Case Report

Severe Hemophilia A in a Male Old English Sheep Dog with a C→T Transition that Created a Premature Stop Codon in Factor VIII

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Animals with hemophilia are models for gene therapy, factor replacement, and inhibitor development in humans. We have actively sought dogs with severe hemophilia A that have novel factor VIII mutations unlike the previously described factor VIII intron 22 inversion. A male Old English Sheepdog with recurrent soft-tissue hemorrhage and hemarthrosis was diagnosed with severe hemophilia A (factor VIII activity less than 1% of normal). We purified genomic DNA from this dog and ruled out the common intron 22 inversion; we then sequenced all 26 exons. Comparing the results with the normal canine factor VIII sequence revealed a C→T transition in exon 12 of the factor VIII gene that created a premature stop codon at amino acid 577 in the A2 domain of the protein. In addition, 2 previously described polymorphisms that do not cause hemophilia were present at amino acids 909 and 1184. The hemophilia mutation creates a new *Taq*I site that facilitates rapid genotyping of affected offspring by PCR and restriction endonuclease analyses. This mutation is analogous to the previously described human factor VIII mutation at Arg583, which likewise is a CpG dinucleotide transition causing a premature stop codon in exon 12. Thus far, despite extensive treatment with factor VIII, this dog has not developed neutralizing antibodies ('inhibitors') to the protein. This novel mutation in a dog gives rise to severe hemophilia A analogous to a mutation seen in humans. This model will be useful for studies of the treatment of hemophilia.

Many animal models of human hemorrhagic disorders have been described,[32] including severe hemophilia A,[15,18,24,31] severe hemophilia B,[12,35] and von Willebrand disease in dogs;[10,33] sheep with hemophilia A;[38] factor VIII and factor IX knockout mice;[2,26,28,48] rats with hemophilia A;[4,5,34] and pigs with von Willebrand disease.[14,20] All have phenotypic features of severe hemorrhage disease analogous to human disease and have various advantages for studying gene therapy, factor replacement therapy, immune tolerance, and curative organ transplantation.[40]

An inversion of the factor VIII gene analogous to one commonly found in humans with severe hemophilia A has been described in at least 2 independent dog colonies.[24,31] These dogs have been used to test gene therapy, factor VIII replacement therapy, inhibitor development, and the induction of immune tolerance to treat factor VIII inhibitors. Evidence indicates that, in humans, the likelihood of factor VIII inhibitor development depends in large part on the type of mutation that causes hemophilia A.[17,36] Whether polymorphisms in the amino-acid sequence of the factor VIII gene that are not related to the hemophilia mutation contribute to inhibitor risk[47] is a topic of debate.[21,44]

We study the immunogenicity of factor VIII with mismatches in amino acids of the factor VIII protein in dogs with hemophilia A due to the gene inversion involving intron 22. These dogs have polymorphic forms of factor VIII that differ from the normal, consensus sequence at amino acids 909 and 1184 of the mature protein.[6,24,31] These polymorphic sites differ from normal canine factor VIII that is transfused to treat bleeding in hemophilic dogs and may represent novel epitopes that can be recognized in recipients of mismatched donor factor VIII. As part of our studies of hemophilia A and the immunogenicity of the factor VIII protein, we have sought to identify dogs with hemophilia A due to mutations other than the intron 22 gene inversion, to study the immune response to 'foreign' proteins in the setting of other factor VIII mutations. We describe here a nonsense C→T point mutation in a dog with severe hemophilia A and no circulating factor VIII activity. Like the dogs with hemophilia A due to intron 22 gene inversion, this animal model recapitulates a known human mutation that causes hemophilia A.[13]

Case Report

A male Old English Sheep Dog had frequent severe spontaneous bleeding in joints and soft tissues that did not respond to vitamin K therapy (empirical treatment for possible inadvertent broudifacoum ingestion). Baseline laboratory studies showed prolongation of the activated partial thromboplastin and whole-blood clotting times, with normal prothrombin time and von Willebrand factor levels. Results of testing at Cornell University Veterinary School showed both the factor VIII activity and antigen were less than 1% of normal, and a diagnosis of severe hemophilia A was made (Table 1).

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Table 1. Results of baseline coagulation laboratory studies in the subject dog

	Result	Normal reference range (canine)
Prothrombin time	12.5 s	11–15.5 s
Activated partial thromboplastin time	32.4 s	8.5–15.5 s
Thrombin clotting time	6.5 s	no reference range
Fibrinogen	310 mg/dL	150–490 mg/dL
Factor VIII (activity)	<0.3% (chromogenic assay)	no reference range
Von Willebrand factor (antigen)	94%	70%—180%

The dog had 14 bleeding events in 10 mo that required administration of approximately 10,000 units of factor VIII, prior to his donation to the University of North Carolina at Chapel Hill due to the high cost of treatment of his bleeding disorder. Since his donation, he has had 41 bleeding episodes resulting in 72 treatments with either plasma (2560 units) or recombinant canine factor VIII (40,168 units) for a total exposure of 42,728 units of factor VIII protein.

