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Canine factor VII deficiency: lessons learned in applying methods-based laboratory proficiency testing

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Abstract. Canine inherited factor VII deficiency is a mild-to-moderate, inherited coagulopathy that affects several breeds of dog. We identified 2 polymorphisms near the disease-causing *F7* gene mutation, one of which interfered with testing in several Beagles by causing allele dropout of the normal, wild-type allele. In the absence of an external proficiency program among veterinary genetic testing laboratories, implementation of an internal proficiency program, which requires 2 independent methods for genotyping dogs at any given locus, was further enhanced by ensuring minimally non-overlapping primer pairs between the 2 assays. After redesign of our clinical tests, all dogs were re-examined, and the correct genotypes were identified. These changes ensure higher accuracy in future testing of the *F7* mutation.

Key words: Dogs; factor VII deficiency; genetic testing; laboratory proficiency testing.

Factor VII, encoded by the *F7* gene, is a vitamin K–dependent glycoprotein that participates in the blood coagulation cascade. Clotting disorders attributed to factor VII deficiency have been identified in several breeds of dog, including Beagles,[1,2,9,14,16] Alaskan Malamutes,[8] Alaskan Klee Kai dogs,[4] and others.[3,7] Canine inherited factor VII deficiency is an autosomal recessive condition caused by a G to A missense mutation in the *F7* gene, resulting in substitution of glycine 96 by glutamic acid in exon 5.[1] This amino acid change results in mild-to-moderate bleeding in these dogs, with postsurgical hemorrhage in most dogs without prior history of spontaneous bleeding.[1] Unlike more serious coagulopathies, such as type 3 von Willebrand disease in which dogs have a history of impaired hemostasis, owners and veterinarians have no reason to expect postsurgical complications in dogs with factor VII deficiency.[1,15] A phenotypic diagnosis can be made based on increased prothrombin time (PT), defined as any value greater than the valid upper reference limit of the laboratory performing the testing, in conjunction with a normal activated partial thromboplastin time (aPTT).[1,15] Without genetic testing for factor VII deficiency, other differential diagnoses, such as the early stages of hepatic disease or anticoagulant rodenticide exposure, cannot be ruled out.[15] Additionally, given that coagulation times can vary among affected dogs, and carriers would be normal with these clinical assays, a molecular genetic test that can accurately identify both the heterozygous and the homozygous mutant individuals is more useful for dog breeders. Genetic testing laboratories recognize that the presence of a common mutation in multiple breeds of dog is amenable to the development of a single, reliable test for factor VII deficiency across many breeds.

Initial identification of the factor VII deficiency mutation was achieved by direct sequencing of the *F7* gene in an affected Beagle; additional dogs were screened by using a restriction digest run on polyacrylamide gel.[1] For laboratory assays, robust tests can be developed to directly interrogate the mutation site and distinguish wild-type alleles from mutant alleles. For example, a PCR-based assay was developed in which the digested PCR products were visualized on agarose gel.[2] This method was simpler than an earlier method,[1] and was used to screen a large colony of dogs for heterozygous and homozygous mutant individuals. In our laboratory, we utilized in-house, primer-prediction software to identify primers that flanked the *F7* mutation at chr22:60,578,895 (CanFam3.1) and developed 2 routine genotyping methods to be used as assays, in keeping with our internal proficiency quality assurance protocols.[10–12]

From February 2015 through November 2017, we tested DNA extracted from buccal swabs via routine methods[12] from 43 Beagles using our 2 assays for factor VII deficiency. We identified 11 homozygous normal dogs, 12 heterozygous (carrier) dogs, and 20 homozygous mutant (at-risk/affected) dogs. In November 2017, we were contacted by a client who received 5 homozygous mutant (at-risk/affected) results and

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Figure 1. Partial sequence of the *F7* gene showing the location of the polymorphisms relative to the mutation and primer sequences (UCSC Genome Browser Dog Sep. 2011, Broad/CanFam3.1/canFam3, Assembly, http://genome.ucsc.edu). Lowercase nucleotides indicate intronic sequence; uppercase nucleotides indicate exonic sequence. The G>A disease-associated mutation at chr22:60,578,895 is shown in bold and underlined. The 2 polymorphisms at positions chr22:60,578,848 and chr22:60,578,849 are in red and bold. Orange indicates the non-overlapping sequence from the original primer pairs for assay 1. Green indicates the non-overlapping sequence from the original primer pairs for assay 2. Gray indicates overlap between primers used for original assays 1 and 2. Blue indicates new primer pairs for assay 1. Yellow indicates new primer pairs for assay 2. Blue and yellow primers were also used for direct sequencing as indicated in Table 1.

