DEVELOPING A CANINE GENETIC TESTING PROGRAM AT THE ATLANTIC VETERINARY COLLEGE: PILOT STUDY ON DISEASE SUSCEPTIBILITY LOCI IN LABRADOR RETRIEVERS

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Abstract

Canine genetic testing for disease susceptibility loci provides invaluable guidance for dog breeders, enabling them to eliminate known disease carriers from breeding programs, and thereby avoid generating affected progeny. Despite the recognized risks associated with breeding dogs of unknown genotypes, many breeders forgo genetic testing due to prohibitively high costs and long turnaround times associated with these tests. Our overall objective was to develop and validate a rapid turnover, cost-effective canine genetic testing program at the Atlantic Veterinary College (AVC). To this end, we conducted a pilot study to develop and validate a complete genetic test panel for heritable diseases in Labrador retrievers, including (1) exercise-induced collapse, (2) progressive rod-cone degeneration, and (3) degenerative myelopathy, which are each associated with a single nucleotide polymorphism (SNP) at the disease locus, and (4) centronuclear myopathy which is associated with a short interspersed nuclear element (SINE) insertion. First, plasmids were designed containing wild type or mutant alleles of each gene of interest and served as genotyping controls. Next, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)-based tests were designed for SNP and SINE typing. Following PCR-RFLP test optimization with plasmid DNA controls, approximately 300 canine buccal swabs obtained from Labrador retriever breeders were tested. Our blinded test results were validated by comparing our test results with results previously obtained by breeders. Our results corresponded 94-98% to the genotype status for the four genetic tests and 100% to the disease statuses of the dogs. Pending final validation, we will be able to offer these tests at the AVC. Ultimately, results from this study will support a grant application to develop a full genetic testing program.

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I would like to thank Dr. Ian Gardner, veterinary epidemiologist, who we consulted regarding study design for test validation, and Dr. Henrik Stryhn, who we consulted regarding statistical parameters for the study.

I would also like to thank the Labrador retriever breeders for their time and for providing samples for the validation portion of this project. Without them, this project would not have been possible.

I would like to dedicate this thesis to my parents, Randy Cooper and Beth Smith Cooper; my brother, Dylan Cooper; and my grandparents, Ian and Lorna Smith and David and Glenda Cooper, who have provided unwavering support and encouragement to me throughout my academic pursuits.

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List of Abbreviations

°C – Degrees Celsius

AmpR – Ampicillin resistance

bp – Base pair

CNM – Centronuclear myopathy

DM – Degenerative myelopathy

Dnm1 – Dynamin 1

EIC – Exercise-induced collapse

MCS – Multiple cloning site

NCBI – National Center for Biotechnology Information

pBS – pBluescript

PCR – Polymerase chain reaction

PCR-RFLP – Polymerase chain reaction-restriction fragment length polymorphism

Prcd – Progressive rod-cone degeneration

PRCD – Progressive rod-cone degeneration

Ptpla - Protein tyrosine phosphatase-like, member A

RFLP – Restriction fragment length polymorphism

SINE – Short interspersed nuclear element

SNP – Single nucleotide polymorphism

Sod1 – Superoxide dismutase 1

TE – Tris-EDTA

INTRODUCTION

Ongoing studies of the canine genome are generating new insights and knowledge into the genetic basis of disease. In pure bred dogs, genetic studies have determined a recessive inheritance pattern for many diseases. Consequently, phenotypically normal carriers of potentially debilitating diseases may be present in the population. Selective inbreeding of pure bred dogs is a common practice, often with direct relatives. Canine genetic testing for disease susceptibility loci is therefore of particular importance for pure bred dogs to avoid breeding carriers that may generate affected offspring. Knowledge of the genetic status of canines is not only useful in guiding breeding programs but is also crucial for maintaining sufficient genetic diversity in the population (Donner et al. 2018). Genetic testing also allows veterinarians to diagnose inherited diseases and provide appropriate treatments. Collectively, the benefits associated with genetic testing will lead to a healthier canine population with a greater quality of life.

Despite the known risks associated with breeding dogs of unknown genotypes, many breeders decline having their dogs tested due to the high costs and long turnaround times associated with genetic testing. This may result in long term adverse consequences for affected dogs and their owners, including, but not limited to, illness and reduced quality of life for the dog as well as high costs to their owners who seek treatments for them. The overall objective of Dr. Hartwig's research program is to develop and validate a rapid turnover, cost-effective canine genetic testing program, and to establish the

Atlantic Veterinary College (AVC) as a regional and potentially national center of excellence for canine genetic testing.

As a first step towards developing this program, the purpose of this proof-of-principle study is to develop and validate a complete genetic test panel for Labrador retrievers. For the purpose of test validation, a large number of samples is required to reach a high level of statistical confidence. As the most popular dog breed in Canada (Patterson et al. 2008), Labrador retrievers were chosen for this pilot study in part because they would provide the largest sample population for the study, contingent upon breeder support. Given the high cost and slow turnaround time currently associated with these tests, it was anticipated that Canadian Labrador retriever breeders would be strongly motivated to support this study by providing samples.

Additionally, Labrador retrievers are susceptible to multiple heritable diseases that cause serious health problems in the canine population, including exercise-induced collapse (EIC), progressive rod-cone degeneration (PRCD), degenerative myelopathy (DM), and centronuclear myopathy (CNM). Each of these autosomal recessive diseases is linked to a specific gene locus and can be detected by genetic testing. At present, these diseases are among the most frequently tested for in this breed (Donner et al. 2018).

Test development for these diseases was initiated previously in Dr. Hartwig's laboratory. The objective of this study was to optimize and validate these four genetic tests. An integral component of the research was to develop gold-standard DNA controls for our polymerase chain reaction (PCR)-based tests, by designing plasmid vectors containing either the wild type or mutant allelic sequence of interest. Once plasmid

design and test optimization were complete, tests could be validated (details in the Data Analysis and Statistical Analysis sections of Methods). For the validation portion of the study, Labrador retriever breeders across Canada were contacted and asked to submit canine buccal DNA samples for our study. The validation process was started by testing the collected samples. For this validation process, our test results were compared to results previously obtained by the breeders from genetic testing providers. Ultimately, the development, optimization, and validation of these four canine genetic tests will provide support for a grant application for a full genetic testing program at the AVC (Figure 1).

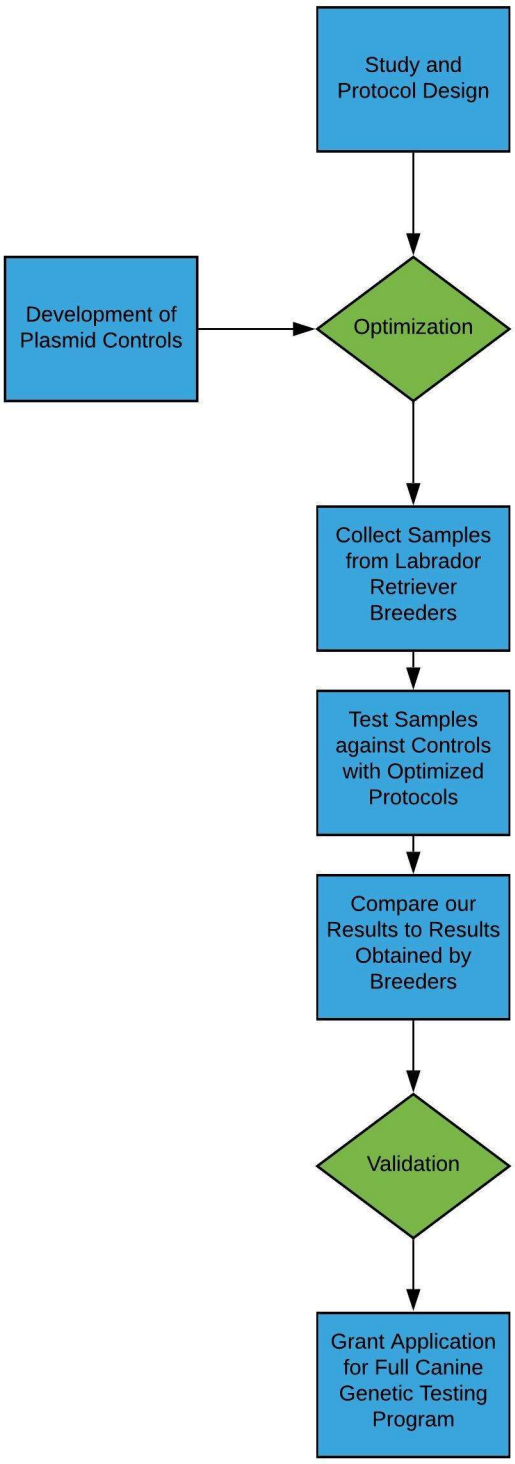

Figure 1. Overview of the steps required to obtain results to support a grant application for a full canine genetic testing program at the Atlantic Veterinary College.

LITERATURE REVIEW

Exercise-Induced Collapse

EIC is a disease that causes uncoordinated movements in dogs following periods as short as five minutes of exercise (Patterson et al. 2008) or extreme excitement (Takanosu et al. 2012), ultimately leading to uncontrolled hind limb movement and collapse. Patterson et al. (2008) found that this autosomal recessive disease results from a point mutation in exon 6 of the *Dynamin 1* (*Dnm1*) gene, located on canine chromosome 9. The substitution of a G to a T at nucleotide position 767 (Figure 2) creates a codon for the amino acid leucine rather than arginine (R-L substitution). While most affected dogs tend to return to their normal movement and activity approximately 30 minutes after collapsing, in rare instances, the condition can be fatal (Patterson et al. 2008).

Progressive Rod-Cone Degeneration

PRCD is a form of progressive retinal atrophy caused by a point mutation in exon 1 of the *Progressive rod-cone degeneration* (*Prcd*) gene, located on chromosome 9 (Sussadee et al. 2014). A G to A nucleotide substitution occurs at nucleotide position 5 (Figure 3) (Kohyama et al. 2015), causing an amino acid substitution of cysteine to tyrosine (C-Y substitution) (Spencer et al. 2016). PRCD is a late-onset disease that results in the degeneration of rods and cones in the eye, leading to blindness (Kohyama et al.

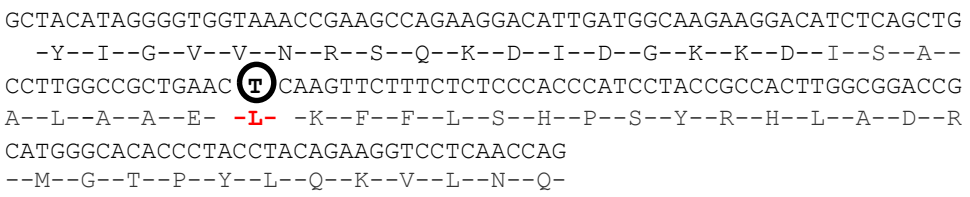

Figure 2. Exon 6 of the *Dnm1* gene. The amino acid sequence is indicated below each row of the nucleotide sequence. The circled nucleotide is the location of the point mutation where the T nucleotide replaces a G nucleotide to form the mutant allele. The amino acid highlighted in red indicates the amino acid substitution caused by the mutation.

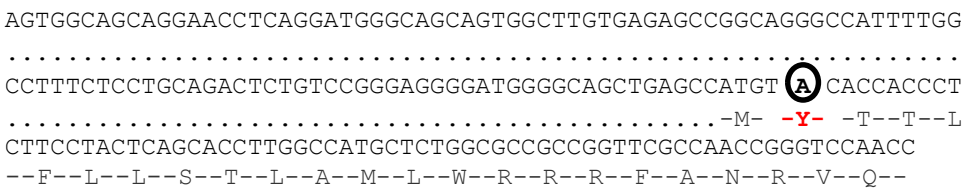

Figure 3. Exon 1 of the *Prcd* gene. The amino acid sequence is indicated below each row of the nucleotide sequence. The circled nucleotide is the location of the point mutation where the A nucleotide replaces a G nucleotide to form the mutant allele. The amino acid highlighted in red indicates the amino acid substitution caused by the mutation.

