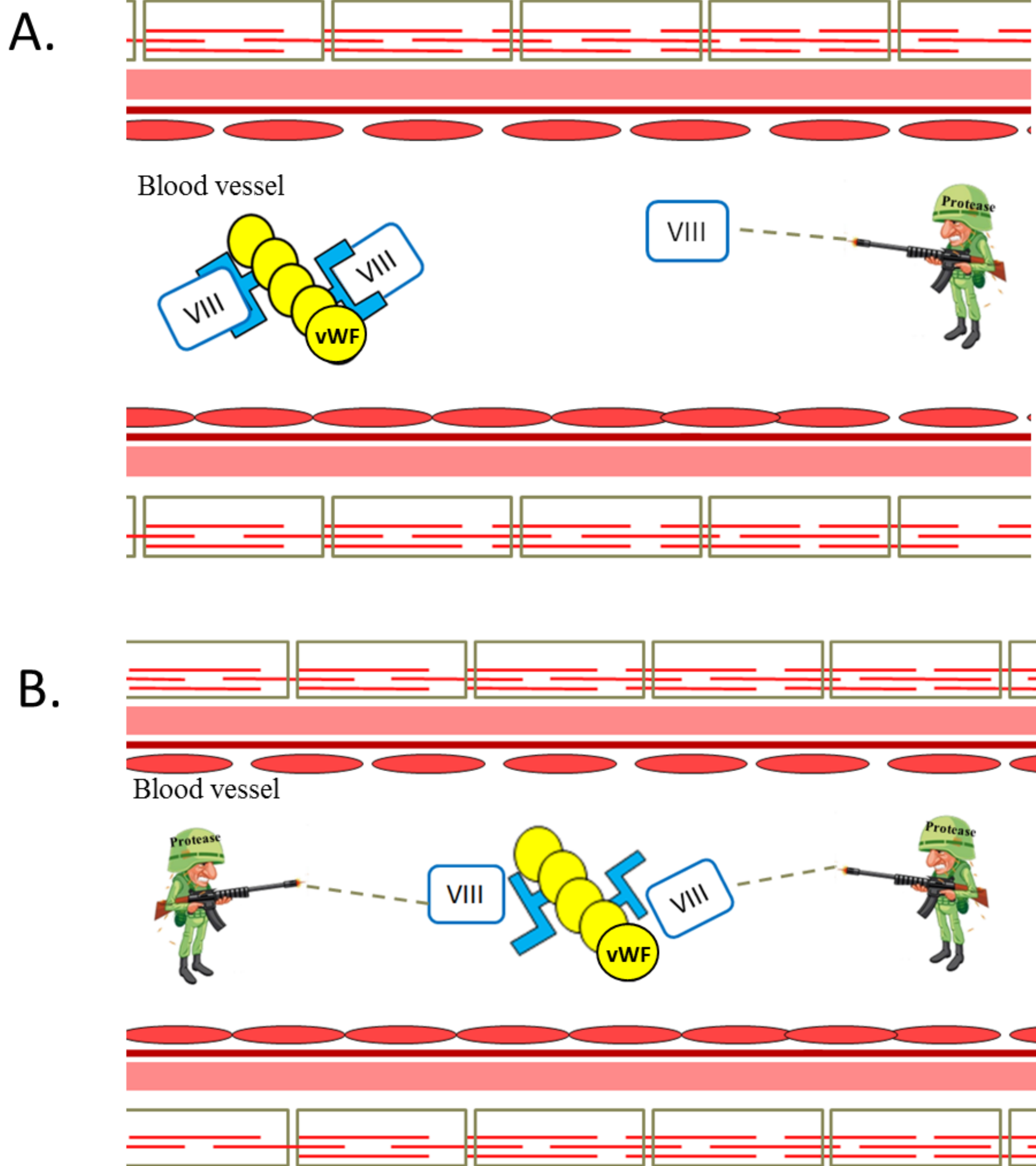


Figure 5: In circulation, coagulation FVIII is tightly bound non-covalently to von Willebrand factor (vWf). Unbound FVIII is immediately degraded by phospholipid binding proteases. (B) Qualitative defect in the FVIII binding site to vWF causes FVIII to remain unbound and subject to degradation, leading a to secondary FVIII deficiency and hemophilia-like phenotype.

unique FVIII binding mutations, or compound heterozygotes for a FVIII binding mutation with a vWF null mutation.[30,84] Notably, 80% of the missense mutations identified have been located within exons 18-20. Therefore, in humans, genetic analysis can initially be started in these regions.[84]

Preliminary diagnosis of type 2N VWD is based on clinical signs and decreased FVIII:C activity (5-40%) with concurrent decreased plasma FVIII:vWF antigen ratios. If diagnosed only on these criteria, type 2N VWD may be misidentified as mild to moderate hemophilia A in males or hemophilia A carriers in females.[85] Also, people with heterozygous mutations in the FVIII binding site and a null mutation in vWF may have low levels of circulating vWF, leading to a misdiagnosis of type 1 VWD.[86] Differentiation between these three diseases can be made by mutational analysis or vWF:FVIII binding assays. vWF:FVIII binding assays are typically performed by washing patient vWF to remove any endogenous bound FVIII. A standard amount of exogenous FVIII is added to facilitate a uniform exposure; amounts of vWF and bound FVIII are normalized to account for decreased amounts of plasma vWF. In cases of hemophilia A or type 1 VWD, the exogenous FVIII will bind vWF; however, in cases of type 2N VWD little to no exogenous FVIII will be bound.[85]

It has become increasingly imperative to correctly differentiate type 2N VWD versus hemophilia A since treatment consequences of a misdiagnosis can be detrimental to the patient. Hemophilia A patients treated for an acute bleeding diathesis are often given rFVIII and those with type 1 VWD are treated with desmopressin to increase the release of endogenous vWF from endothelial cells. However, patients with type 2N VWD cannot bind endogenous FVIII or rFVIII and both are quickly degraded, ergo reducing the half-life of FVIII and inciting a

treatment failure. Treatment of type 2N VWD requires infusion of vWF-FVIII concentrates with quick response to prevent further bleeding.[86]

Type 2N VWD has not been reported in animals. However, based on the limited specific hemostatic factor assays and mutational analyses available to veterinarians, it may be that type 2N VWD occurs in animals but has been misdiagnosed as type 1 VWD or hemophilia A. Further investigation for cases with hemorrhagic diatheses and low FVIII:C activities should be pursued to ensure accurate diagnosis, prognosis, and future breeding recommendations for the affected line.

Combined Deficiency of Coagulation Factor V and Factor VIII

Combined deficiency of coagulation factor V and factor VIII (CDF5F8) is a rare autosomal recessive disorder characterized by mutations in either transport protein lectin mannose binding protein type 1 (LMAN-1) or multiple coagulation factor deficiency 2 (MCFD2).[87] LMAN-1 is a 53kDa integral membrane protein which cycles between the endoplasmic reticulum and golgi apparatus; whereas, MCFD2 is a 16kDA protein which acts as a cofactor for LMAN-1. Both proteins form a calcium-dependent cargo transporter which facilitates movement of FV and FVIII from the endoplasmic reticulum to the golgi apparatus during the early secretory pathway through mannose-selective and calcium dependent binding and release (see figure 6).[87,88] Although LMAN-1 and MCFD2 are responsible for the transport of other cargo proteins, CDF5F8 is the only known clinical manifestation of mutations within either protein.[89]

CDF5F8 has been estimated to affect 1:1,000,000 human beings, although the exact prevalence of CDF5F8 is geographically dependent increasing to 1:100,000 in regions where there is a predominance of consanguineous marriages.[89,90] This disorder manifests as mild to

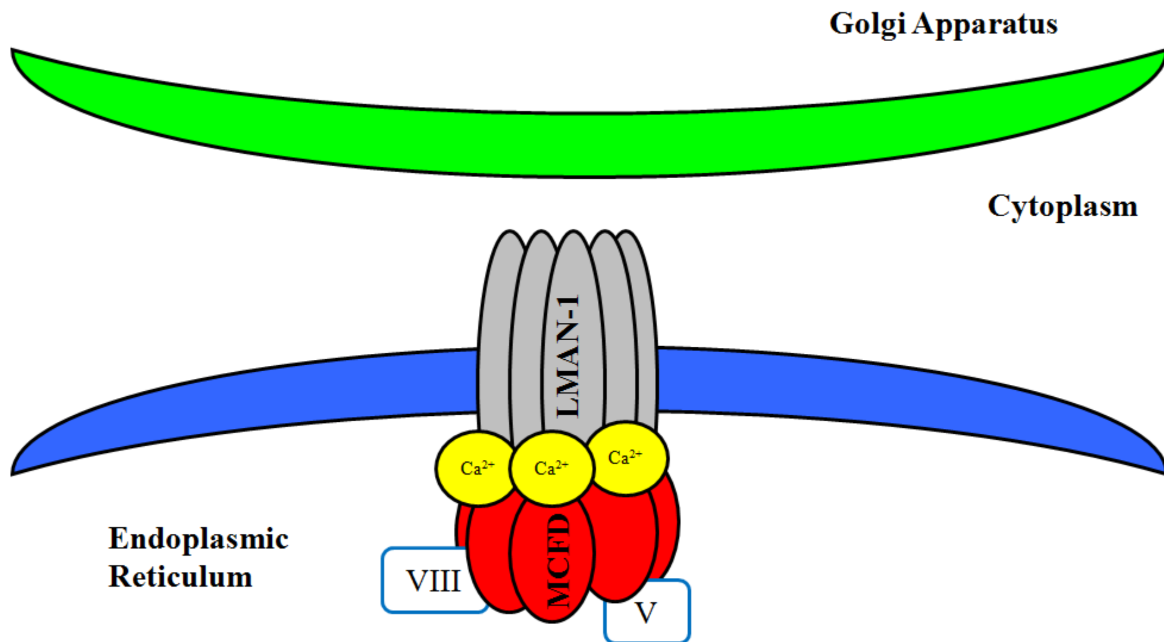


Figure 6: Transport protein lectin mannose binding protein type 1 (LMAN-1) and multiple coagulation factor deficiency 2 (MCFD2) form a cargo receptor which facilitates movement of FV and FVIII from the endoplasmic reticulum to the golgi apparatus during the early secretory pathway through mannose-selective and calcium dependent binding and release

moderate clinical bleeding with a percent coagulation activity of 5-30% for FVIII and FV.[91] Typical clinical signs include epistaxis, mucosal bleeding from the gums or gastrointestinal tract, menorrhagia, and excessive bleeding after trauma or during surgery. Diagnosis is based on a prolonged aPTT and prothrombin time (PT) with specific coagulation factor assays identifying a concurrent decrease in FV and FVIII.[87]

In human beings, the genes encoding LMAN-1 has been localized to chromosome 18q21 and contains 13 coding exons. MCFD2 is a much smaller gene located on chromosome 2p21, and contains 4 exons.[89] Mutations in both transport proteins have been identified, with the majority of mutations consisting of homozygous nonsense, frameshifts, splicing defects, or missense mutations resulting in a null allele. Although there are ongoing efforts to identify mutations in afflicted individuals, as of 2011, approximately 70% of the mutations reported were located in LMAN-1.[89,91] However, geographical considerations must be taken into account, with reports of a predominance of MCFD2 mutations in patients from India and Italy.[90,91] The founder effect was also suspected in family lineages where a single, unique mutation predominated.[91] While patients with mutations in LMAN-1 or MCFD2 have virtually indistinguishable clinical phenotypes, a statistically significant difference in lower coagulation factor activity for FV and FVIII was discovered in patients with MCFD2 mutations.[91] Based on these results, it has been hypothesized that MCDF2 may initiate the interaction with FV and FVIII in the endoplasmic reticulum, thus functioning as a selective cargo adaptor.[91,92]

CDF5F8 has not been reported in animals. However, mild bleeding diathesis or limited specific coagulation factor assays may hinder diagnosis of this disease in veterinary medicine. Further investigation is warranted to determine if CDF5F8 is present in animals.

Section 4: DNA and Genetic Mutations

The genome is a dynamic entity and is subject to mutations from spontaneous errors during DNA replication or repair, somatic *de novo* stochastic events, inherited mutations, or damage from environmental or endogenous insults. Mutations may be synonymous, which do not change the sequence or gene product, or non-synonymous, which result in altered DNA and RNA sequence. Pathogenic mutations exhibiting an aberrant phenotype may occur within the coding sequence of the gene, noncoding sequence, or regulatory sequence outside of the coding area of the gene. Although most medical emphasis is placed on non-synonymous mutations which have a deleterious effect, it should be noted that non-synonymous mutations may also lead to a beneficial effect to the individual or have no consequence to protein function.

