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A Novel Frameshift Mutation in F9 in a Mixed Breed Dog With Clinical Hemophilia B

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Case Report

Keywords: genetic, coagulopathy, Great Pyrenees, mixed breed, direct to consumer genetic testing

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Title:

A novel frameshift mutation in *F9* in a mixed breed dog with clinical Hemophilia B.

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Abstract:

A male mixed breed dog with clinical Hemophilia B was tested with a direct-to-consumer dog genomics service. After being genotyped as “normal” at three previously published Hemophilia B variants, all coding regions of the dog’s F9 gene were sequenced. A single basepair deletion in exon 5 of F9 led to a frameshift mutation leading to a premature truncation of FIX, including loss of the catalytic domain. The dam for this individual is a Great Pyrenees suggesting this X chromosome variant could segregate within the Great Pyrenees and possibly related breeds. The discovery of this novel variant could be applicable to the Great Pyrenees community should hemophiliac Great Pyrenees be observed at appreciable frequency and emphasizes the utility of genetic testing in mixed breed dogs.

Key words:

genetic, coagulopathy, Great Pyrenees, mixed breed, direct to consumer genetic testing

Background:

Hemophilia B, or Christmas Disease, is a deficiency of coagulation Factor IX (FIX). An X-linked gene in both humans and dogs, males are more commonly clinically affected, with signs including easy bruising, delayed blood clotting, or excessive bleeding after trauma; indeed, most cases of hemophilia in the dog are detected when puppies begin to shed their deciduous teeth, or the time of spay/neuter [1]. Distinct loss of function mutations in canine F9 have been characterized in the Rhodesian Ridgeback [2], the Labrador Retriever [3], the German Shorthaired Pointer [4], and the Lhasa Apso [5]; a

more recent publication describes a mutation in a family of Hovawarts that reduces expression of F9 via transcription factor binding site disruption [6]. Reports of Hemophilia B in non-purposely bred dogs are incredibly rare [7]. Here, we report a novel mutation in the coding sequence of *F9*. To our knowledge, this is the first F9 mutation to be discovered in a mixed breed dog. This emphasizes the utility of genetic testing in mixed breed dogs; further, combined with high resolution breed identification, could also inform proactive testing for genetic health risk variants in contributing dog breeds.

Case Presentation:

An adult male intact mixed breed dog with clinical Hemophilia B presented for genetic testing for breed composition and health risk variants. At 4 months of age, the dog underwent placement of a microchip and subsequently developed a large hematoma at the site of the microchip. The dog was taken to an emergency veterinary practice, where a complete blood count revealed severe regenerative anemia (HCT = 12.4%, Ref 37.3-61.7%; Retics 112.1 K/µL; Ref 10.0-110.0 K/µL). A coagulation panel revealed a normal prothrombin time (13.0s, Ref 11.0-17.0s) and a prolonged partial prothrombin time (236.0s, Reference 72.0-102.0s). Further testing revealed subnormal levels of Factor IX, leading to a diagnosis of Hemophilia B or Factor IX Deficiency [8]. The dog was reported to be the result of an accidental breeding of a Great Pyrenees dam (Figure 1) and a mixed breed sire. The dam had had one to two litters prior to the proband's, with no reported health issues; however, the owners of a male littermate of the proband did report abnormal bleeding upon deciduous teeth shedding. Prior to diagnosis of Hemophilia B, the proband had an episode of lameness in his front leg and

an episode of swelling underneath the chin, both of which resolved spontaneously within several days.

Figure 1. Photo of the proband's dam, courtesy of S. Nath.

Testing with a direct-to-consumer genomics company, Embark Veterinary, confirmed the dog's mixed Great Pyrenees ancestry, but revealed that the dog lacked three genetic variants for Hemophilia B included in the Embark Vet health testing repertoire (embarkvet.com/health-list). Upon discussion with the owner, consent was given to collect an additional DNA sample for direct sequencing of *F9*. All exons of *F9* were Sanger sequenced (**Tables 1** and **2**). We identified a single base pair (c.650delC; p.D143Ifs) deletion at chrX:109,518,978 (CanFam3.1) causing a frameshift mutation in exon 5 of the canine orthologue of *F9* (**Figure 2, Table 3**), leading to premature protein truncation and loss of the entire catalytic domain of FIX, and is almost certainly the cause of this dog's clinical Hemophilia B.

Figure 2. Sanger sequence trace of F9 exon 5 from A) the proband and B) a clinically unaffected male control. The site of a single cytosine deletion in the affected sequence as indicated by the black arrowhead. Predicted amino acid residue is shown in single letter code below each codon in red.

Discussion:

We have described a novel mutation in exon 5 of canine *F9* causing clinical Hemophilia B in a mixed breed dog. *F9* codes for the proenzyme of the serine protease Factor IX

[9]. Part of the intrinsic coagulation pathway, Factor IX is cleaved to its active form by Factor XIa or tissue factor-activated VIIIa in the presence of bivalent calcium ions and Factor VIIIa [10]. Factor IX then cleaves Factor X, prothrombin, to its active form thrombin; thrombin converts fibrinogen to fibrin, which polymerizes to generate the hemostatic plug [11].

