# Evaluation of 10 genes encoding cardiac proteins in Doberman Pinschers with dilated cardiomyopathy

M. Lynne O'Sullivan, DVM, DVSc; Michael R. O'Grady, DVM, MS; W. Glen Pyle, PhD; John F. Dawson, PhD

**Objective**—To identify a causative mutation for dilated cardiomyopathy (DCM) in Doberman Pinschers by sequencing the coding regions of 10 cardiac genes known to be associated with familial DCM in humans.

**Animals**—5 Doberman Pinschers with DCM and congestive heart failure and 5 control mixed-breed dogs that were euthanized or died.

**Procedures**—RNA was extracted from frozen ventricular myocardial samples from each dog, and first-strand cDNA was synthesized via reverse transcription, followed by PCR amplification with gene-specific primers. Ten cardiac genes were analyzed: cardiac actin, α-actinin, α-tropomyosin, β-myosin heavy chain, metavinculin, muscle LIM protein, myosin-binding protein C, tafazzin, titin-cap (telethonin), and troponin T. Sequences for DCM-affected and control dogs and the published canine genome were compared.

**Results**—None of the coding sequences yielded a common causative mutation among all Doberman Pinscher samples. However, 3 variants were identified in the α-actinin gene in the DCM-affected Doberman Pinschers. One of these variants, identified in 2 of the 5 Doberman Pinschers, resulted in an amino acid change in the rod-forming triple coiled-coil domain.

**Conclusions and Clinical Relevance**—Mutations in the coding regions of several genes associated with DCM in humans did not appear to consistently account for DCM in Doberman Pinschers. However, an α-actinin variant was detected in some Doberman Pinschers that may contribute to the development of DCM given its potential effect on the structure of this protein. Investigation of additional candidate gene coding and noncoding regions and further evaluation of the role of α-actinin in development of DCM in Doberman Pinschers are warranted. (*Am J Vet Res* 2011;72:932–939)

Dilated cardiomyopathy is the most common myocardial disease in dogs and is characterized by reduced contractility (systolic dysfunction) and eccentric hypertrophy (dilation) of the left or both ventricles. It is responsible for a considerable degree of morbidity and death in this species, with the primary consequences being CHF or arrhythmic sudden death.[1] Most cases are idiopathic, and an underlying cause remains to be identified. Comparatively few cases are associated with known underlying causes, such as nutritional deficiencies (eg, taurine deficiency), drugs or toxins (eg, doxorubicin), or primary arrhythmias (tachycardiomyopathy).[1,2] The high prevalence of DCM in specific dog breeds strongly suggests that genetic factors have a role in the pathogenesis of some and probably most forms of DCM in dogs.[3]

**Abbreviations**

| | |
|---|---|
| CHF | Congestive heart failure |
| cTnT | Cardiac troponin T |
| DCM | Dilated cardiomyopathy |
| MYBP-C | Myosin-binding protein C |
| MYHC | β-Myosin heavy chain |
| NCBI | National Center for Biotechnology Information |
| SNP | Single nucleotide polymorphism |
| Tcap | Titin-cap (telethonin) |

The Doberman Pinscher is one such highly affected breed, with almost 50% of dogs affected over their lifetime in some populations.[a] Prospective pedigree analyses in Doberman Pinschers have revealed that DCM is indeed a familial disease inherited as an autosomal dominant trait.[4] The Doberman Pinscher breed in North America is a relatively closed breed; by the 1950s, approximately half of the registered Doberman Pinschers in the United States were descended from a single family.[5] Thus, it is possible that the genetic basis for DCM in that breed stems from the same causative mutation or mutations. The fact that familial forms of DCM are known to exist in humans further suggests

Received February 10, 2010.
Accepted May 3, 2010.
From the Departments of Clinical Studies (O'Sullivan, O'Grady), Biomedical Sciences (Pyle), and Molecular and Cellular Biology (Dawson), University of Guelph, Guelph, ON N1G 2W1, Canada.
Supported by OVC Pet Trust, Ontario Veterinary College, University of Guelph.
The authors thank Shu Chen and Jiping Li for technical assistance.
Address correspondence to Dr. Dawson (jdawso01@uoguelph.ca).

a genetic cause, and several genetic mutations responsible for these forms of DCM have been described.[6–8]

Dilated cardiomyopathy in humans is characterized by the same structural and functional changes in affected Doberman Pinschers, and at least 30% to 50% of cases are familial (genetic).[7] Mutations that affect genes encoding proteins of the contractile apparatus may cause defective force generation, and genetic mutations that affect actin, myosin, MYBP-C, and troponin T have been described.[6–8] Similarly, mutations that affect genes encoding cardiac structural proteins may cause defective force transmission and mutations affecting titin, desmin, dystrophin, dystroglycans, and sarcoglycans have been described.[6–8] Other genetic mutations may affect proteins involved in calcium handling, thereby altering availability of or sensitivity to calcium (eg, phospholamban and SERCA2), or proteins involved in regulating energy use, thereby causing energy deficits. Evaluation of genetic mutations known to be associated with human familial DCM may provide a framework within which to begin investigating the causes of DCM in dogs.