Base substitutions, or point mutations, occur when one nucleotide is substituted for another and can be due to transitions, where a pyrimidine is substituted for the opposite pyrimidine (C or T) or of purine by a purine (A or G), or transversions, with a substitution of a pyrimidine by purine or vice versa.[93] Individual base substitutions within the non-coding sequence typically do not alter gene expression except in highly conserved areas such as the gene promoter, splice site junctions, or splice branch sites.[94] Point mutations within the coding sequence may result in a silent mutation which is a synonymous nucleotide substitution encoding the same amino acid and does not change the protein produced. This is consistent with the concept of a degenerate genetic code and a strong conservative selective pressure to avoid amino acid changes.[95] A missense mutation is a non-synonymous substitution that results in encoding of a different amino acid, altering the translated protein with variable effects on protein function due to varying protein tolerance of single amino acid changes.[96] Other nucleotide substitutions can result in the introduction of a nonsense mutation, a premature stop codon leading to a

truncated protein or nonsense-mediated decay of RNA, or a readthrough mutation that causes a breaking of the original stop codon so the translated protein is extended at the c-terminus.[97]

Insertions are mutations in which extra nucleotides are added into a portion of the DNA; whereas, deletions occur when sections of DNA are lost. Although insertions and deletions can occur in any portion of the genome, they are particularly prevalent in regions with short, tandem repeats due to replication slippage.[98] Replication slippage occurs due to mispairing of the replicating DNA which forces the repeat DNA fragment to loop out at the mismatched site; most DNA loops are recognized by the mismatch repair system but if they escape elimination then alterations of the DNA are maintained.[98] Insertions and deletions have variable effects on the coding capacity of the gene depending on the length, location, and presence of frameshifts. Frameshifts result in alteration of the message and are often deleterious due to the resultant nonfunctional transcript or protein.[99] However, not all frameshifts are pathogenic due to the relief of selective protein pressure from a second copy of the gene which may compensate for the loss of protein production or function from the frameshift mutation.[100] It should be noted that although an insertion or deletion may result in a frameshift, these terms are not synonymous since the insertion or deletion of three nucleotides may add or remove codons without affecting the reading frame.[97]

Mutations can also affect the component of normal splicing leading to alterations known as aberrant splicing. It has been suggested that aberrant splicing represents up to 50% of mutations that cause gene dysfunction.[101] Aberrant spicing may occur due to alterations of the consensus splice sites due to mutations at the G-T 5' splice donor site, A-G 3' splice acceptor site, conserved flanking sequence elements, or from activation of a cryptic splice site due to mutations within the intronic or exonic enhancers, silencers or branching points.[102] Failure of

splicing can lead to retention of intronic sequence resulting in a frameshift or the addition of amino acids within the translated protein.[100] Alterations in the consensus splice donor sequence can lead to skipping of the upstream exon; whereas, mutation to the splice acceptor sequence results in skipping of the downstream exon. Similarly, activation of a cryptic exonic splice site often results in deletion of a portion of the exon. Extraction of an exon or portions of the exon often has deleterious effects due to the resultant frameshift and unstable transcript or nonfunctional protein due to loss of critical amino acids.[96,97,102]

Other mutations identified within genes include inversions, which occur predominantly in areas with high sequence similarity resulting from the bending backwards of the chromatid and subsequent breakage and rejoining mispaired repeats.[96,97] A classic example of a pathogenic inversion is the coagulation factor VIII inversion of intron 22 and is estimated to be the cause of 40% of severe cases of hemophilia A. Intron 22 inversion results from flipping of the tip of the X-chromosome, causing intrachromosomal recombination and resulting in the translocation of exons 1-22 away from exons 23-26, rendering the gene nonfunctional.[51,63,66]

The genome faces numerous damaging events daily, but not every insult results in a permanent mutation to the genetic code due to proofreading mechanisms which constantly screen and repair inaccurate or damaged DNA.[103] Direct repair of the DNA reverses the damage by converting the damaged nucleotides back to the original sequence. Only minor alterations in the genome can be corrected by direct repair including nicks and some forms of alkylation damage.[96,97] More often, excision repair occurs where the damaged section of DNA is removed with the subsequent resynthesis of the correct nucleotide sequence. Base excision and nucleotide excision repair both work to identify mutagens which have led to alterations in the chemical structure of the nucleotide. Base excision repair involves removal of one or more damaged

nucleotides by the cleavage of the damaged base from the nucleotide complex via DNA glycosylase and AP endonucleases, creating a single nucleotide gap. The single nucleotide gap is filled by DNA polymerase and stabilized by DNA ligase. Nucleotide excision repair deals with more extensive damage to the DNA strand and acts by removing a larger portion of the single stranded DNA; correction of the gap occurs similarly to base excision.[104]

Mismatch repair works in the same way as nucleotide excision repair except that it deals with errors in replication, such as the absence of base pairing between the parent and daughter strand. The mismatch repair system is responsible for the detection of mismatched base pairs and insertion or deletion loops within the transcript. Differentiation and identification of the parent versus daughter strand occurs during a small window after DNA replication and prior to methylation of the daughter strand, ensuring conservation of the parent strand.[105] Post-replication repair is used to fix a double-strand break which is a critical injury to the DNA helix. This can occur by a genetic exchange from a homologous chromosome (also described as a gene conversion) or recombinational repair or by the rejoining of broken ends in a process known as nonhomologous end-joining, which often leads to permanent mutations.[106]

Sequence variation within the exon or intron may alter the mRNA transcript by disrupting normal splicing. Nonsense mediated mRNA decay, nonstop decay, no-go decay, and nonfunctional rRNA decay are all quality control systems which recognize alterations in transcript elongation or termination and degrade aberrant mRNA.[107] For example, creation of a premature termination codon within the aberrant splice site may lead to translation of a C-terminally truncated protein. Nonsense mediated mRNA decay recognizes and degrades the truncated transcript; whereas, nonsense-associated altered splicing increases the level of an alternatively spliced transcript that does not contain the premature termination codon.[108]

The genome exists as a non-static entity in a genotoxic environment. Alterations in the genetic code occur constantly and are typically corrected by DNA damage response mechanisms. However, permanent alterations within the genome or inherited defects do occur and can lead to deleterious non-synonymous mutations with clinical manifestations occurring due to effects on genetic sequencing and alterations in protein function. The understanding of genetic diseases has made remarkable advances in the past 50 years with the fundamental discoveries made in molecular biology. Since then, mutational analysis has become a vital component in identification and characterization of genetic diseases as well as determining the relationships between genotype and phenotype.

Section 5: Justification of the project

In human beings, inherited coagulopathies have been well documented and extensively studied. One of the most significant contributions to the understanding of inherited coagulopathies occurred in the 1980s when the genes encoding the coagulation factors were characterized.[64] Since then, extensive effort has been made in discovering the causative mutations associated with inherited coagulopathies and linking the genotype with clinical manifestations and treatment outcomes. For example, a direct relationship has been established with genotype-phenotype correlations in clinical cases of VWD.[30,31] Identification of mutations in LMAN1 and MCFD2 has allowed for the recognition of the underlying cause of a relatively rare inherited coagulopathy, CDF5F8, which instigated the appropriate diagnosis and treatment of many patients previously labeled as mild hemophiliacs or type 1 VWD.[89] In patients with hemophilia A, identification of genetic mutations have led to a clinical understanding of direct relationship of particular mutations with severity of clinical signs, prognosis, and future

development of anti-factor VIII antibodies.[64,109] Predominant causative mutation within a lineage has also allowed for ease in determining carrier status and prenatal diagnosis.[64] More importantly, genetic counseling has become a mainstay in educating families with hemophilic children, enhancing patient understanding in what was coined the 'untapped expertise of learning how to be successfully ill' in the hemophiliac patient.[110,111] In a study evaluating the knowledge of genetics and inheritance in hemophiliac children, it was determined that hemophiliac children 13-25 years of age who had received genetic counseling had a better understanding and acceptance of their disease and risk of inheritance; genetic counseling was also found to have helped to enhance not only the individual knowledge but the support of the family unit and sibling relationship.[110]

In horses, genetic linkage studies of inherited coagulopathies have been sparse in the literature and absent in cases of inherited FVIII deficiencies. Consequently, equine practitioners have been reliant on diagnosing foals with FVIII deficiencies based on clinical presumption, coagulation panel findings (prolonged aPTT with a normal platelet count and PT), and decreased FVIII coagulant activity (FVIII:C). With no previous identification of mutations associated with hemophilia A in horses, practitioners have attempted to identify carrier mares based on FVIII:C activities. Unfortunately, carrier dams often have FVIII:C activities within the reference range, particularly immediately post-partum, decreasing the chance of identification of carrier mares.[46] Furthermore, the lack of genetic confirmation in hemophiliac foals may be leading to the inability to recognize other coagulation FVIII deficiencies such as type 2N VWD and CF5F8D.

Making a correct diagnosis for inherited coagulation FVIII deficiencies, and distinguishing between maternal inheritance and a *de novo* stochastic event is essential for making recommendations on future breeding of the affected foal, dam and her female

offspring.[24] Sequencing of the coding region of the equine FVIII gene would allow for the identification of mutations associated with hemophilia A in horses, giving practitioners an accurate method of identifying carriers within a herd and preventing the perpetuation of the disease within a specific lineage. If the causative mutation is unable to be identified in the FVIII gene, then further investigation is warranted into other disorders associated with inherited FVIII deficiencies. It should be acknowledged that many genetic mutations may exist in horses, as demonstrated in human beings, and identification of the causative mutation in one horse does not imply that the mutation is causative for hemophilia A in all blood lines. However, mutational analysis in individual horses will lead to a better understanding of equine inherited coagulopathies and more accurate recommendations and genetic counseling for owners with an affected foal and potential carrier mare. For these reasons, mutational analysis was performed on a two day old Tennessee Walking Horse colt with clinical signs of coagulation type bleeding, prolonged aPTT and decreased FVIII:C activity.

Chapter 2: Mutational analysis and coagulation factor VIII sequence in a colt with Hemophilia A

Section 1: Case Presentation

A 2-day-old, 45kg, Tennessee Walking Horse colt presented to the Auburn University Large Animal Teaching Hospital for lethargy and multiple joint swellings. The colt was born at 345 days of gestation to a multiparous mare. The delivery was unassisted and the placenta appeared grossly normal. The colt had difficulty standing and required assistance for nursing. He was subjectively noted to be weaker and less coordinated than other foals at 24 hours of age. He was witnessed to have stumbled into a bucket, striking the frontal bone, resulting in the formation of a large swelling. Approximately 36 hours after birth, the colt developed multiple swollen joints and bilateral epistaxis.

Upon presentation, the colt was alert and responsive. He was reluctant to stand but was able to nurse normally and walked without appreciable lameness. His was tachycardic at 140 beats per minute and tachypneic at 96 breaths per minute with a rectal temperature of 101.7^{0}F (38.7^{0}C). Mucous membranes were icteric but moist with a capillary refill time of less than two seconds. Unilateral epistaxis was present from the right nostril, and petechial hemorrhages were noted within the pinnae and internal nares. The umbilicus was dry and palpated normally. Gastrointestinal borborgymi and cardiopulmonary auscultation were within normal limits. Large, soft, non-painful swellings were present on the frontal bone and on both stifles, carpi, and hind fetlocks (see figure 7). Thoracic, abdominal, and umbilical ultrasound examinations were unremarkable.

Foal ELISA SNAP IgG test revealed an IgG level less than 400mg/dL, indicating failure of passive transfer. The left jugular vein was clipped and aseptically prepped; blood was obtained and submitted for culture, complete blood count, coagulation panel, blood gas, and biochemical analysis. A 14 gauge central venous catheter (MILA) was placed and the colt was administered 1L of commercial plasma. A treatment regimen of amikacin (25mg/kg IV Q24H) and potassium penicillin (22,000IU/kg IV Q6H) was initiated.

Hematology and total protein values were consistent with acute blood loss or hemolysis characterized by a low PCV (14%, reference interval(RI) 33-48%), decreased red blood cell count (3.92 X 10^6/μL, RI: 6.5-9.99 X 10^6/μL), decreased hemoglobin (5.4g/dL, RI: 10-16g/dL), normal MCV (35.7/fL, RI: 34-58/fL), and decreased total protein (3.62g/dL; RI: 6.0-8.6g/dL). He was leukopenic (3.81 X 10^3 cells/μL, RI: 6-12 X 10^3 cells/μL) characterized by a neutropenia (2.74 X 8.19^3 cells/μL, RI: 3-6 X 10^3 cells/μL) and lymphopenia (1.029 X 10^3 cells/μL, RI: 1.75-5.67 X 10^3cells/μL). Clinical chemistry values were consistent with acute blood loss or hemolysis, anorexia and failure of passive transfer with a hyperbilirubinemia (9.19 mg/dL, RI: 0.7-3.6mg/dL), hyponatremia (118mEq/L, RI: 134-150mEq/L), hypochloremia (87mEq/L, RI: 97-111mEq/L), hyperglycemia (208mg/dL, RI: 81-127mg/dL), hypoglobulinemia (1.3g/dL, RI: 2.8-4.4g/dL) and hypoalbuminemia (2.3g/dL, RI: 2.7-4.1g/dL). Venous blood gas results included a partial pressure of oxygen of 28.5 mmHg. This result (<30mmgHg), in conjunction with the PCV and clinical signs, indicated a blood transfusion would be warranted. Coagulation panel results from citrated blood showed a significantly prolonged aPTT (greater than 60 seconds, RI: 31-37 seconds) and a decreased fibrinogen (151mg/dL, RI: 264-1000mg/dL); the remainder of the coagulation profile was within normal limits (see table 1). Arthrocentesis from the left carpal joint yielded frank blood and cytology confirmed hemarthrosis.