Exon 5 of *F9* codes for the second EGF-like domain of FIX, which is required for contact with FVIIIa [12]; hydrophobic interactions between the two EGF-like domains of FIX are also thought to contribute to its catalytic activity [13]. Given that the mutation described here not only disrupts the second EGF-like domain, but also causes premature protein truncation (from a 482aa ORF to a 210aa ORF), including complete loss of the catalytic domain of FIX, it is very likely that this is the causal mutation for this dog's clinical Hemophilia B.

For the past twenty years, the bread and butter of genomic discovery in canine genomics has been the genome wide association study or GWAS, where genotype data of dogs sharing a common trait or disease phenotype (preferably as unrelated as possible) are compared to that of healthy controls. In recent years, more health risk variants are being identified through whole genome sequencing (WGS) of just a few individuals [14,15,16,17]. As the cost of WGS continues to reduce, we expect that the rate of HRV discovery will increase rapidly in coming years. In this case, however, a well-informed candidate gene screen proved to be as fruitful as more

cutting-edge methods, as to our knowledge, all known instances of Hemophilia B in every species map to defects in the *F9* gene.

Common knowledge of coagulopathies in the Great Pyrenees consists of a Great Pyrenees-specific mutation for Glanzmann's thrombasthenia [18]. Our examination of the veterinary literature did not yield any documentation of Hemophilia B in Great Pyrenees; in fact all mentions of hemophilia in this breed are restricted to a single case report of Hemophilia A [19]. However, the dog's history combined with the physical position of F9 on CFAX, strongly support inheritance of this mutation from the Great Pyrenees dam. It is possible that this could have been a *de novo* germline mutation; alternatively, the dog's dam as a heterozygote for this mutation would be expected to display few or no clinical signs of coagulopathy. Adoption of genetic testing for this mutation may not be necessary in purebred Great Pyrenees, though retroactive testing of Great Pyrenees DNA samples in existing repositories such as those maintained by the Orthopedic Foundation for Animals would bolster a more definitive recommendation.

Abbreviations:

FIX, Factor IX or Christmas Factor; Retics, Reticulocytes; ORF, open reading frame; aa, amino acid; CFA, *Canis familiaris* chromosomal nomenclature

Declarations

Ethics Approval

The owners of the proband consented to the Embark Veterinary Inc.'s Terms of Service at time of registration (https://embarkvet.com/tos/) and personally submitted a second buccal swab for sequencing.

Consent for Publication

The owners of the probands consented to and participated in the publication of this case report, and provided the photo of the proband's dam and the proband's laboratory values.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing Interests

ER and ETC are former employees of Embark Veterinary, Inc. The authors declare no competing interests, financial or non-financial.

Funding

This work was funded by Embark Veterinary, Inc.

Author Contributions:

ER and ETC conceptualized the experiments. ER performed the PCR and sequence analysis. ETC performed protein functional prediction analysis. SN owns the proband, provided the proband's clinical history, presentation and diagnosis, and edited the manuscript. ER and ETC wrote the manuscript.

Acknowledgements:

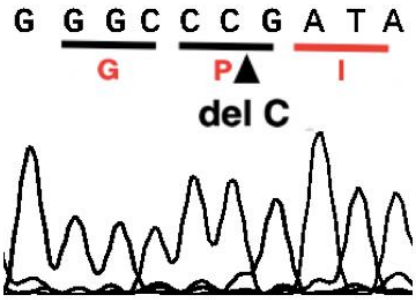
We thank Adam Boyko for editing and feedback on this manuscript.

Figures

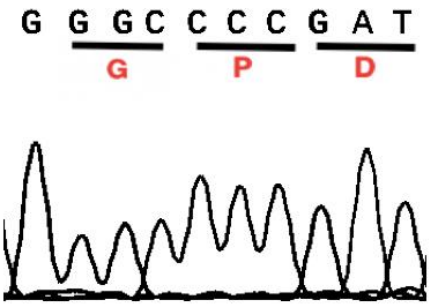
Figure 1. Photo of proband's dam, courtesy of S. Nath.

Figure 2. Sanger sequence trace of F9 exon 5 from A) the proband and B) a clinically unaffected male control. The site of a single cytosine deletion in the affected sequence as indicated by the black arrowhead. Predicted amino acid residue is shown in single letter code below each codon in red.

A. Proband



B. Control



Tables

Table 1. Primer Sequences for Sanger Sequencing.