Comparatively little information exists with respect to the genetics of DCM in dogs; however, mutations of genes encoding for contractile, regulatory, and structural cardiac proteins are all potential candidates. With respect to isolated DCM (in the absence of skeletal muscle involvement), studies investigating genetic causes in dogs have revealed neither mutations in specific segments of the cardiac actin, desmin, sarcoglycan delta, phospholamban, troponin C, lamin A/C, cysteine- and glycine-rich protein 3, cTnT, or MYHC7 genes in Doberman Pinschers[9–13] nor abnormalities in myocardial dystrophin, α-sarcoglycan, or β-dystroglycan in various breeds, including Doberman Pinschers.[14] No changes were found in the sequences of Tcap, α-tropomyosin, vinculin, or taffazin in Newfoundlands or Irish Wolfhounds with DCM.[15–17] Results of other research have suggested that DCM in a European line of Doberman Pinschers may be associated with a mutation of the titin gene.[b] To our knowledge, this was the first evidence supporting a potential specific genetic cause for DCM in Doberman Pinschers.

The purpose of the study reported here was to identify a causative mutation for DCM in Doberman Pinschers by sequencing the coding regions of 10 cardiac genes associated with familial DCM in humans. The 10 genes were selected on the basis of known mutations associated with DCM in humans who had the characteristics of the disease that develops in Doberman Pinschers, combined with information from the scarce literature regarding genetic linkage to DCM in dogs and from the recently available dog genome.[18–20]

## Materials and Methods

The coding sequences of 10 genes from 5 control dogs without overt signs of cardiac disease and 5 Doberman Pinscher dogs with DCM were determined by use of reverse transcriptase PCR analysis of mRNA and sequencing of the resulting cDNA. The resulting sequences were compared with the sequences in the dog genome obtained from the Boxer breed.[21] The sequences in the dog genome database were accepted as wild type.[20]

**Dogs**—The experimental group included 4 male Doberman Pinschers (2 sexually intact and 2 neutered) and 1 spayed female Doberman Pinscher. The dogs' ages ranged from 7.1 to 10.9 years (mean age, 8.7 years) and weights ranged from 27.6 to 42.0 kg (mean weight, 36.2 kg). From the last available echocardiographic examinations for each dog, the mean left ventricular internal dimension in diastole was 55.7 mm (range, 48.23 to 65.10 mm), mean left ventricular internal dimension in systole was 48.57 mm (range, 40.57 to 55.32 mm), and mean fractional shortening was 12.7% (range, 7.9% to 15.8%). The Doberman Pinschers had been treated for CHF for 1 to 273 days.

The control group included 4 male dogs (3 sexually intact and 1 neutered) and 1 sexually intact female dog. All of these pound-source dogs were adults, although their exact ages were unknown. The dogs' weights ranged from 21.5 to 36 kg (mean weight, 27.4 kg). Breeds included German Shepherd Dog (n = 1), Border Collie (1), and mixed (3). The history and clinical data for these dogs were unknown, except that the hearts were grossly normal with no evidence of DCM or CHF on postmortem examination. Dogs of breeds other than Doberman Pinscher were used as controls instead of Doberman Pinschers with no signs of DCM to avoid the possibility of misclassification of dogs with subclinical disease, given the adult-onset nature of the disease.

**Selection of target genes**—Ten candidate genes were selected for cDNA sequencing to determine whether changes in the coding sequence of the gene might be linked to the development of DCM in Doberman Pinschers. Candidate genes known to be associated with familial forms of isolated DCM (DCM unassociated with skeletal myopathy or conduction system disease) in humans were selected. The genes investigated included those encoding sarcomeric proteins involved in contraction (cardiac actin, α-tropomyosin, MYHC7, cTnT, and MYBP-C), sarcomere-associated proteins of the stretch sensor machinery (Tcap and muscle LIM protein), and cytoskeletal proteins involved in force transmission (α-actinin and metavinculin). These genes are all associated with autosomal dominant familial DCM in humans, which is also the mode of inheritance of DCM in Doberman Pinschers.[4] The exception is taffazin, which comes from a unique group of proteins whose function is largely unknown and is associated with X-linked recessive forms of DCM in infants, although a late-onset form has also been described in 1 family.[8,22] Although a potential association between DCM in Doberman Pinschers and the protein titin has been reported,[b] the size of the coding sequence (encompassing 363 exons) excluded this candidate gene from the present study.

**Myocardial samples**—Myocardial samples were obtained from 5 client-owned Doberman Pinschers with DCM and CHF (experimental group) and 5 dogs of breeds other than Doberman Pinscher (control group). Four of the 5 Doberman Pinschers in the experimental group were euthanized by IV injection of pentobarbital sodium (102 mg/kg) at the University of Guelph Veterinary Teaching Hospital at the request of their owners after being treated for CHF secondary to DCM for

a period of 1 to 194 days. The remaining Doberman Pinscher had been treated for CHF due to DCM for 273 days and was brought to the hospital immediately following death at home. Written consent for postmortem examination was obtained from all owners. For each dog, a diagnosis of DCM and CHF had been determined on the basis of a history of respiratory signs, previous thoracic radiographic or postmortem evidence of pulmonary edema, and previous echocardiographic examination findings supportive of DCM. For the latter, the criteria included left ventricular internal dimension in diastole > 0.1749 × body weight (kg) + 40.255 mm or left ventricular internal dimension in systole > 0.1402 × body weight (kg) + 34.99 mm (both derived on the basis of unpublished observations of the relation between left ventricular dimension and body weight in 51 clinically normal Doberman Pinschers) and no other concurrent cardiac disease.

For the control group, myocardial samples were collected from pound-source dogs arriving at the Animal Health Laboratory immediately following euthanasia at the end of veterinary surgical laboratories at the teaching hospital. Dogs were euthanized via IV injection of pentobarbital sodium (102 mg/kg). There was no evidence of cardiac disease on gross postmortem examination in all dogs.