Based on the history, physical exam and diagnostic tests, failure of passive transfer, neonatal septicemia, and acute hemorrhage secondary to an inherited coagulopathy of the intrinsic pathway (coagulation factors VIII, IX, XI, and XII) were suspected. Citrated plasma was collected from the colt, the mare and a normal horse (determined by a normal physical examination, complete blood count, biochemical profile, and coagulation profile) from the large animal teaching herd and submitted to Cornell University Animal Health Diagnostic Center for determination of coagulation factor percent activity. Blood was collected from the colt and the mare into EDTA tubes and stored at -20^{0}C. Due to financial constraints and a poor prognosis for athletic function, the owners elected humane euthanasia, and no further treatment options were pursued.

Necropsy findings included acute, extensive hemorrhage within the subcutis, skeletal muscle, intermuscular fascia, small intestine, and other soft tissues consistent with a diagnosis of a hemorrhagic diathesis. Gram stain and blood culture results from admission were negative after seven days of growth in the laboratory. Results of the percent coagulation factor activity revealed significantly decreased coagulation FVIII activity in the colt, consistent with a diagnosis of hemophilia A (see table 2). Notably, coagulation FXI was also decreased in the colt (26%) when compared to the dam (91%) and normal horse (55%). Combined deficiencies in coagulation FVIII and FXI have not been described in the literature and primary FXI deficiencies in people typically present as a mild bleeding diathesis with a poor correlation between coagulation factor activity and clinical manifestation.[112] Consultation with Dr. Marjory Brooks from the Cornell University Veterinary Diagnostic lab indicated that the low value may have been secondary to consumption, *ex vivo* loss of activity, or artifact of the assay system (a heterologous mixture of human and equine plasma).

Section 2: Materials and Methods

Specimens

Prior to euthanasia of the foal and discharge of the mare, blood was collected from the colt and dam in EDTA anti-coagulant blood tubes and stored at -20^{0}C. Banked equine genomic DNA was available from seven normal horses, determined by coagulation profiles and percent factor activity within normal limits. Three Tennessee Walking Horses were included for breed matching. Liver tissue from the colt was obtained from necropsy and stored at -80^{0}C; additional banked liver samples stored at -80^{0}C were available from one normal horse.

Isolation of genomic DNA from whole blood

Genomic DNA was harvested from EDTA-anticoagulated whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Incorporated, Valencia, CA, USA). Briefly, 20 µL of Qiagen protease, 200µL of whole blood, and 200µL of Buffer AL were added to a 1.5mL microcentrifuge tube and vortexed for 15 seconds then incubated at 56^{0}C for 10 minutes. The sample was centrifuged and 200µL of 96% ethanol was added to the microcentrifuge tube and vortexed for 15 seconds and then centrifuged. The sample was transferred to a QIAamp mini spin column and centrifuged at 6000 x g for 1 minute, and then transferred to a new spin column. 500µL Buffer AW1 was added and the sample was centrifuged at 6000 x g for 1 minute. The spin column was transferred into a new collection tube, 500µL Buffer AW2 was added and then centrifuged at 20000 x g for 3 minutes. The spin column was moved to a new collection tube and 200µL of Buffer AE was added and incubated at room temperature for one minute then centrifuged at 6000 x g for an additional minute. Isolated genomic DNA was stored at -20^{0}C

Polymerase chain reactions (PCR) for genomic DNA

Primers were designed from predicted sequence for the promoter and coding region (exons 1-26) of equine coagulation factor VIII (NCBI reference number XM_001498904.1),

verified sequence for equine FVIII binding site on vWF, incorporating exons 17-28 of the vWF gene (NCBI reference number NM_001242566.1), and on predicted sequence for equine LMAN-1 (NCBI reference number XM_001489477.1) and equine MCFD2 (NCBI reference number XM_001498157.2). Primers contained 21 base pairs (bp), maintained a 50-58% GC content, and the 5' primer was within 2-3^{0}C optimum annealing temperature of the antisense primer. When primers were within the non-coding (intronic) region, they were designed with 60-100 bp of freedom from the desired exon to incorporate all conventional splice sites and complete exonic sequence. For larger sequence (>1200 bp), primers were designed to achieve 50-100 bp of overlap between amplicons to ensure accurate sequence. See tables 3-6 for complete list of primers and PCR conditions.

PCR was performed as previously described.[113] Reactions were combined with hot start or HiFidelity TAQ polymerase, 10X reaction buffer, magnesium chloride, deoxynucleotide triphosphates (dNTP), RNAse free water, gene specific primers and the extracted genomic DNA. Reactions were performed on C1000 thermal cycler (Bio-Rad, Hercules, CA, USA). Three step PCR protocols were designed with the annealing temperature at or within two degrees of the lowest primer melting temperature and repeated for 35 cycles. Purified PCR products were loaded on a 1.5% agarose gel, separated with electrophoresis, and viewed under UV light. Amplified DNA bands of appropriate target size were extracted from the agarose gel utilizing the QIAquick Gel Extraction Kit (Qiagen, Incorporated, Germantown, MD, USA).

Overlapping PCRs were performed on genomic DNA for intron 1 of equine coagulation factor VIII for the normal horse, colt and dam. Intron 1 was predicted to contain 16.9 kilobases (kb); primers were designed with 50-100 bp of overlap to ensure accurate sequencing (table 9).

Amplified products were submitted to the Auburn University Genomics and Sequencing Laboratory (AU-GSL), Auburn, AL for sequencing using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The final sequenced data was compared both manually and using a computerized basic local alignment search tool (BLAST) program to evaluate for genetic mutation and maternal inheritance from the colt, dam and normal horse.[114]

Western Immunoblot

Homogenates from 100mg of liver from both the colt and a normal horse were generated using standard lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% NP40, protease inhibitor cocktail). Gel electrophoresis was performed using a 3-15% tris-acetate gel for 90 minutes at 200 volts. The gel was transferred to a polyvinylidene fluoride (PVDF) membrane for 60 minutes at 100 volts. The PVDF membrane was stained with Ponceau solution to confirm protein transfer and rinsed with distilled water.

The membrane was blocked with 5% milk (5 grams dry milk in 100mL of 0.1% tween20 tris-buffered saline [TBS-T]) at room temperature for one hour. For immunoblot, a polyclonal human anti-FVIII antibody directed against the C-terminal portion of the FVIII protein was used due to nearly 100% amino acid homology between the equine and human FVIII gene in that region (Abcam, Cambridge, MA, USA). The membrane was incubated with the primary antibody diluted in 5% milk at 1:1000 at 4^0C for 12 hours. The membrane was also blotted with anti-rat β-actin (1:10,000) as an internal loading control. The membrane was rinsed with TBS-T three times prior to the addition of the secondary antibody, a horse-radish peroxidase conjugated anti-rabbit IgG (1:10,000), diluted in 5% milk. The membrane was incubated at room temperature with the secondary antibody for one hour and then rinsed with TBS-T five times for 5 minutes each. Detection was performed by enhanced chemiluminescence. Results obtained

from the colt and normal horse samples were compared for evaluation of molecular weight and amount of protein expression.

Isolation of hepatic mRNA

Hepatic mRNA was isolated from liver samples stored at -80^{0}C from both the affected colt and a normal horse. Approximately 100mg of liver was homogenized in 2mL Trizol reagent (Invitrogen, Grand Island, NY, USA). The mixture was transferred to a 2mL micro centrifuge tube and centrifuged at 12000xg for 10 minutes at 42^{0}C. The supernatant was transferred to a clean micro centrifuge tube and incubated at room temperature for 5 minutes. A total of 0.4mL of chloroform was added to the supernatant, shaken for 15 seconds, incubated at room temperature for 2-3 minutes, and then centrifuged at 12000xg for 15 minutes at 4^{0}C. The aqueous portion was collected and transferred to a clean genomic DNA eliminator spin column and centrifuged at 9000 x g for 30 seconds.

The remainder of the RNA extraction was according to the RNase-Free DNase Set followed by RNeasy Plus Mini Kit manufacturer's protocol (Qiagen, Inc., Germantown, MD, USA). Briefly, 600µL of 50% ethanol was added to the flow through from the previous step; 700µL of total solution was transferred to a new collection tube and centrifuged for 15 seconds. For RNA purification, 50µL of total RNA solution, 10µL of Buffer RRD, 2.5µL DNase 1 stock solution, and 37.5µL RNase free water were combined and incubated at room temperature for 10 minutes. 900µL of 100% ethanol was added and mixed; 700uL of the sample was transferred to a spin column, placed in a collection tube and centrifuged at 8000 x g for 15 seconds. In a stepwise fashion 700µL of Buffer RW1 followed by 500uL Buffer RPE repeated twice were added to the spin column and centrifuged between each step with the flow through discarded. A dry spin was performed for one minute prior to the addition of 30uL of RNase free water and a final centrifugation for one minute. The purified RNA was stored at -80^{0}C.

Generation of first strand cDNA from hepatic mRNA

Reverse transcription PCR was used to create first strand cDNA from the RNA template using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The reaction mixture consisted of 4µL 5X reaction mix (mixture oligo(dT) primers, dNTP, and magnesium chloride), 1µL reverse transcriptase, 14µL RNase free water, and 1µL purified RNA template (1µg/µL). The reaction was incubated at 25^0C for 5 minutes, 42^0C for 30 minutes, and then 85^0C for 5 minutes. Hepatic cDNA was stored at -20^0C.

PCR reactions from hepatic cDNA

Primers were designed across the junctions of the equine coagulation factor VIII gene coding sequence; for several sequences, multiple primer sets were designed (see table 7-8). PCR reactions were performed on hepatic cDNA from the affected colt and normal horse and the products were extracted as described above; high-fidelity iProof DNA polymerase was used in all reactions (Bio-Rad, Hercules, CA, USA). Purified products were submitted to Operon DNA Sequencing Laboratory (Eurofins MWG Operon, Huntsville, AL, USA) and compared for mutational analysis.

Evaluation of aberrant splice sites in intron 1 of the equine coagulation factor VIII gene was performed on the hepatic cDNA from the normal horse and colt. Multiple primer sets were designed across the 5' splice site (forward primer within exon 1 and reverse primer in the 5' region of intron 1) and 3' splice site (forward primer near the 3' region of intron 1 and reverse primer in exon 2) for intron 1 (see table 10). PCR reactions were performed on hepatic cDNA for the affected colt and normal horse as described above using high fidelity iProof DNA polymerase.

Long and accurate PCR reactions on hepatic cDNA

Gene specific first strand cDNA was made from the hepatic mRNA with qScript Flex cDNA Synthesis Kit (VWR, Radnor, PA, USA). The gene specific antisense primer (TCCAGTAGGATACACCAACAG) was designed from exon 3 of coagulation factor VIII. The initial mixture contained 1µL hepatic mRNA, 10µL RNase free water, 2µL gene specific primer, and 2µL of gene specific primer enhancer. The mixture was vortexed, centrifuged for 10 seconds, then incubated for 5 minutes at 65^0C. The reaction was moved to a 42^0C hot plate and 4µL qScript Flex reaction and 1µL qScript reverse transcriptase were added and then incubated at 42^0C for 60 minutes followed by 85^0C for 5 minutes. Hepatic cDNA was stored at -20^0C.

PCR reactions were performed across exon 1 (GCTATCCACCTGCTTCTTTC: forward primer) and exon 2 (TGTGAACTCTACAAACACAGTC: antisense primer) with the gene specific hepatic cDNA from the affected colt and normal horse. PCR reactions were performed using TaKaRa Long and Accurate PCR kit (Clontech, Mountain View, CA). Reaction mixtures contained 10x LA PCR Buffer, dNTP, double filtered distilled water, TaKaRa LA Taq polymerase, primers, and gene specific hepatic cDNA template. The PCR reactions were set at 94^0C denaturation for 30 seconds, $65\text{-}68^0$C annealing temperature for 30 seconds, and 72^0C extension for 15 minutes, which was repeated for 30 cycles. Purified PCR products were loaded on a 0.8% agarose gel. The remainder of the PCR procedure was performed as described above.

Section 3: Results

Genomic sequence for the promoter, exons and conventional splice sites of the FVIII gene, were identical in all animals. The previously predicted sequences for the equine coagulation FVIII coding region and promoter were determined. Mutational analysis of exons

17-28 of the vWF gene, encoding for the coagulation FVIII binding site, was performed to evaluate for type 2N VWD. A single nucleotide polymorphism (SNP) (A>C) was detected in exon 22 of the vWF gene in the area encoding the FVIII binding site in the colt, leading to a predicted amino acid change from lysine to asparagine in the encoded protein. The SNP was also identified in genomic sequence from the dam and three normal horses, including one Tennessee walking horse, suggesting that it was not a functional mutation (see figure 8). Further mutational analysis was performed for the coding regions of equine LMAN-1 and MCFD2 to evaluate the transport proteins associated with combined factor V and factor VIII deficiency. Genomic sequences for LMAN-1 and MCFD2 were identical in all animals.

FVIII protein expression in liver from the colt and a normal horse was compared by Western immunoblot. Western blot revealed an appropriately sized product for coagulation FVIII in the normal horse at approximately 200kDa; no product was obtained for the colt indicating lack of appropriate protein expression (see figure 9).