Name	Primer Sequence	Direction	Purpose
F9_ex1_F	AACGGGTGAATTTTGCTGCC	Forward	Amplifies exon 1
F9_ex1_R	ACAGCTAGAAGACAAGCATACTCA	Reverse	
F9_ex2+3_F	ACAGTACCATGAAGACATCACAG	Forward	Amplifies exon 2 and 3
F9_ex2+3_R	CTAGGGAGGAGTCGGCTGAT	Reverse	
F9_ex4_F	CCCCCTCCGTCTCTCATGAA	Forward	Amplifies exon 4

F9_ex4_R	TCCATTAAGTGTTCCTTACCACA	Reverse	
F9_ex5_F	GCTCCCAAGCCTCTTTCCAT	Forward	Amplifies exon 5
F9_ex5_R	AGGAAGTAGAAACAAACAGGGT	Reverse	
F9_ex6_F	TTGATGGGCCTGCTTCTCAG	Forward	Amplifies exon 6
F9_ex6_R	ACCCTGTCTGGTAGCTGTCT	Reverse	
F9_exon7_variant1_F	GCTGGGGGAGAGTCTTCAAC	Forward	Amplifies chrX:109,531,802-109,532,355 in exon 8
F9_exon7_variant1_R	GGGAGTGGTCACAAGTTCCC	Reverse	
F9_exon7_variant2_F	ACTCATGTGCAAAGCTGACAC	Forward	Amplifies exon 7
F9_exon7_variant2_R	CTCCTCTAGCATTAGCCCAACT	Reverse	
F9-E8-1F	ACCATGACATCGCCCTTCT	Forward	Amplifies chrX:109,531,676-109,532,258 (exon 8)
F9-E8-1R	CCCCAGAAAGAGAAAAAGCA	Reverse	
F9-E8-2F	TTACCAAGATTGCGCTTGTG	Forward	Amplifies chrX:109,531,458-109,532,258 (exon 8), use with F9-E8-1R
F9-E8-3F	TGGCACATCATAGTTTCCTGA	Forward	Amplifies chrX:109,531,007-109,532,258 (exon 8), use with F9-E8-1R

Table 2. FASTQ data from Sanger sequencing of proband and control with both forward and reverse primers. Sequence was trimmed of low quality base reads (depicted as “N”) from the beginning and end of each trace.

Subject	Primer	Sequence Trace
Proband	Forward	GTACTCTTGAGAAATGCATGTGAAATGANGCTGTTACTGT TGATTTTGCTTTCTTTCAGATGTAACATGCAACATTAAGAATGGC AGATGCAAGCAGTTTTGTAAATTGGGCCCGATAAC AAGGTGNNTTGTTCCTGTACTACGGGATACCAACTTGCGGAAGA CCAAAGGTCCTGTGAACCAGCAGGTCAGAATCTAAT AAAGTTTTTTTAACACTCTCCAAACCTATATTTGAACCTTCAGTAT TTTAACTAGTTCACATACTTTCATAAACCCTGTT
Proband	Reverse	TGAAGGTCNATATAGGTTTGGAGAGTGTTAAAAAAACTTTATTAG ATTCTGACCTG CTGGTTCACAGGACCTTTGGTCTTCCGCAAGTTGGTATCCCGTA GTACAGGAACAAACCACCTTGTTATCGGGCCCAATT TACAAAACTGCTTGCATCTGCCATTCTTAATGTTGCATGTTACAT CTGAAAGAAAGCAAAATCAACAGTAACAGCATCAT TTCACATGCATTTCTCAAGAGTACATGAAACCAGTGACTCATGTA TAGGTCAAGTGTAAGCAGGGAGCATTCATGGAAAG
Control	Forward	GTGAAATGATNGCTGTTACTGT TGATTTTGCTTTCTTTCAGATGTAACATGCAACATTAAGAATGGC AGATGCAAGCAGTTTTGTAAATTGGGCCCCGATAA CAAGGTGGTTTGTTCCTGTACTACGGGATACCAACTTGCGGAAG ACCAAAGGTCCTGTGAACCAGCAGGTCAGAATCTAA TAAAGTTTTTTTAACACTCTCCAAACCTATATTTGAACCTTCAGTA TTTTAACTAGTTCACATACTTTCATAAACCCTGT TTGTTNCTTACTTTCCTNNNNNNCCGGCTGANNNNACTNTGGAG CANNGCGAATGGACAGNGNNGGTCNANGATGTNGTA GATCCTGCNAANNTCTGGGCTCTGNTTCCTGNNNTGTGAATGCT NNAACCTGANTGGCTAANCNAGANNNANCAACAGGN AGNAGANNACCNTCGTCTGNCTCTGAGCCTGNNCTCNGNNNNN NTCTCCAAAAGANCTGNNNATCNAGGGNANAGNNTNN NNAACNCTCTAANCTGCTNNAGGAGGAGNTCNNGNNCNTGCNT GNTCATGAGCTC
Con	Rev	ATATAGGTTTGGAGAGTGTTAAAAAAACTTTATTAGATTCTG

trol	erse	ACCTGCTGGTTCACAGGACCTTTGGTCTTCCGCAAGTTGGTATC CCGTAGTACAGGAACAAACCACCTTGTTATCGGGGC CCAATTTACAAAACTGCTTGCATCTGCCATTCTTAATGTTGCATG TTACATCTGAAAGAAAGCAAAATCAACAGTAACAG CATCATTTCACATGCATTTCTCAAGAGTACATGAAACCAGTGACT CATGTATAGGTCAAGTGTAAGCAGGGAGCATTCAT

Table 3. Consensus cDNA and predicted amino acid sequence, and that of proband.