This dog has been tested for neutralizing antifactor VIII antibodies (by Bethesda inhibitor antibody assay) and for factor VIII binding antibodies (by ELISA) and has not developed any detectible antibody to factor VIII despite his extensive treatment with canine factor VIII while at the University of North Carolina. The dog was fertile and generated a litter with 5 confirmed female carriers of hemophilia A, as is expected for a sex-linked disorder.

Materials and Methods

Animals. All animals were handled in strict accordance with the USDA regulations and the standards described in the 2010 *Guide for the Care and Use of Laboratory Animals* (http://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf). All procedures and protocols were in accordance with institutional guidelines and approved by the IACUC at the University of North Carolina at Chapel Hill (Animal Subject Assurance no. A3410-01).

Genomic DNA isolation. DNA was prepared from whole blood anticoagulated with EDTA by using the Gentra Puregene Blood Kit (Qiagen, Valencia, CA). DNA was stored in 10 mM Tris, 1 mM EDTA (pH 8.0) at –20 °C after purification.

Preparation of cDNA for intron 22 inversion analysis. mRNA was prepared from peripheral blood mononuclear cells (purified from EDTA-anticoagulated whole blood) by using a Versagene RNA Purification Kit (Gentra Systems), and DNA was eliminated from the preparation by using DNase I (Sigma, St Louis, MO). cDNA was produced by reverse transcription of mRNA (High Capacity cDNA Reverse Transcriptase Kit, Life Technologies, Carlsbad, CA).

PCR amplification of genomic DNA and cDNA. PCR amplification was performed as described previously,[41] by using an automated thermal cycler and AmpliTaq enzyme (Perkin-Elmer Cetus, Norwalk, CT). Primers for PCR analysis are listed in Table 2.

DNA sequencing and analysis. Amplified PCR fragment DNA was sequenced directly by using a capillary sequencer (Prism 3100, ABI, Waltham, MA). Sequence data were analyzed by using Sequencher software (Gene Codes, Ann Arbor, MI). Factor VIII sequences from our hemophilia A dog and normal dogs were compared with current GenBank submissions by using the Entrez Genomes BLASTN and BLASTP programs.[1] The canine factor VIII polypeptide product was numbered with amino acid 1 as the alanine residue after the 19-amino-acid signal peptide of the protein encoded by the dog factor VIII mRNA (GenBank accession number, AF016234.[6]

Restriction endonuclease digests. PCR products from genomic DNA were digested with *Mlu*CI (recognition sequence, 5′ AATT 3′) under conditions specified by the manufacturer (New England Biolabs, Beverly, MA) before electrophoresis on 1% or 5% NuSieve agarose gels (Lonza Rockland, Rockland, ME) prepared with TBE buffer (pH 8.2).

Bethesda inhibitor-antibody assay. Neutralizing antibodies to factor VIII activity were evaluated by using the Nijmegen modifications to the Bethesda inhibitor assay, in which subject sample plasma underwent serial 2-fold dilutions (beginning at 1:2) in factor-VIII–deficient plasma; samples then were mixed with pH-buffered normal pooled canine plasma and incubated for 2 h at 37 °C, after which residual factor VIII activity was assayed. The reciprocal of the dilution at which 50% of the residual factor VIII seen in a control reaction (mix of normal plasma and factor VIII deficient plasma) remained is defined as the Bethesda inhibitor assay titer. This test is typically sensitive to approximately 0.5 Bethesda inhibitor assay units.[11]

Factor VIII binding antibody assay. The assay for IgG antibodies that bind factor VIII was adapted for canine factor VIII antibody detection from a previously published method for quantifying IgG antibodies that bind factor VIII in mice.[43] Briefly, plates were first coated with 0.1 unit recombinant canine factor VIII per well, blocked with bovine serum albumin, and dilutions of dog serum were then applied to the plate. After washing, bound antifactor VIII antibodies were detected by using peroxidase-conjugated sheep antidog IgG heavy chain (A40-118P, Bethyl Laboratories, Montgomery Texas), goat antidog IgG1 (A40-120P, Bethyl Laboratories), or sheep antidog IgG2 (A40-121P, Bethyl Laboratories). After incubation with 3,3′,5,5′-tetramethylbenzidine (peroxidase substrate), antibody binding was quantified by measuring the absorbance of light at 450 nm after adding sulfuric acid to stop the reaction.

