Evaluation of the phospholamban gene in purebred large-breed dogs with dilated cardiomyopathy

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Objective—To evaluate the role of the phospholamban gene in purebred large-breed dogs with dilated cardiomyopathy (DCM).

Animals—6 dogs with DCM, including 2 Doberman Pinschers, 2 Newfoundlands, and 2 Great Danes.

Procedure—All dogs had clinical signs of congestive heart failure, and a diagnosis of DCM was made on the basis of echocardiographic findings. Blood samples were collected from each dog, and genomic DNA was isolated by a salt extraction method. Specific oligonucleotides were designed to amplify the promoter, exon 1, the 5′-part of exon 2 including the complete coding region, and part of intron 1 of the canine phospholamban gene via polymerase chain reaction procedures. These regions were screened for mutations in DNA obtained from the 6 dogs with DCM.

Results—No mutations were identified in the promoter, 5′ untranslated region, part of intron 1, part of the 3′ untranslated region, and the complete coding region of the phospholamban gene in dogs with DCM.

Conclusions and Clinical Relevance—Results indicate that mutations in the phospholamban gene are not a frequent cause of DCM in Doberman Pinschers, Newfoundlands, and Great Danes. (*Am J Vet Res* 2005;66:432–436)

Dilated cardiomyopathy (DCM) is a myocardial disease that is an important cause of congestive heart failure and sudden death in dogs. The disease primarily affects large and giant breeds.[1] Familial disease in dogs has been identified in Doberman Pinschers, Boxers, English Cocker Spaniels, Irish Wolfhounds, Portuguese Water Dogs, Great Danes, and Newfoundlands.[2-8] Causal mutations have not yet been identified. The high prevalence of DCM in specific breeds suggests a genetic background.

Within each breed, DCM has unique characteristics, and between breeds, it is probably a genetically heterogeneous disease. In Great Danes and Newfoundlands, notable clinical characteristics of DCM include ventricular dilatation, congestive heart failure (left sided or biventricular), and atrial fibrillation, whereas in Doberman Pinschers, DCM is associated with left-sided heart failure, ventricular arrhythmias, and sudden death.[7,9,10] Although clinical signs of DCM usually become evident in adulthood, puppies can be affected within the first weeks or months after birth, as has been described in Portuguese Water Dogs[6] and more recently in a litter of Doberman Pinschers.[11] However, DCM in Doberman Pinschers typically has a late onset.[2,10] In several breeds, a preclinical phase of DCM has been recognized.[7,10,12] In dogs, the diagnosis of DCM is not problematic once clinical signs are apparent, but diagnosis of the preclinical phase of DCM may be challenging. In a recent publication,[12] application of a scoring system for the identification of dogs in preclinical stages of DCM highlighted the problem of false-positive and false-negative diagnosis of preclinical DCM in dogs without clinical signs. Difficulty in detection of preclinical DCM in dogs, coupled with late onset of the disease, frustrates early diagnosis and removal of disease carriers from the breeding population.

Dilated cardiomyopathy in humans closely resembles the canine form of the disease and has a genetic background in as many as 35% of all cases.[13] To date, 15 genes have been implicated as the cause of DCM in humans.[14-16] Most of these genes code for proteins of the cytoskeleton and destabilize the cardiomyocyte membrane or cytoskeleton via mechanical instability or force transduction, resulting in poor systolic cardiac function.[17] In contrast to genes encoding cytoskeletal proteins, humans with an Arg-to-Cys missense mutation at residue 9 (R9C) in the phospholamban protein develop dominantly inherited DCM through a mechanism involving direct disturbance of the myocellular Ca^{2+} metabolism.[15] Phospholamban is a small transmembrane phosphoprotein of 52 amino acids that plays an important role in cardiac contraction and relaxation. Cardiac contraction occurs with Ca^{2+} release from the sarcoplasmic reticulum into the cytosol, and relaxation begins with Ca^{2+} uptake into the sarcoplasmic reticulum through the Ca^{2+}-ATP (SERCA2a) pump. Whereas the wild-type phospholamban protein directly inhibits SERCA2a pump, the phospholamban^{R9C} protein fails to do so. The consequence is delayed decay of calcium transport in cardiac myocytes that initiates DCM in humans.[15] The canine phospholamban gene (*PLN*) consists of 2 exons that are transcribed into phospholamban messenger RNA. The exon 1 RNA sequence of 83 nucleotides and the initial RNA

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sequence of 101 nucleotides of exon 2 are not translated into the phospholamban protein and represent the 5′ **untranslated region (UTR)**. The 159-bp coding sequence starts at nucleotide position 102 and ends at position 260 of exon 2 (**Figure 1**).[18] The part of exon 2 after position 260 that is not translated into the protein is the 3′UTR. Gene transcription is regulated by the promoter region that has been well defined for *PLN*.[19] The purpose of the study reported here was to evaluate the role of the *PLN* in purebred large-breed dogs with DCM. To achieve this goal, we sequenced the promoter, the 5′UTR, the border regions of intron 1 at which splicing occurs, part of the 3′UTR, and the complete coding region of the *PLN* in 6 purebred large-breed dogs with DCM.