Subject	**Sequence**
Consensus mRNA	gccacagtgacagaagcccacagaatcagaggcgagctttaaagatgaac actgcctacactcttaacccaagaggccactggaaatagcccaaagaccc actgagggagatggacactgtttcccagaagtaaatacagctcaacttgt actttggaacaactggtcaaccttgctccttgcacaattcatcagcaaag gttaggcagcgcctgaataggatcatggcagaagcatcgggcctcgtcac cgtctgccttttaggatatctactcagtgccgaatgtgcagtttttcttg atcgtgaaaatgccaccaaaattctgagtcggccaaagaggtataattca ggtaaactggaagagtttgttcgagggaaccttgagagagaatgtataga agaaaagtgcagttttgaagaagcacgggaagtttttgaaaacactgaaa aaaccactgaattttggaagcaatatgttgatggagatcaatgtgaatcc aatccatgtttaaatgacggtgtatgcaaggatgacattaattcctatga atgttggtgtcgagctggatttgaaggaaagaactgtgaattagatgtaa catgcaacattaagaatggcagatgcaagcagttttgtaaattgggcccc gataacaaggtggtttgttcctgtactacgggataccaacttgcggaaga ccaaaggtcctgtgaaccagcagtgccatttccatgtggaagagtttctg tccctcacatttctatgacacgcacccgtgctgaaactctttttttccaat atggactatgaaaattcaactgaagtggaaaaaattttggataacgtcac ccaaccgcttaacgacttcactcgagttgttggtggaaaagatgccaaac caggtcaattcccttggcaggtccttttgaatgggaaagttgatgcattc tgcggaggttccatcatcaatgaaaaatgggtggtaactgcagcccactg tattgagcctgatgttaaaattaccatagttgcaggtgagcataacaccg agaagagggaacatacagagcagaagcgaaacgtgattcgcactattctt caccacagctataatgcaactattaataagtacaaccatgacatcgccct

	tctggaactggatgagcccttaacgctgaacagctatgtaacacctattt gcattgctgacagggaatactcgaacatcttcctcaaatttgggtctggc tatgtgagtggctgggggagagtcttcaacaaagggcgatcggcttcaat tcttcaataccttaaagttccacttgttgaccgagccacgtgccttcggt ccacgaagttcaccatttataacaacatgttctgtgctggcttccatgag ggaggtaaagattcatgccagggcgatagtgggggaccccatgtcaccga agtagaaggcataagtttcttaactgggattattagctggggtgaagagt gtgcgatgaaagggaagtatggaatatataccaaggtgtcccggtatgtc aactggattaaagaaaagacgaagctcacctaaagaataatgtatttcca aggttgacacgtttagggtagaaaatggacaaggtcctttactaactaat cacttttttatctctttagatttgactatatacattctctgaatactgc tttttctctttctggggagaaatctatctagaattcctattttactagac taagtgaattagaaaatgtaatcactacgggaatgtactgtgatgggaac ttgtgaccactcccacaggtctagcccttggcaccattgtgaggttaggt tattcctcctgcccgtcaggtgttaagtttctccactggggcaactccct gattctccctccttggcagcattccatgttccagacctttcttacctttc ccatggaaatcatcaaaatgtgttagatctacatccaggatatttgatct agttcataacaagtctaacacctcacttgttaaggaagaacacaggagca actgacaggttgcaactcaccagaaaacactatttccttttctgtatgct tattcctgcttcctttatctcttctgtttcctaatcctgaaatcagtgtc tctctttctctttctctctctcttttcctacagaggtttaaaggaggga agggacacatcatgctgttttactactgtccacagttatacatgtctatc aaacccagacttgctttcagtttggtctttgacttgcttttcggagcata gggatgaagccaggtgcctgaagcacttgcaggaaaatttgtctgaaaga gtcatgttactgaaatacatgcatcgtggaaggaatgaccgacccatcag aatacgtccaataagcatttgtaattgtgttgtgatagaggtaactaaga agagtgacatcaactcctgcgtctcatcccccatgtgaaaaaactgtgaa ctaaaagagaacagccagtgtgcaacgtagaactagtagagtcttcaagg aagaattcattggtgggtctccagtactggccggagctaaggaagaagtt gccctcgaccaaagaacatgagcaccctgtctcttaaactagcatgtccc cacagtggagaaggatgtactggtggcttaaaggcatgagtcaggggtgt ctgggtggctcagtggttgagcctttggctcagggcatgatcccagcgtc ctgggattgagtcccgcatcaggcttcctgcagggggcctgcttctccct ctgcttatgtctctgcttctctgtgtgtgtgtgtgtgtctctcatgaaaa aaataaataaaatctttaaaaaaaaaaaaggtatgagtcattccagtgag ccatctcactccccctttctggtttcaggttctctaggcaacctttga ttataattaggccttccatattgaattttctaaagagttgctgaccaacc tctgcatgtactttagtgtgttaggctggctccctttatgaattaataaa ctgttgttctggttgatactgtagcttttgtgaatgtggctgatgtgaa tcagtcatcccacatgttgttatatatgagactactgacaaaatcatgtt