Results

Analysis of intron 22 inversion as a potential cause of hemophilia A in the subject dog. The first step in our investigation was to amplify canine factor VIII cDNA sequence across exons 22 and 23, a region that is disrupted in dogs with the intron 22 inversion previously described in hemophilia A dogs at Chapel Hill[31] and Queens University.[24] Figure 1 A shows amplification of a 236-bp product in cDNA from a normal dog and the subject of this study, by using primers listed in Table 2. The amplified sequence in the subject hemophilia A dog was identical to the normal canine fac-

Table 2. Oligonucleotide primers used to amplify genomic DNA and create cDNA from hemophilia A and normal dogs

Primer location	Primer sequence	Amplimer size (bp)
Canine factor VIII exon 22	5′-GATCTCTTGGCACCGATGAT-3′	236
Canine factor VIII exon 23	5′-TGCAAACGGATGTACTGAGC-3′	
Canine factor VIII exon 22	5′-TCCAGCCTCTACGTGTCTCA-3′	200
ch8 (sequence following exon 22 in intron 22 inversion)	5′-TGGCTTTAAGCAGTGGACCT-3′	
Canine factor VIII exon 12	5′-TGATGTCAGACAAGAGAAATGTCA-3′	150
Canine factor VIII exon 12	5′-TGTGCATGATGTTAGAGAGTTGG-3′	

tor VIII cDNA sequence including exons 22 and 23. As expected, this portion of the canine factor VIII cDNA did not amplify in the dog with hemophilia A due to intron 22 inversion.

Conversely, PCR with primers from canine factor VIII exon 22 and the novel *ch8* sequence (AF361485) seen in the Chapel Hill intron 22 inversion mutation transcript failed to amplify in genomic DNA from a normal dog and the subject of this study but did amplify from cDNA derived from a descendant of the Chapel Hill hemophilia A dog, as expected (Figure 1 B). The sequence of the 200-bp amplimer derived from the Chapel Hill dog with intron 22 inversion hemophilia A corresponded exactly to that previously described[31] (that is, normal exon 22 sequence followed by *ch8* sequence at the exon 22–exon23 boundary).

Together, these findings indicate the hemophilia A mutation in our subject was not the intron 22 inversion previously reported. We then proceeded to determine the nucleotide sequence of the factor VIII cDNA.

Sequence analysis of entire factor VIII gene from the subject dog. All 26 exons of the canine factor VIII gene were sequenced in the subject of this report. Aside from various polymorphisms in the nucleic acid sequence that have been seen in normal dogs[6,24,31] or would be predicted not to change the underlying amino-acid coding sequence (Table 3), the cDNA sequence contained a C-to-T transition at nucleotide 1786 that changes a CGA (arginine) codon in exon 12 to a TGA (stop) codon (Figure 2). The mutated nucleotide is a member of a CpG dinucleotide, and the mutation eliminates a *Taq*I restriction endonuclease cleavage site (TCGA) in the genomic DNA, as can occur with such transitions in hemophilia A and other diseases.[13,37,39] The mutation also creates a new *Mlu*CI restriction endonuclease cleavage site (AATT). Figure 3 shows the results of *Mlu*CI digestion of a 150-bp amplimer containing the site of the mutation for the subject of our study as well as normal and heterozygous female carrier hemophilia A dogs, indicating the gain of a *Mlu*CI cleavage site in the subject of our study.

The DNA sequence of the subject hemophilia A dog contained two additional mutations (besides the stop codon) that altered the amino acid sequence of factor VIII. Both affected amino acids are in exon 14, an exon that is part of the B domain of the factor VIII protein, which is dispensable for coagulation factor activity. These variant forms (serine 909 and proline 1184) have both been seen in the normal dog factor VIII sequence and have no apparent significance with regard to factor VIII activity (Table 3 and Figure 4).

Analysis for inhibitor antibodies to factor VIII in the subject dog. Tests for inhibitor antibodies to factor VIII were performed on 2 samples from 2010 (shortly after the dog was diagnosed with hemophilia A) and on 2 samples from 2015 (after extensive exposure to factor VIII) by using a Bethesda inhibitor antibody assay. All 4 samples displayed no inhibition of factor VIII activity (less than 0.8 Bethesda inhibitor assay units). Similarly, ELISA measurement of factor VIII binding antibodies on the same samples showed no change in baseline IgG2 antibody levels (approximately 250 ng/mL) in all samples.