The equine FVIII transcript was evaluated by utilizing primers designed across the coding sequence from hepatic cDNA from the colt and the normal horse. Several reactions were designed to include multiple, smaller exons per amplicon (see figure 10). Appropriately sized products and sequence were obtained in the colt and the normal horse across exons 4 through 26. Product was obtained in the normal horse but not in the colt for exons 1-4 (see figure 11), which was reproducible with two different forward and reverse primers sets (forward primers in exon 1 and antisense primers in exon 4). Additional primers were designed across exons 1 through 4 to cross each exon junction individually (see table 8); PCR reactions were performed on the colt and normal horse (see figure 10). Appropriately sized product and sequence were obtained in the colt crossing the junctions between exon 2 to 3 and exon 3 to 4, but no product was obtained in

the colt for exons 1 to 2; appropriate sized bands were obtained in the normal horse for all reactions (see figure 12). PCR reactions with the forward and reverse primers located in exon 1 (see table 8), yielded a normal product for exon 1 in the colt, confirming that exon 1 was being appropriately transcribed.

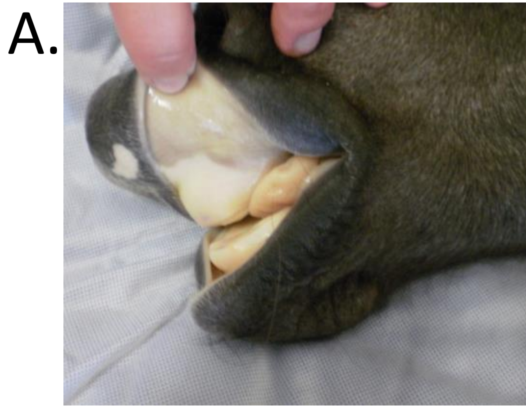
Based on these results, a causative mutation was localized to the non-coding region of the FVIII gene, specifically intron 1 (approximately 16.9kb in length). Overlapping PCR reactions were performed on genomic DNA across intron 1 for the affected colt, mare, and normal horse. Genomic sequencing analysis identified several SNPs throughout intron 1. A four base pair deletion was identified in the colt approximately 9.9kb from the 5' splice site; a SNP (C<G) was located 11 base pairs 3' to the deletion and a second SNP (C<T) was located 32 base pairs 5' to the deletion. The mare was heterozygous for the deletion and two SNPs, which were not present in the DNA from a total of seven normal horses including three Tennessee Walking Horses (see figure 13).

To determine if portions of intron 1 were being transcribed in the affected colt due to alternative splicing, PCR reactions utilizing hepatic cDNA were performed across the 5' and 3' splice sites of intron 1 for equine coagulation FVIII. No product was obtained for the colt or the normal horse across the 3' splice site utilizing four unique forward and reverse primer sets. Product was obtained in the colt across the 5' splice site utilizing three separate forward and reverse primer sets (see figure 14). Sequencing confirmed portions of intron 1 were present in the transcript across the 5' splice site. Bands of non-target size were obtained in the normal horse; sequencing was consistent with non-specific primer binding (see figure 14). Occasionally, two primer sets (sets A and C) yielded faint bands of appropriate size in the normal horse across

the 5' splice site; sequencing was inconclusive but genomic contamination of the cDNA or during PCR set-up was suspected.

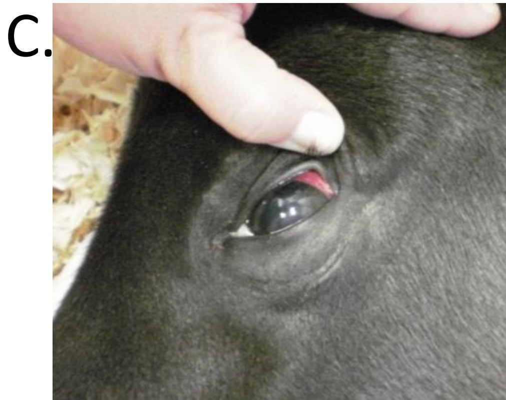
PCRs with TaKaRa long and accurate Taq polymerase (TaKaRa LA taq) (Clontech, Mountain View, CA), were performed utilizing gene specific priming of hepatic cDNA to attempt to amplify the extent of the intron inserted in the transcript. A product was not obtained in the colt, but more optimization for this technique is likely needed.

A.



B.

C.



D.

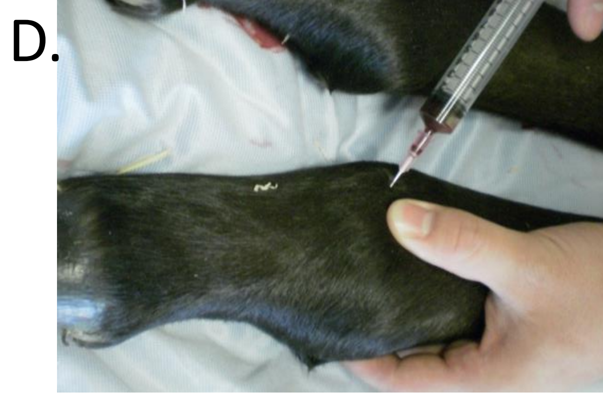


Figure 7: A 2-day-old Tennessee Walking Horse colt presented for non-specific signs of lethargy and bleeding from the right nostril. On presentation the colt had (A) icteric, pale mucous membranes, (B) a hematoma over the frontal sinus, (C) episcleral hemorrhage, and (D) hemarthrosis. All findings were consistent with a bleeding diathesis.

Test	Affected Colt	Reference Interval	Role in Coagulation
Platelets	159	77-230	Primary
MPV	9.0	5.8-11.5	Primary
PT	12.4	11.2-13.4	Extrinsic
aPTT	>60	31-37	Intrinsic
ATIII	152	>150	Anticoagulant
D-Dimers	500-1000	250-1000	Fibrinolysis
Plasma FDP	<5	0-5	Fibrinolysis

Table 1: Coagulation profile from a 2-day-old Tennessee Walking Horse colt with clinical signs of coagulation type bleeding. Results show prolongation only in aPTT, consistent with alterations in the intrinsic pathway of coagulation.

Factor	Normal Horse	Dam	Affected Colt
Factor VIII	67%	57%	15%
Factor IX	77%	110%	64%
Factor XI	55%	91%	26%
Factor XII	250%	170%	150%
vWF	102%	117%	110%

Table 2: Coagulation factor activity from a normal horse, a colt diagnosed with hemophilia A, and the colt's dam. The colt had significantly decreased coagulation factor VIII activity compared to a normal horse and the colt's dam.

Primers and PCR conditions for amplification of equine FVIII promoter and exons 1-12

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Promoter			
Prom 1A F	CCCCAACACATTCTTATCTGT	15sec, 1min, 1min	880BP
Prom 1A R	GCGGCTTTAATCAGAACCTTT	anneal 58^0C, 35 cycles, HF	
Prom 1B F	GGCAGAGTAGAAGCAGAAAGA	15sec, 1min, 1min	912BP
Prom 1B R	GGCAGCATGTGACAAAGAGAA	anneal 60^0C, 35 cycles, HF	
Exon 1			
UTR F	GGCTGCTTCCCATGGCCAAAA	30sec, 30sec, 45sec	505BP
Intron 1R	GCATGCATGCCTTACCTGGAA	anneal 59^0C, 40 cycles, HS	
Exon 2			
Intron 1F	GGAGAGTAGGATGGAAGCATT	30sec, 30sec, 30sec	343BP
Intron 2R	GCCTGGCCAAGAGAACATTCT	anneal 57^0C, 40 cycles, HS	
Exon 3			
Intron 2F	GGCTGAGCTGGTGATAGTAGT	15sec, 1min, 30sec	398BP
Intron 3R	GCCTAGGTTTGAATCCTGACT	anneal 57^0C, 35 cycles, HF	
Exon 4			
Intron 3F	CTGTACATCACCCGCTCCCTGA	15sec, 1min, 1min	720BP
Intron 4R	CACGGGCTTGATGCTAGGGAA	anneal 61^0C, 35 cycles, HF	
Exon 5			
Intron 4F	CGTGCCTACTCACGTGGTTTA	30sec, 30sec, 30sec	409BP
Intron 5R	GGGAATGAGGCCAACCTGTTA	anneal 59^0C, 40 cycles, HS	
Exon 6			
Intron 5F	CCCATGGGAGGAATGGAAAGA	30sec, 30sec, 30sec	287BP
Intron 6R	GTGCAGAGCTGTCTGAGGAGA	anneal 61^0C, 40 cycles, HS	
Exon 7			
Intron 6F	CAGCATGACAGTCATTGGTGA	30sec, 30sec, 1min	625BP
Intron 7R	CTCCCTCTGTCACAGGTATTT	anneal 57^0C, 40 cycles, HS	
Exons 8 and 9			
Intron 7F	GGCCTGATGATATATAACAAA	15sec, 1min, 1min	978BP
Intron 9R	GGAGATTAGCCACTTGTCAAT	anneal 57^0C, 35 cycles, HF	
Exon 10			
Intron 9F	CCTAAGTAGCTGTCTGAACAA	30sec, 30sec, 30sec	388BP
Intron 10R	GTGCCTGCATAGTACATAACA	anneal 55^0C, 40 cycles, HS	
Exon 11			
Intron 10F	CCCCTTGCAATAACAACACAA	30sec, 30sec, 30sec	431BP
Intron 11R	GCCAACATTGCAGACTACCAT	anneal 55^0C, 40 cycles, HS	
Exon 12			
Intron 11F	GACCCTAGGGAGAAATGGAAT	30sec, 30sec, 30sec	398BP
Intron 12R	CCAGGATCCGAACTGGCAAAT	anneal 60^0C, 40 cycles, HS	

Table 3A: Primers and PCR conditions for equine coagulation factor VIII gene promoter and exons 1 to 12
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute, UTR: Untranslated region, HS: hot start polymerase; HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 94°C and extension at 72°C for the indicated number of cycles.

Primers and PCR conditions for amplification of equine FVIII exons 13-26

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Exon 13			
Intron 12F	CACGATGGTCTATGGCTCACT	30sec, 30sec, 1min	694BP
Intron 13R	GCCTCCACATGTTAACTGCAT	anneal 59^{0}C, 40 cycles, HS	
Exon 14			
Intron 13 F	CAGAACTTGCTTGGGACCTGA	15sec, 1min, 1min	1057BP
Exon 14A R	GGGCCATCAATGTGAGTCTTT	anneal 60^{0}C, 35 cycles, HF	
Exon 14B F	GGAAGGAATTCATGGACCTGT	30sec, 30sec, 1min	958BP
Exon 14B R	GCTTGGATGCTTCTCTACCAT	anneal 60^{0}C, 40 cycles, HS	
Exon 14C F	GGGACGTATACTCCAATACTT	30sec, 30sec, 1min	864BP
Exon 14C R	GGCTATTGTCCCAGGTCTATT	anneal 56^{0}C, 40 cycles, HS	
Exon 14D F	CTGGATCTCATGGAAGAGATT	15sec, 1min, 1min	687BP
Exon 14D R	GCTGCAGATTGGCTATGCATT	anneal 60^{0}C, 35 cycles, HF	
Exon 15			
Intron 14F	CCTACCTAGTACACTTCCAAT	30sec, 30sec, 30sec	476BP
Intron 15R	GGAACACATAAAGAGAAGACT	anneal 56^{0}C, 40 cycles, HS	
Exon 16 and 17			
Intron 15F	GGGATGTAAACCCTAAGCTAA	30sec, 30sec, 1min	911BP
Intron 17R	GAGGACTCTGCTCCCATATAT	anneal 56^{0}C, 40 cycles, HS	
Exon 18			
Intron 17F	GGAGCAGAGTCCTCATAGGAA	30sec, 30sec, 1min	357BP
Intron 18R	CCCAGTGCCTAGACCATTTAA	anneal 58^{0}C, 40 cycles, HS	
Exons 19 and 20			
Intron 18F	GCCAATGTAGCTCATGCTCAT	15sec, 1min, 1min	980BP
Intron 19R	GGGTTTACAATGTACACCTGA	anneal 56^{0}C, 35 cycles, HF	
Exon 21			
Intron 20F	CCACAGCTAGGATTAACCTTT	30sec, 30sec, 30sec	506BP
Intron 21R	GGCAGTTGGAGGCATCTAATA	anneal 57^{0}C, 40 cycles, HS	
Exon 22			
Intron 21F	GGTATCTGCCTGACCTACACA	30sec, 30sec, 1min	369BP
Intron 22R	GCTAGGAGTGTTTGTCCCATT	anneal 58^{0}C, 40 cycles, HS	
Exons 23 and 24			
Intron 22F	CCAGACCTAATCTCCCTTGTT	30sec, 30sec, 1min	1054BP
Intron 24R	CCCATAGTCAACCTTCCTTGA	anneal 58^{0}C, 40 cycles, HS	
Exon 25			
Intron 24F	CCCTGAGGATACAGAGATGAA	30sec, 30sec, 1min	538BP
Intron 25R	CCTTGCTTGGAGTCACTGTGT	anneal 58^{0}C, 40 cycles, HS	
Exon 26			
Intron 25F	CCCAGATGCATAGGACACAGA	30sec, 30sec, 1min	579BP
Intron 26R	GGCAGTGTTTACTAGGACTGT	anneal 58^{0}C, 40 cycles, HS	

Table 3B: Primers and PCR conditions for equine coagulation factor VIII gene exons 13 to 26
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute, UTR: Untranslated region, HS: hot start; polymerase HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 94°C and extension at 72°C for the indicated number of cycles.