	tgggaccactttgacctgaccaagctgctgccttcacctcacccccagcc aggcatttactttagtccttcaaatattaagtaccaataaatgtcttttc aaatttcaaaaaaaaaaaaaaaaaaaaaaaaa
Consensus AA	MAEASGLVTVCLLGYLLSAECAVFLDRENATKILSRPKRYNSGKLEEFVRGN LERECIEEKCSFEEAREVFENTEKTTEFWKQYVDGDQCESNPCLNDGVCK DDINSYECWCRAGFEGKNCELDVTCNIKNGRCKQFCKLGPDNKVVCSCTT GYQLAEDQRSCEPAVPFPCGRVSVPHISMTRTRAETLFSNMDYENSTE VEKILDNVTQPLNDFTRVVGGKDAKPGQFPWQVLLNGKVDAFCGGSIINEK WVVTAAHCIEPDVKITIVAGEHNTEKREHTEQKRNVIRTILHHSYNATINKYN HDIALLELDEPLTLNSYVTPICIADREYSNIFLKFGSGYVSGWGRVFNKGRSA SILQYLKVPLVDRATCLRSTKFTIYNNMFCAGFHEGGKDSCQGDSGGPHVT EVEGISFLTGIISWGEECAMKGKYGIYTKVSRYVNWIKEKTKLT
Proband predicted mRNA	gccacagtgacagaagcccacagaatcagaggcgagctttaaagatgaac actgcctacactcttaacccaagaggccactggaaatagcccaaagaccc actgagggagatggacactgtttcccagaagtaaatacagctcaacttgt actttggaacaactggtcaaccttgctccttgcacaattcatcagcaaag gttaggcagcgcctgaataggatcatggcagaagcatcgggcctcgtcac cgtctgccttttaggatatctactcagtgccgaatgtgcagttttcttg atcgtgaaaatgccaccaaaattctgagtcggccaaagaggtataattca ggtaaactggaagagtttgttcgagggaaccttgagagagaatgtataga agaaaagtgcagttttgaagaagcacgggaagtttttgaaaacactgaaa aaaccactgaattttggaagcaatatgttgatggagatcaatgtgaatcc aatccatgtttaaatgacggtgtatgcaaggatgacattaattcctatga atgttggtgtcgagctggatttgaaggaaagaactgtgaattagatgtaa catgcaacattaagaatggcagatgcaagcagttttgtaaattgggccc gataacaaggtggtttgttcctgtactacgggataccaacttgcggaaga ccaaaggtcctgtgaaccagcagtgccatttccatgtggaagagtttctg tccctcacatttctatgacacgcacccgtgctgaaactctttttttccaat atggactatgaaaattcaactgaagtggaaaaaattttggataacgtcac ccaaccgcttaacgacttcactcgagttgttggtggaaaagatgccaaac caggtcaattcccttggcaggtccttttgaatgggaaagttgatgcattc tgcggaggttccatcatcaatgaaaaatgggtggtaactgcagcccactg tattgagcctgatgttaaaattaccatagttgcaggtgagcataacaccg agaagagggaacatacagagcagaagcgaaacgtgattcgcactattctt caccacagctataatgcaactattaataagtacaaccatgacatcgccct tctggaactggatgagcccttaacgctgaacagctatgtaacacctattt gcattgctgacagggaatactcgaacatcttcctcaaatttgggtctggc tatgtgagtggctgggggagagtcttcaacaaagggcgatcggcttcaat