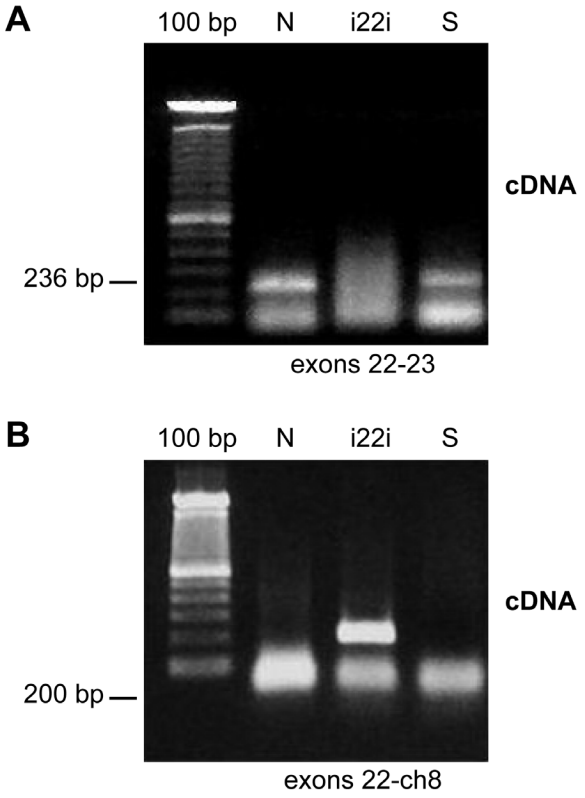

Figure 1. (A) Amplification of exons 22 and 23 of the canine factor VIII cDNA. cDNA purified from a normal dog (N), the Chapel Hill intron 22 inversion hemophilia A dog (i22i), and the subject of this report (S) underwent PCR analysis using primers in exons 22 and 23. The expected length of the exon 22–23 product is 236 bp. (B) Amplification of canine factor VIII exon 22 and *ch8* from genomic DNA. Genomic DNA purified from a normal dog (N), the Chapel Hill intron 22 inversion hemophilia A dog (i22i), and the subject of this report (S) underwent PCR analysis using primers in exons 22 and 23. The expected length of the exon 22–ch8 product is 200 bp.

Discussion

Our investigation revealed a point mutation in the canine factor VIII gene that creates a termination codon in place of arginine 577

Table 3. Summary of variants in canine factor VIII coding sequence of normal and hemophilia A dogs

	Exon number																
	1	7	7	8	10	12	12	12	14	14	14	14	14	14	15	16	25
Nucleotide number in reference sequence (NM_001003212)	141	877	909	1094	1440	1774	1786	1807	2782	2868	2943	3608	4278	5086	5292	5490	6866
Predicted normal dog coding sequence (XM_005640988.2) from dog X chromosome genomic sequence (NW_003726126.1)	c	a	a	a	t	t	c	a	a	t	g	c	a	a	c	g	g
mRNA from kidney and spleen (NM_001003212) and liver (reference 24) of normal dog	t	g	g	g	a	c	c	g	g	c	a	t	g	g	t	a	a
EST DN369010.1 (nucleotides 5013–5653)	–	-	-	-	-	-	-	-	-	-	-	-	-	a	t	g	-
Male hemophilia A dog (current study)	c	a	a	a	t	t	t	a	a	t	g	c	a	a	c	g	g
Heterozygous normal female dog (current study)	c/t	a	a	a	t	t	c	a	a/g	t	g/a	c/t	a	a	c/t	g	g
Amino acid change	syn	R>G	syn	N>G	N>K	F>L	R>X	N>D	S>G	syn	syn	P>L	syn	N>D	syn	syn	G>D

syn, synonymous

in exon 12 of the normal factor VIII protein. This mutation has not been seen in previously characterized hemophilia A dogs, specifically the intron 22 inversion dogs characterized at Chapel Hill[31] or at Queens University (Ontario, Canada).[24] We also detected 2 additional polymorphisms (serine 909 and proline 1184) in the subject dog that have been seen in normal dogs and that do not result in reduced factor VIII activity or contribute to the hemophilia A phenotype.[6]

The subject dog's factor VIII mutation (arginine 577→stop) replicates the equivalent point mutation (arginine 602→stop, nucleotide 1804) that has previously been seen in exon 12 of the factor VIII gene in humans with severe hemophilia A.[37] Both this subject dog and humans with the corresponding mutation have severe hemophilia A, no detectable factor VIII activity, and no detectable factor VIII antigen circulating in the blood. According to the Factor VIII Variant Database (http://www.factorviii-db.org/; formerly the Haemophilia A Mutation, Structure, Test and Resource Site),[13] this mutation has been detected in at least 20 individual human patients with severe hemophilia A from 9 different countries; of the 20 patients reported therein, 11 are stated to lack inhibitor antibodies to factor VIII, 2 have inhibitor antibodies to factor FVIII, and there is no reported information on the remainder.[3,7,9,19,25,27,29,30,36,37,42,46,51] The apparently spontaneous occurrence of this mutation in multiple individuals might be explained as a consequence of the inherent instability of CpG dinucleotides, which are prone to deamination of cytosine resulting in its transition to thymidine.[8,50] It is therefore unsurprising that the first point mutation found to cause hemophilia A in dogs would be in a CpG dinucleotide sequence, as we describe in the current report.