Primers and PCR conditions for amplification of equine LMAN-1 coding regions using genomic DNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Exon 1			
5' UTR F	GGCGGAATCACAGGTGTTAGA	15sec, 1min, 1min	891BP
Intron 1R	CGCTTGGAAGCGTAGGAAGTA	anneal 62^0C, 35 cycles, HF	
Exons 2 and 3			
Intron 1F	CCATCCTGCCCCTTGAGTTTA	30sec, 30sec, 1min	844BP
Intron 3R	GCAGGAGGTCACTGCCCAGTT	anneal 63^0C, 40 cycles, HS	
Exon 4			
Intron 3F	GCGGCCGTGTTTGTCCTGTTA	30sec, 30sec, 1min	980BP
Intron 4R	GCCCAGGCACGTCTACTCCAA	anneal 63^0C, 40 cycles, HS	
Exon 5			
Intron 4F	CCGGCCCAGGATCTGCATTTT	30sec, 30sec, 30sec	492BP
Intron 5R	CGGCCACTTACATGGAGCTTT	anneal 63^0C, 40 cycles, HS	
Exon 6			
Intron 5F	CCGAAGAAGCGAGGTGATTTA	30sec, 30sec, 30sec	514BP
Intron 6R	GCAGTCCCAGCCAACTGTTCA	anneal 63^0C, 40 cycles, HS	
Exon 7			
Intron 6F	GGCTAGCCAGGATTGGTATTT	15sec, 1min, 1min	1025BP
Intron 7R	GGCCAGGACGAAACCTCAGTT	anneal 60^0C, 35 cycles, HF	
Exon 8			
Intron 6F	CCCCACTGCCTTTACTAAATT	30sec, 30sec, 1min	883BP
Intron 8R	GCCAGGTAAGAGAGTGAGGAT	anneal 58^0C, 40 cycles, HS	
Exons 9 and 10			
Intron 8F	GCCCTGTGTTGATGATGTTCT	30sec, 30sec, 1min	609BP
Intron 10R	CTCCCACAGGAATCTTACAAT	anneal 58^0C, 40 cycles, HS	
Exon 11			
Intron 10F	GGCCTGATGGGTTAATGCTTT	30sec, 30sec, 1min	626BP
Intron 11R	CACTGGCAGCAGAATAAAGTA	anneal 58^0C, 40 cycles, HS	
Exon 12 and 13			
Intron 11F	GGCCTTCCAGTGTCAACTACT	15sec, 1min, 1min	1189BP
3' UTR R	GGGTGGCCCCCACTTTCTATT	anneal 62^0C, 35 cycles, HF	

Table 4: Primer and PCR conditions for equine lectin mannose-binding 1 (LMAN-1)
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute, UTR: Untranslated region, HS: hot start; polymerase HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 94°C and extension at 72°C for the indicated number of cycles.

Primers and PCR conditions for amplification of equine MCFD2 coding regions using genomic DNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Exon 1			
5' UTR F	GGAGTGCACTGTGTCAAGTCT	30sec, 30sec, 1min	376BP
Intron 1R	CCCATCTAGGCCTCAAGATTC	anneal 60^0C, 40 cycles, HS	
Exon 2			
Intron 1F	GAGTGTGGTCTGCCACTGTTT	30sec, 30sec, 1min	482BP
Intron 2R	GCCACAGCAGGTCACTCTTTT	anneal 62^0C, 40 cycles, HS	
Exon 3			
Intron 2F	CCCTCGGGAAAGCCTAGTCTA	15sec, 1min, 1min	875BP
3' UTR R	GCGTCCACTTGGCAAAGCTTA	anneal 62^0C, 35 cycles, HF	

Table 5: Primer and PCR conditions for equine multiple coagulation deficiency 2 (MCFD-2)
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute, UTR: Untranslated region, HS: hot start polymerase; HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 94°C \and extension at 72°C for the indicated number of cycles.

Primers and PCR conditions for vWF exons 17-28 encoding FVIII binding site

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Exon 17			
Intron 16F	CAGCGGGACCAGTGTGTGGAA	15sec, 1min, 2min	1320BP
Intron 17R	CCGGACACGGAAACTGTGCAG	anneal 64^0C, 35 cycles, HF	
Exon 18			
Intron 17F	GGAGGTCGGGCAGTACTTTAT	30sec, 30sec, 30sec	485BP
Intron 18R	GGCAGCTAACCCAGCCACAAA	anneal 61^0C, 40 cycles, HS	
Exon 19			
Intron 18F	CCAGGGCTTCCGACACATGTT	30sec, 30sec, 30sec	436BP
Intron 19R	CCACGTCTGCTTCCACAGCAT	anneal 63^0C, 40 cycles, HS	
Exon 20			
Intron 19F	GCTCCACAGGCCGCAGATCTT	30sec, 30sec, 1min	719BP
Intron 20R	GCTGTTCACTGCACAAGGGTA	anneal 61^0C, 40 cycles, HS	
Exon 21			
Intron 20F	CACACTTGCACGGACATGGTA	30sec, 30sec, 30sec	544BP
Intron 21R	GCGCTGATTCTGCCTCAGCTT	anneal 61^0C, 40 cycles, HS	
Exon 22			
Intron 21F	GGGCAAACGACTTGTGGTTCT	30sec, 30sec, 30sec	475BP
Intron 22R	CTCAGAGTGAGGAAGGTGAAA	anneal 59^0C, 40 cycles, HS	
Exons 23 and 24			
Intron 22F	CCAACCCGTCCTCCTCTTCTT	30sec, 30sec, 1min	775BP
Intron 24R	CCAGGCATCGCTCGCCTCATA	anneal 63^0C, 40 cycles, HS	
Exon 25			
Intron 24F	CCCTTCCCTCTTTGGGAGCAT	30sec, 30sec, 30sec	498BP
Intron 25R	CCCTTCCCTCCTCACTCACAT	anneal 63^0C, 40 cycles, HS	
Exon 26			
Intron 25F	GGTTCCTGGTTTAGGTGCTTT	30sec, 30sec, 30sec	588BP
Intron 26R	GCAAATGGAAGCACCGGTATA	anneal 59^0C, 40 cycles, HS	
Exon 27			
Intron 26F	GCCACATGTGGTTCCTGGCTA	30sec, 30sec, 1min	621BP
Intron 27R	GCGATCTCGTCTGCCCTCAAA	anneal 63^0C, 40 cycles, HS	
Exon 28			
Intron 27U	GGTTCGGGTGGGCAGGCAGTT	30sec, 30sec, 30sec	536BP
Exon 28A R	GGCCATCAGGAGCAAGGCGAT	anneal 65^0C, 40 cycles, HS	
Exon 28B F	CCATGATGGCTCCCACGCCTA	30sec, 30sec, 1min	634BP
Exon 28B R	GCCCACATCCATCCGCTGATT	anneal 63^0C, 40 cycles, HS	
Exon 28C F	GGCACCCAAGAGGAACTCCAT	30sec, 30sec, 1min	720BP
Intron 28R	GCACTCTGAAGGCAGTGGTTT	anneal 61^0C, 40 cycles, HS	

Table 6: Primers and PCR condition for exon 17-28 of the von Willebrand factor gene encoding the binding site for coagulation factor VIII
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute, UTR: Untranslated region, HS: hot start; polymerase HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 94°C and extension at 72°C for the indicated number of cycles.

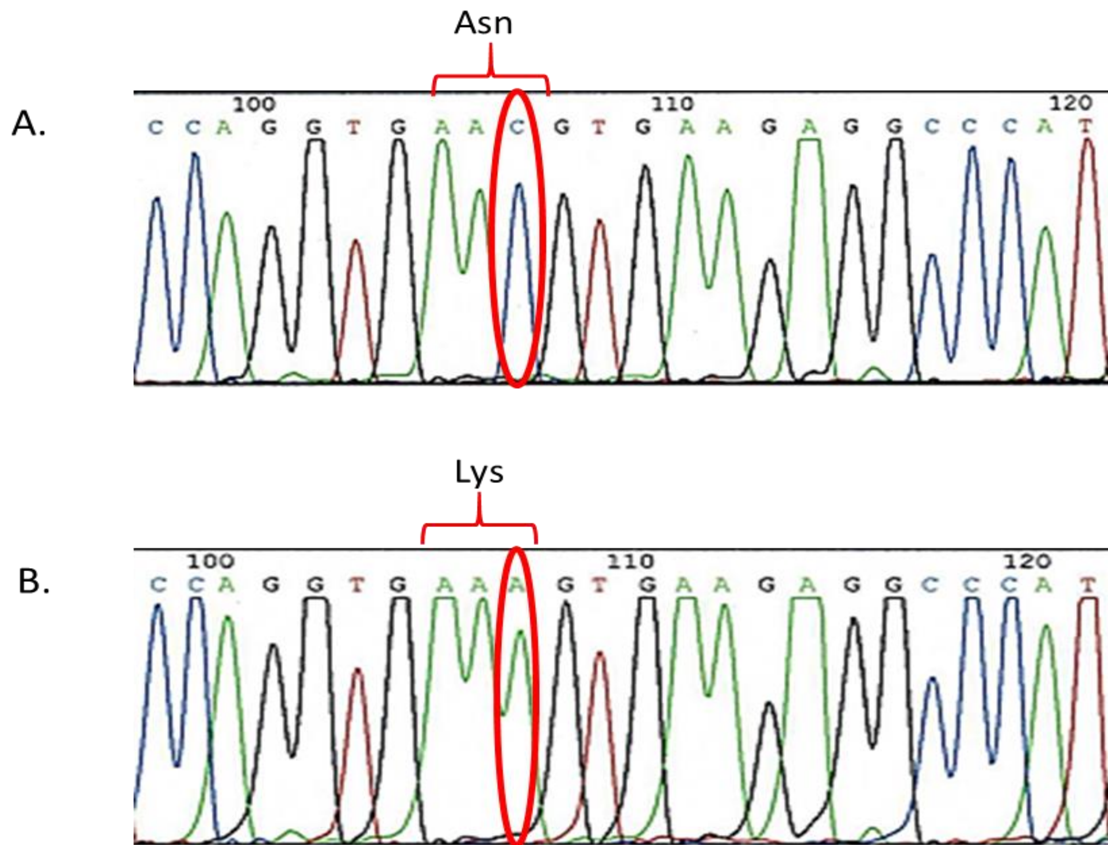


Figure 8: Genomic sequence data from exon 22 of the von Willebrand factor gene encoding the factor VIII binding site read 5' to 3' (A) Sequence from the affected colt with a single nucleotide polymorphism from A to C and a predicted amino acid changed from lysine to asparagine (B) Sequence from a normal horse with the correct predicted sequence.

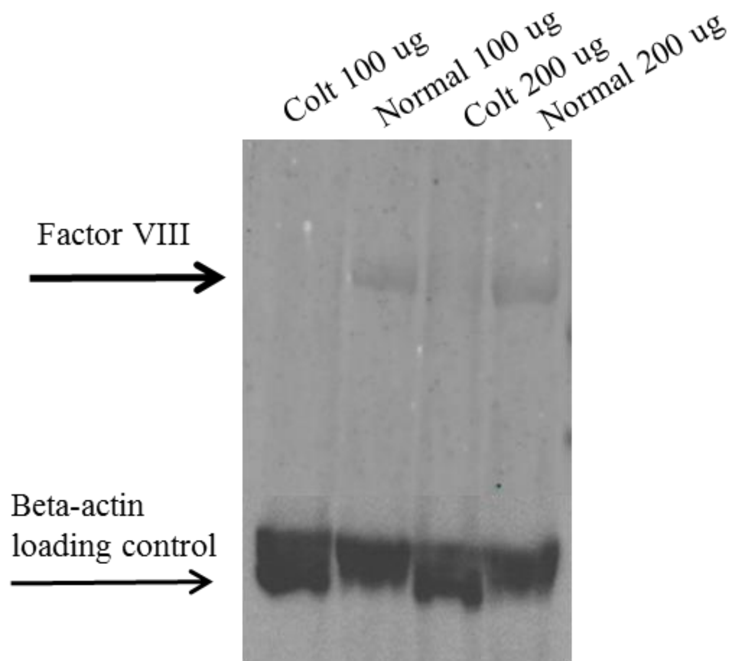


Figure 9: Western immunoblot using 100 and 200µg of hepatic lysate from the colt and normal horse. An appropriately sized product was obtained in the normal horse (lane 2 and 4) with no product obtained in the colt (lanes 1 and 3).