For each of the study dogs, full-thickness myocardial tissue samples from the left ventricular free wall were obtained within 30 minutes after death, snap frozen in liquid nitrogen, and stored at –70°C until processing.

**RNA extraction**—The frozen myocardial tissue samples were transferred to a reagent solution[c] and stored in a –20°C freezer overnight prior to RNA extraction. Total RNA was extracted from 150 mg of tissue by use of 1 of 2 RNA isolation kits[d,e] following the manufacturers' protocols for RNA extraction from tissue samples.

**cDNA synthesis and amplification**—Gene-specific primers (**Appendix 1**) were designed by use of the NCBI database, a search of the canine genome,[20] and software.[f] The primers were synthesized at University of Guelph Laboratory Services by use of a high-throughput column.[g] When possible, primers were synthesized corresponding to mRNA sequences outside of the coding region of each cDNA of interest to ensure complete coverage of all exons.

Difficulties with amplification led to redesign of primers for the metavinculin cDNA. The new forward primer corresponded to nucleotides 27,633,897 to 27,633,915 on chromosome 4 of the dog genomes, 39 nucleotides upstream of the start codon in the accession file (Appendix), whereas the reverse primer encompassed the predicted stop codon. Despite the redesign, only the reverse primer provided good-quality sequence, with incomplete coverage of all exons.

Two micrograms of total RNA was used to synthesize cDNA via reverse transcription with random hexamers.[h] Two microliters of the first-strand cDNA was then amplified via the gene-specific primers by use of PCR procedures.[i] Reactions (25 µL) contained 1× PCR buffer I, 400nM each of the primers, and 1.0 U of *Taq*. Next, PCR thermal cycling was conducted by use of a sequence detector system[j] under the following conditions: initial incubation at 95°C for 15 minutes; 35 cycles of 94°C for 20 seconds; 52° to 60°C for 30 seconds, depending on the melting temperature of the primers; and 72°C for 2 to 5 minutes, depending on the length of the amplicon, followed by a final extension at 72°C for 7 minutes. The PCR fragments were confirmed by use of ethidium bromide–stained agarose gels.[k]

**DNA sequencing**—The PCR fragments were purified by use of a PCR clean-up plate.[l] The purified fragments were sequenced from 5′ and 3′ ends until 2 strands reached 100% overlap. Approximately 5 to 30 ng of each purified PCR fragment was sequenced by use of the dye deoxy terminator method[m] and a sequence detector system[j] or thermal cycler.[n] Cycle sequencing reaction conditions were performed as described by the manufacturer.[m] Dye terminators and primers were removed from the cycle sequencing reactions by use of filtration plates[o] loaded with gel filtration medium.[p] The purified reaction products underwent electrophoresis by use of an automated sequencer.[q] The chromatograms were analyzed by use of software[r] to generate high-quality target sequences within the software's clear confidence range. The edited sequences were assembled with software[g] by use of the default settings to produce a consensus sequence. Gaps in the DNA sequences were closed by use of primer walking techniques, where each sequencing primer was designed on the basis of the end of the results obtained with the previous sequencing primer until the entire gene sequence was determined. The amplification primers were also used for producing sequences. For the forward and reverse sequencing primers, the overall mean coverage of each primer was 673 bp (**Appendix 2**).

## Results

**Analysis of cDNA sequences**—The coding regions of the 10 genes of interest were sequenced, including some untranslated regions of the cDNAs (Appendix 1). The sequence of the tafazzin coding region from 1 control dog was removed because of priming problems where little sequence was available. The remaining 9 tafazzin sequences covered the entire coding sequence with substantial coverage of the 3′ and 5′ untranslated region. The myosin sequences were found to be the result of PCR-mediated recombination. As in all other mammals, the cardiac myosin gene in dogs consists of a tandem gene cluster; on dog chromosome 8, the first cardiac myosin gene is the α isoform (designated MYHC6) and the second is the β isoform (designated MYHC7). Analysis of the myosin gene sequencing results revealed a chimeric sequence containing the first 33 exons from the β-cardiac myosin gene and the last 5 exons from the α-cardiac myosin gene. In the middle of the β-cardiac myosin sequence, a short stretch of α-cardiac myosin sequence (corresponding to exons 18 to 21) was uncovered in several samples, including samples of 4 of the 5 Doberman Pinschers. An error in the design was identified: the 3′ primer used annealed to the α-cardiac myosin cDNA. This error in design occurred because of an inconsistency in the NCBI

dog genome database at the time of the primer design process, where the β- (MYHC7) and α-cardiac myosin (MYHC6) genes were both designated as MYHC6.

The coding regions of the actin, tafazzin, and Tcap genes were 100% identical in tissue samples from all the dogs. Moreover, the sequences from all of the dogs examined were 100% identical within the β-cardiac myosin gene exons (consisting of over 4,500 nucleotides, covering > 75% of the β-cardiac myosin sequence). For cTnT, reported degenerate primers[23] failed to produce product and new primers were designed to encompass the start and stop codons of the gene. A single SNP in the cTnT sequence resulted in a silent (ie, not causing a change in the amino acid) mutation in the coding region of 1 control dog. For muscle LIM protein and tropomyosin, the coding sequences of the respective gene were 100% identical among the samples; however, 1 SNP was detected in the 5′ untranslated region of the LIM coding sequence for 1 dog. One SNP was detected in the 5′ untranslated region of the tropomyosin coding sequence for 2 dogs (**Table 1**): one of these dogs had a homozygous change from C to A in the 5 untranslated region, and the other had a combination of 2 nucleotides (C and A) at this position. We interpreted sequenced combinations of 2 nucleotides at the same position as allelic heterozygosity (**Figure 1**). Such heterozygosity was observed in the sequences of other genes. The coding regions of 3 genes revealed multiple DNA sequence differences. These genes included MYBP-C, metavinculin, and α-actinin.