We predict that a mutation at amino acid 577 of the canine factor VIII in which the arginine is converted to glutamine could occur by deamination in the cytosine in the opposite strand of DNA (for example, CGA→CAA, Arg→Gln). Instability of a CpG dinucleotide has been shown to convert arginine to either a stop codon or to glutamine (depending on which DNA strand undergoes cytosine deamination) in the factor VIII gene in humans at a different arginine (amino acid 2307 in exon 26) of the human factor VIII protein.[16] The severity of the bleeding phenotype that might result from a mutation leading to conversion of arginine to

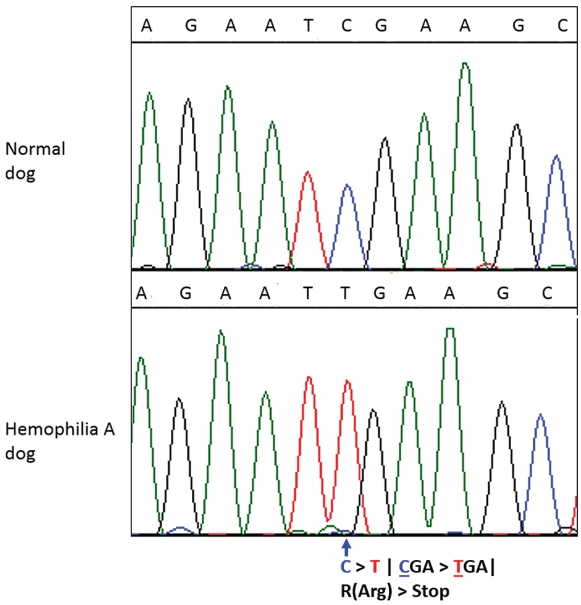

Figure 2. Nucleotide sequences of factor VIII cDNA from a normal dog and a hemophilia A dog. The hemophilia A dog shows substitution of T for C (resulting in a new stop codon) at position 1768 (Arg577) of the factor VIII cDNA.

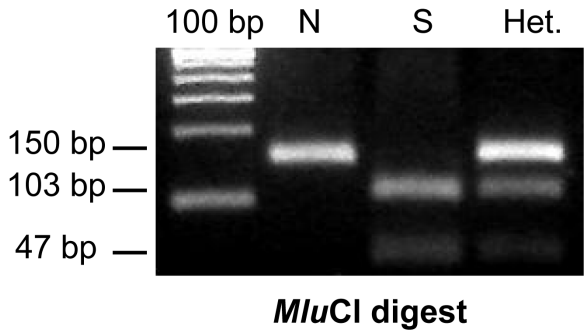

Figure 3. *Mlu*CI digest of the 150-bp PCR amplimer from exon 12 that contains the C→T mutation (Arg577→ stop) in a normal dog (N), the subject of this study (S), and a heterozygous carrier for the Arg577→stop mutation (Het). The mutation creates an *Mlu*CI cleavage site, resulting in digest fragments of 103 and 47 bp in the subject's DNA, whereas the normal DNA remains undigested, and the DNA from a heterozygous female dog yields fragments corresponding to both undigested (normal) and digested (hemophilia A) DNA sequences.

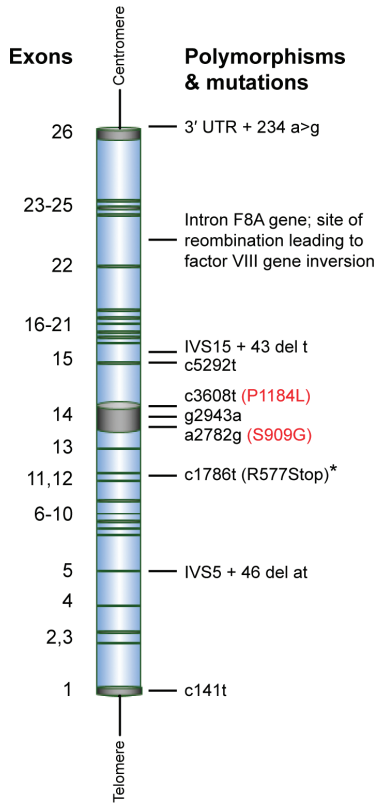

Figure 4. Canine factor VIII gene on the X chromosome, with exons indicated at left and mutations and polymorphisms indicated on the right. Factor VIII gene is oriented such that exons 1 through 26 run from the telomere to the centromere of the X-chromosome. The asterisk indicates the early termination codon found in subject of this study that was associated with hemophilia A. Red text indicates polymorphisms that lead to changes in the amino acid sequence of the factor VIII protein that are not associated with hemophilia A.

glutamine in a hemophilic dog is unclear. Notably, humans with a mutation at the adjacent nucleotide convert arginine 583 to glycine, and the bleeding phenotype is mild.[23,45,49]

