Screening for *COL5A1* Variants Responsible for Canine Ehlers Danlos Syndrome

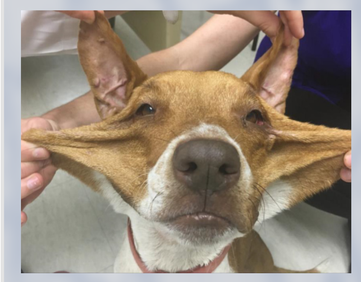
Allison Sanders,[1] Gary S. Johnson,[1] Tendai Mutangadura,[1] Garrett Bullock,[1] Sarah Brickey,[1] Leah Cohn,[2] Jared Jaffey[2]

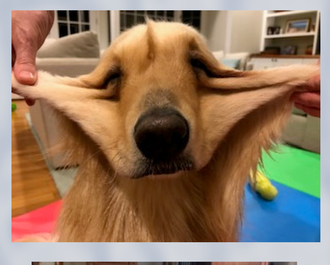
Departments of Veterinary Pathobiology,[1] and Veterinary Medicine and Surgery,[2] College of Veterinary Medicine, University of Missouri, Columbia, Missouri

Veterinary Research Scholars Program
University of Missouri

Abstract

The Ehlers-Danlos syndromes (EDS) are a heterogeneous group of hereditary connective tissue disorders characterized by joint hypermobility and skin hyper-extensibility and fragility. Our laboratory has been working to identify the molecular genetic causes for EDS by generating whole genome sequences with DNA from affected dogs. This strategy has facilitated our identification of novel causal variant alleles in *COL5A1* in three of six dogs analyzed so far. Budgetary constraints have not allowed us to generate whole genome sequences for all 27 of the affected dog samples sent to our lab from around the world. The focus of this study is to develop a new method to pre-screen DNA from dogs for *COL5A1* variants so that our limited funds for whole genome sequencing can be used to identify canine-EDS causing variants in other genes. We have devised a method for pre-screening for *COL5A1* variants based on a combination of (1) long PCR amplification of *COL5A1* exon clusters, (2) templates of pooled DNA from affected dogs, and (3) Illumina next generation sequencing.





Background

The EDSs are a group of hereditary disorders that affect connective tissues supporting the skin, bones, blood vessels, as well as other organs and tissues. At least 19 genes have been found to harbor mutations responsible for human EDS. Heterozygous mutations in human *COL5A1* cause the classic clinical signs of hypermobile joints and elastic, fragile skin. So far, no mutations responsible for canine EDS have been reported. We have generated whole genome sequences with DNA from 6 dogs with EDS and identified heterozygous truncating mutations in *COL5A1* in 3 of them.

Methods

DNA concentrations were determined with a UV spectrometer. PCR primers were designed with Primer-BLAST from the National Center for Biotechnology Information. Amplification was conducted with a TaKaRa PrimeSTAR long PCR kit according to the manufacturer's instructions except that we used 38 thermocycles instead of the recommended 30 cycles. Amplification mixtures were evaluated by electro-phoresis with a Qiagen micrcapillary system.

Rationale

We have collected DNA from 27 unrelated dogs with EDS as diagnosed by the referring veterinarians. We want to determine the molecular genetic causes of the EDS in as many of these dogs as we can. Our primary strategy for discovering causal variants is whole genome sequencing. Of the 6 whole genome sequences generated so far for dogs with EDS, 3 have had truncating mutations in *COL5A1*. The other 3 are still under investigation. Based on this result, we expect some of our other EDS cases are caused by *COL5A1* variants. We have devised a protocol intended to allow us to simultaneously screen all of our EDS samples for *COL5A1* variants in the following four steps.

Step 1: Make a pool of DNA from EDS dogs

We took DNA from 26 dogs with EDS (including 2 samples with known COL5A1 mutations as internal controls) and measured the concentrations with a spectrometer. The DNA from each dog was diluted to 150 µg/mL and equal volumes were mixed to create a pooled PCR template. **COMPLETED**

Step 2: Amplify COL5A1 exon clusters by long range PCR

Twenty-six PCR primer pairs were designed to flank clusters of 1 to 4 of the 66 *COL5A1* exons and used to amplify the pooled PCR template. Electrophoretograms showing single major bands of the expected size can be seen in the figure below. So far we have produced 18 useful amplicons. We are waiting for replacement primers for the 8 amplifications that did not yield satisfactory results on the first attempt. Details about the current progress of step 2 are provided in Table 1. **IN PROGRESS**