	tcttcaataccttaaagttccacttgttgaccgagccacgtgccttcggt ccacgaagttcaccatttataacaacatgttctgtgctggcttccatgag ggaggtaaagattcatgccagggcgatagtgggggaccccatgtcaccga agtagaaggcataagtttcttaactgggattattagctggggtgaagagt gtgcgatgaaagggaagtatggaatatataccaaggtgtcccggtatgtc aactggattaaagaaaagacgaagctcacctaaagaataatgtatttcca aggttgacacgtttagggtagaaaatggacaaggtcctttactaactaat cacttttttatctctttagatttgactatatacattctctgaatactgc tttttctctttctggggagaaatctatctagaattcctattttactagac taagtgaattagaaaatgtaatcactacgggaatgtactgtgatgggaac ttgtgaccactcccacaggtctagcccttggcaccattgtgaggttaggt tattcctcctgcccgtcaggtgttaagtttctccactggggcaactccct gattctccctccttggcagcattccatgttccagacctttcttacctttc ccatggaaatcatcaaaatgtgttagatctacatccaggatatttgatct agttcataacaagtctaacacctcacttgttaaggaagaacacaggagca actgacaggttgcaactcaccagaaaacactatttccttttctgtatgct tattcctgcttcctttatctcttctgtttcctaatcctgaaatcagtgtc tctctttctctttctctctctcttttttcctacagaggtttaaaggaggga agggacacatcatgctgttttactactgtccacagttatacatgtctatc aaacccagacttgctttcagtttggtctttgacttgcttttcggagcata gggatgaagccaggtgcctgaagcacttgcaggaaaatttgtctgaaaga gtcatgttactgaaatacatgcatcgtggaaggaatgaccgacccatcag aatacgtccaataagcatttgtaattgtgttgtgatagaggtaactaaga agagtgacatcaactcctgcgtctcatcccccatgtgaaaaaactgtgaa ctaaaagagaacagccagtgtgcaacgtagaactagtagagtcttcaagg aagaattcattggtgggtctccagtactggccggagctaaggaagaagtt gccctcgaccaaagaacatgagcaccctgtctcttaaactagcatgtccc cacagtggagaaggatgtactggtggcttaaaggcatgagtcaggggtgt ctgggtggctcagtggttgagcctttggctcagggcatgatcccagcgtc ctgggattgagtcccgcatcaggcttcctgcaggggcctgcttctccct ctgcttatgtctctgcttctctgtgtgtgtgtgtgtctctcatgaaaa aaataaataaaatctttaaaaaaaaaaaaggtatgagtcattccagtgag ccatctcactcccccttttctggtttcaggttctctaggcaacctttga ttataattaggccttccatattgaattttctaaagagttgctgaccaacc tctgcatgtactttagtgtgttaggctggctccctttatgaattaataaa ctgttgttctggttgatactgtagctttttgtgaatgtggctgatgtgaa tcagtcatcccacatgttgttatatatgagactactgacaaaatcatgtt tgggaccactttgacctgaccaagctgctgccttcacctcacccccagcc aggcatttactttagtccttcaaatattaagtaccaataaatgtcttttc Aaatttcaaaaaaaaaaaaaaaaaaaaaaaaa

Proband predicted AA	MAEASGLVTVCLLGYLLSAECAVFLDRENATKILSRPKRYNSGKLEEFVRGN LERECIEEKCSFEEAREVFENTEKTTEFWKQYVDGDQCESNPCLNDGVCK DDINSYECWCRAGFEGKNCELDVTCNIKNGRCKQFCKLGPITRWFVPVLRD TNLRKTKGPVNQQCHFHVEEFLSLTFL

Additional Files

Methods and Materials:

Clinical Assessment

The subject presented to his general care veterinarian with a primary complaint of prolonged and excessive bleeding. A coagulopathy panel was run by IDEXX (idexx.com). Prolonged PTT was diagnosed (Fig S1).

Breed analysis and health risk variant genotyping

A buccal swab was submitted for genotyping on the Embark Vet platform (Embark Veterinary, Inc., Boston, MA). Mixed breed analysis was performed as previously described [20]. Genotyping of health risk variants was performed on a custom Illumina Infinium BeadChip array, according to the manufacturer's recommendation (Illumina, Inc., San Diego, CA). Quality control measures for health risk variant genotyping included 1) validation of health risk genotypes with known positive and negative controls; 2) technical replicates of each health risk genotyping assay; 3) manual review of health risk genotypes; and 4) a standard genotyping quality threshold requiring high confidence calls for over 98% of all genotype calls.

Genomic DNA isolation

Owners submitted buccal swabs directly to Embark Veterinary, Inc for DNA isolation. Genomic DNA was extracted using the Zymo Quick-DNA Microprep kit (Zymo Research, Irvine, CA) according to the manufacturer's instructions. DNA quality and concentration were assessed using a NanoDrop™(Thermo Fisher Scientific, Waltham, MA).

Polymerase Chain Reaction

All exons of F9 were amplified via PCR for the subject dog and one clinically normal, male mixed breed dog as a negative control (Table S1). For all primer pairs, PCR was performed using with 15 ng gDNA, 0.4ul of 10mM dNTPs, 1ul of each 10uM PCR primer, 0.15ul Q5® High-Fidelity DNA Polymerase (New England Biolabs Inc, Ipswich, MA), 4ul of Q5® Reaction Buffer, and 4ul of Q5® High GC Enhancer in a total volume of 20 uL. PCR was performed on a BioRad thermocycler (BioRad, Foster City, CA) with the following parameters: 95 C for 30 seconds followed by 40 cycles of 95 C for 10 s, 60 C for 30 s, and 72 C for 1 min, then a final extension of 72 C for 7 mins. PCR products were assessed by agarose gel electrophoresis (1% agarose at 100V for 40 minutes), then submitted for Sanger sequencing.