**MYBP-C**—Several SNPs within the MYBP-C coding region were identified from the sequencing analysis (**Table 2**). All the changes led to silent mutations, except a heterozygous difference in 1 control dog, where 1 allele coded for a protein amino acid substitution (aspartic acid at residue 913 replaced by glycine [p.Asp913Gly]).

**Metavinculin**—The initial primer pair used to produce the metavinculin cDNA failed to amplify any product. When new primers were designed, several rounds of PCR procedures were required to produce product and then only the reverse primer covering the end of the coding sequence for the predicted metavin-

Table 1—Genes with few SNPs identified in ventricular myocardial samples obtained from dogs of breeds other than Doberman Pinscher without overt signs of cardiac disease (control dogs) and Doberman Pinschers with DCM and CHF in a study to identify a causative mutation for DCM in Doberman Pinschers by sequencing the coding regions of 10 cardiac genes associated with familial DCM in humans.

| Gene | DNA sequence change | Dogs (allelic status) |
|---|---|---|
| cTnT | c.18A>G | Control dog 1 (G/G) |
| Muscle LIM protein | –63C>T | Control dog 1 (T/T) |
| Tropomyosin | –8C>A | Control dog 2 (A/A); control dog 1 (C/A) |

Tissue samples were collected for analysis from 5 control dogs and 5 Doberman Pinschers with DCM and CHF; dogs in each group were designated 1 through 5. The DNA sequence change is presented as the substitution at the specified nucleotide (eg, 18A>G is an A to G substitution at nucleotide 18 of the coding sequence).
Prefix c. = Coding.

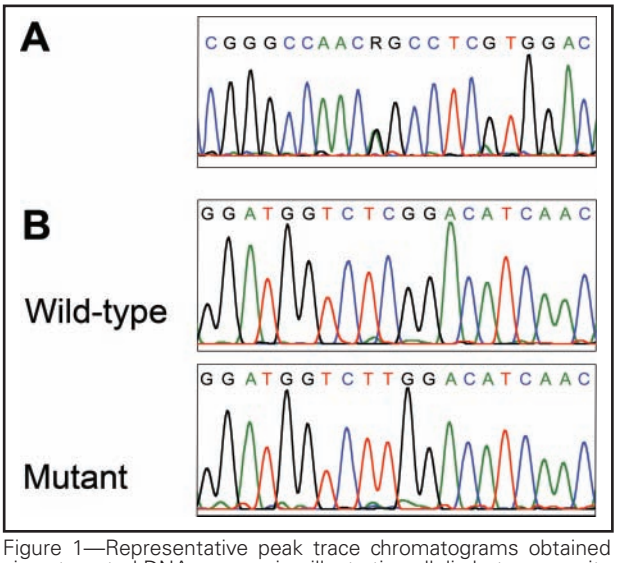


Figure 1—Representative peak trace chromatograms obtained via automated DNA sequencing illustrating allelic heterozygosity and SNP in cDNA obtained from ventricular myocardial samples from non–Doberman Pinscher dogs without overt signs of cardiac disease (control dogs) and Doberman Pinscher dogs with DCM and CHF. A—Evidence of heterozygosity on the noncoding strand complementary to c.789T>C (T to C substitution at nucleotide 789 in the coding sequence) in the MYBP-C gene from a control dog. B—The c.1085C>T mutated allele coding for a p.Ser362Leu substitution (serine at residue 362 replaced by leucine) in the α-actinin protein from a Doberman Pinscher with DCM, compared with the wild-type sequence.

Table 2—Single nucleotide polymorphisms identified in the MYBP-C gene in ventricular myocardial samples obtained from dogs of breeds other than Doberman Pinscher without overt signs of cardiac disease (control dogs) and Doberman Pinschers with DCM and CHF in a study to identify a causative mutation for DCM in Doberman Pinschers by sequencing the coding regions of 10 cardiac genes associated with familial DCM in humans.

| DNA sequence change | Amino acid change | Dogs (allelic status) |
|---|---|---|
| c.789T>C | — | Control dog 4 and DP 4 (C/C); control dog 2 and DPs 2 and 3 (T/C) |
| c.1671G>A | — | Control dogs 1 and 2 (A/A); control dog 4 (G/A) |
| c.2741A>G | p.Asp913Gly | Control dog 1 (A/G) |
| c.3165A>G | — | Control dog 3 (G/G); control dog 4 and DP 1 (A/G) |
| c.3507C>T | — | Control dog 3 (T/T) |
| c.3540G>A | — | DP 5 (G/A) |
| c.3585A>T | — | Control dogs 1 and 3 (T/T) |
| c.3855C>G | — | Control dog 4 and DP 4 (G/G) |

— = No amino acid change. Asp = Aspartic acid. DP = Doberman Pinscher. Gly = Glycine. Prefix p. = Protein amino acid numbers.
*See* Table 1 for remainder of key.