Dogs with severe hemophilia A, like humans, may develop neutralizing antibodies (inhibitors) to factor VIII after exposure to normal factor VIII through replacement therapy to treat bleeding episodes or as prophylaxis against bleeding. This particular dog has not, thus far, demonstrated evidence of an inhibitor antibody despite 72 treatments with canine factor VIII for bleeding (40,168 units of recombinant B-domain and 2560 units of plasma-derived canine factor VIII over 5 y). No prediction of susceptibility to inhibitor development can be made from the (negative) results of a single dog, but it would be informative to see whether other hemophilia A dogs with this mutation made inhibitor antibodies, as occurs in some members of the 2 dog colonies with intron 22 inversions.[6,24,31] The type of genetic mutation that causes hemophilia A in humans seems to be a strong predictor of the risk to develop inhibitors after exposure to factor VIII protein.[17,37] This same scenario is likely to be the case for dogs with hemophilia A and can now be tested in dogs with the mutation described in this report. Furthermore, changes in the factor VIII nucleic acid sequence that do not cause hemophilia but that change the amino acid sequence of the factor VIII protein have recently been argued to contribute to the risk of inhibitor antibody development when patients with hemophilia A are exposed to factor VIII that does not match their underlying sequence.[47] With that understanding, dogs with the hemophilia A mutation that we describe here may ultimately prove to be useful animal models (along with those with the intron 22 gene inversion) in which to test this hypothesis through provocative transfusion strategies and characterization of the specificity of antibodies that arise against canine factor VIII after such maneuvers.

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References

1. **Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res **25:**3389–3402.
2. **Bi L, Lawler AM, Antonarakis SE, High KA, Gearhart JD, Kazazian HH Jr.** 1995. Targeted disruption of the mouse factor 8 gene produces a model of haemophilia A. Nat Genet **10:**119–121.
3. **Bogdanova N, Markoff A, Pollmann H, Nowak-Gottl U, Eisert R, Wermes C, Todorova A, Eigel A, Dworniczak B, Horst J.** 2005. Spectrum of molecular defects and mutation detection rate in patients with severe hemophilia A. Hum Mutat **26:**249–254.
4. **Booth CJ, Brooks MB, Rockwell S.** 2010. Spontaneous coagulopathy in inbred WAG/RijYcb rats. Comp Med **60:**25–30.
5. **Booth CJ, Brooks MB, Rockwell S, Murphy JW, Rinder HM, Zelterman D, Paidas MJ, Compton SR, Marks PW.** 2010. WAG-F8^{m1Ycb} Rats harboring a factor 8 gene mutation provide a new animal model for hemophilia A. J Thromb Haemost **8:**2472–2477.
6. **Cameron C, Notley C, Hoyle S, McGlynn L, Hough C, Kamisue S, Giles A, Lillicrap D.** 1998. The canine factor 8 cDNA and 5′ flanking sequence. Thromb Haemost **79:**317–322.
7. **Chan V, Pang A, Chan TPT, Chan VW-Y, Chan TK.** 1996. Molecular characterization of haemophilia A in southern Chinese. Br J Haematol **93:**451–456.
8. **Cooper DN, Youssoufian H.** 1988. The CpG dinucleotide and human genetic disease. Hum Genet **78:**151–155.
9. **David D, Ventura C, Moreira I, Diniz MJ, Antunes M, Tavares A, Araújo F, Morais S, Campos M, Lavinha J, Kemball-Cook G.** 2006.