2015). Since rod degeneration tends to occur first, night blindness is often the earliest symptom; however, the condition continues to progress to complete blindness (Sussadee et al. 2014).

Degenerative Myelopathy

DM is a late-onset disease with symptoms typically appearing after eight years of age. It is caused by a point mutation of a G nucleotide to an A nucleotide at position 118 (Figure 4). This nucleotide is located in exon 2 of the *Superoxide dismutase 1* (*Sod1*) gene on canine chromosome 31 (Awano et al. 2009). This mutation results in an amino acid substitution from glutamic acid to lysine (E-K substitution). The reduction in negative charge associated with this amino acid substitution may contribute to the aggregation of neurons, and subsequent motor neuron degeneration in the spinal cord leads to paraplegia. DM is currently an incurable disease, and many owners choose to euthanize affected dogs within a year of symptom onset as the hind limbs weaken significantly within this time (Awano et al. 2009).

Centronuclear Myopathy

CNM is an autosomal recessive disease resulting in atrophied and weakened skeletal muscle (Pelé et al. 2005). A key feature of this disease is the displacement of the nucleus from the edges of the muscle cell towards a more centralized location in the cell as the muscle cells atrophy. The disease is caused by a 236 base pair (bp) short

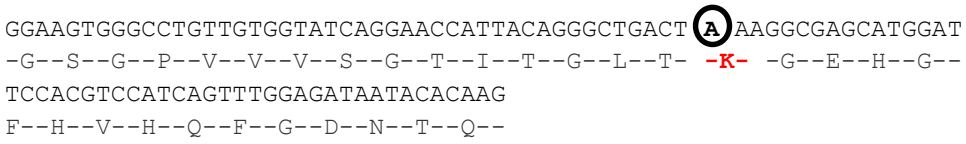

Figure 4. Exon 2 of the *Sod1* gene. The amino acid sequence is indicated below each row of the nucleotide sequence. The circled nucleotide is the location of the point mutation where the A nucleotide replaces a G nucleotide to form the mutant allele. The amino acid highlighted in red indicates the amino acid substitution caused by the mutation.

interspersed nuclear element (SINE) insertion (Figure 5) in exon 2 of the *Protein tyrosine phosphatase-like, member A* (*Ptpla*) gene located on canine chromosome 2 (Pelé et al. 2005). The two identical 13 bp underlined nucleotide sequences located at either end of the insertion allows the SINE insertion into the gene seamlessly since proofreading mechanisms detect the same short nucleotide sequence at the end of the SINE insertion as is found in the wild type allele (Pelé et al. 2005).

The locations of the mutations involved in the study are summarized in Table 1. Where available, published primer sequences were used to guide the development and optimization of PCR protocols. Alternatively, primer design software was used to design primers (detailed in the PCR Optimization section of Methods).

Figure 5. Short interspersed nuclear element insertion nucleotide sequence in the *Ptpla* gene that causes centronuclear myopathy. The inserted nucleotides are indicated from the end of the first underlined sequence to the end of the second underlined sequence (Pelé et al. 2005).

Table 1. Summary of mutations in disease susceptibility loci in Labrador retrievers.

	Exercise-Induced Collapse	**Progressive Rod-Cone Degeneration**	**Degenerative Myelopathy**	**Centronuclear Myopathy**
Mutation	G to T nucleotide substitution at position 767	G to A nucleotide substitution at position 5	G to A nucleotide substitution at position 118	236 bp SINE insertion
Amino Acid Substitution	Arginine to leucine (R-L substitution)	Cysteine to Tyrosine (C-Y substitution)	Glutamic Acid to Lysine (E-K substitution)	N/A
Chromosome	9	9	31	2
Gene	*Dnm1*	*Prcd*	*Sod1*	*Ptpla*
Exon	6	1	2	2
Test[1]	Restriction enzyme digest of PCR product	Restriction enzyme digest of PCR product	Restriction enzyme digest of PCR product	PCR
Restriction Enzyme[1]	SmlI	RsaI	AcuI	N/A

[1]Refer to the Methods section for a description of these tests and use of restriction enzymes

METHODS

Plasmid Vector Subcloning

Mutant gene sequences identified in the primary literature for the four diseases were first verified against published wild type sequences in the National Center for Biotechnology Information (NCBI) and Ensembl databases to safeguard against possible publication errors. For the purpose of subcloning sequences into plasmid vectors, two unique restriction flanking sites corresponding to restriction sites in the plasmid vector pBluescript (pBS) SK II (+) multiple cloning site (Figure 6) were designed around wild type and mutant sequences of interest (Appendix A). These modified sequences were sent to the biotechnology company GenScript, which synthesized and subcloned the modified sequences into pBS SK II (+) plasmids.

Molecular Cloning of Chemically Competent Cells for Plasmid Propagation

The lyophilized plasmids containing the gene sequences of interest were received from GenScript and resuspended in sterilized double distilled water. Plasmids were propagated by transforming Lucigen *E. Cloni* 10G Chemically Competent Cells. For each gene sequence, a chemically competent cell tube was thawed on ice for 15 minutes. Four microliters of plasmid were added to the competent cells, and the tube was placed on ice for thirty minutes. The cells were heat shocked for 45 seconds in a 42°C water bath, and

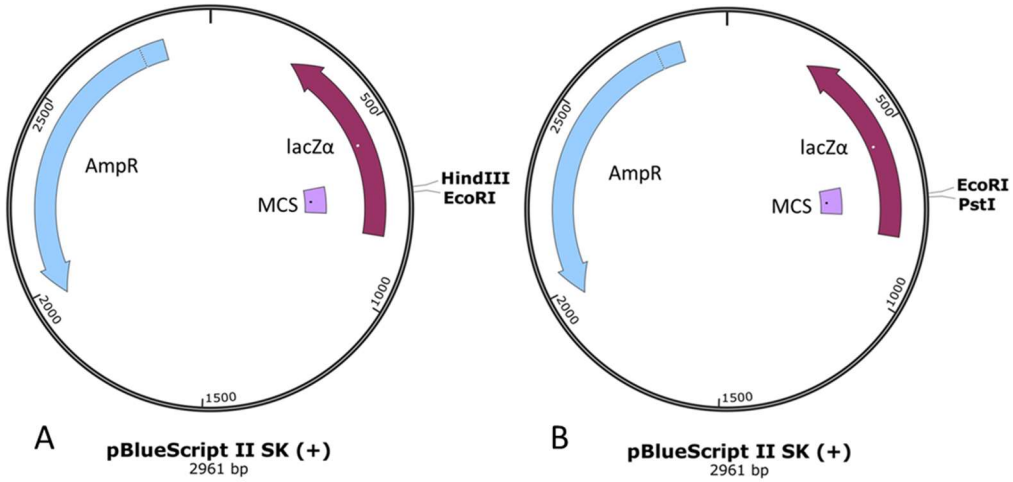

Figure 6. Subcloning gene sequences into pBluescript II SK (+) vectors. Restriction sites in the multiple cloning site (MCS) were selected to insert the genes of interest and interrupt the lacZ operon for blue/white screening. Ampicillin resistance (AmpR) gene expression permits the plasmids to be grown on LB ampicillin agar following competent cell transformation. (A) HindIII and EcoRI restriction sites were used to insert the *Prcd* and *Sod1* genes into the plasmids and (B) EcoRI and PstI restriction sites were used to insert the *Ptpla* and *Dnm1* genes into the plasmids.

then the cells were placed on ice for 2 minutes. Next, 960 µL of LB broth was added to the cells, and the tube was placed in a shaking incubator for 1 hour at 250 rpm and 37°C. Transformed cells were plated and grown on LB ampicillin (100 µg/mL) agar with 40 µL of 40 mg/mL X-Gal (Teknova) to allow for blue/white screening.

A single white bacterial colony, corresponding to recombinant bacteria containing the plasmid, was selected. Plasmid isolation from the bacterial colony was performed by maxipreparation using an E.Z.N.A.® Plasmid DNA Maxi Kit (Omega Bio-Tek – D6922-02) and their E.Z.N.A.® Plasmid DNA Maxi Kit Centrifugation Protocol with slight modifications to obtain a large yield of purified plasmid DNA. This plasmid DNA served as controls for the wild type and mutant alleles in the PCR tests of genomic canine buccal swab DNA. Approximately 200 mL of overnight culture was transferred to a centrifuge bottle and was centrifuged at 4,000 x *g* for 10 minutes at 4°C. The culture media was decanted, and the centrifuge bottle was inverted to dry the pellet.

Once the pellet was dry, 12 mL of Solution I/RNase A was added to the centrifuge bottle and was pipetted up and down until the pellet was entirely resuspended. The mixture was moved to a 50 mL centrifuge tube, and 12 mL of Solution II (lysis buffer) was added to the tube; it was inverted gently 12 times. After incubation at room temperature for 2 minutes, 16 mL of Solution III (neutralization buffer) was added to the tube, and it was inverted gently until white precipitates formed. The maxipreparation for the *Dnm1* gene was performed first, and the solutions were split into two tubes prior to the addition of Solution III. In one tube, room temperature Solution III was used, and in the other, ice-cold Solution III was used. The ice-cold Solution III provided greater DNA concentrations (212.01 ng/µL vs. 190.52 ng/µL for the wild type sequence and 250.72

ng/µL vs. 180.00 ng/µL for the mutant sequence), so ice-cold Solution III was used for the *Prcd*, *Sod1*, and *Ptpla* gene maxipreparations.

The mixture was transferred to two 30 mL ultracentrifuge tubes and was centrifuged at 15,000 x *g* for 10 minutes at 4°C. A HiBind® DNA Maxi Column from the kit was placed in a 50 mL centrifuge tube. The supernatant was decanted into the column and was centrifuged at 4,000 x *g* for 5 minutes at 4°C. The filtrate was discarded and 10 mL of HBC Buffer (containing 100% isopropanol) was added to the column and centrifuged at 4,000 x *g* for 5 minutes at 4°C. The filtrate was discarded, and 15 mL DNA Wash Buffer (containing 100% ethanol) was added to the column and centrifuged at 4,000 x *g* for 5 minutes at 4°C twice. The column was centrifuged at 4,000 x *g* for 10 minutes at 4°C, it was transferred to a new 50 mL centrifuge tube, and 3 mL of Elution Buffer was added to the column membrane. Next, the column was left to stand at room temperature for 5 minutes. The column was then centrifuged at 4,000 x *g* for 5 minutes at 4°C. The DNA was transferred to microcentrifuge tubes and was stored at -20°C.

Glycerol stocks were prepared by adding 0.5 mL sterile 50% glycerol and 0.5 mL of transformed competent cells to a cryogenic tube. The mixture was vortexed and stored at -80°C for future maxipreparations as necessary.

Progressive Rod-Cone Degeneration

Unexpectedly, the transformation of chemically competent cells with *Prcd* wild type plasmid resulted in the propagation of blue colonies when grown on LB ampicillin (100 µg/mL) agar with 40 µL of 40 mg/mL X-Gal (Teknova). Since blue colonies

correspond to non-transformed colonies, these results indicated that the *Prcd* wild type allele had not integrated into the plasmid, or alternatively, that it had integrated into an unintended location in the plasmid where it did not interrupt the lacZ operon.