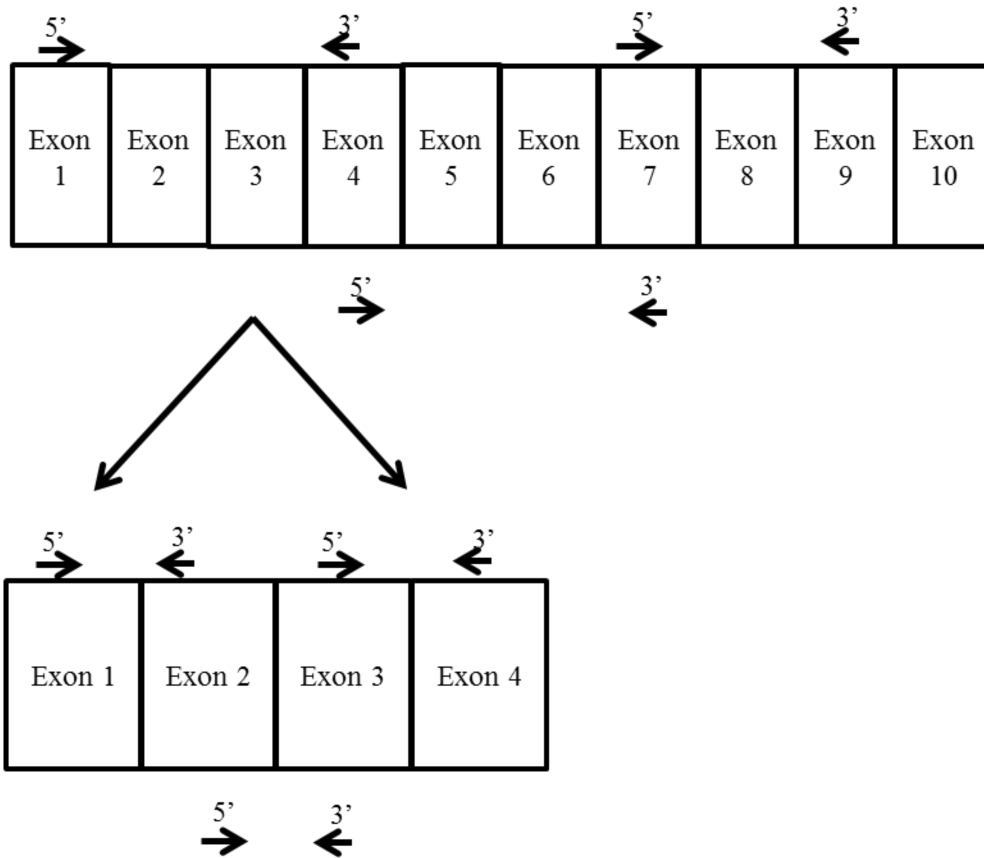


Figure 10: Primers were designed and PCR reactions performed across FVIII coding region (exons 1-26) using hepatic cDNA. Initial primer sets were designed to cross several exon-exon junctions in one PCR reaction. When no product was obtained in the colt across exons 1-4, small PCR reactions were designed to cross each exon junction individually

Primers and PCR conditions for amplification of equine FVIII exons 1-13 using hepatic cDNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Exons 1-4			
Exon 1F Set A	GCTTCTTTCTGTGCCTTTTG	10sec, 1min, 30sec	415BP
Exon 4R Set A	TCATCTTCTTTCTCGCTTTGG	anneal 56^0C, 35 cycles, HF	
Exon 1F Set B	TGCCACCAGAAGATACTACC	10sec, 1min, 30sec	351BP
Exon 4R Set B	TCTTCATATTCAGCACCTTCAG	anneal 56^0C, 35 cycles, HF	
Exons 4-7			
Exon 4F	GGCAGATCCTGAAAGAGAAC	10sec, 30sec, 30sec	425BP
Exon 7R	CCTCACAAGAAATGTGTGACC	anneal 55^0C, 35 cycles, HF	
Exons 7-9			
Exons 7F	CTTTCCTTACTGCTCAGACAC	10sec, 30sec, 30sec	390BP
Exon 9R	TCTTACCAATCTGCTGAGGAC	anneal 55^0C, 35 cycles, HF	
Exons 9-10			
Exon 9F	GCCCGATTTATAGCATACACAG	10sec, 30sec, 30sec	176BP
Exon 10R	CTGACATCAGTGATTCCATGAG	anneal 55^0C, 35 cycles, HF	
Exons 10-11			
Exon 10F	AAGCAAGCCGACCATATAAC	10sec, 30sec, 30sec	182BP
Exon 11R	GCACCGAGGATCTGATTTAG	anneal 55^0C, 35 cycles, HF	
Exons 11-12			
Exon 11F	ATTGGCCCTCTCCTCATCTG	10sec, 30sec, 30sec	165BP
Exon 12R	CTGCACTCCATCTGCATTG	anneal 55^0C, 35 cycles, HF	
Exons 12-13			
Exon 12F Set A	CCTCACAGAGAATATGCAGC	10sec, 30sec, 30sec	208BP
Exon 13R Set A	TCCAGAGAAGAAGACAGAGAG	anneal 55^0C, 35 cycles, HF	
Exon 12F Set B	CAATGCAGATGGAGTGCAG	10sec, 30sec, 30sec	170BP
Exon 13R Set A	TCCAGAGAAGAAGACAGAGAG	anneal 55^0C, 35 cycles, HF	

Table 7a: Primers and PCR condition using hepatic cDNA for equine coagulation factor VIII exons 1-13
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute; HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 98°C and extension at 72°C for the indicated number of cycles.

Primers and PCR conditions for amplification of equine FVIII exons 13-26 using hepatic cDNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Exons 13-14			
Exon 13F	CACACTTACCCTATTCCCATTC	10sec, 30sec, 30sec	152BP
Exon 14R	CACCAGTGTTCCTGTTACAAC	anneal 55^0C, 35 cycles, HF	
Exons 14-15			
Exon 14F	GGTGAGGAGGAAAATCAAGAC	10sec, 30sec, 30sec	207BP
Exon 15R	ACGGTATAAGGGCTGAGTAAAG	anneal 55^0C, 35 cycles, HF	
Exons 15-16			
Exon 15F Set A	AATTCACTGATGGCTCCTTTAC	10sec, 30sec, 30sec	204BP
Exon 16R Set A	TTTTTCTAGGTTCTGCTCCTTG	anneal 55^0C, 35 cycles, HF	
Exons 16-17			
Exon 16F Set A	TGGCACCCACTAAAGATGAG	10sec, 30sec, 30sec	207BP
Exon 17R Set A	TGAAGTACCAACTCTTGGTCTC	anneal 55^0C, 35 cycles, HF	
Exons 17-22			
Exon 17F Set A	CCATGCTAACACGCTGAAC	10sec, 30sec, 30sec	746BP
Exon 22R Set A	AGGCTGGAGAACTTCTGAC	anneal 55^0C, 35 cycles, HF	
Exon 17F Set B	ATGCTAACACGCTGAACCC	10sec, 10sec, 30sec	769BP
Exon 22R Set B	GAGAGATGTAGAGGCTGGAG	anneal 55^0C, 35 cycles, HF	
Exons 22-24			
Exon 22F SetA	TTATTCACAGCATCATGACCC	10sec, 30sec, 30sec	315BP
Exon 24R SetA	TTGCTTTATTCTCCATTCCCAG	anneal 56^0C, 35 cycles, HF	
Exon 22F Set B	CTCCAGCCTCTACATCTCTC	10sec, 30sec, 30sec	352BP
Exon 24R Set B	TCTGTGCATCTGCTATTGC	anneal 56^0C, 35 cycles, HF	
Exons 24-25			
Exon 24F	CAGATGCACAGATAACTGCC	10sec, 30sec, 30sec	230BP
Exon 25R	GATGAGGAACTCCTTCACATAC	anneal 55^0C, 35 cycles, HF	
Exons 25-26			
Exon 25F	TCACAGGAATAACCACCCAG	10sec, 30sec, 30sec	199BP
Exon 26R	AGGTAGCGAGTCAGTAAAGG	anneal 55^0C, 35 cycles, HF	

Table 7b: Primers and PCR condition using hepatic cDNA for equine coagulation factor VIII exons 13-26
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute; HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 98°C and extension at 72°C for the indicated number of cycles.

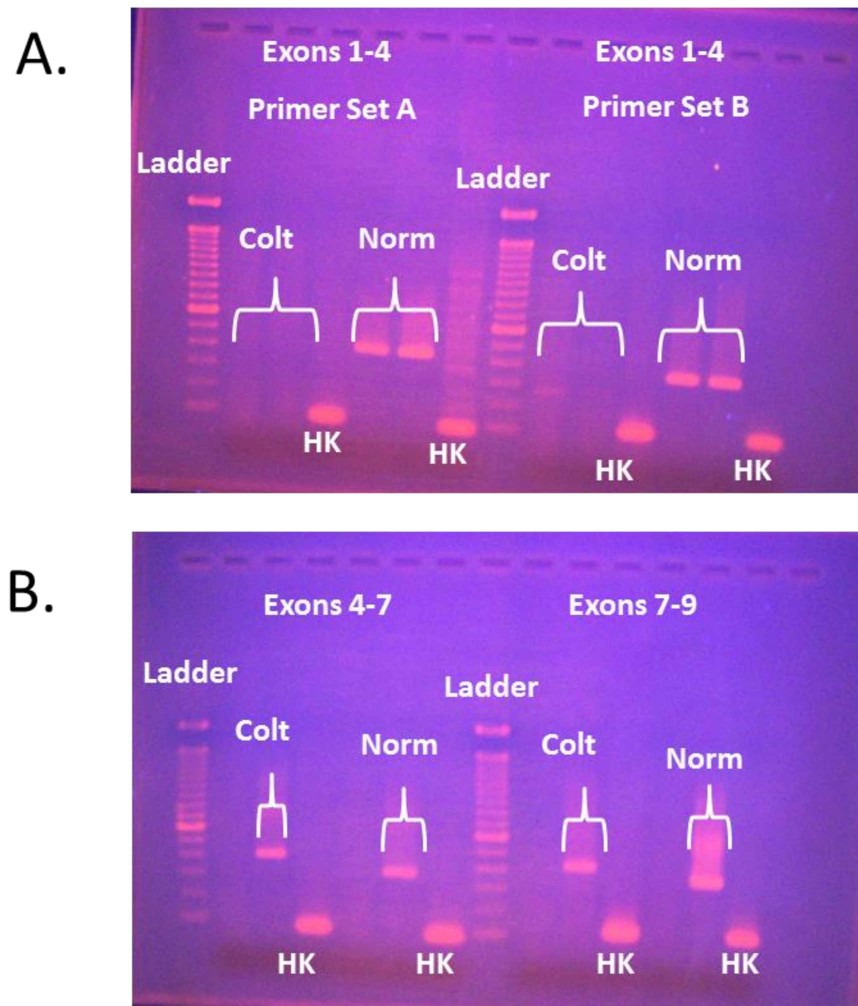


Figure 11: Gel electrophoresis on PCR products amplified from hepatic cDNA for the equine coagulation factor VIII transcript (A) Exons 1-4 with product in the normal horse but not in the colt with two separate primer sets and (B) for exons 4-7 and exons 7-9 with a product obtained in the normal horse and the colt.
HK: Housekeeping gene

Primers and PCR conditions for amplification of equine FVIII exons 1-4 Using hepatic cDNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Exon 1			
Exon 1F Set A	GCTATCCACCAGCTTCTTTC	10sec, 1min, 30sec	100BP
Exon 1R Set A	AGTTCGCTTTGCATATAGTCC	anneal 60^0C, 35 cycles, HF	
Exon 1F Set B	TCTTTCTGTGCCTTTTGCC	10sec, 30sec, 30sec	100BP
Exon 1R Set B	TCACTGAGAAGTTCGCTTTG	anneal 60^0C, 35 cycles, HF	
Exons 1-2			
Exon 1F Set A	GCTATCCACCTGCTTCTTTC	10sec, 30sec, 30sec	214BP
Exon 2R Set A	TGTGAACTCTACAAACACAGTC	anneal 55^0C, 35 cycles, HF	
Exon 1F Set B	TCTTTCTGTGCCTTTTGCC	10sec, 30sec, 30sec	200BP
Exon 2R Set B	TGTGAACTCTACAAACACAGTC	anneal 55^0C, 35 cycles, HF	
Exons 2-3			
Exons 7F	TTTCCATTCAACCCTTCAGTC	10sec, 30sec, 30sec	208BP
Exon 9R	TCCAGTAGGATACACCAACAG	anneal 55^0C, 35 cycles, HF	
Exons 3-4			
Exon 9F	TTAAGAACATGGCTTCTCATCC	10sec, 30sec, 30sec	283BP
Exon 10R	CCTTCTCTACAAATCAGCAGG	anneal 55^0C, 35 cycles, HF	

Table 8: Primers and PCR condition using hepatic cDNA for equine coagulation FVIII exons 1-4
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute; HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 98°C and extension at 72°C for the indicated number of cycles.