Sanger Sequencing and Sequence Analysis

Sanger sequencing was performed by Genewiz (genewiz.com). Sequence analysis was performed with SnapGene Viewer (https://www.snapgene.com/snapgene-viewer/). Sequence traces were aligned using Clustal Omega (EMBL-EBI), and compared to CanFam3.1 canonical sequence for *F9* (Figure S1, S2, Table S2). (http://genome.ucsc.edu/cgi-bin/hgGateway?hgsid=767040331_34TdcLrihjhgCKXDOS2vCAVeaywa).

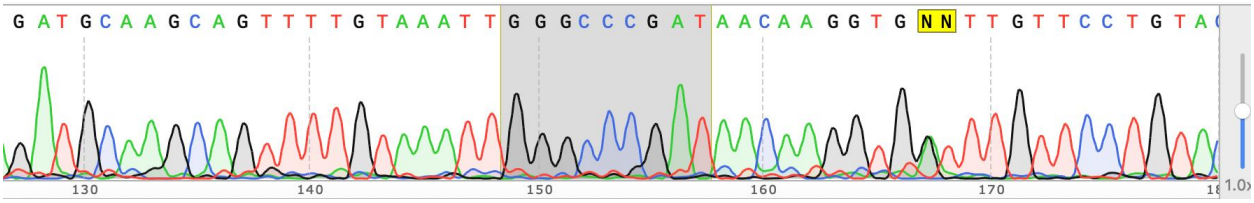
cDNA and protein prediction

The proband's cDNA sequence was manually inferred from canine F9 mRNA in RefSeq (NM_001003323.2) and translated with EMBL SixPack (https://www.ebi.ac.uk/Tools/st/emboss_sixpack/). Predictive functional analysis was performed with EMBL InterProScan (http://www.ebi.ac.uk/interpro/).

Supplementary Figures:

Figure S1. SnapGene sequence trace views of the proband (top) and a healthy male control (bottom). Forward and reverse primer traces are shown. A C/G deletion is evident in both sequence traces of the proband.