The transformation of competent cells with the *Prcd* wild type plasmid took place at the same time and under the same conditions as the transformation of competent cells with five other plasmids, for which all grown colonies were white. To determine whether the transformed competent cells contained plasmids with the gene of interest, a maxipreparation was performed, followed by a double digest of the purified plasmid DNA. The double digest product was added to loading dye and run on a 2% agarose gel. The expected band size for the gene of interest was detected; this indicated that the plasmid contained the gene of interest and the DNA had been isolated and purified for use as a DNA control for the genetic tests.

Additionally, we contacted GenScript and requested that they sequence the entire plasmid to confirm that the gene of interest had been inserted. Their sequencing showed that the gene of interest had indeed inserted into the lacZ operon of the plasmid, and the sequence of the entire plasmid was found to be a complete match except for a single nucleotide polymorphism (SNP) in the backbone of the plasmid. Neither the technical staff at GenScript, nor any other resources were able to provide a satisfactory explanation for the failure of blue/white screening in this instance.

Test Validation

For obvious reasons, it was anticipated that there would be low numbers of canine buccal DNA samples obtained from carrier or affected dogs. In consultation with veterinary epidemiologist Dr. Ian Gardner, who has expertise in diagnostic validation, multiple samples were obtained from some dogs to be tested as separate samples, thus increasing the sample size for these genotypes. Increasing the number of carrier and affected samples randomly throughout the sample population to be tested increases the test's power.

Breeder Consent

A University of Prince Edward Island Gmail account was created for the development of the AVC genetic testing program. Through this email, Labrador retriever breeders across Canada were contacted to ask for their participation in the study (Appendix B). Their email addresses and/or websites were found on the Canadian Kennel Club website. Some respondents provided the information to veterinarians, family members, friends, or other members of Labrador retriever clubs who were interested in participating, and their emails were obtained. Buccal swabs (DNA Genotek), along with instructions, a consent form, an information sheet, and return postage, were mailed to participants. Thirty-five participants collected buccal swabs (n=375) from their Labrador retrievers and returned the swabs with their written consent to participate in the study (Figure 7). The genetic status of the dogs, when known and provided by the breeder, was catalogued with the number of the swab. As described below, samples were subsequently

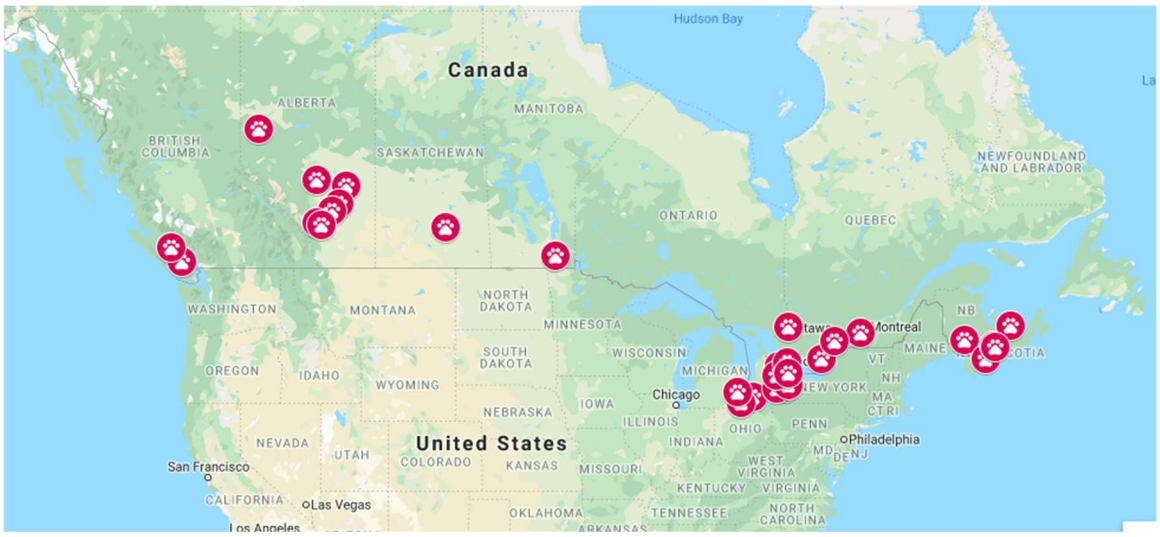

Figure 7. Locations of the breeders and owners who submitted buccal samples for the study.

randomly assigned a working number to ensure the study was conducted in a double-blinded design.

DNA Extraction

Canine DNA from the buccal swabs was extracted and purified using the Performagene™ laboratory protocol (DNA Genotek), previously optimized in Dr. Hartwig's laboratory. Each sample was mixed and placed in a water bath at 50°C for at least 1 hour. The collection sponge was pressed against the tube to extract the sample, and 500 µL of the sample was added to a microcentrifuge tube. Twenty microliters of PG-L2P purifier was added to the sample, and the mixture was vortexed and placed on ice for ten minutes. The mixture was centrifuged at 15,000 x *g* for 5 minutes, the supernatant was moved to a new microcentrifuge tube, and the pellet was discarded. To the supernatant, 25 µL of 5 M NaCl was added, followed by 600 µL of 100% ethanol. The mixture was gently inverted 10 times to initiate precipitation. The sample was incubated at room temperature for 10 minutes and was centrifuged at 15,000 x *g* for 2 minutes. The supernatant was decanted, and the DNA was washed with 250 µL of 70% ethanol. The sample was incubated at room temperature for 1 minute. Next, the ethanol was decanted, and the tube was inverted to allow the pellet to dry. The pellet was suspended in 100 µL of TE buffer, and the mixture was vortexed. The samples were left to stand at room temperature overnight and were heated the following day in a heat block at 50°C for 10 minutes with the microcentrifuge tube caps open and for 50 more minutes with the microcentrifuge tube caps closed.

To design a double-blinded study, all DNA samples were randomly assigned a working number by an individual (other than myself) who had no knowledge of the genetic status of each sample. PCR experiments were then performed (by myself) on these samples using these working numbers.

PCR Optimization

At the commencement of this project in the summer of 2019, it was discovered that the PCR tests developed by previous lab members had not been optimized. Therefore, test optimization comprised a major initial step of my thesis project prior to test validation.

Exercise-Induced Collapse

Takanosu et al. (2012) designed primers for their PCR protocol that result in the amplification of a 337 bp DNA fragment. They noted that the point mutation creates a restriction enzyme cut sequence for SmlI. Therefore, when the alleles are digested with this enzyme, the wild type allele remains the same size while the mutant allele is cut into two segments (Table 2).

Progressive Rod-Cone Degeneration

Dostal et al.'s (2011) primers and PCR protocol result in the amplification of a

Table 2. Expected band sizes following restriction enzyme digestion of PCR-amplified alleles.

	Dnm1 **WT**	***Dnm1*** **MT**	***Prcd*** **WT**	***Prcd*** **MT**	***Sod1*** **WT**	***Sod1*** **MT**	***Ptpla*** **WT**	***Ptpla*** **MT**
Restriction Enzyme	SmlI	SmlI	RsaI	RsaI	AcuI	AcuI	N/A	N/A
Band Size(s) (bp)	337	173 164	512	396 116	317 179 49	317 228	116	352

512 bp DNA fragment. Sussadee et al. (2014) found that the G to A nucleotide substitution in mutant *Prcd* creates a restriction enzyme cut sequence for RsaI. When a restriction enzyme digest is performed with the PCR product and is then visualized by gel electrophoresis, a genetically clear dog will have a single band at 512 bp, an affected dog will have bands at 396 bp and 116 bp, and a heterozygote will have all three bands (Table 2) (Dostal et al. 2011).

Degenerative Myelopathy

Zeng et al. (2014) designed primers for each of *Sod1*'s exons. Therefore, primers flanked the mutation in exon 2, resulting in its amplification. Within this amplified sequence, various cut sequences for particular restriction enzymes can be identified.

Centronuclear Myopathy

The initial primers and conditions designed for the PCR reactions resulted in the detection of nonspecific bands and the inability to clearly detect the band of interest representing the mutant allele (Figure 8). The uncut plasmid, linearized plasmid, and double digested plasmid were run on a 2% agarose gel to confirm that the band sizes correlated to those expected for each. The plasmid was also sequenced by the biotechnology company GenScript, who synthesized the wild type and mutant plasmids. GenScript confirmed that the correct gene sequences of interest were present in the synthesized plasmids. This led to the conclusion that the nonspecific bands and inability

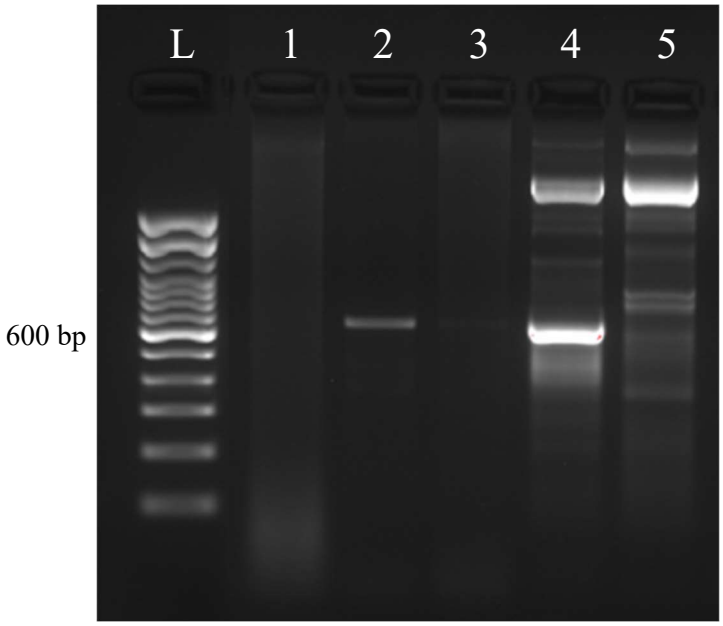

Figure 8. PCR for *Ptpla* gene with initial primers and conditions. L: Invitrogen TrackIt 100 bp DNA Ladder. Lanes 1-3: clear genomic samples. Lane 4: wild type plasmid control. Lane 5: mutant plasmid control. Bands were not detected for all genomic samples, and many nonspecific bands were detected for the plasmid controls.

to detect the mutant allele were not due to the wrong sequence being inserted into the plasmid and cloned, but that the PCR protocol was failing to amplify the region of interest of the gene.

To optimize this protocol, the original PCR protocol, including primers, was retained. However, instead of using the uncut plasmid as DNA template, linearized and double digested plasmid DNA was used to determine whether the primers could bind to the linearized or double digested plasmid DNA more effectively than the uncut plasmid in order to amplify the region of interest of the gene. Next, genomic samples collected in 2018 were tested under the same conditions to determine whether the protocol produced nonspecific bands only for the plasmid DNA or both plasmid DNA and genomic DNA. In all three cases, either the band of interest could not be detected, or nonspecific bands were detected, which helped identify that the reaction components or conditions likely needed to be modified to optimize the protocol. The annealing temperature was increased from 57°C to 60°C to attempt to increase the specificity of the primers and reduce or eliminate the presence of nonspecific bands. The presence of nonspecific bands continued, and as a result, the PCR reaction components were changed.

Primer 3 software was used to design new primers (Sigma-Aldrich) to amplify a smaller region of the *Ptpla* gene surrounding the SINE insertion area (Figure 9). The PCR product for the mutant allele was 236 bp larger than the wild type allele (Table 2) (Pelé et al. 2005).