The spectrum of mutations and molecular pathogenesis of hemophilia A in 181 Portuguese patients. Haematologica **91:**840–843.
10. **Dodds WJ.** 1970. Canine von Willebrand's disease. J Lab Clin Med **76:**713–721.
11. **Duncan E, Collecutt M, Street A.** 2013**.** Nijmegen-Bethesda assays to measure factor 8 inhibitors, Chapter 24, p 321–333. In: Monagle P, editor. Haemostasis: methods and protocols, methods in molecular biology, vol 992, New York (NY): Springer Science and Business Media.
12. **Evans JP, Brinkhous KM, Brayer GD, Reisner HM, High KA.** 1989. Canine hemophilia B resulting from a point mutation with unusual consequences. Proc Natl Acad Sci USA **86:**10095–10099.
13. **University College London**. [Internet]. Factor 8 variant database**.** [Cited 22 October 2015]. Available at: http://www.factorviii-db.org/.
14. **Fass DN, Bowie EJ, Owen CA Jr, Zollman PE.** 1979. Inheritance of porcine von Willbrand's disease: study of a kindred of over 700 pigs. Blood **53:**712–719.
15. **Giles AR, Tinlin S, Greenwood R.** 1982. A canine model of hemophilic (factor 8:C deficiency) bleeding. Blood **60:**727–730.
16. **Gitschier J, Wood WI, Shuman MA, Lawn RM.** 1986. Identification of a missense mutation in the factor 8 gene of a mild hemophiliac. Science **232:**1415–1416.
17. **Gouw SC, van den Berg HM, Oldenburg J, Astermark J, de Groot PG, Margaglione M, Thompson AR, van Heerde W, Boekhorst J, Miller CH, le Cessie S, van der Bom JG.** 2012. F8 gene mutation type and inhibitor development in patients with severe hemophilia A: systematic review and meta-analysis. Blood **119:**2922–2934.
18. **Graham JB, Buckwalter JA, Hartley LJ, Brinkhous KM.** 1949. Canine hemophilia: Observations on the course, the clotting anomaly, and the effect of blood transfusions. (1949). J Exp Med **90:**97–111.
19. **Green PM, Bagnall RD, Waseem NH, Giannelli F.** 2008. Haemophilia A mutations in the UK: results of screening one-third of the population. Br J Haematol **143:**115–128.
20. **Griggs TR, Webster WP, Cooper HA, Wagner RH, Brinkhous KM.** 1974. Von Willebrand factor: gene dosage relationships and transfusion response in bleeder swine—a new bioassay. Proc Natl Acad Sci USA **71:**2087–2090.
21. **Gunasekera D, Ettinger RA, Nakaya Fletcher S, James EA, Liu M, Barrett JC, Withycombe J, Matthews DC, Epstein MS, Hughes RJ, Pratt KP, Personalized Approaches to Therapies for Hemophilia (PATH) Study Investigators.** 2015. Factor 8 gene variants and inhibitor risk in African American hemophilia A patients. Blood **126:**895–904.
22. **Haberichter SL, Merricks EP, Fahs SA, Christopherson PA, Nichols TC, Montgomery RR.** 2005. Reestablishment of VWF-dependent Weibel–Palade bodies in VWD endothelial cells. Blood **105:**145–152.
23. **Hill M, Deam S, Gordon B, Dolan G.** 2005. Mutation analysis in 51 patients with haemophilia A: report of 10 novel mutations and correlations between genotype and clinical phenotype. Haemophilia **11:**133–141.
24. **Hough C, Kamisue S, Cameron C, Notley C, Tinlin S, Giles A, Lillicrap D.** 2002. Aberrant splicing and premature termination of transcription of the FVIII gene as a cause of severe canine hemophilia A: similarities with the intron 22 inversion mutation in human hemophilia. Thromb Haemost **87:**659–665.
25. **Jayandharan GR, Nair SC, Poonnoose PM, Thomas R, John J, Keshav SK, Cherian RS, Devadarishini M, Lakshmi KM, Shaji RV, Viswabandya A, George B, Mathews V, Chandy M, Srivastava A.** 2009. Polymorphism in factor 8 gene modifies phenotype of severe haemophilia. Haemophilia **15:**1228–1236.
26. **Kundu RK, Sangiorgi F, Wu LY, Kurachi K, Anderson WF, Maxson R, Gordon EM.** 1998. Targeted inactivation of the coagulation factor 9 gene causes hemophilia B in mice. Blood **92:**168–174.
27. **Liang Y, Zhao Y, Yan M, Fan XP, Xiao B, Liu JZ.** 2009. Prenatal diagnosis of haemophilia A in China. Prenat Diagn **29:**664–667.
28. **Lin HF, Maeda N, Smithies O, Straight DL, Stafford DW.** 1997. A coagulation factor 9-deficient mouse model for human hemophilia B. Blood **90:**3962–3966.
29. **Lin SY, Su YN, Hung CC, Tsay W, Chiou SS, Chang CT, Ho HN, Lee CN.** 2008. Mutation spectrum of 122 hemophilia A families from Taiwanese population by LD-PCR, DHPLC, multiplex PCR and evaluating the clinical application of HRM. BMC Med Genet **9:**53–66.
30. **Liu J, Zhang Y, Wang H, Huang W, Cao W, Wang X, Qu B, Wang H, Shao H, Wang Z, Chen L, Huang W.** 1997. Molecular characterization of genetic defects in hemophilia in Shanghai. Zhonqhua Xue Ye Xue Za Zhi **18:**464–467. [Article in Chinese].
31. **Lozier JN, Dutra A, Pak E, Zhou N, Zheng Z, Nichols TC, Bellinger DA, Read M, Morgan RA.** 2002. The Chapel Hill hemophilia A dog colony exhibits a factor 8 gene inversion. Proc Natl Acad Sci USA **99:**12991–12996.
32. **Lozier JN, Nichols TC.** 2013. Animal models of hemophilia and related bleeding disorders. Semin Hematol **50:**175–184.
33. **Nichols TC, Hough C, Agerso H, Ezban M, Lillicrap D.** 2016. Canine models of inherited bleeding disorders in the development of coagulation assays, novel protein replacement and gene therapies. J Thromb Haemost.**14:**894-905.
34. **Nielsen LN, Wiinberg B, Hager M, Holmberg HL, Hansen JJ, Roepstorff K, Tranholm M.** 2014. A novel F8 (-/-) rat as a translational model of human hemophilia A. J Thromb Haemost **12:**1274-1282.
35. **Mauser AE, Whitlark J, Whitney KM, Lothrop CD Jr.** 1996. A deletion mutation causes hemophilia B in Lhasa Apso dogs. Blood **88:**3451–3455.
36. **Oldenburg J, El-Maarri O, Schwaab R.** 2002. Inhibitor development in correlation to factor 8 genotypes. Haemophilia **8 Suppl 2:**23–29.
37. **Pattinson JK, Millar DS, McVey JH, Grundy CB, Wieland K, Mibashan RS, Martinowitz U, Tan-Un K, Vidaud M, Goossens M, Sampietro M, Mannucci PM, Krawczak M, Reiss J, Zoll B, Whitmore D, Bowcock S, Wensley R, Ajani A, Mitchell V, Rizza C, Maia R, Winter P, Mayne EE, Schwartz M, Green PJ, Kakkar VV, Tuddenham EGD, Cooper DN.** 1990. The molecular genetic analysis of hemophilia A: a directed search strategy for the detection of point mutations in the human factor 8 gene. Blood **76:**2242–2248.
38. **Porada CD, Sanada C, Long CR, Wood JA, Desai J, Frederick N, Millsap L, Bormann C, Menges SL, Hanna C, Flores-Foxworth G, Shin T, Westhusin ME, Liu W, Glimp H, Zanjani ED, Lozier JN, Pliska V, Stranzinger G, Joerg H, Kraemer DC, Almeida-Porada G.** 2010. Clinical and molecular characterization of a re-established line of sheep exhibiting hemophilia A. J Thromb Haemost **8:**276–285.
39. **Reiner AP, Thompson AR.** 1992. Screening for nonsense mutations in patients with severe hemophilia A can provide rapid, direct carrier detection. Hum Genet **89:**88–94.
40. **Sabatino DE, Nichols TC, Merricks E, Bellinger DA, Herzog RW, Monahan PE.** 2012. Animal models of hemophilia. Prog Mol Biol Transl Sci **105:**151–209.
41. **Saiki RK, Scharf S, Faloona F, Mullis KB, Hort GT, Erlich HA, Arnheim N.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230:**1350–1354.
42. **Salviato R, Belvini D, Radossi P, Sartori R, Pierobon F, Zanotto D, Zanon E, Castaman G, Gandini G, Tagariello G.** 2007. F8 gene mutation profile and ITT response in a cohort of Italian haemophilia A patients with inhibitors. Haemophilia **13:**361–372.
43. **Sherman A, Su J, Lin S, Wang X, Herzog RW, Daniell H.** 2014. Suppression of inhibitor formation against F8 in a murine model of hemophilia A by oral delivery of antigens bioencapsulated in plant cells. Blood **124:**1659–1668.
44. **Schwarz J, Astermark J, Menius ED, Carrington M, Donfield SM, Gomperts ED, Nelson GW, Oldenburg J, Pavlova A, Shapiro AD, Winkler CA, Berntorp E, Hemophilia Inhibitor Genetics Study Combined Cohort.** 2013. F8 haplotype and inhibitor risk: results