Proband, ex5_F



Control, ex5_F

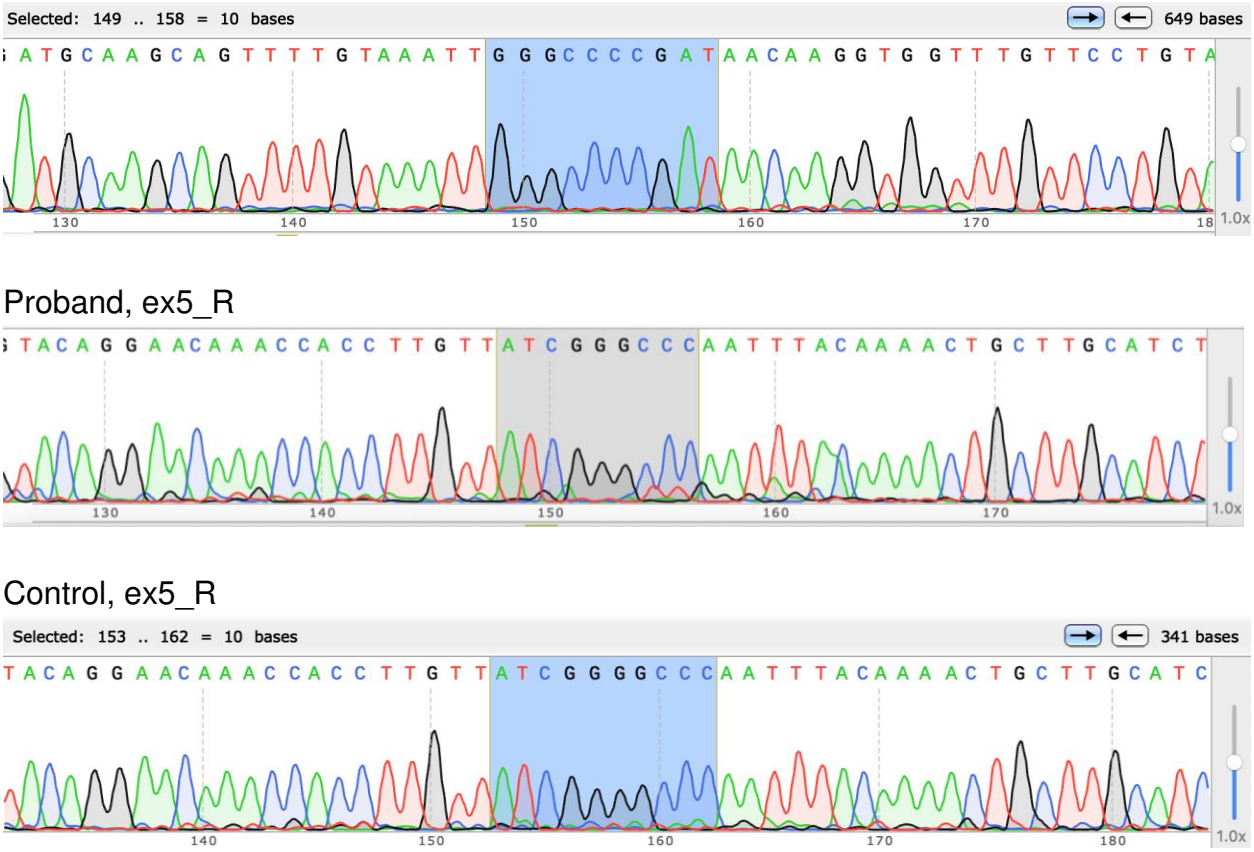
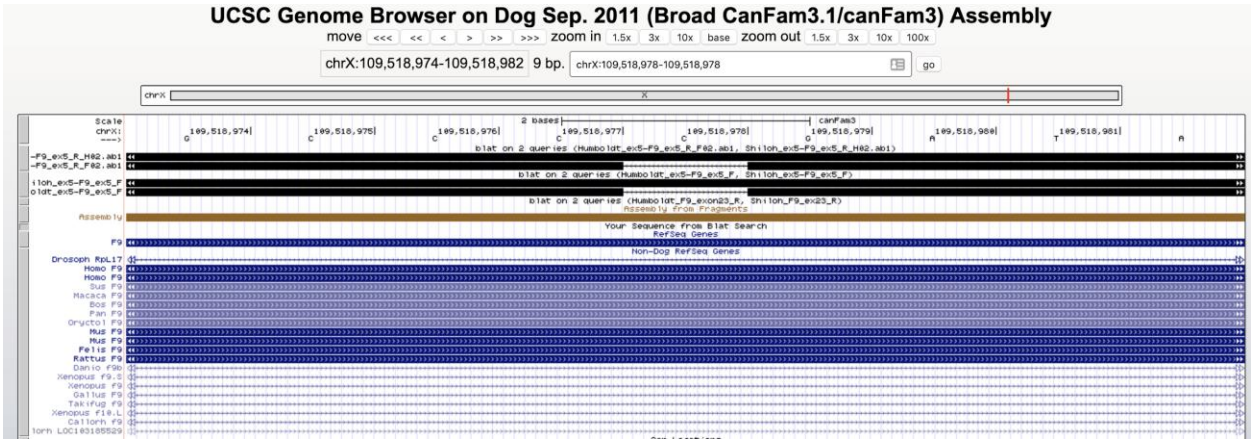


Figure S2. Forward and reverse sequencing trace alignment to CanFam3.1 in the UCSC Genome Browser. The dash in the proband sequence demonstrates the deletion.



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Figures

Figure 1

Photo of proband's dam, courtesy of S. Nath.

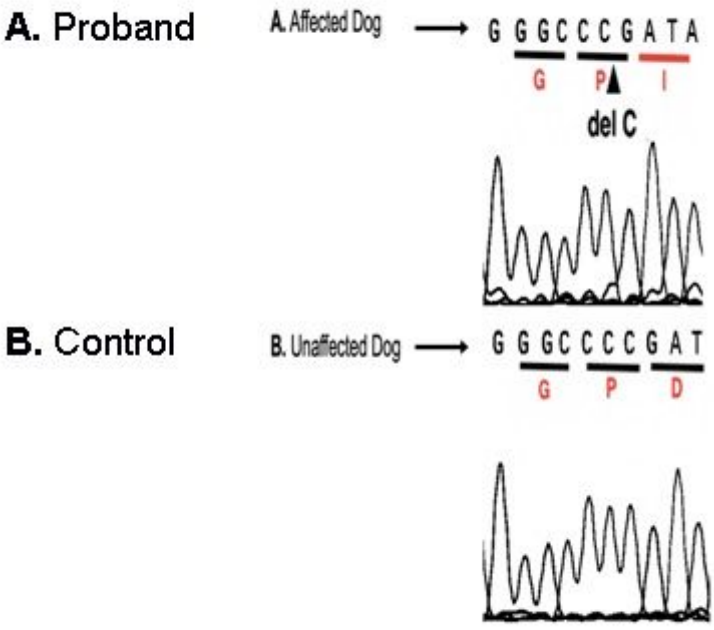

Figure 2

Sanger sequence trace of F9 exon 5 from A) the proband and B) a clinically unaffected male control. The site of a single cytosine deletion in the affected sequence as indicated by the black arrowhead. Predicted amino acid residue is shown in single letter code below each codon in red.

Supplementary Files

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