Table 1. Genotyping primers used to detect F7 mutations for canine factor VII deficiency.

Source	Forward	Reverse
Original primers		
Assay 1	CTGTCCCCTT***CC***TGTCCTCAGA	CCTCTGCGTGGTCACTG
Assay 2	CCTT***CC***TGTCCTCAGATAAG	TGGTCACTGCAGTACTGCTG
New primers		
Assay 1 and sequencing primers—set 2	AAGCACCACTATTGTGCAGAAGG	GCTCCTTGGGAATGAAGCAAAAC
Assay 2 and sequencing primers—set 1	CAGGTCACCCTTCTGGATCAAAC	TTACCTATGGGCATGCAGGACA
Carlstrom et al.[2]	CTCTCAGCCTGTCCCCTT***CC***TGTTCTCAGATAAG	GAACGCCTGGTCTCTGCGTGGTCAC

Nucleotides in bold italic indicate variant C>T single nucleotide polymorphisms at positions chr22:60,578,848 and chr22:60,578,849.

2 heterozygous (carrier) results for a litter of 7 puppies, although the dam and sire were tested at another laboratory and genotyped as homozygous mutant and homozygous normal, respectively. Such a breeding should only produce heterozygous carrier puppies. Based on our internal protocol for investigating discrepant results, we performed a parentage analysis between the offspring in question, the homozygous mutant dam, and the homozygous normal sire. The parentage analysis confirmed the homozygous normal dog to be the sire (data not shown). To investigate further, DNA samples from the 5 offspring, the dam, and the sire were sent for sequencing of the region of the *F7* gene encompassing the primers used for our F7 assays (Macrogen USA, Rockville, MD; Fig. 1). Sequencing identified an intronic C>T variant at site chr22:60,578,848 that sits under the annealing site of the forward primer used in our original PCR assays as well as the forward primer described elsewhere[2] (Table 1). The sire is heterozygous for this variant, and all 5 puppies originally genotyped as homozygous mutant are also heterozygous. Presumably, this polymorphism caused allele dropout (non-amplification) of the normal, paternally inherited allele.

The discovery of this polymorphism located within our forward primers for both genotyping assays prompted us to develop new primer pairs outside of this region to avoid possible allele dropout. The new primer pairs (Table 1, Fig. 1) were used to rescreen 36 Beagles that were previously reported as homozygous normal or homozygous mutant by our laboratory and therefore had the potential to have been misclassified because of allele dropout. After retesting, 7 Beagles were reclassified from homozygous mutant (at-risk /affected) to heterozygous (carriers). Five of these were the original puppies that we sequenced and 2 were unrelated Beagles from 2 different owners.

Additional targeted sequencing was performed on 429 dogs of various breeds (MiSeq system, Illumina, San Diego, CA) using primers shown in Table 1. Raw sequences were aligned using BWA-MEM, and regions of interest were examined using SAMtools.[6] Sequence data revealed an adjacent C>T polymorphism at chr22:60,578,849 that was not found in the Beagles studied (Table 2). Of 429 dogs screened, 90 were found to carry one or both of the C>T polymorphisms. The chr22:60,578,848 polymorphism identified in the Beagle was also identified in 69 individuals from 18 other breeds. Among these, 56 were heterozygous and 13 were homozygous. The chr22:60,578,849 polymorphism was identified in 19 individuals from 15 breeds. Among

Table 2. Breeds and individuals identified with one or both polymorphisms for the *F7* gene mutation causing canine factor VII deficiency.