Using the primers and protocols described in the literature, in addition to primer development software, PCR protocols were optimized (Appendix C) for use with

ttcatttttatagaactttacaaaacacttttatttttctagGTGGTTGGTTCTAGCTATTGCCATGGT
ACGTTTTTATATGGAAAAAGGAACACACAAAGGTTTTTTTTTTTTTTTTTAAATTTTTTTTTTTAAATTT
TTTTTTTTTAATTTTTTTTTATTTATTTATGATAGTCACACACAGATAGAGAGAGAGGCAGAGACACAGG
CAGAGGGAGAAGCAGGCTCCATGCACCGGGAGCCCGACGTGGGACTCGATCCCGGGTCTCCAGGATCGCG
CCCTGGGCCAAAGGCAGGCGCCAAACCGCTGCGCCACCCAGGGATCCCCCACACAAAGGTTTATATAAAA
GTATTCAGAAGACACTTAAATTTTTCCAGACATTTGCCTTGCTTGAGgtaagttttcagtgatgctgttt

Figure 9. Exon 2 and surrounding introns of the *Ptpla* gene on canine chromosome 2. The SINE insertion is denoted in red. The designed primers are highlighted in yellow. Exon 2 is indicated by blue nucleotides.

designed primers (Appendix D) to amplify plasmid and genomic DNA for the four diseases of interest.

Restriction Enzyme Digests and Gel Electrophoresis

Restriction fragment length polymorphism (RFLP) is useful when combined with PCR and gel electrophoresis to determine whether a particular sequence is present in a nucleotide or gene sequence (Sharifiyazdi et al. 2016). For diseases that are caused by point mutations, the mutations can create or eliminate cut sequences for restriction enzymes, and the fragments created by the enzymes can be visualized by gel electrophoresis (Figure 10).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) SNP typing tests were performed using SmlI (New England Biolabs – R0597L), AcuI (New England Biolabs – R0641L), and RsaI (New England Biolabs – R0167L) restriction enzymes for EIC, DM, and PRCD, respectively (Appendix E), to determine whether a particular restriction sequence was present in the gene sequences. The mutations created or eliminated restriction sites, so these digests resulted in RFLP. The fragments created were visualized by gel electrophoresis (Appendix F) alongside Invitrogen TrackIt 100 bp DNA Ladder (Invitrogen – 10488058) or Invitrogen TrackIt 1 Kb Plus DNA Ladder (Invitrogen – 10488085), and the banding pattern was determined by the wild type or mutant status of each allele.

Distinctly, a SINE insertion changes the length of the *Ptpla* nucleotide sequence (Pelé et al. 2005). Therefore, the difference in the size of the fragments of the PCR

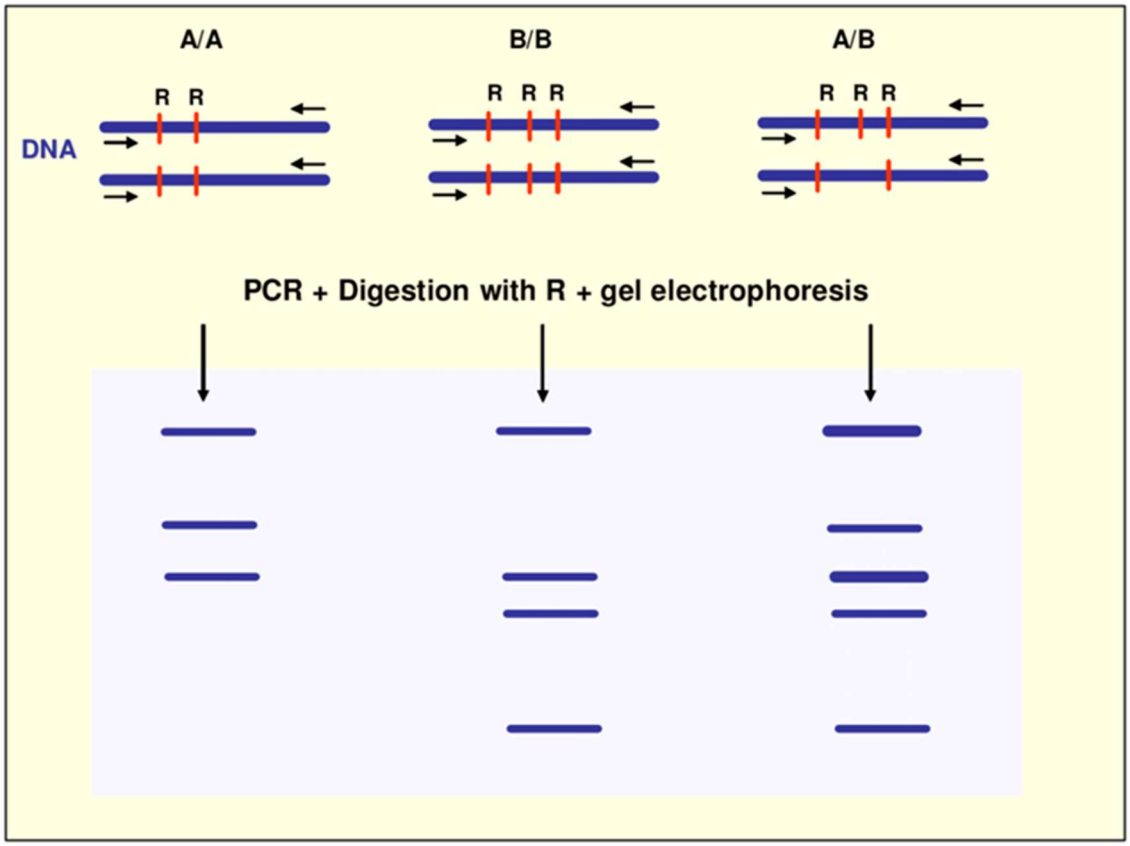

Figure 10. General schematic showing PCR product restriction enzyme digest visualized by gel electrophoresis (Ocampo et al. 2008). A represents one allele of a gene, and B represents another allele of the same gene. R represents the restriction sites present on each allele that create the fragments that are visualized by gel electrophoresis.

product indicates the canine's genetic status. Thus, performing PCR targeting a section of the gene containing the insertion made it possible to determine the genetic status based on the size of the PCR product run on an agarose gel.

The expected banding patterns for each genotype were confirmed using the online New England Biolabs NEBcutter® V2.0. The various fragment lengths were detected by agarose gel electrophoresis with ethidium bromide as the intercalating agent. Two percent agarose gel was used for EIC and PRCD genetic tests, and three percent agarose gel was used for DM genetic tests. Gel electrophoresis using 2% agarose gel was also performed with the PCR product for CNM to detect the presence or absence of a SINE insertion.

Degenerative Myelopathy

A nonspecific band of approximately 550 bp was repeatedly detected following PCR-RFLP of the *Sod1* gene, preventing the optimization of the *Sod1* protocols. This band was most prominent in the restriction enzyme digest of the *Sod1* mutant plasmid.

The *Sod1* PCR amplicons were run alongside the *Sod1* restriction enzyme digest products on a 3% agarose gel (Figure 11), which showed that the nonspecific band in the restriction enzyme digest product was the PCR product of 545 bp, indicating the band was a result of incomplete digestion.

In an attempt to completely digest DNA, the digestion time was increased to 2 hours (Figure 12). Accordingly, the 545 bp band appeared fainter when run on a gel, confirming that this band was due to incomplete digestion of the PCR product. Next, the

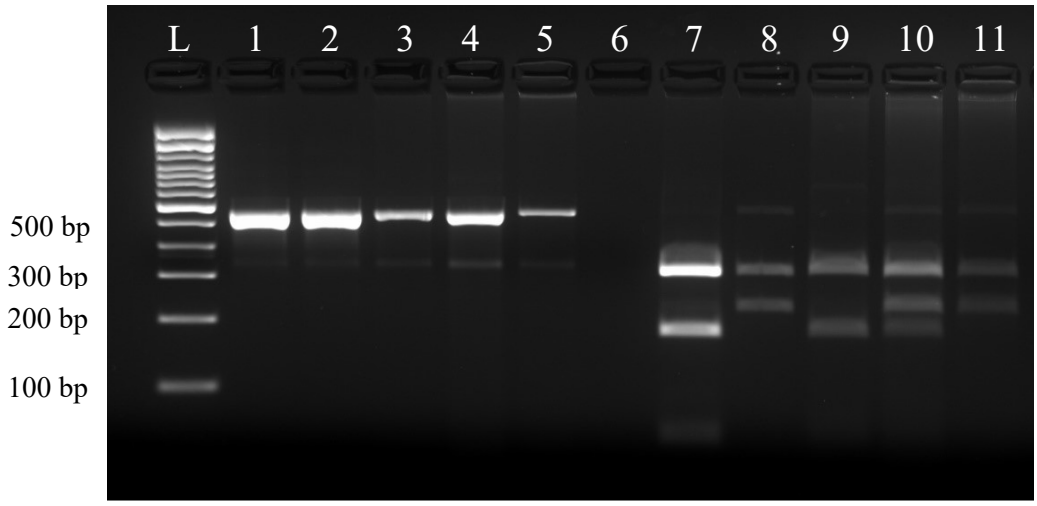

Figure 11. PCR and PCR-RFLP SNP typing test for Degenerative Myelopathy - *Sod1* gene. *Sod1* PCR amplicons (lanes 1-5) were subjected to restriction enzyme digest (lanes 7-11) and run on a 3% agarose gel. Wild type and mutant PCR amplicons are both 545 bp prior to restriction enzyme digestion (faint bands may be seen and represent incomplete restriction enzyme digestion). L: Invitrogen TrackIt 100 bp DNA ladder. Lanes 1 and 7: wild type plasmid control. The PCR amplicon is digested into 317 bp, 179 bp, and 49 bp fragments. Lanes 2 and 8: mutant plasmid control. The PCR amplicon is digested into 317 bp and 228 bp fragments. Lanes 3 and 9: clear genomic sample. Lanes 4 and 10: carrier genomic sample. Lanes 5 and 11: affected genomic sample.

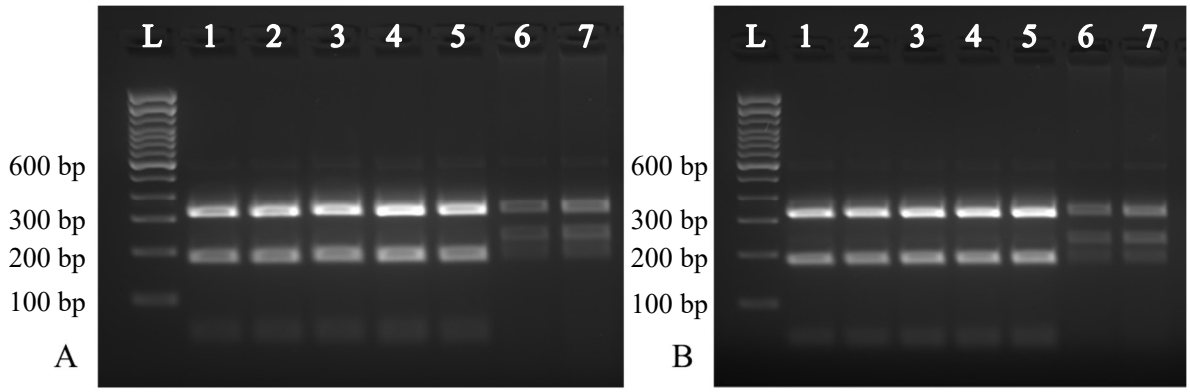

Figure 12. PCR-RFLP SNP typing test for the *Sod1 gene* with (A) 1-hour restriction enzyme digest and (B) 2-hour restriction enzyme digest. L: Invitrogen TrackIt 100 bp DNA Ladder. Lanes 1-5: wild type plasmid control amplicon is digested into 317bp, 179 bp, and 49 bp. Lanes 6-7: carrier genomic samples have the wild type allele amplicon digested into 317 bp, 179 bp, and 49 bp, and the mutant allele amplicon digested into 317 bp and 228 bp. Wild type and mutant amplicons are 545 bp before digestion, and the faint band is incomplete digestion.

concentration of the restriction enzyme in the restriction enzyme digest mixture was doubled; however, no effect on the strength of the band was identified (Figure 13).