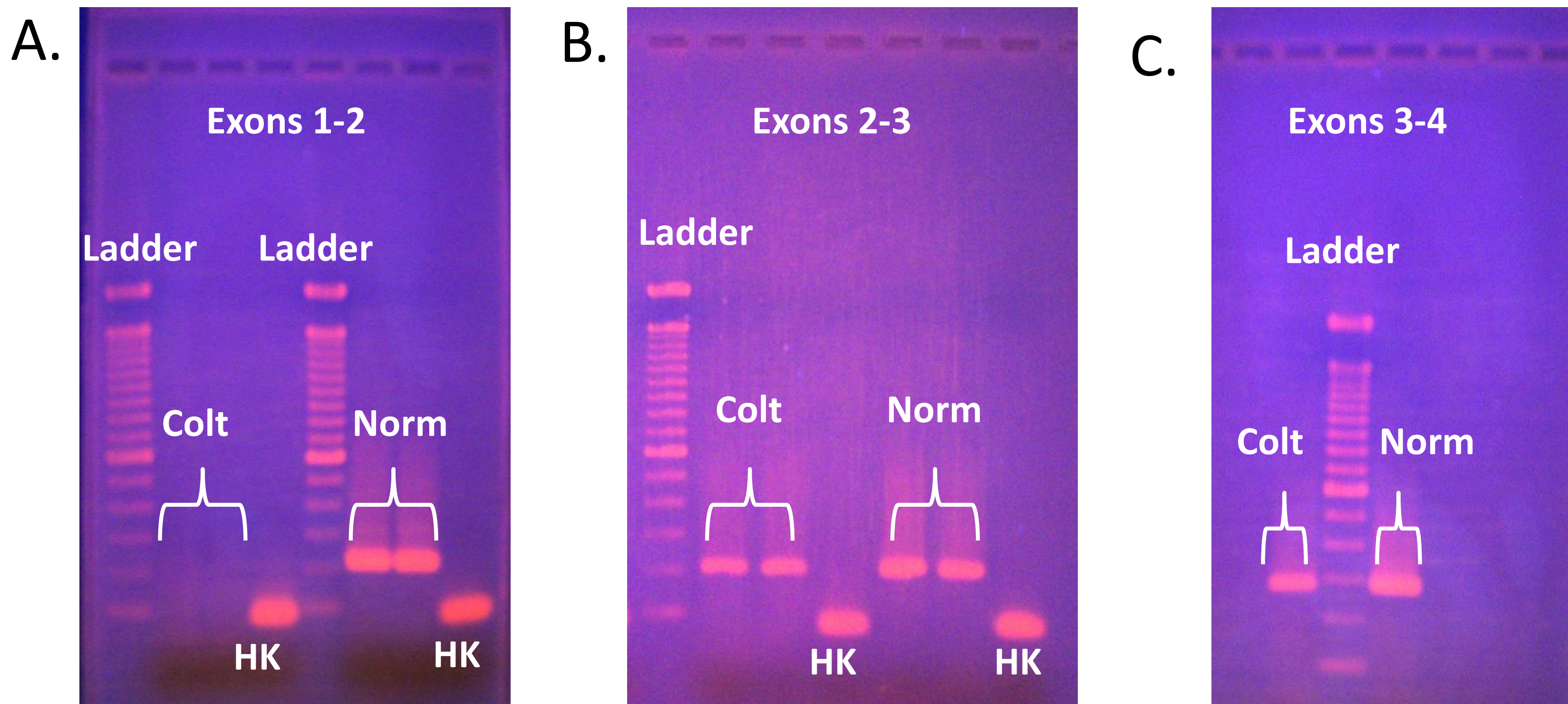


Figure 12: Gel electrophoresis from PCRs performed on hepatic cDNA with primers design to amplify the equine coagulation factor VIII transcript (A) Exons 1-2 with product in the normal horse but not in the colt (B) for exons 2-3 with a product obtained in the normal horse and the colt (C) for exons 3-4 with a product obtained in the normal horse and the colt

Primers and PCR conditions for amplification of equine FVIII intron 1 using genomic DNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
Intron 1 F1	CTCAGTGAACTGCACGTGGAC	30sec, 30sec, 60 sec	1064BP
Intron 1 R1	CCATGGTGTGGCAGGTGTCCA	anneal 61^{0}C, 40 cycles, HS	
Intron 1 F2a	CTGACTAGCCCTGAGCTAACA	30sec, 30sec, 30sec	422BP
Intron 1 R2a	CCTGCTCAGGTGACCTAGTTG	anneal 60^{0}C, 40 cycles, HS	
Intron 1 F2b	GGCTCCCCACCCTTCTTCTAC	30sec, 30sec, 30sec	416BP
Intron 1 R2b	GGGAATGCAGGGAGAAGAGCA	anneal 63^{0}C, 40 cycles, HS	
Intron 1 F2c	GGGACAGATGCTCAAGAAGGA	15sec, 1min, 30sec	524BP
Intron 1 R2c	GGGAAGATTAGCCCTGAGCTA	anneal 61^{0}C, 35 cycles, HF	
Intron 1 F3	GCCAGTGTAGTAGTGATAGTG	15sec, 30sec, 90sec	1488BP
Intron 1 R3	CGCGAACAGTACATTGTACTC	anneal 58^{0}C, 40 cycles, HS	
Intron 1 F4	CAGCATCTAGCATCATCTGAG	15sec, 1min, 75sec	1135BP
Intron 1 R4	CCCACTGACACTGTGAGATAA	anneal 60^{0}C, 35 cycles, HF	
Intron 1 F5	CCACATCCCATAGGTTATGGT	30sec, 30sec, 60 sec	1061BP
Intron 1 R5	GCGTGGTGGTTATGTAACTTG	anneal 58^{0}C, 40 cycles, HS	
Intron 1 F6	CCAAAGGGCAAAGGTCATGAG	30sec, 30sec, 60 sec	1080BP
Intron 1 R6	GGGACCTTGGATCTGGCAATG	anneal 61^{0}C, 40 cycles, HS	
Intron 1 F7	CTCCTTTGGTTGCTTGTGCTT	30sec, 30sec, 60 sec	1090BP
Intron 1 R7	GGTTGTGAAACGTGGTTCAGA	anneal 58^{0}C, 40 cycles, HS	
Intron 1 F8	CCCTGAAACTAAGACCAGCAC	15sec, 1min, 75sec	1179BP
Intron 1 R8	GGAGAGGGCTTAATGACTGAT	anneal 58^{0}C, 35 cycles, HF	
Intron 1 F9	GGAGCTCTGGAGCTGGAATGA	30sec, 30sec, 60 sec	1100BP
Intron 1 R9	GGGTCAGCTTGATATGCAGGT	anneal 60^{0}C, 40 cycles, HS	
Intron 1 F10	GGACAGCTAACAGCATTGGCT	15sec, 1min, 75sec	1144BP
Intron 1 R10	CCACGGAAATGCTGTGGTGAA	anneal 60^{0}C, 35 cycles, HF	
Intron 1 F11	CCACCCTGATGTGCACCTGAA	30sec, 30sec, 60 sec	992BP
Intron 1 R11	GCAAGCAAGAAGAGAGTGGAC	anneal 59^{0}C, 40 cycles, HS	
Intron I F12	GCAGGGTACAGAATTCTGCAT	30sec, 30sec, 75sec	1235BP
Intron 1 R12	CCAGGAGCTCTACTAGGTCTT	anneal 58^{0}C, 40 cycles, HS	
Intron 1 F13	CCCTGGGTAGGTTAGGCTGTG	30sec, 30sec, 75sec	1097BP
Intron 1 R13	GGCATCCTTGCCTTGTTCCTG	anneal 58^{0}C, 40 cycles, HS	
Intron 1 F14	CCACAGCGAACATCACACTCA	30sec, 30sec, 60 sec	1073BP
Intron 1 R14	GCCTATGCCTTTGGTGTCATA	anneal 59^{0}C, 40 cycles, HS	
Intron 1 F15	GGGTTCCAAGACCATTCAATG	30sec, 30sec, 75sec	1295BP
Intron 1 R15	GCAGCCACACCAGAGAATAAC	anneal 58^{0}C, 40 cycles, HS	
Intron 1 F16	CACCCATGTTCATAGCAGCAT	30sec, 30sec, 75sec	1257BP
Intron 1 R16	GCTCATTCTTGTCTCCAGTCA	anneal 58^{0}C, 40 cycles, HS	
Intron 1 F17	CCAAGATACAACCACCTTCAC	30sec, 30sec, 75sec	1021 BP
Intron 1 R17	CCTGGGCTTGGCAATGTTGAA	anneal 58^{0}C, 40 cycles, HS	

Table 9: Primers and PCR condition for equine coagulation factor VIII intron 1
BP: base pairs; F: forward; R: reverse; sec: seconds; HS: hot start; HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 94°C and extension at 72°C for the indicated number of cycles.

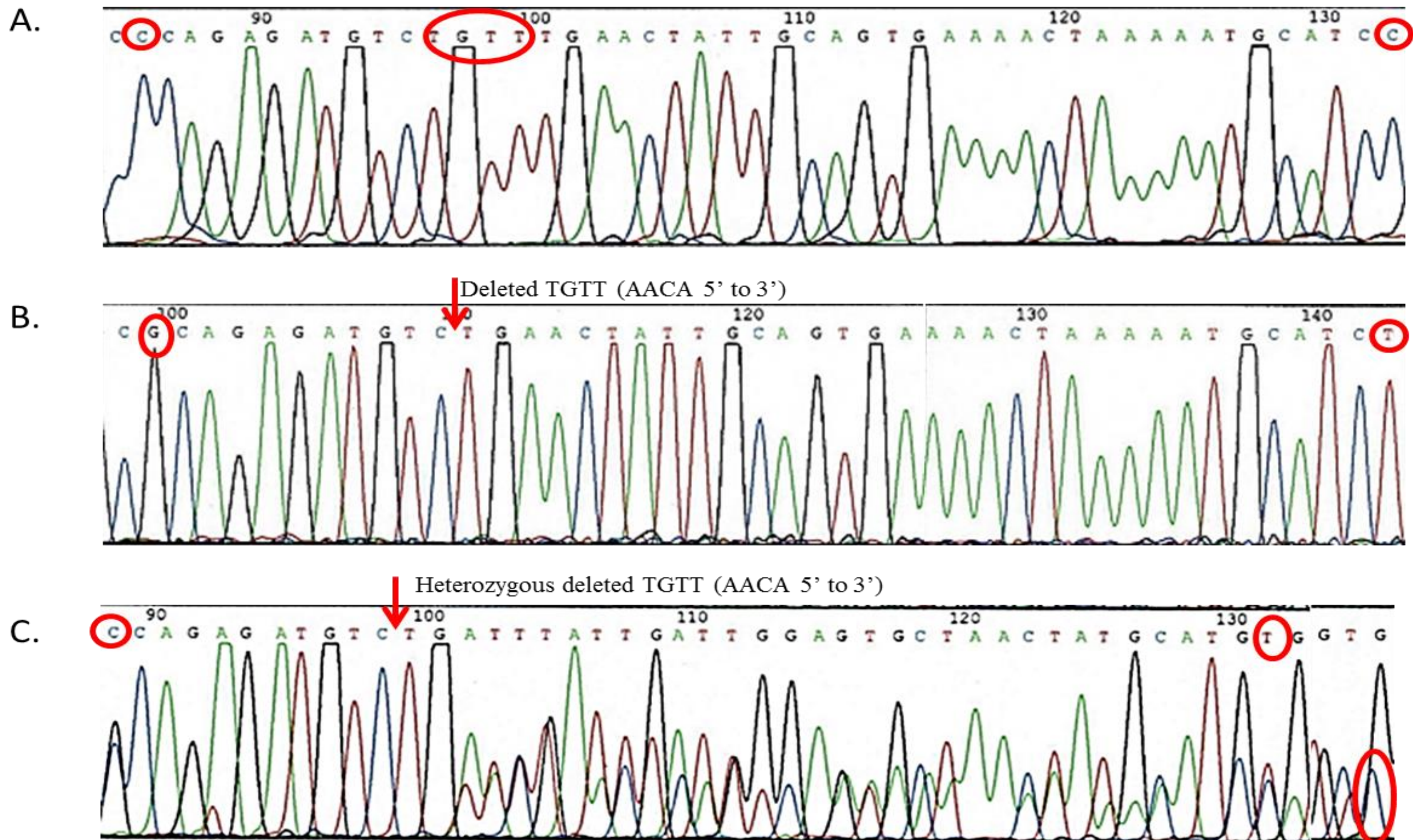


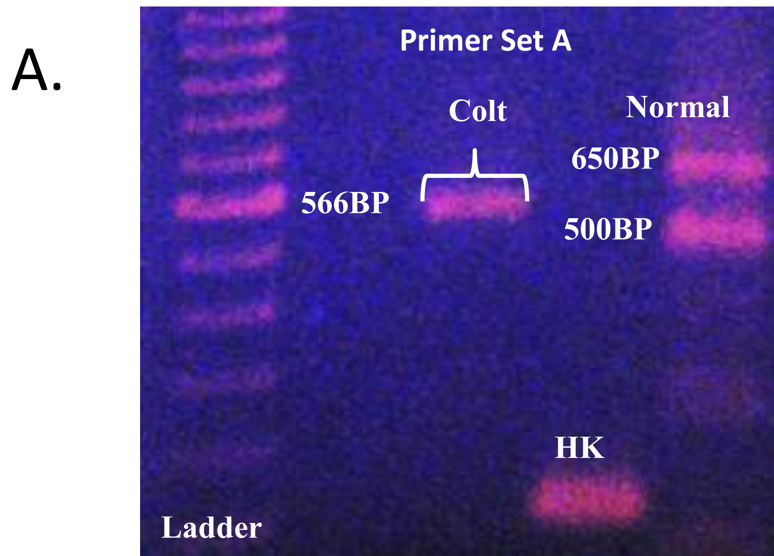
Figure 13: Genomic sequence data for the FVIII intron 1 reading 3’ to 5’ for (A) normal horse with sequence matching the predicted sequence (B) hemophiliac colt with a 4 base pair deletion 9.9KB from the 5’ splice site and 1 SNP (G<C) 11BP 3’ to the deletion and one SNP (T<C) 33BP 5’ to the deletion (C) dam heterozygous for the deletion and both SNPs