Breed	60578848C>T	60578849C>T	60578848C>T & 60578849C>T
American Staffordshire Terrier	2		
Australian Labradoodle	13	2	1
Australian Shepherd	6		
Belgian Malinois	9	1	
Border Collie	3	1	
Boykin Spaniel		1	
Brittany		1	
Cane Corso		1	
Chesapeake Bay Retriever	1		
Chinese Crested	1		
Cocker Spaniel	1		
Doberman Pinscher		1	
English Shepherd	1		
German Shepherd Dog	4		
Giant Schnauzer		1	
Golden Retriever	7	2	
Labrador Retriever	1	2	
Miniature American Shepherd	8		
Miniature Australian Shepherd	6		
Miniature Poodle	1		1
Nova Scotia Duck Tolling Retriever		1	
Poodle		1	
Standard Poodle	2	1	
Toy Australian Shepherd	1		
Toy Poodle	2		
Wirehaired Pointing Griffon		1	
Yorkshire Terrier		1	
Total	69	19	2

these, 17 were heterozygous and 2 were homozygous. Finally, 2 additional individuals from different breeds were heterozygotes for both polymorphisms. Although these polymorphisms were found in 27 breeds, most of the breeds are not known to carry the mutation associated with factor VII deficiency. However, the chr22:60,578,849 C>T polymorphism was identified in the Giant Schnauzer, which is a breed that carries the disease-associated G>A missense mutation in *F7*. Thirteen Giant Schnauzers previously tested for the *F7* mutation were retested with the new primer sets and all were homozygous normal, as originally reported to clients.

We developed our own internal proficiency program that includes 2 independent testing methods for each disease or trait test offered in our laboratory.[10] Although we cannot completely eliminate the risk of allele dropout, this program allows us to recognize potential allele dropout by comparing genotyping results across testing platforms. Because predicting every polymorphism in the canine genome is impossible, comparing results across methods within an individual sample can help ensure that normal genomic variation is less likely to affect target amplification. However, an essential component to this program is the use of minimal or non-overlapping primers between the 2 methods. In the case presented herein, we failed to recognize that the 2 genotyping methods that we were using for the *F7* mutation had significant overlapping primers that, unfortunately, also overlapped an apparently, relatively frequent polymorphism in the Beagle population. This led to reporting errors and significant time and cost involved in correcting the assays and regaining the trust of our clients.

Because genetic testing is relatively simple, quick, and affordable, its use is increasing and can provide additional information to aid in decisions regarding breeding, as well as diagnosis and treatment of other genetic disorders. The importance of assay development that minimizes allele dropout is crucial to accurate testing. Although it is impossible to completely avoid all sites of polymorphism during primer development, laboratories can reduce the chance of developing primers overlapping regions of polymorphism by screening repositories that contain this information, such as the Database of Single Nucleotide Polymorphisms (dbSNP) and European Bioinformatics Institute (EMBL-EBI) databases.[5,13] The dbSNP was a repository for non-human mam-

malian SNPs until 2018, and EMBL-EBI has a repository for non-human SNPs. Both polymorphisms were reported in the dbSNP in 2015 (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD: dbSNP accession rs851310581and rs850788751; dbSNP Build ID: 151. Available from: http://www.ncbi.nlm.nih.gov/SNP/). Although our assay was developed in 2013, regular review of these databases would have alerted us to the possible risk for allele dropout and allowed us to identify potential cases to review and confirm the results.

Laboratories should be committed to the highest accuracy possible and develop safeguards, including multiple independent assays, to prevent and clarify discrepant results.[10] Additionally, laboratories should work with clients to understand whether any discrepancies reflect true biology, as in our case, non-paternity, or sample mishandling by either the client or the laboratory. Because veterinary genetic testing laboratories are not, to date, required to be accredited, and have no regulatory oversight, it is up to individual laboratories to implement systems and protocols to minimize errors. We recognize that genetic testing results are used to make breeding decisions and sometime irreversible decisions, such as neutering or euthanasia. As such, for every mutation for which we offer testing, we chose to implement an on-going methods-based proficiency testing program.[10] This testing program includes interrogating every mutation region utilizing 2 independent methods regardless of the genotypes and is applied across all samples, not just when heterozygous or at-risk results are obtained. To prevent the future development of primers overlapping areas with known polymorphisms, SNP databases such as dbSNP and EMBL-EBI should be reviewed during the development of primers, and any new genetic variants should be submitted to public SNP databases such as EMBL-EBI European Variation Archive (https://www.ebi.ac.uk/eva/?Home). Additionally, there should be collaboration among genetic testing laboratories to share the identification of polymorphic variants or other technical issues that may impact ongoing test performance, because the ultimate goal of all testing laboratories is to provide accurate genetic results and thereby improve the health of dogs.

Declaration of conflicting interests

LG Shaffer is the owner, and all authors are employees of Genetic Veterinary Sciences, DBA Paw Print Genetics, which provides genetic screening for inherited disorders to breeders, owners, and veterinarians.

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