Finally, since the *Sod1* mutant plasmid control produced the strongest band at 545 bp following restriction enzyme digestion, 5 µL of the *Sod1* mutant plasmid PCR product was diluted with 20 µL of nuclease-free water before being added to the restriction enzyme digest mixture. Along with increasing digestion time to 16 hours, this dilution resulted in the detection of a fainter band that represented the incomplete digestion of the PCR amplicon (Figure 11). Although dilution of the mutant plasmid PCR product reduced the strength of the nonspecific band, the PCR products of the wild type plasmid control and genomic samples were not diluted, because doing so would have prevented visualization of a 49 bp band present in genetically clear and carrier samples.

Ethanol Precipitation

For some genomic samples, no banding patterns were detected following PCR-RFLP tests. To test the possibility that the absence of detected bands was due to contamination or a low concentration of DNA, ethanol precipitation was performed to concentrate and purify genomic DNA. A 50 µL aliquot of DNA sample suspended in Tris-EDTA (TE) buffer was added to 5 µL of 3 M sodium acetate and 150 µL of ice-cold 100% ethanol, and the mixture was vortexed. The mixture was precipitated at -20°C overnight and was centrifuged for 30 minutes at 13,000 rpm and 4°C. The pellet was washed with 500 µL ice-cold 75% ethanol and was centrifuged for 10 minutes at 4°C. The wash was repeated once. Once the ethanol was decanted, the tubes were centrifuged

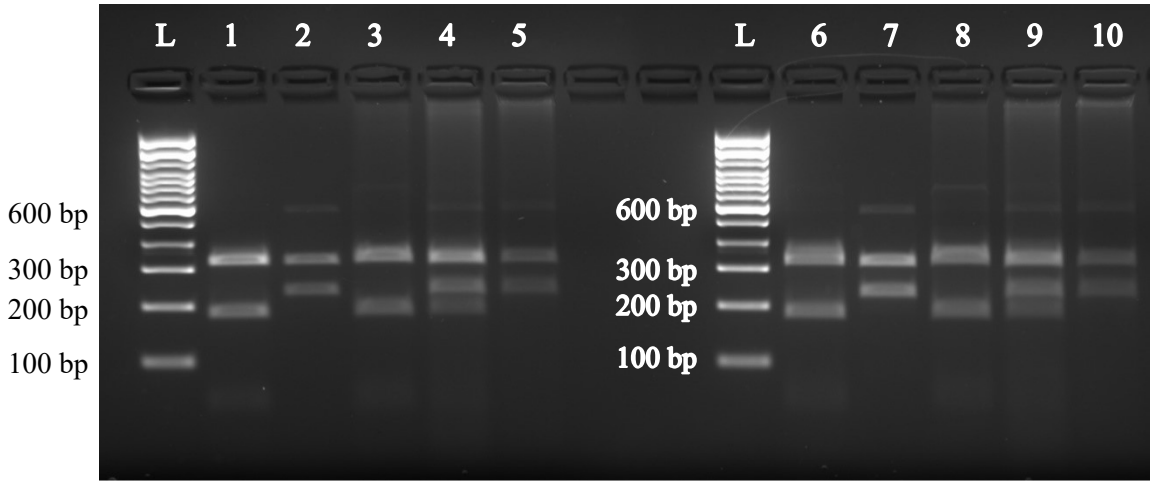

Figure 13. PCR-RFLP SNP typing test of the *Sod1* gene with two restriction enzyme concentrations. Restriction enzyme concentration was doubled in lanes 6-10 compared to lanes 1-5. L: Invitrogen TrackIt 100 bp DNA Ladder. Lanes 1 and 6: wild type plasmid control amplicon is digested into 317 bp, 179 bp, 49 bp. Lanes 2 and 7: mutant plasmid control amplicon is digested into 317 bp and 228 bp. Lanes 3 and 8: clear genomic samples. Lanes 4 and 9: carrier genomic samples. Lanes 5 and 10: affected genomic samples. Wild type and mutant amplicons are 545 bp before digestion, and the faint band is incomplete digestion.

quickly at maximum speed to remove the remaining ethanol. The pellets were air-dried and resuspended in 50 μL of nuclease-free water. PCR and PCR-RFLP testing were then repeated with the samples.

Data analysis

The presence of a specific banding pattern corresponding to the genetically clear, affected, or carrier banding pattern for each disease indicated the genotype of the individual. The results of the blinded genetic tests were compared with the known genetic status of samples that were supplied by breeders who previously had third-party genetic testing performed on their dogs.

Statistical Analysis

The correspondence obtained by comparing the known genetic statuses of the dogs to our results was used to determine the validity of the tests.

RESULTS

Optimization

Exercise-Induced Collapse

The detection signal for the plasmid DNA controls was too strong, so the plasmid DNA had to be diluted. The addition of approximately 0.21 ng and 0.25 ng of wild type and mutant plasmid controls to the PCR reaction mixture and diluting the PCR product before adding it to the restriction enzyme digest mixture prevented saturation of the detected bands for the positive and negative controls. This modification resulted in an optimized PCR protocol to detect the alleles present in both plasmid controls and genomic samples (Figure 14).

Progressive Rod-Cone Degeneration

The original PCR and restriction enzyme digest protocols for this disease had been previously optimized and did not require further optimization. These conditions were maintained while the amount of plasmid sample and genomic sample to be loaded to the gel for gel electrophoresis was established to detect the bands indicating positive and negative plasmid controls alongside genomic samples (Figure 15).

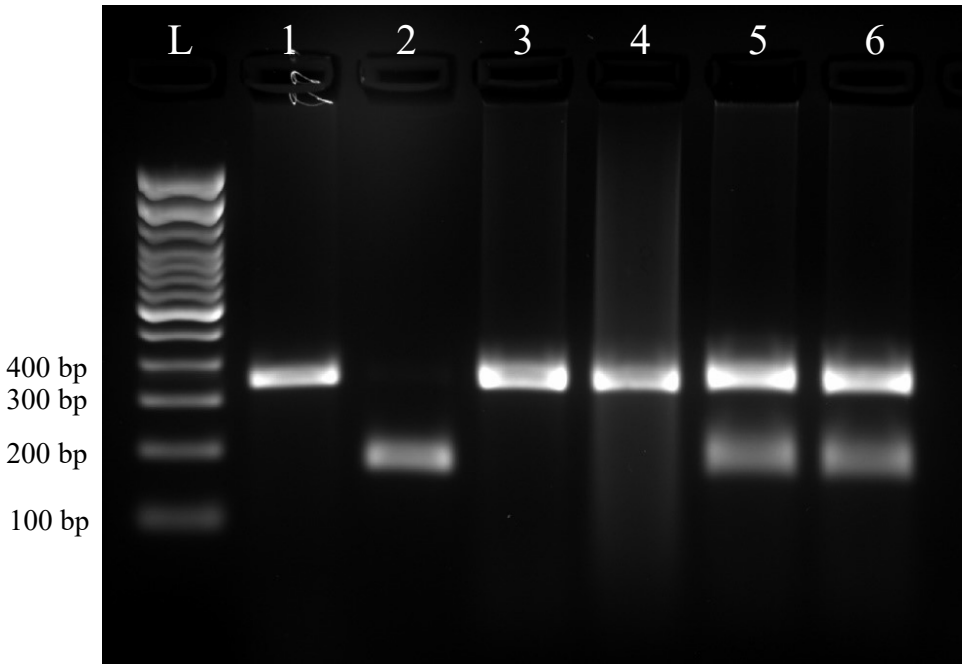

Figure 14. PCR-RFLP SNP typing test for exercise-induced collapse – *Dnm1* gene. Optimized protocols run on 2% agarose gel. L: Invitrogen TrackIt 100 bp DNA ladder. Lane 1: *Dnm1* wild type plasmid control amplicon remains 337 bp. Lane 2: *Dnm1* mutant plasmid control amplicon is digested into 173 bp and 164 bp fragments. Lanes 3 and 4: clear genomic samples. Lanes 5 and 6: carrier genomic samples.

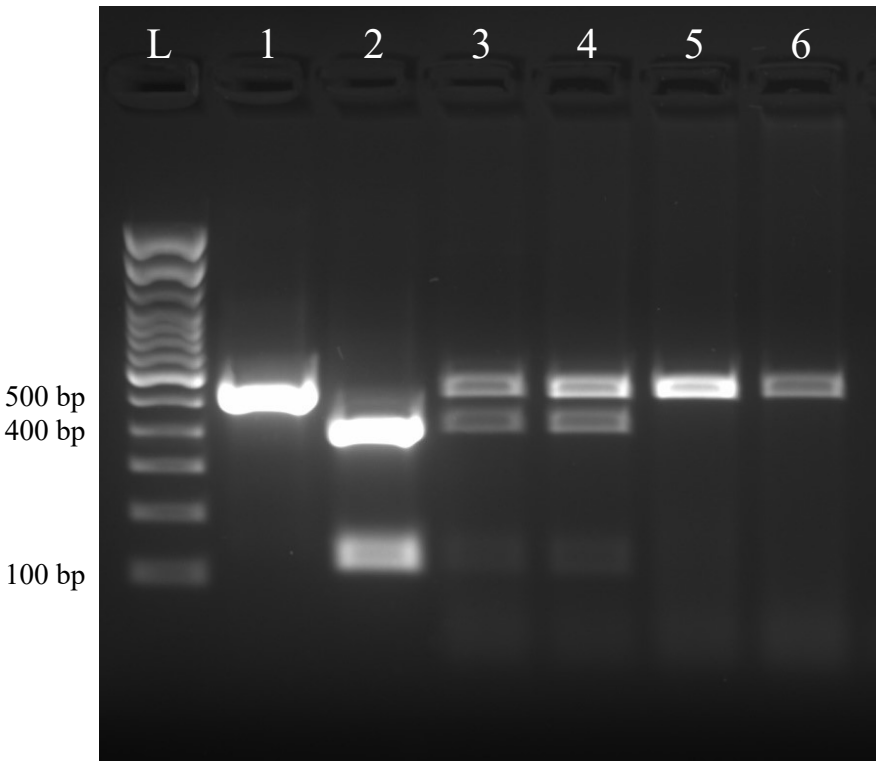

Figure 15. PCR-RFLP SNP typing test for progressive rod-cone degeneration – *Prcd* gene. Optimized protocols run on 2% agarose gel. L: Invitrogen TrackIt 100 bp DNA ladder. Lane 1: *Prcd* wild type plasmid control amplicon remains 512 bp. Lane 2: *Prcd* mutant plasmid control amplicon is digested into 396 bp and 116 bp fragments. Lanes 3 and 4: carrier genomic samples. Lanes 5 and 6: clear genomic samples.

Degenerative Myelopathy

The detection signal for the plasmid DNA controls was too strong, so the plasmid DNA had to be diluted. The addition of approximately 0.15 ng and 0.12 ng of wild type and mutant plasmid controls to the PCR reaction mixture reduced the saturation of the detected bands for the positive and negative controls. Diluting the mutant plasmid control PCR product before adding it to the restriction enzyme digest mixture reduced the strength of the 545 bp band representing incomplete digestion. These modifications resulted in an optimized protocol to detect the alleles present in both plasmid controls and genomic samples (Figure 16).