Table 3—Single nucleotide polymorphisms identified in the metavinculin gene in ventricular myocardial samples obtained from dogs of breeds other than Doberman Pinscher without overt signs of cardiac disease (control dogs) and Doberman Pinschers with DCM and CHF in a study to identify a causative mutation for DCM in Doberman Pinschers by sequencing the coding regions of 10 cardiac genes associated with familial DCM in humans.

| DNA sequence change | Amino acid change | Dogs (allelic status) |
|---|---|---|
| c.440C>A | p.Asn147Thr | Control dog 4 (A/A) |
| c.501A>G | — | Control dogs 1, 2, 3, and 4 and DP 5 (G/G); DP 1 (A/G) |
| c.666C>T | — | Control dogs 3 and 4 and DP 5 (C/T) |
| c.1887G>A | — | DP 2 (G/A) |
| c.1970C>T | — | Control dog 4 and DP 1 (C/T) |
| +38G>T | — | Control dog 4 and DP 1 (G/T) |

Asn = Asparagine. Thr = Threonine.
*See* Tables 1 and 2 for remainder of key.

Table 4—Single nucleotide polymorphisms identified in the α-actinin gene in ventricular myocardial samples obtained from dogs of breeds other than Doberman Pinscher without overt signs of cardiac disease (control dogs) and Doberman Pinschers with DCM and CHF in a study to identify a causative mutation for DCM in Doberman Pinschers by sequencing the coding regions of 10 cardiac genes associated with familial DCM in humans.

| DNA sequence change | Amino acid change | Dogs (allelic status) |
|---|---|---|
| c.367G>A | p.Val123Ile | Control dog 2 (A/A) |
| c.594T>C | — | DPs 2, 4, and 5 (C/C) |
| c.966T>C | — | DPs 1, 2, and 5 (C/C) |
| c.1068A>G | — | Control dogs 1, 2, 3, and 5 (G/G) |
| c.1085C>T | p.Ser362Leu | DPs 2 and 5 (T/T) |

Ile = Isoleucine. Leu = Leucine. Ser = Serine. Val = Valine.
*See* Tables 1 and 2 for remainder of key.

culin protein provided good sequence information. As a result, the coding region data started at protein amino acid residue 75 (serine) of the full coding sequence. All of the changes observed were silent differences, except for a p.Asn147Thr substitution (asparagine at residue 147 replaced by threonine) found on 1 chromosome of a control dog (**Table 3**).

Although the reverse primer was designed to sequence the end of the coding sequence reported in accession file XM_536395 in the NCBI database, the sequences from all samples revealed 1 long exon at the 3′ end of the gene encompassing exons 15 to 17 of the prediction from the dog genome database. The long sequence codes for a stop codon immediately after the end of exon 15, resulting in a coding sequence that is 85 amino acids shorter than predicted (GenBank accession No. HM163574).

**α-Actinin**—Various transcript variants were identified in the sequencing of the α-actinin gene. The sequence portion (2,331 to 2,411 bp) of one of the control dogs and one of the Doberman Pinschers with DCM matched the transcript variant 11 XM_861890, rather than XM_848010 in the other subjects. In the control dog sequence, a p.Val123Ile substitution (valine at residue 123 replaced by isoleucine) was noted.

Within the remaining dogs, 3 homozygous variants in the α-actinin gene were uncovered that segregated with the Doberman Pinschers (**Table 4**); however, none of these changes were present in all of the Doberman Pinschers studied. Of these variants, 2 represented silent mutations and 1 coded for a p.Ser362Leu substitution (serine at residue 362 replaced by leucine; Figure 1). The Ser362Leu substitution was in the rod domain in a turn region between major α-helical regions of the first spectrin repeat.

## Discussion

A genetic test for DCM in Doberman Pinschers and other canine breeds would be invaluable to clinicians and breeders for the purposes of identifying carriers to potentially exclude from breeding, to aid in the diagnosis of equivocal cases, and for early detection of disease allowing intervention that may impact disease progression. Development of such a genetic test first requires identification of the genetic mutations underlying the disease in families or breeds. In the present study of DCM in Doberman Pinschers, no consistent mutation was observed in the coding sequences of the 10 cardiac genes investigated.

To our knowledge, Tcap, α-tropomyosin, metavinculin, MYBP-C, tafazzin, or α-actinin have not previously been investigated for association with DCM in Doberman Pinschers. As in Newfoundlands and Irish Wolfhounds,[15–17] causative mutations were not found in the Tcap, α-tropomyosin, or taffazin coding regions in Doberman Pinschers with DCM. Mutations in MYBP-C are typically associated with hypertrophic cardiomyopathy, as is the case in Maine Coon and Ragdoll cats.[24,25] One mutation has been associated with a sporadic case of human DCM[26]; however, no notable changes in the MYBP-C coding region in Doberman Pinschers with DCM were found in the present study.

The sequence of the metavinculin coding region obtained with the reverse primer revealed 1 large exon at the end of the gene, contrary to the predicted exon structure from the gene prediction[s] (chr4, 27,614,605 to 27,729,357) provided by a bioinformatics search tool[t] when the sequence from the accession XP_536395 is used. The coding sequence obtained in the present study starts in the middle of the predicted exon 3 and ends at a TGA stop codon immediately following exon 15, as opposed to the 1,127 amino acid predicted protein sequence, which also possessed 7 exons beyond the end of the predicted exon structure that correspond to expressed sequence tags. As a result, the predicted sequence of the protein possesses a different N-terminus and different exons at the C-terminus. The amplification primers used for metavinculin were based on the

shorter sequence in the accession file; they would not amplify sequences that correspond to the longer predicted sequence.