from the Hemophilia Inhibitor Genetics Study (HIGS) combined cohort. Haemophilia **19:**113–118.

45. **Theophilus BDM, Enayat MS, Williams MD, Hill FGH.** 2001. Site and type of mutations in the factor 8 gene in patients and carriers of haemophilia A. Haemophilia **7:**381–391.
46. **Timur AA, Gurgey A, Aktuglu G, Kavalki K, Canatan D, Olek K, Caglayan SH.** 2001. Molecular pathology of Hemophilia A in Turkish patients: identification of 36 independent mutations. Haemophilia **7:**475–481.
47. **Viel KR, Ameri A, Abshire TC, Iyer RV, Watts RG, Lutcher C, Channell C, Cole SA, Fernstrom KM, Nakaya S, Kasper CK, Thompson AR, Almasy L, Howard TE.** 2009. Inhibitors of factor 8 in black patients with hemophilia A. N Engl J Med **360:**1618–1627.
48. **Wang L, Zoppe M, Hackeng TM, Griffin JH, Lee K-F, Verma IM.** 1997. A factor 9-deficient mouse model for hemophilia B gene therapy. Proc Natl Acad Sci USA **94:**11563–11566.
49. **Waseem NH, Bagnall R, Green PM, Gianelli F.** 1999**.**Start of UK confidential haemophilia A database: analysis of 142 patients by solid phase fluorescent chemical cleavage of mismatch. Haemophilia Centres. Thromb Haemost **81:**900–905.
50. **Youssoufian H, Kazazian HH Jr, Phillips DG, Aronis S, Tsifitis G, Brown VA, Antonarakis SE.** 1986. Recurrent mutations in haemophilia A give evidence for CpG mutation hotspots. Nature **324:**380–382.
51. **Zhang YZ, Liu JX, Shao HZ, Chi ZW, Wang HL, Chen SJ, Chen Z, Wang ZY, Huang W.** 1999. Characterization of genetic defects of hemophilia A in mainland China. Genet Anal **15:**205–207.