Centronuclear Myopathy

The new primers designed for the *Ptpla* gene were used in a modified PCR protocol to establish the optimized protocol to detect wild type and mutant alleles in both plasmid and genomic samples (Figure 17).

Validation

Exercise-Induced Collapse

Double-blinded genetic testing was performed for *Dnm1* on 300 genomic samples. Of these samples, 157 had previously been tested by third-party genetic testing providers. Comparing our results with these known samples showed a 94% (n=148/157) genotype match (Table 3). Both wild type and carrier genotypes are disease negative, so

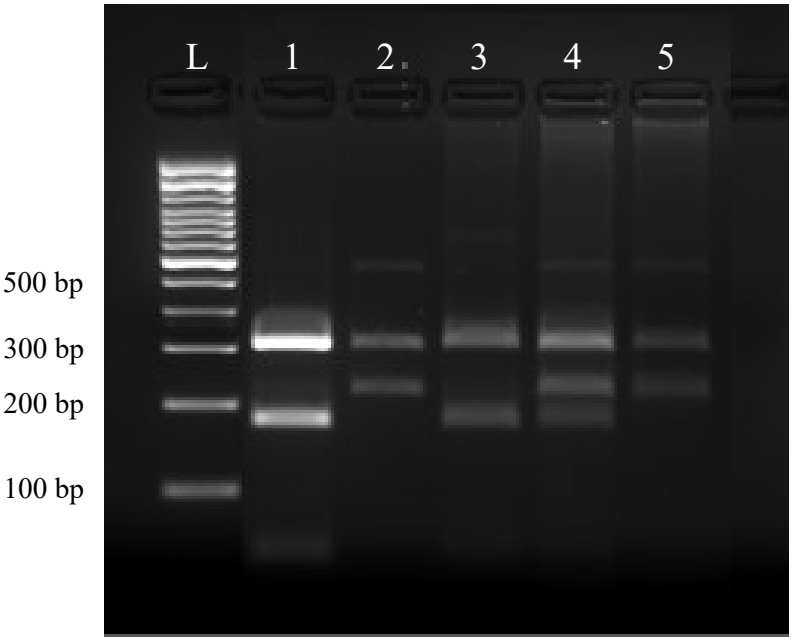

Figure 16. PCR-RFLP SNP typing test for degenerative myelopathy - *Sod1* gene. Optimized protocols run on 3% agarose gel. Wild type and mutant PCR amplicons are 545 bp prior to restriction digestion. L: Invitrogen TrackIt 100 bp DNA ladder. Lane 1: *Sod1* wild type plasmid control amplicon is digested into 317 bp, 179 bp, and 49 bp fragments. Lane 2: *Sod1* mutant plasmid control amplicon is digested into 317 bp and 228 bp fragments. Lane 3: clear genomic sample. Lane 4: carrier genomic sample. Lane 5: affected genomic sample.

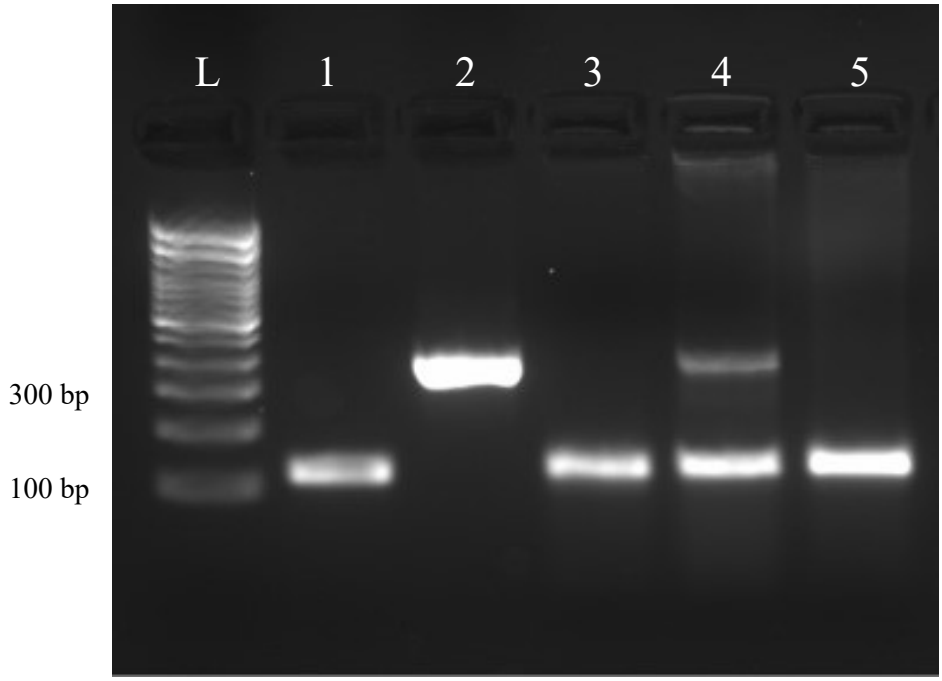

Figure 17. PCR SINE typing for centronuclear myopathy – *Ptpla* gene. Optimized protocol run on 2% agarose gel. L: Invitrogen TrackIt 100 bp DNA ladder. Lane 1: *Ptpla* wild type plasmid control amplicon is 116 bp. Lane 2: *Ptpla* mutant plasmid control amplicon is 352 bp. Lanes 3 and 5: clear genomic samples. Lane 4: carrier genomic sample.

Table 3. *Dnm1* gene genetic testing results from samples of known genetic status (n=157).

		Test Result		
		Mutant	Carrier	Wild Type
True Genetic Condition	Mutant	6	0	0
	Carrier	0	21	0
	Wild Type	0	9	121

100% of our samples' results for EIC disease status matched the results provided by breeders (Table 4).

Progressive Rod-Cone Degeneration

Double-blinded genetic testing was performed for *Prcd* on 275 samples. Subsequent comparison of our results for 129 samples that had been previously tested at third-party genetic testing centers showed a 94% (n=121/129) match to the results provided by breeders (Table 5). Both wild type and carrier genotypes are disease negative, so our results provided a 100% match to the PRCD disease status of the dogs (Table 6).

Degenerative Myelopathy

Double-blinded genetic testing was performed for *Sod1* on 295 samples. Subsequent comparison of our results for 106 samples that had been previously tested at third-party genetic testing centers showed a 97% (n=103/106) match to the *Sod1* genotyping results provided by breeders (Table 7). Both wild type and carrier genotypes are disease negative, so our results provided a 100% match to the DM disease status of the dogs (Table 8).

Table 4. EIC diagnostic results from samples of known genetic status (n=157).

		Test Result	
		Disease Positive	Disease Negative
True Condition	Disease Positive	6	0
	Disease Negative	0	151

Table 5. *Prcd* gene genetic testing results from samples of known genetic status (n=129).

		Test Result		
		Mutant	Carrier	Wild Type
True Genetic Condition	Mutant	0	0	0
	Carrier	0	16	2
	Wild Type	0	6	105

Table 6. PRCD diagnostic results from samples of known genetic status (n=129).

		Test Result	
		Disease Positive	Disease Negative
True Condition	Disease Positive	0	0
	Disease Negative	0	129

Table 7. *Sod1* gene genetic testing results from samples of known genetic status (n=106).

		Test Result		
		Mutant	Carrier	Wild Type
True Genetic Condition	Mutant	0	0	0
	Carrier	0	0	0
	Wild Type	0	3	103

Table 8. DM diagnostic results from samples of known genetic status (n=106).

		Test Result	
		Disease Positive	Disease Negative
True Condition	Disease Positive	0	0
	Disease Negative	0	106

Centronuclear Myopathy

Double-blinded genetic testing was performed for *Ptpla* on 259 samples. Subsequent comparison of our results for 127 samples that had been previously tested at third-party genetic testing centers showed a 98% (n=124/127) match to the results provided by breeders (Table 9). Both wild type and carrier genotypes are disease negative, so our results provided a 100% match to the CNM disease status of the dogs (Table 10).

Table 9. *Ptpla* gene genetic testing results from samples of known genetic status (n=127).

		Test Result		
		Mutant	Carrier	Wild Type
True Genetic Condition	Mutant	0	0	0
	Carrier	0	0	0
	Wild Type	0	3	124

Table 10. CNM diagnostic results from samples of known genetic status (n=127).

		Test Result	
		Disease Positive	Disease Negative
True Condition	Disease Positive	0	0
	Disease Negative	0	127

DISCUSSION

Gene Sequences

There was a lack of correspondence between two major genome browser sites, Ensembl and NCBI, for the *Sod1* gene. The amino acid sequence obtained from the primary literature (Awano et al. 2009) had complete identity with NCBI upstream of the mutation of interest for DM, but there were two amino acids that differed downstream of the mutation of interest. As a result, the position of the mutation from a G to A nucleotide does not correspond between the two major genomic databases, and the position 118 G to A mutation of the *Sod1* gene indicated in the Awano et al. (2009) paper does not correspond to the mutation site displayed on Ensembl. However, the 745 bp sequence containing 100 bp of intron followed by forward primer, gene portion of interest, reverse primer, and subsequent 100 bp of intron, matched 100% with chromosome 31 of *Canis lupus familiaris* on NCBI. For our purposes of a genetic test for DM, a complete match of this 745 bp region of the gene sequence to *Canis lupus familiaris*'s chromosome 31 was considered sufficient evidence that the necessary mutation could be detected, so test development and optimization continued.

Blue/White Screening

Blue/white screening was used following competent cell transformation to

distinguish the propagated colonies as being transformed and containing the gene of interest or non-transformed and, therefore, not containing the gene of interest. For all of the genes except the *Prcd* wild type gene, all the colonies grown on LB ampicillin agar were white. This clearly indicated that the transformation efficiency of the competent cells was high for all genes except for the *Prcd* wild type plasmid, and the gene sequences had inserted and interrupted the lacZ operon of the plasmids.

For the *Prcd* wild type plasmid, blue colonies were propagated on the LB ampicillin agar. The blue colonies are indicative of non-transformed colonies, so these results indicated that the gene had not inserted into the plasmid, or less likely, that it had inserted into an unexpected location in the plasmid without disrupting the lacZ operon.

Since a maxipreparation was performed to isolate the plasmid DNA and a double digest performed with the restriction enzymes used to insert the gene sequence into the plasmid provided the expected band sizes when the double digest product was run on a gel, it was determined that the gene sequence had integrated into the plasmid. Sequencing of the entire plasmid also provided evidence that the *Prcd* gene sequence of interest was within the lacZ operon of the plasmid, and there was a single point mutation in the backbone of the plasmid.

A clear explanation for the growth of blue colonies when the plasmid contained the gene of interest in the lacZ operon has not been convincingly determined. Although it is unlikely, there is a possibility that the single mutation in the backbone of the plasmid may have had an impact on the plasmid's gene expression, causing the lacZ gene to express, leading the colonies to be blue. Another possibility is that the gene sequence

may have been inserted in such a way that it did not largely impact the translation mechanisms for the lacZ gene in the plasmid, allowing the gene to be translated and expressed.

The Four Genetic Tests are Optimized

Protocols have now been developed and optimized for PCR-RFLP SNP typing and PCR SINE typing to detect and unambiguously distinguish wild type from mutant alleles of *Dnm1*, *Prcd*, *Sod1*, and *Ptpla* genes. The detection of these alleles serves as genetic tests for EIC, PRCD, DM, and CNM, respectively.