There were discrepancies between what was sequenced and what has been predicted and observed with expressed sequence tags. Complicating matters is the fact that the metavinculin coding sequence is a splicing variant of the vinculin gene. It is possible that the predicted metavinculin sequence in the accession file is not accurate and that the N-terminus sequence provided by the bioinformatics search tool[t] is correct. The existence of this different N-terminus sequence would help explain why multiple rounds of PCR procedures were required to produce product and no reliable sequence data were obtained by use of the forward sequencing primer. Together with the different C-terminus obtained in the sequencing, it is possible that part of a splice variant of the gene that has not been reported previously was amplified and sequenced.

The present study revealed a point mutation in the α-actinin rod sequence in 2 of the 5 dogs with DCM (a spayed female and a sexually intact male). Mutations in the coding region for the α-actinin gene are linked to heart failure.[27] In particular, the rod region of α-actinin is crucial for dimerization of the protein[28,29] and has been linked to cardiomyopathy because signaling molecules known to be involved in heart failure directly interact with α-actinin through this segment.[30,31] The failure to find the same mutation in α-actinin in all affected dogs indicated that this mutation is not the sole causative change for DCM in Doberman Pinschers; however, an effect of this change in the 2 affected dogs cannot be ruled out.

The study of this report had several limitations. First, only the exonic regions of the genes of interest were sequenced; therefore, changes cannot be excluded in the intronic regions and regions outside of the sequenced 5′ and 3′ regions that could affect the regulation and expression of the genes and ultimately impact protein expression, structure, or function. Second, the number of dogs studied was small, such that it is possible that amino acid changes in the population were not detected. The North American Doberman Pinscher breed has a fairly tight and closed gene pool, so it seems probable that there is a common mutation in the breed that is unlikely to be missed, even in a small group. However, even within the closed gene pool of the Doberman Pinscher breed, there may be variations and subgroups that could complicate the search for mutations that cause DCM.

The candidate gene approach is most useful when there are few genes of interest of large effect. There are > 20 DCM-causing genes identified to date in humans, making those genes rational targets to investigate in dogs with DCM, but most are not considered to have a large effect. The genetic mutations identified to date only account for a very small percentage of familial DCM cases in humans, suggesting there are many other mutations associated with the disease yet to be discovered.[6] Linkage analysis may be useful to help identify loci of interest by determining whether coinherited regions of the genome exist in affected individuals; however, this technique requires adequate multigeneration information and, ideally, large numbers of families with many affected individuals for it to be most useful. To illustrate this challenge, Meurs et al[4] were unable to find a major region of interest in their prospective genetic evaluation of a Doberman Pinscher family despite examination of 41 dogs in 4 generations in which there were 25 DCM-affected dogs. Linkage analysis may also be difficult in dog breeds because of limited family information and the difficulty of accurately identifying affected individuals with adult-onset disease.

Results of the present study did not indicate that there was a common and completely segregating amino acid change in the coding sequences of the 10 genes linked with DCM in the Doberman Pinschers. The study findings add to the growing body of knowledge suggesting that the development of DCM in this breed of dog is linked to more than a single gene mutation. In support of this added complexity, there is a gradation of DCM disease phenotypes, depending on the combination of multiple gene mutations, in humans. Alternatively, there may be a single gene mutation responsible for DCM in affected Doberman Pinschers, but that gene has not been among the genes studied to date.

a. O'Grady MR, Horne R. The prevalence of dilated cardiomyopathy in Doberman Pinschers: a 4.5 year follow-up (abstr). *J Vet Intern Med* 1998;12:199.
b. Stabej P, Leegwater PA, Imholz S, et al. The giant myofilament protein titin is associated with dilated cardiomyopathy in the Doberman (abstr), in *Proceedings*. 2nd Int Conf Adv Canine Feline Genomics 2004.
c. RNAlater-ICE solution, Ambion Inc, Streetsville, ON, Canada.
d. PureYield RNA Midiprep System, Promega Corp, Madison, Wis.
e. RNeasy Midi Kit, Qiagen, Mississauga, ON, Canada.
f. Gene Tools Software, BioTools, Edmonton, AB, Canada.
g. ABI 3900 High-Throughput DNA Synthesizer, Applied Biosystems, Foster City, Calif.
h. SuperScript First-Strand Synthesis System, Invitrogen, Carlsbad, Calif.
i. AccuPrime Taq DNA Polymerase High Fidelity kit, Invitrogen, Carlsbad, Calif.
j. GeneAmp PCR System 9700, Invitrogen, Carlsbad, Calif.
k. Bioshop, Burlington, ON, Canada.
l. NucleoFast 96 PCR clean-up plate, Biolynx, Brockville, ON, Canada.
m. BigDye Terminator Cycle Sequencing Ready Reaction kit, version 3.1, Invitrogen, Carlsbad, Calif.
n. GeneAmp PCR System 2720 Thermal Cycler, Invitrogen, Carlsbad, Calif.
o. Multiscreen-HV plates, Millipore, Mississauga, ON, Canada.
p. Sephadex G-50 superfine, Sigma-Aldrich, Oakville, ON, Canada.
q. 3730 DNA Analyzer, Invitrogen, Carlsbad, Calif.
r. ABI Prism DNA Sequencing Analysis Software, version 3.7, Invitrogen, Carlsbad, Calif.
s. N-SCAN gene prediction system, UCSC Genome Bioinformatics, University of California-Santa Cruz, Santa Cruz, Calif. Available at: genome.ucsc.edu/. Accessed Apr 16, 2009.
t. BLAT, UCSC Genome Bioinformatics, University of California-Santa Cruz, Santa Cruz, Calif. Available at: genome.ucsc.edu/. Accessed Apr 16, 2009.