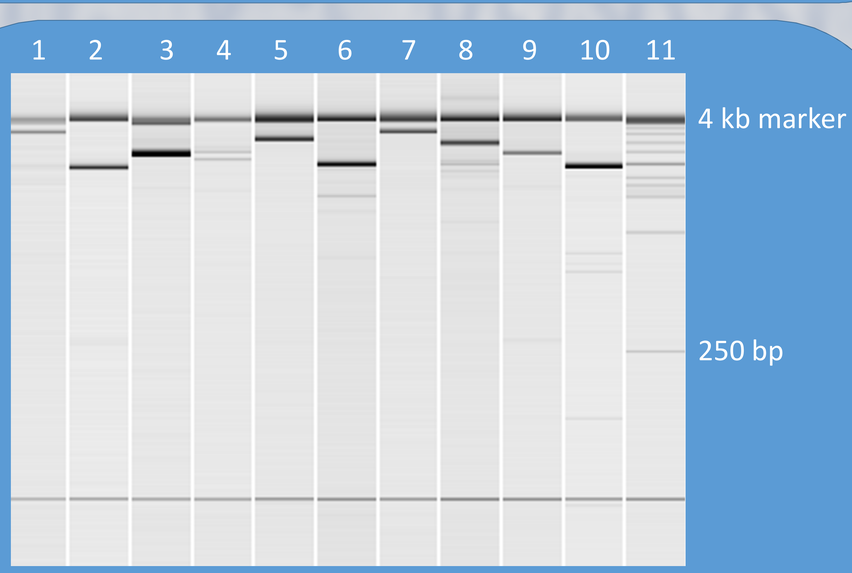


Figure 1. Lane 1 expected length: 3,092 bp. Lane 2: 1,399 bp. Lane 3: 2,041 bp. Lane 4: failed due to multiple bands and low amplification. Lane 5: 2,571 bp. Lane 6: 1,406 bp. Lane 7: 3,074 bp. Lane 8: 2,342 bp. Lane 9: 1,767 bp. Lane 10: 1,401 bp. Lane 11: marker length ladder.

Table 1: Long PCR Progress Report

Primer Pair #	Exon #'s	Amplification	Amplicon Size
1	1	In Progress	Approx. 1600 bp
2	2	Completed	1222 bp
3	3 & 4	Completed	1753 bp
4	5 to 7	In Progress	Approx. 2200 bp
5	8 & 9	Completed	1175 bp
6	10 & 11	Completed	1463 bp
7	12 & 13	Completed	1677 bp
8	14 to 16	Completed	2342 bp
9	17 & 18	Completed	1793 bp
10	19 & 20	Completed	3047 bp
11	21 to 25	In Progress	Approx. 2800 bp
12	26 & 27	In Progress	Approx. 1700 bp
13	28 & 29	In Progress	Approx. 2400 bp
14	30 to 32	In Progress	Approx. 3400 bp
15	33 to 37	Completed	3092 bp
16	38 & 39	Completed	1399 bp
17	40 to 42	Completed	2041 bp
18	43 to 46	Completed	2568 bp
19	47 to 49	In Progress	Approx. 1700 bp
20	50 to 52	In Progress	Approx. 1500 bp
21	53 to 57	Completed	2483 bp
22	58 to 60	Completed	2921 bp
23	61 to 63	Completed	2571 bp
24	64	Completed	1401 bp
25	65	Completed	1079 bp
26	66	Completed	1406 bp
Total		**18 of 26 Completed**	**52,733 bp**

Step 3: Sequence *COL5A1* amplicon pool

Equimolar amounts of DNA from each of the 26 amplifications will be combined into an amplicon pool, which will be submitted to the University of Missouri for library preparation and 2 X250 base sequencing on the Illumina MiSeq. The same data processing pipeline we use for analysis of whole genome sequences will be used to identify likely causal *COL5A1* variants. **PLANNED**

Step 4: Link causal variants to specific dogs

Individual DNA samples from each of the 24 dogs with EDS of unknown etiology will be genotyped for each of the identified likely causal *COL5A1* variants to determine which dog has each variant. **PLANNED**

Anticipated Results and Significance

We expect to identify likely causal *COL5A1* variants for approximately half of the 24 dogs with EDS of unknown etiology. Medical histories from this cohort will provide a basis for management of future cases of EDS due to COL5A1 mutations. The results of this study will enable us to focus diagnostic efforts on the cohort of EDS dogs that do not carry COL5A1 variants.

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