Band Saturation Resolution

Reduction of the amount of plasmid DNA in the PCR mixture, followed by dilution of the PCR mixture prior to restriction enzyme digest, resolved the issue of gel band saturation for EIC and DM genetic tests. When imaged by ultraviolet light, optimal exposure of all DNA bands of interest in both plasmid and genomic samples was achieved.

PCR-RFLP SNP Typing Tests

The optimized PCR-RFLP SNP typing test for EIC produced the unambiguous bands of the expected size for both the wild type and mutant alleles in both plasmid DNA

controls and genomic samples. A single band is detected at 337 bp for the wild type allele. In contrast, two smaller bands (173 bp and 164 bp) are produced by restriction enzyme digestion for the mutant allele because the mutation creates a novel restriction site for SmlI (Patterson et al. 2008). Since these two bands are very similar in size, they are indistinguishable on the gel and appear as a single band.

In the optimized PCR-RFLP SNP typing test for PRCD, bands of the expected size are unambiguously detected in plasmid DNA controls as well as in genomic samples. A single 512 bp band is detected for the wild type allele, and two bands (396 bp and 116 bp) are detected for the mutant allele, corresponding to the creation of a novel RsaI restriction site by the mutation.

The optimized PCR-RFLP SNP typing test for DM produced unambiguous bands of the expected size for both the wild type and mutant alleles in both plasmid DNA controls and genomic samples. Three bands (317 bp, 179 bp, and 49 bp) are detected for the wild type allele, and two bands (317 bp and 228 bp) are detected for the mutant allele, corresponding to the loss of an AcuI restriction site due to the mutation.

PCR SINE Typing Test

The PCR SINE typing test for CNM clearly distinguishes wild type from mutant alleles in both plasmid and genomic DNA samples. A substantial (236 bp) increase in amplicon size is detected in mutant alleles due to the SINE insertion (Pelé et al. 2005), that when present in duplicate, causes the disease.

As a result of the unambiguous bands detected for wild type and mutant alleles in plasmid DNA samples and genomic samples in each of the four developed tests, we conclude that all four genetic tests have been fully optimized for use in our laboratory.

Validation Process

The first arm of the validation process has been completed by comparing the double-blinded results from our optimized tests to results previously obtained by breeders. In all four diseases, the tested samples have corresponded 100% to previous results in terms of the disease condition of each sample. A disease positive test indicates that disease symptoms, if not already present, will likely develop as the dog ages.

Although a larger number of samples will need to be tested in order to reach higher levels of statistical confidence, the completed tests have corresponded 94% for detecting genotypes for the *Dnm1* gene and *Prcd* gene, corresponded 97% for detecting genotypes for the *Sod1* gene, and corresponded 98% for detecting genotypes for the *Ptpla* gene. The correspondence of each test was determined by directly comparing our test results to the results provided by the breeders. The total number of our results that matched the breeders' results compared to the total number of samples of known genotype that were tested allowed the determination of correspondence as a percentage.

When a dog with a wild type genotype is tested and results indicate the genotype as carrier, there are no health risks to the dog since a carrier remains disease negative; however, breeding to this dog may be unnecessarily avoided to other carriers. The consequences of a carrier being tested and results indicating the genotype as wild type

may be more serious, as the carrier presumed to have a wild type genotype may be bred to another carrier, which may result in the inadvertent generation of affected progeny.

Provided the correspondence values obtained for the four genetic tests are maintained with the remaining samples to be tested and our results are reproducible, they will provide sufficient support for an application to develop the AVC into a full canine genetic testing center.

Future Studies

Second Arm of Test Validation

The samples remaining from the 375 samples initially collected will be tested, and results will be compared to the results provided by breeders from third-party genetic testing providers to continue the validation process. Additionally, in consultation with veterinary epidemiologist Dr. Ian Gardner, who has expertise in diagnostic validation, mutant plasmid DNA will be used to spike known wild type canine buccal DNA samples obtained from breeders to generate large numbers of 'genomic carrier' DNA samples. For further validation, randomly selected samples (n=30) will be sent to Cornell University College of Veterinary Medicine and/or Ontario Veterinary College to assess the reproducibility of our results.

Analytic Sensitivity

The upper and lower limits of analytic sensitivity may be determined once the

tests are validated by performing serial dilutions of the genomic and plasmid DNA that will be added to the PCR reaction mixture. The highest and lowest concentrations of genomic and plasmid DNA added to the mixture visualized by gel electrophoresis will represent the upper and lower limits of analytic sensitivity, respectively.

Conclusion

The overall objective of this study was to develop a canine genetic testing program for four diseases of interest with the aim of establishing the AVC as a center of excellence for canine genetic testing. The four tests were optimized within our lab, and the validation process has been started, with the first arm of test validation completed. Ultimately, the results from this study and further validation from Cornell University College of Veterinary Medicine and/or Ontario Veterinary College will support a grant application to establish a complete genetic testing program at the AVC.

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APPENDICES

Appendix A – Gene Sequences

Dnm1 **Wild Type**

GAATTCGATGCCCGAGATGTGCTAGAGAATAAGCTCCTTCCCCTGCGCAGAGgtaggtaggctct
ccgacccactccacctgcccttcttcaccccaccctgtgcgaggctggttgcccctgacttcggc
ccccttccacagGCTACATAGGGGTGGTAAACCGAAGCCAGAAGGACATTGATGGCAAGAAGGAC
ATCTCAGCTGCCTTGGCCGCTGAACGCAAGTTCTTTCTCTCCCACCCATCCTACCGCCACTTGGC
GGACCGCATGGGCACACCCTACCTACAGAAGGTCCTCAACCAGgtaaggaactcaggcctgggga
aagcagcgcggggacaggtatatttaaatgtgttttgtgaaggtgaccaacaggggttagtgtcc
tcatcatgcagatgaacagagacccagaacacaaagcaacttgcccgcagcccCTGCAG

Dnm1 **Mutant**

GAATTCGATGCCCGAGATGTGCTAGAGAATAAGCTCCTTCCCCTGCGCAGAGgtaggtaggctct
ccgacccactccacctgcccttcttcaccccaccctgtgcgaggctggttgcccctgacttcggc
ccccttccacagGCTACATAGGGGTGGTAAACCGAAGCCAGAAGGACATTGATGGCAAGAAGGAC
ATCTCAGCTGCCTTGGCCGCTGAACTCAAGTTCTTTCTCTCCCACCCATCCTACCGCCACTTGGC
GGACCGCATGGGCACACCCTACCTACAGAAGGTCCTCAACCAGgtaaggaactcaggcctgggga
aagcagcgcggggacaggtatatttaaatgtgttttgtgaaggtgaccaacaggggttagtgtcc
tcatcatgcagatgaacagagacccagaacacaaagcaacttgcccgcagcccCTGCAG

Prcd **Wild Type**

AAGCTTTGCACAAGGTCGGGTTGGCTGACCCCACTAATCAGCTTGAGCCTCCTAATccAGTGGCA
GCAGGAACCTCAGGATGGGCAGCAGTGGCTTGTGAGAGCCGGCAGGGCCATTTTGGCCTTTCTCC
TGCAGACTCTGTCCGGGAGGGGATGGGGCAGCTGAGCCATGTGCACCACCCTCTTCCTACTCAGC
ACCTTGGCCATGCTCTGGCGCCGCCGGTTCGCCAACCGGGTCCAACCgtgagaagctgatggggc
catgggcagggatggggagagaggagaagctaggggtgaggggtggtgcaggggctgcctggac
ctcctgggaggctggagggcggggaggatttgcagggaggtccagagaggtttcccatcagagca
cgcgggggcgggggctcgcaggtgctccgagactggctggagtccccggtcccccagcccaacac
ggccaggagaggggttctgggcccgggcgctgcccacagctcttccagcctcttcctcccgccc
acagGGAGCCCAGCGGAGCAGACGGGGCAGTCGTGGGCAGCAGGTCGGAGAGAGACCTCCAGTCC
TCGGGCAGGTAAGGCAGAGTCTGGGCTGGGGGAGAATTC

Prcd **Mutant**

AAGCTTTGCACAAGGTCGGGTTGGCTGACCCCACTAATCAGCTTGAGCCTCCTAATccAGTGGCA
GCAGGAACCTCAGGATGGGCAGCAGTGGCTTGTGAGAGCCGGCAGGGCCATTTTGGCCTTTCTCC
TGCAGACTCTGTCCGGGAGGGGATGGGGCAGCTGAGCCATGTACACCACCCTCTTCCTACTCAGC
ACCTTGGCCATGCTCTGGCGCCGCCGGTTCGCCAACCGGGTCCAACCgtgagaagctgatggggc
catgggcagggatggggagagaggagaagctaggggtgaggggtggtgcaggggctgcctggac

ctcctgggaggctggagggcggggaggatttgcagggaggtccagagaggtttcccatcagagca
cgcgggggcgggggctcgcaggtgctccgagactggctggagtccccggtcccccagcccaacac
ggccaggagaggggttctgggcccgggcgctgcccacagctcttccagcctcttcctcccgccc
acagGGAGCCCAGCGGAGCAGACGGGGCAGTCGTGGGCAGCAGGTCGGAGAGAGACCTCCAGTCC
TCGGGCAGGTAAGGCAGAGTCTGGGCTGGGGGAGAATTC

Sod1 Wild Type

AAGCTTtgtaaggaggtcaaatcctatgaagtctgtaaacctaccaccttagtgctcactctcct
tgccctactgctagatgcttttcctgtgctattttattaattgtgatgcgggctgcagggctcct
gggctgtgagtgttagaacaaggtcacatctcctttgtgttgggtccccagcctagaatggttaa
atagcttgctagaaagtggtgggcctaggatttgggcacagatctttaaactctcaagtccatgt
tccttccactttcttgtgattggtaaagataatacagctgttttctttgttcagaagcacttgct
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ACAGGGCTGACTGAAGGCGAGCATGGATTCCACGTCCATCAGTTTGGAGATAATACACAAGgtgg
gtgttgtgttggtctagtgactcttctatttgtttcatctagtaagataggactgagtagagcta
ctcctaaacattgaaaatcctcaacatgccaaaaaaaaaaaaaggtcaataggattactaaggtc
agccttagggaaatgatccacaaagccggtttttgactctattgaagtgtggaaaggaaagggaa
gGAATTC

Sod1 Mutant

AAGCTTtgtaaggaggtcaaatcctatgaagtctgtaaacctaccaccttagtgctcactctcct
tgccctactgctagatgcttttcctgtgctattttattaattgtgatgcgggctgcagggctcct
gggctgtgagtgttagaacaaggtcacatctcctttgtgttgggtccccagcctagaatggttaa
atagcttgctagaaagtggtgggcctaggatttgggcacagatctttaaactctcaagtccatgt
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ACAGGGCTGACTAAAGGCGAGCATGGATTCCACGTCCATCAGTTTGGAGATAATACACAAGgtgg
gtgttgtgttggtctagtgactcttctatttgtttcatctagtaagataggactgagtagagcta
ctcctaaacattgaaaatcctcaacatgccaaaaaaaaaaaaaggtcaataggattactaaggtc
agccttagggaaatgatccacaaagccggtttttgactctattgaagtgtggaaaggaaagggaa
gGAATTC