Primers and PCR conditions for amplification of equine FVIII intron 1 splice sites using hepatic cDNA

Primer Name	Primer Sequence 5' to 3'	PCR Conditions*	Target BP
5' Splice Site			
Ex1_Int1 F Set A	GCTATCCACCTGCTTCTTTC	10sec, 1min, 30sec	566BP
Ex1_Int1 R Set A	ACCAAAACCACACCATACATAC	anneal 55^0C, 35 cycles, HF	
Ex1_Int1 F Set A	GCTATCCACCTGCTTCTTTC	10sec, 1min, 30sec	650BP
Ex1_Int1 R Set B	TTACTCTCTCAAAATGCACCAG	anneal 55^0C, 35 cycles, HF	
Ex1_Int1 F Set C	TGCCACCAGAAGATACTACC	10sec, 30sec, 30sec	991BP
Ex1_Int1 R Set C	CCCCACCCAAAATAGATCAAC	anneal 55^0C, 35 cycles, HF	
3' Splice Site**			
Int1_Ex2 F Set A	TCACATCATGGAAGAGGTGAG	10sec, 30sec, 30sec	200BP
Int1_Ex2 R Set A	TTGTACATGACTGAAGGGTTG	anneal 55^0C, 35 cycles, HF	
Int1_Ex2 F Set B	TGGCACCTAATGTATCAGGTC	10sec, 30sec, 30sec	500BP
Int1_Ex2 R Set B	TGTGAACTCTACAAACACAGTC	anneal 55^0C, 35 cycles, HF	
Int1_Ex2 F Set C	CAAGATACAACCACCTTCACC	10sec, 30sec, 30sec	1000BP
Int1_Ex2 R Set C	TGTGAACTCTACAAACACAGTC	anneal 55^0C, 35 cycles, HF	
Int1_Ex2 F Set D	AGACATAAGAAACAACACCCAC	10sec, 30sec, 30sec	1500BP
Int1_Ex2 R Set D	ACTGAAGGGTTGAATGGAAAAG	anneal 55^0C, 35 cycles, HF	

Table 10: Primers and PCR condition using hepatic cDNA across the equine coagulation factor VIII intron 1 5’ and 3’ splice sites
BP: base pairs; F: forward; R: reverse; sec: seconds; min: minute; HF: HiFidelity polymerase.
* With all PCR conditions, denaturation was at 98°C denature and extension at 72°C for the indicated number of cycles.
** Although product was not obtained with cDNA, primers were evaluated with genomic DNA

A.



B.

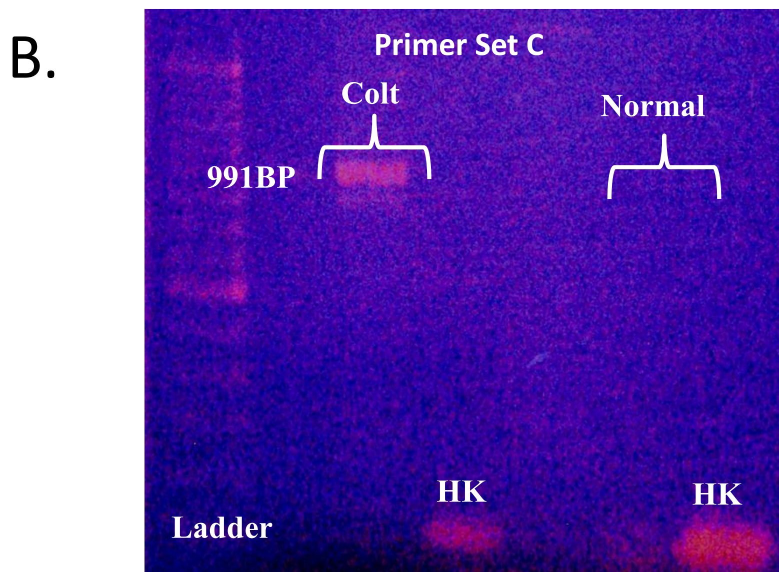


Figure 14: Gel electrophoresis of PCR products amplified from cDNA for exon 1 into intron 1 with (A) primer set A appropriate sized product in the colt at 566BP and non-specific bands in the normal horse (B) primer set C with appropriate product in the colt at 991BP with no product in the normal horse.
HK: Housekeeping gene

Chapter 3: Discussions and Conclusions

To our knowledge, this is the first verification of the equine FVIII coding sequence as well as identification of a genetic mutation associated with hemophilia A in a horse. The colt in this report was appropriately diagnosed with a bleeding diathesis due to deficiency in coagulation FVIII based on clinical signs, necropsy findings, coagulation panel results, and FVIII:C activity. Since the mutation was not identified within the promoter sequence or coding region (exons) of the FVIII gene, mRNA was isolated from liver samples of a normal horse and the affected colt. Normal liver tissue was chosen based on availability of samples and Hollestelle, et al's study showing that FVIII mRNA was expressed in the liver, kidney, lung, brain, and heart of mice. In this study, particularly strong expression was documented in hepatocytes and hepatic sinusoidal endothelial cells, suggesting that the liver could account for 75% of plasma FVIII concentrations.[115]

PCR on hepatic cDNA revealed products of appropriate size on the normal horse and the colt when primer sets were designed to amplify exons 2-26; no product was obtained for the colt from exon 1-2, indicating that a normal sized product was likely not being transcribed (see figures 11 and 12). This was supported by the lack of an appropriately sized product for the colt via Western immunoblot. A PCR product was obtained on the colt across exon 1 to exon 2 when extension time and the number of cycles on the PCR protocol were doubled, however, this result was not able to be repeated on 3 more attempts. If the one result was real, it is not surprising since the colt had 15% FVIII:C, indicating that a small amount of the FVIII gene was being transcribed appropriately. Doubling the extension time and number of cycles effectively

increased the PCR sensitivity and allowed for amplification of a small amount of product, which could not be obtained with the standard PCR protocol or western immunoblot.

Due to the location of the mutation in intron 1 and the presence of 15% FVIII:C activity, aberrant splicing across the 5' splice site was suspected. During normal transcription of mRNA, intronic sequence is removed from the pre-mRNA by spliceosomes, which cut the exon-intron-exon junction at the 5' and 3' splice sites. Correct splicing is essential for accurate coding sequence and translation of the transcript into a functional protein.[102] However, all intronic sequence contains pseudo exons (intronic sequence flanked by 5' and 3' splicing signals) and decoy splice sites (alternative splice sites within the exon or intron).[101] Therefore, recognition of authentic coding sequence and consensus splice sites is dependent on *cis*-elements including both exonic and intronic splicing enhancers and silencers.[102] Mutations can affect any of these components leading to alterations in splicing known as aberrant splicing. It has been suggested that aberrant splicing represents up to 50% of mutations which ultimately lead to gene dysfunction.[101]

Aberrant splicing was assessed by PCRs performed on the transcript (cDNA) for the colt and normal horse across the 5' splice site (forward primer located within exon 1 and the reverse primer within 1kb of the intron) and across the 3' splice site (forward primer within the end of the intron and reverse primer within exon 2). A product was obtained for the colt across the 5' splice site with multiple unique primer sets. Sequence analysis confirmed that intronic sequence was present at the end of exon 1, indicating inappropriate transcription of the coding sequence in the colt. The RNA was cleaned with an RNase-free DNase and PCRs repeated with distinct product obtained only in the foal. However, occasionally a faint band was obtained on two primer sets across the 5' splice site in the normal horse; failed DNA sequencing reactions made

analysis inconclusive; however, genomic contamination of the cDNA or contamination of the laboratory were suspected. Although the products obtained in the colt were consistently present, obtained with multiple unique primer sets, and sequencing analysis yielded successful DNA sequencing reads, it cannot be confirmed that genomic contamination had not contributed to the results obtained in the colt.

Further evaluation of alterations of splicing include online *in silico* tools which predict potential splice sites, enhancers, silencers, or branching sites. Two prediction programs, Exonic Sequence Finder (http://rulaie.cshl.edu/tools/ESE3) and the Human Splicing Finder (http://zeus2.itb.cnr.it/~webgene/wwwspliceview_ex.html), were used to evaluate the intronic sequence in the affected foal. Both programs base splice prediction from statistical analysis of alterations in several consensus sequences.[116] Results were inconclusive upon evaluation of the intronic deletion in this foal. This was not surprising based on reported inability of *in silico* tools to predict deep intronic mutations.[102]

The full length of the inserted transcript could not be accurately amplified with conventional PCR. It was hypothesized that due to the length of the intron and location of the deletion, the insertion may have been too large to be amplified by normal PCR techniques. TaKaRA long and accurate taq polymerase (TaKaRa LA taq) was used in a PCR reaction attempting to amplify cDNA using a gene specific primer, but the amplification was unsuccessful. Several factors could account for the lack of product including non-specific binding of the gene specific primer or inappropriate parameters for the PCR protocol. An extension time that is too short may generate no amplification product or non-specific bands; whereas, extension times that are too long can cause diffusely smeared electrophoresis bands. The TaKaRa LA PCR protocol recommended an extension time of 30 seconds to 1 minute per

1kB of amplification size; however, since the length of the insert was unknown an initial extension time was estimated at 15 minutes. Additionally, annealing temperatures which are too high may yield no amplification product, while a temperature that is too low enhances non-specific reactions. The TaKaRA LA taq was recommended to be used with an annealing temperature of 60-68^{0}C for sufficient activity. The PCR primers designed for exon 1 to exon 2 had melting temperature of 60^{0}C and 58^{0}C, respectively; therefore, the annealing temperature with TaKaRa LA Taq was set at 60^{0}C. However, the annealing temperature from the initial hepatic cDNA PCR reactions with this primer set had been successful at 55-56^{0}C, consistently yielding a product in the normal horse, which may have been required for primer optimization with the TaKaRa LA taq. PCR reactions in the colt and normal horse either yielded no product or smeared bands, indicating optimization of the extension time or annealing temperature may have been necessary to obtain a product.

The inability to fully amplify the aberrant transcript, lack of product on Western blot and normal 15% FVIII: C activity reported in this colt could also be explained by DNA repair and alternative activation of the consensus splice site with degradation of the aberrant mRNA. Aberrant splice sites remain in competition with consensus splice sites, which can lead to variations in phenotype between individuals due to variable levels of normal transcript. Nonsense mediated mRNA decay, nonstop decay, no-go decay, and nonfunctional rRNA decay are all quality control systems which recognize alterations in translation elongation or termination and degrade aberrant mRNA.[107] For example, creation of a premature termination codon within the aberrant splice site may lead to translation of a C-terminally truncated protein. Nonsense mediated mRNA decay recognizes and degrades the truncated transcript; whereas,

nonsense-associated altered splicing increases the level of alternatively spliced transcript that does not contain the premature termination codon.[108]

Although hemophilia A is the most common coagulopathy associated with factor VIII deficiency, several other hemophilia-like phenotypes have been identified including mutations in the FVIII binding site to vWF or FVIII transport proteins.[51] In normal circulation, FVIII is tightly bound to vWF until hemostasis is initiated; once FVIII becomes activated, it is cleaved from vWF. Premature dissociation of FVIII from vWF leads to immediate degradation of FVIII to prevent alterations in coagulation. Therefore, a qualitative defect in the FVIII binding site to vWF, characterized as type 2N VWD, leads to secondary FVIII deficiency and hemophilia-like phenotype. In horses, exons 17-28 of the vWF gene code for the FVIII binding site. Analysis of the coding region of the FVIII binding site on vWF identified a SNP (A<C) in the colt in exon 22 which was predicted to the change a lysine (positively charged amino acid) to an asparagine (neutral amino acid). The SNP was also noted in the mare and three breed matched normal horses, suggesting that the mutation did not significantly alter the FVIII binding site.

Combined deficiency of FVIII and FV (CDF5F8) is a disorder in humans characterized by mutations in either transport protein LMAN-1 or MCFD2. Both proteins form a calcium dependent cargo transporter which facilitates movement of FV and FVIII from the endoplasmic reticulum to the Golgi apparatus.[88] In people, this disorder manifests as mild to moderate clinical bleeding with a percent coagulation activity of 5-30% for FVIII and FV.[91] In the colt, FV activity was not evaluated so CDF5F8 could not be ruled out. Comparison of genomic DNA for the foal, mare and normal horse for both equine LMAN-1 and MCFD2 did not reveal any genetic mutations. CDF5F8 has not been identified in animals, therefore, additional transport

mechanisms for FV and FVIII may exist in horses, which were not evaluated. However, based on cDNA analysis of FVIII gene in the foal, CDF5F8 was considered highly unlikely.

In conclusion, this is the first report of identification of a genetic mutation in a colt with hemophilia A. Based on cDNA analysis, the mutation was localized to the non-coding region of the FVIII gene (specifically intron 1). Genomic sequence of intron 1 suggested maternal inheritance. Transcription of intron 1 across the 5' splice site suggested activation of an aberrant splice site in intron 1. It is yet to be determined if this defect is responsible for hemophilia A in other horses, but this is the first genetic analysis of a horse with FVIII deficiency and has been an initial step in paving the way for future understanding of coagulation abnormalities in horses.

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