## References


1. O'Grady MR, O'Sullivan ML. Dilated cardiomyopathy: an update. *Vet Clin North Am Small Anim* 2004;34:1187–1207.
2. Tidholm A, Haggstrom J, Borgarelli M, et al. Canine idiopathic dilated cardiomyopathy. Part I: aetiology, clinical characteristics, epidemiology and pathology. *Vet J* 2001;162:92–107.

3. Meurs KM. Insights into the heritability of canine cardiomyopathy. *Vet Clin North Am Small Anim Pract* 1998;28:1449–1457.
4. Meurs KM, Fox PR, Norgard M, et al. A prospective genetic evaluation of familial dilated cardiomyopathy in the Doberman Pinscher. *J Vet Intern Med* 2007;21:1016–1020.
5. Walker J. *New Doberman Pinscher*. Hoboken, NJ: Howell Book House, 1981;18–25.
6. Hare JM. The dilated, restrictive, and infiltrative cardiomyopathies. In: Libby P, Bonow RO, Mann DL, et al, eds. *Braunwald's heart disease: a textbook of cardiovascular medicine*. 8th ed. Philadelphia: WB Saunders Co, 2008;1739–1762.
7. Karkkainen S, Peuhkurinen K. Genetics of dilated cardiomyopathy. *Ann Med* 2007;39:91–107.
8. Fatkin D, Graham RM. Molecular mechanisms of inherited cardiomyopathies. *Physiol Rev* 2002;82:945–980.
9. Meurs KM, Magnon AL, Spier AW, et al. Evaluation of the cardiac actin gene in Doberman Pinschers with dilated cardiomyopathy. *Am J Vet Res* 2001;62:33–36.
10. Stabej P, Imholz S, Versteeg SA, et al. Characterization of the canine desmin (DES) gene and evaluation as a candidate gene for dilated cardiomyopathy in the Doberman. *Gene* 2004;340:241–249.
11. Stabej P, Leegwater PA, Imholz S, et al. The canine sarcoglycan delta gene: BAC clone contig assembly, chromosome assignment and interrogation as a candidate gene for dilated cardiomyopathy in Dobermann dogs. *Cytogenet Genome Res* 2005;111:140–146.
12. Stabej P, Leegwater PA, Stokhof AA, et al. Evaluation of the phospholamban gene in purebred large-breed dogs with dilated cardiomyopathy. *Am J Vet Res* 2005;66:432–436.
13. Meurs KM, Hendrix KP, Norgard MM. Molecular evaluation of five cardiac genes in Doberman Pinschers with dilated cardiomyopathy. *Am J Vet Res* 2008;69:1050–1053.
14. Spier AW, Meurs KM, Coovert DD, et al. Use of western immunoblot for evaluation of myocardial dystrophin, α-sarcoglycan, and β-dystroglycan in dogs with idiopathic dilated cardiomyopathy. *Am J Vet Res* 2001;62:67–71.
15. Wiersma AC, Stabej P, Leegwater PA, et al. Evaluation of 15 candidate genes for dilated cardiomyopathy in the Newfoundland dog. *J Hered* 2008;99:73–80.
16. Philipp U, Vollmar A, Distl O. Evaluation of the titin-cap gene (TCAP) as candidate for dilated cardiomyopathy in Irish wolfhounds. *Anim Biotechnol* 2008;19:231–236.
17. Philipp U, Broschk C, Vollmar A, et al. Evaluation of tafazzin as candidate for dilated cardiomyopathy in Irish wolfhounds. *J Hered* 2007;98:506–509.
18. Kirkness EF, Bafna V, Halpern AL, et al. The dog genome: survey sequencing and comparative analysis. *Science* 2003;301:1898–1903.
19. Parker HG, Kim LV, Sutter NB, et al. Genetic structure of the purebred domestic dog. *Science* 2004;304:1160–1164.
20. BLAST, the Dog Genome, National Center for Biotechnology Information, National Institutes of Health, Bethesda, Md. Available at: www.ncbi.nlm.nih.gov/genome/seq/CfaBlast.html. Accessed Nov 6, 2004.
21. Lindblad-Toh K, Wade CM, Mikkelsen TS, et al. Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* 2005;439:803–819.
22. Ichida F, Tsubata S, Bowles KR, et al. Novel gene mutations in patients with left ventricular noncompaction or Barth syndrome. *Circulation* 2001;103:1256–1263.
23. Biesiadecki, BJ, Elder, BD, Yu, ZB, et al. Cardiac troponin T variants produced by aberrant splicing of multiple exons in animals with high instances of dilated cardiomyopathy. *J Biol Chem* 2002;277:50275–50285.
24. Meurs KM, Sanchez X, David RM, et al. A cardiac myosin binding protein C mutation in the Maine Coon cat with familial hypertrophic cardiomyopathy. *Hum Mol Genet* 2005;14:3587–3593.
25. Meurs KM, Norgard MM, Ederer MM, et al. A substitution mutation in the myosin binding protein C gene in ragdoll hypertrophic cardiomyopathy. *Genomics* 2007;90:261–264.
26. Daehmlow S, Erdmann J, Knueppel T, et al. Novel mutations in sarcomeric protein genes in dilated cardiomyopathy. *Biochem Biophys Res Commun* 2002;298:116–120.
27. Mohapatra B, Jimenez S, Lin JH, et al. Mutations in the muscle LIM protein and alpha-actinin-2 genes in dilated cardiomyopathy and endocardial fibroelastosis. *Mol Genet Metab* 2003;80:207–215.
28. Djinovi-Carugo K, Young P, Gautel M, et al. Structure of the alpha-actinin rod: molecular basis for cross-linking of actin filaments. *Cell* 1999;98:537–546.
29. Franzot G, Sjöblom B, Gautel M, et al. The crystal structure of the actin binding domain from alpha-actinin in its closed conformation: structural insight into phospholipid regulation of alpha-actinin. *J Mol Biol* 2005;348:151–165.
30. Klaavuniemi T, Ylänne J. Zasp/Cypher internal ZM-motif containing fragments are sufficient to co-localize with alpha-actinin—analysis of patient mutations. *Exp Cell Res* 2006;312:1299–1311.
31. Klaavuniemi T, Kelloniemi A, Ylänne J. The ZASP-like motif in actinin-associated LIM protein is required for interaction with the alpha-actinin rod and for targeting to the muscle Z-line. *J Biol Chem* 2004;279:26402–26410.