Ptpla Wild Type

GAATTCtggacattgacatttgaatccagttgatctccaatatgttctattcatcccctcgaaga
agggtcagtgtaaaaaccatgtcatttttccagtgggttttgttaaaaagaataatgaactgag
aatgtaataaaacatacatattattactatgaattatgccatttcctttcaggaagatcactggt
attcatttttatagaactttacaaaacacttttatttttctagGTGGTTGGTTCTAGCTATTGC
CATGGTACGTTTTTATATGGAAAAAGGAACACACAAAGGTTTATATAAAAGTATTCAGAAGACAC
TTAAATTTTTCCAGACATTTGCCTTGCTTGAGgtaagttttcagtgatgctgtttttccattgta
ctatttttttgaaaaattatttgttgagcaaaatcaatcttatttttctgatgacatatatcacc
ttcaaatttgtagaaaaccagcattcacgggtttatttttatgttgcatgctattgacttgtgatt
tttttttttatttgcttacagATAGTCCACTGTTTAATTGgtgagtttctgatttaatttttatag
tcggtataaattgcctttagggcaatggttgacttgtttttctctttaagGGATTGTACCTACTT
CTGTGATTGTGGCTGGGGTCCAAGTGAGCTCAAGAATCTTTATGGTGTGGCTCATTACTCACAGT
ACTGCAG

Ptpla Mutant

GAATTCtggacattgacatttgaatccagttgatctccaatatgttctattcatcccctcgaaga
agggtcagtgtaaaaaccatgtcatttttccagtgggttttgttaaaaagaataatgaactgag
aatgtaataaaacatacatattattactatgaattatgccatttcctttcaggaagatcactggt
attcatttttatagaactttacaaaacactttatttttttctagGTGGTTGGTTCTAGCTATTGC
CATGGTACGTTTTTATATGGAAAAAGGAACACACAAAGGTTTTTTTTTTTTTTTTTAAATTTTTT
TTTTTAAATTTTTTTTTTTTAATTTTTTTTTATTTATTTATGATAGTCACACACAGATAGAGAGA
GAGGCAGAGACACAGGCAGAGGGAGAAGCAGGCTCCATGCACCGGGAGCCCGACGTGGGACTCGA
TCCCGGGTCTCCAGGATCGCGCCCTGGGCCAAAGGCAGGCGCCAAACCGCTGCGCCACCCAGGGA
TCCCCCACACAAAGGTTTATATAAAAGTATTCAGAAGACACTTAAATTTTTCCAGACATTTGCCT
TGCTTGAGgtaagttttcagtgatgctgttttccattgtactatttttttgaaaaattatttgt
tgagcaaaatcaatcttatttttctgatgacatatatcaccttcaaatttgtagaaaaccagcat
tcacgggtttatttatgttgcatgctattgacttgtgatttttttttttatttgcttacagATAG
TCCACTGTTTAATTGgtgagtttctgatttaatttttatagtcggtataaattgcctttagggca
atggttgacttgtttttctctttaagGGATTGTACCTACTTCTGTGATTGTGGCTGGGGTCCAAG
TGAGCTCAAGAATCTTTATGGTGTGGCTCATTACTCACAGTACTGCAG

GAATTC – EcoRI restriction site

CTGCAG – PstI restriction site

AAGCTT – HindIII restriction site

Primer sequences are highlighted in yellow

SNPs are highlighted in red

The SINE insertion is indicated in red

Introns are indicated by black lowercase letters

Exons are indicated by BLUE UPPERCASE letters

Appendix B – Letter to Breeders

Dear *breeder's name*,

Our names are Jeremy Lightfoot and Alycia Cooper. We are research students under the supervision of Dr. Sunny Hartwig at the Atlantic Veterinary College (AVC), working on the development of a cost-effective canine genetic testing program for Labrador Retrievers, including testing for centronuclear myopathy (**CNM**), degenerative myelopathy (**DM**), exercise-induced collapse (**EIC**), and progressive rod-cone degeneration (**PRCD-PRA**).

With the generous contribution of breeders in 2018, Phase 1 of our project has been completed, and we now have fully optimized tests for all of the aforementioned diseases. Phase 2 of our project is to validate our results so that we can offer these tests at the AVC. In order to complete Phase 2, we are collecting large numbers of saliva samples (approximately 500 samples), and we anticipate completing the validation this year.

For this reason, we would be tremendously grateful if you would be willing to send us buccal swabs this summer. We will send you the swabbing packs and instructions, and cover return postage costs. In addition, you will find a sheet for documenting your swabs and animals, as well as the genetic status of your animals (if known) for the four diseases we are focusing on.

Thank you once again for your generosity and time. Your participation is crucial for the success of this project, and we look forward to offering this test for Canadian Labrador Retriever breeders within the near future.

We would appreciate if you can please reply to this email.

Thank you so much for your consideration.

Jeremy Lightfoot
Dalhousie BSc., DVM Candidate
Alycia Cooper
4th year UPEI Biology Honours Candidate

Atlantic Canada's College of Veterinary Medicine
550 University Avenue, Charlottetown, Prince Edward Island, Canada C1A 4P3
T(902) 566-0804 F(902) 566-088) upei.ca/avc

Appendix C – Optimized PCR Reaction Mixtures and Thermocycler Conditions

Dnm1

1X Reaction Mixture for *Dnm1* PCR

Component	**Volume (µL)**
DNA	5*
Econotaq PLUS GREEN 2X Master Mix (Lucigen)	12.5
Forward Primers (Sigma-Aldrich)	1.25
Reverse Primers (Sigma-Aldrich)	1.25
Nuclease-free water	5
Total	**25**

*For plasmid controls, add 0.5 µL (approximately 0.21 ng wild type and 0.25 ng mutant) plasmid DNA and 4.5 µL ddH_2O

Thermocycler (Bio-Rad C1000 Thermocycler or Bio-Rad C1000 Touch Thermocycler):

Step 1: Hot start at 95°C for 3 minutes

Step 2: 95°C for 2 minutes

Step 3: 95°C for 30 seconds

Step 4: 68°C for 30 seconds

Step 5: 72°C for 1 minute

Step 6: Go to step 3, repeat for 12 cycles

Step 7: 95°C for 30 seconds

Step 8: 62°C for 30 seconds

Step 9: 72°C for 1 minute

Step 10: Go to step 7, repeat for 25 cycles

Step 11: 72°C for 5 minutes

Step 12: 4°C hold

Prcd

1X Reaction Mixture for *Prcd* PCR

Component	**Volume (µL)**
DNA	5
Econotaq PLUS GREEN 2X Master Mix (Lucigen)	12.5
Forward Primers (Sigma-Aldrich)	1.25
Reverse Primers (Sigma-Aldrich)	1.25
Nuclease-Free Water	5
Total	**25**

For plasmid controls, 5 µL of DNA is approximately 2.17 ng wild type plasmid DNA and 1.30 ng mutant plasmid DNA

Thermocycler (Bio-Rad C1000 Thermocycler and Bio-Rad C1000 Touch Thermocycler):

Step 1: 95°C for 2 minutes

Step 2: 95°C for 30 seconds

Step 3: 58°C for 30 seconds

Step 4: 72°C for 1 minute

Step 5: Go to step 2, repeat for 28 cycles

Step 6: 72°C for 7 minutes

Step 7: 4°C hold

Sod1

1X Reaction Mixture for *Sod1* PCR

Component	**Volume (µL)**
DNA	5*
Econotaq PLUS GREEN 2X Master Mix (Lucigen)	12.5
Forward Primers (Sigma-Aldrich)	1
Reverse Primers (Sigma-Aldrich)	1
DMSO (Sigma Aldrich)	0.5
Nuclease-Free Water	5
Total	**25**

*For plasmid controls, add 0.5 µL (approximately 0.15 ng wild type and 0.12 ng mutant) plasmid DNA and 4.5 µL ddH_2O

Thermocycler (Bio-Rad C1000 Thermocycler and Bio-Rad C1000 Touch Thermocycler):

Step 1: Hot start at 95°C for 1 minute

Step 2: 95°C for 5 minutes

Step 3: 95°C for 30 seconds

Step 4: 55°C for 30 seconds

Step 5: 72°C for 30 seconds

Step 6: Go to step 3, repeat for 30 cycles

Step 7: 72°C for 5 minutes

Step 8: 4°C hold

Ptpla

1X Reaction Mixture for *Ptpla* PCR

Component	**Volume (µL)**
DNA	5
Econotaq PLUS GREEN 2X Master Mix (Lucigen)	12.5
Forward Primers (Sigma-Aldrich)	1.25
Reverse Primers (Sigma-Aldrich)	1.25
Nuclease-Free Water	5
Total	**25**

For plasmid controls, 5 µL of DNA is approximately 3.86 ng wild type plasmid DNA and 6.49 ng mutant plasmid DNA

Thermocycler (Bio-Rad C1000 Thermocycler and Bio-Rad C1000 Touch Thermocycler):

Step 1: 94°C for 2 minutes

Step 2: 94°C for 30 seconds

Step 3: 57°C for 30 seconds

Step 4: 72°C for 1 minute

Step 5: Go to step 2, repeat for 34 cycles

Step 6: 72°C for 5 minutes

Step 7: 4°C hold

Appendix D – Primers for Optimized PCR Reactions

Primer	**5' to 3' Sequence**	**Amplicon Size (bp)**
Ptpla Forward Primer	AGGTGGTTGGTTCTAGCTATTG	116 (wild type)
Ptpla Reverse Primer	AGCAAGGCAAATGTCTGGAA	352 (mutant)
Dnm1 Forward Primer	GTAGGCTCTCCGACCCACTC	337
Dnm1 Reverse Primer	TGAGGACACTAACCCCTGTTG	
Sod1 Forward Primer	CACTCTCCTTGCCCTACTGC	545
Sod1 Reverse Primer	TCATTTCCCTAAGGCTGACC	
Prcd Forward Primer	CCAGTGGCAGCAGGAACC	512
Prcd Reverse Primer	CCGACCTGCTGCCCACGACTG	

Appendix E – Optimized Restriction Enzyme Digestion Reaction Mixtures and Conditions

1X Reaction mixture for restriction enzyme digest of ***Dnm1*** with SmlI:

Component	**Volume (µL)**
DNA (PCR product)	25*
Restriction Enzyme (SmlI) (New England Biolabs – R0597L)	1
CutSmart® Buffer (New England Biolabs)	5
Nuclease-Free Water	19
Total	**50**

*For WT and MT plasmid DNA, add 10 µL PCR product + 15 µL ddH_2O

Incubate in a thermocycler with wells at 55°C and lid temperature at 105°C for 16 hours.

1X Reaction mixture for restriction enzyme digest of ***Prcd*** with RsaI:

Component	**Volume (µL)**
DNA (PCR product)	25
Restriction Enzyme (RsaI) (New England Biolabs – R0167L)	1
CutSmart® Buffer (New England Biolabs)	5
Nuclease-Free Water	19
Total	**50**

Incubate in a thermocycler with wells at 37°C and lid temperature at 45°C for 2 hours.

1X Reaction mixture for restriction enzyme digest of ***Sod1*** with AcuI:

Component	**Volume (µL)**
DNA (PCR product)	25*
Restriction Enzyme (AcuI) (New England Biolabs – R0641L)	1
CutSmart® Buffer (New England Biolabs)	5
Nuclease-Free Water	18.9375
S-adenosylmethionine (SAM) (New England Biolabs – B9003S)	0.0625
Total	**50**

*For MT plasmid DNA, add 5 µL MT PCR product + 20 µL ddH_2O

Incubate in a thermocycler with wells at 37°C and lid temperature at 47°C for 16 hours. Heat inactivate at 65°C for 20 minutes.

Appendix F – Optimized Gel Electrophoresis Conditions

	Dnm1	*Prcd*	*Sod1*	*Ptpla*
Agarose Gel	2%	2%	3%	2%
Amount of Sample to Load to Gel	10 µL plasmid samples 20 µL genomic samples	10 µL plasmid samples 20 µL genomic samples	15 µL plasmid samples 20 µL genomic samples	10 µL plasmid samples 10 µL genomic samples