*Appendices appear on the next page*

## Appendix 1

Primers used for amplification of the coding regions of 10 cardiac genes (associated with familial DCM in humans) in ventricular myocardial samples obtained from dogs of breeds other than Doberman Pinscher without overt signs of cardiac disease (control dogs) and Doberman Pinschers with DCM and CHF in a study to identify a causative mutation for DCM in Doberman Pinschers.

| | | | | Untranslated region covered (bp)† | |
|---|---|---|---|---|---|
| **Gene** | **Accession No.** | **Primer 5′-3′*** | **Amplicon size (kbp)** | **5′** | **3′** |
| β-Cardiac myosin | XM_854082 | Forward: TGTCTTTCCTTGCTGCTCTC<br>Reverse: GTTGGCAAGAGTGGGGTTC‡ | 5.8 | 65 | 11 |
| α-Cardiac actin | XM_535424 | Forward: CGCAGCTGAGCCGGG**ATG**TG<br>Reverse: CCTGAATGTAAAGTAGACT | 1.1 | (6) | 9 |
| Cardiac troponin T | NM_001003012 | Forward: GGAGG**ATG**TCTGACGTGGAAGA<br>Reverse: **TTC**TATTTCCAGCGCCCGGTGAC | 0.8 | (18) | (21) |
| α-Tropomyosin | XM_859943 | Forward: GCTCGCCTCGCCGCTCCC<br>Reverse: CGGTCGGCATCTTCAGCAAT | 0.9 | 33 | 334 |
| Metavinculin | XM_536395 | Forward: GGAAAAGATAACCCTGGTAGTGG§<br>Reverse: TCACCACAGGTTCAGCCGGG | 2.5 | (42) | 512‖ |
| Tafazzin | XM_848512 | Forward: **ATG**CCCCTCCACGTGAAGT<br>Reverse: CGAGGACACGCTACCCATCAA | 1.1 | (20) | 203 |
| Myosin binding protein-C | NM_001048106 | Forward: CTCTTTGGGCGGCCTGTGACTG<br>Reverse: GCACACACAACAGCAGGAAGG | 4.0 | 28 | 85 |
| α-Actinin | XM_848010 | Forward: GGGCAGGCGATCCGAGAG<br>Reverse: CGGGCAATGAGGGCGCTG | 3.0 | 187 | 12 |
| Tcap | XM_846319 | Forward: TCCAGTGAGCACCGATC**ATG**<br>Reverse: TGGCTCCCTCCTCCCCTAG | 0.6 | (4) | 103 |
| Muscle LIM protein | XM_859394 | Forward: AGCGGTCTCTGCCCTCTCC<br>Reverse: GGGCGTGAGGCAGAAAGGAG | 2.0 | 42 | 42 |

The sizes of each amplicon and the length of the 5′ and 3′ untranslated region covered by each primer pair are shown.

*Start and stop codons are in bold. †When the primer includes coding sequence, the last base pair in the coding sequence covered by the primer is listed in parentheses. ‡Primer was designed against the α-cardiac myosin gene. §Primer failed to provide good sequence. ‖Coding sequence was truncated at exon 15, compared with predicted sequence in database.

## Appendix 2

Coverage of primers used for sequencing each reading frame of 10 cardiac genes (associated with familial DCM in humans) in ventricular myocardial samples obtained from dogs of breeds other than Doberman Pinscher without overt signs of cardiac disease (control dogs) and Doberman Pinschers with DCM and CHF in a study to identify a causative mutation for DCM in Doberman Pinschers.

| | | Forward primers | | Reverse primers | |
|---|---|---|---|---|---|
| **Gene** | **Amplicon size (kbp)** | **No.of primers** | **Mean coverage (bp)** | **No.of primers** | **Mean coverage (bp)** |
| β-Cardiac myosin | 5.8 | 9 | 652 | 7 | 743 |
| α-Cardiac actin | 1.1 | 1 | NA* | 2 | 590 |
| Metavinculin | 2.5 | 4 | 615 | 3 | 449* |
| Tafazzin | 1.1 | 1 | NA* | 2 | 359 |
| Myosin binding protein-C | 4.0 | 5 | 805 | 3 | 767* |
| α-Actinin | 3.0 | 4 | 750 | 2 | NA* |
| Muscle LIM protein | 2.0 | 3 | 630 | 2 | NA* |

The primers used for amplification were also used for sequencing and are included. Sequences < 1 kbp are not listed because only the primers used for amplification were used for sequencing and provided complete coverage.

*Some primers produced sequences that did not overlap on that strand but provided complement sequences that were obtained by use of primers for the opposite strand.

NA = Not applicable.