Materials and Methods

Dogs—Six dogs were included in the study (2 Doberman Pinschers, 2 Newfoundlands, and 2 Great Danes). All dogs were client-owned dogs with DCM that were evaluated at the Department of Clinical Sciences of Companion Animals of Utrecht University and at the Clinic for Small Animals and Surgery of Ljubljana University. An informed consent for DNA to be collected and analyzed was obtained from the owners. Blood samples were collected from the Doberman Pinschers (DO1 and DO2), Newfoundlands (NF1 and NF2), and Great Danes (GD1 and GD2), and genomic DNA was isolated by the salt extraction method.[20]

Because DCM is probably an inherited disorder in Doberman Pinschers, Newfoundlands and Great Danes, it was highly likely that the cause of DCM was also genetic in the dogs of these breeds that were included in the study.[2,7,8] Diagnosis of DCM was made on the basis of clinical or radiographic signs of congestive heart failure and echocardiographic evidence of DCM (marked left ventricular chamber dilatation and decreased fractional shortening) in the absence of other congestive heart failure–related lesions. The echocardiographic measurements were compared to breed-specific reference values.[21,22,a]

Echocardiographic and ECG evaluations—After positioning of each conscious dog in right lateral recumbency, echocardiography was performed at the Department of Clinical Sciences of Companion Animals of Utrecht University by use of a high-definition ultrasound system[b] equipped with a 5- to 3-MHz broad and phase array transducer. For simultaneous ECG recording, ECG electrodes were placed on the left and right forelimb and the left hind limb. All measurements were performed by use of a trackball-driven cursor and ultrasound software. From the right parasternal approach, 2-dimensional M-mode tracings were obtained for measurements of the aortic root and left atrium diameters and, during diastole and systole, for measurements of the interventricular septum, the left ventricular dimension, and the left ventricular free wall. These measurements were made from the leading edge of the first endocardial surface to the leading edge of the second endocardial surface. Diastolic measurements were made at the onset of the QRS complex of the ECG tracing, and systolic measurements were made at the maximum systolic excursion of the interventricular septum. The diameter of the aortic root was measured at the onset of the QRS complex of the ECG tracing, and the diameter of the left atrium was measured at its maximal upward excursion near the end of systole.

From values of the **left ventricular internal dimension during diastole (LVIDd)** and the **left ventricular internal dimension during systole (LVIDs)**, the **fractional shortening (FS)** was calculated by use of the following equation:

$$FS\ (\%) = ([LVIDd - LVIDs])/LVIDd) \times 100.$$

From the diameter of the left atrium and the aortic root, the **left atrium-to-aorta ratio (LA:AO)** was calculated. At the Clinic for Small Animals and Surgery, Veterinary Faculty, Ljubljana University, echocardiography was performed as described.[2]

Mutation screening—By use of a canine **complementary DNA (cDNA)** sequence (GenBank accession No.

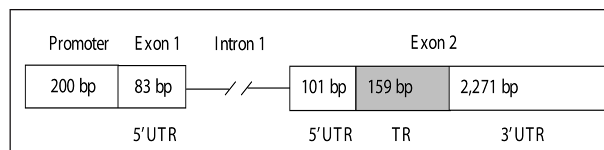

Figure 1—Structure of the canine phospholamban gene (*PLN*) based on the annotated messenger RNA sequence GenBank accession No. Y00399. The gene consists of 2 exons, preceded by a 200-bp promoter region. The 159-bp translated region (TR) is contained in exon 2. Several phospholamban messenger RNAs are present in canine cardiac muscle; the 2614-bp transcript presented here is the major one.[24] UTR = Untranslated region

Table 1—Echocardiographic findings in 2 Newfoundlands (NF1 and NF2), 2 Doberman Pinschers (DO1 and DO2), and 2 Great Danes (GD1 and GD2) with dilated cardiomyopathy, compared with reference values for each variable in that breed.

	Newfoundland			Doberman Pinscher			Great Dane		
	NF1	NF2	Reference values[a] Mean ± SD	DO1	DO2	Reference values[10,21] Mean ± SD	GD1	GD2	Reference values[22] Median (range)
Weight (kg)	61	50	NA	35	35.2	NA	63.6	68	NA
LVIDd (mm)	76*	83*	45.35 ± 4.03	73*	66*	49.7 ± 4.309[21]	96*	92*	53 (44–59)
LVIDs (mm)	69*	70*	34.31 ± 3.00	66*	56*	33.8 ± 4.310[21]	85*	98*	39.5 (34–45)
LVWd (mm)	6*	11	10.28 ± 1.13	9*	6*	10.8 ± 0.987[21]	14	14	12.5 (10–16)
LVWs (mm)	8*	13	13.69 ± 1.38	11*	6.6*	16.2 ± 0.753[21]	18	14	16 (11–19)
IVSd (mm)	9*	10	10.66 ± 1.13	8*	10.4	10.5 ± 0.444[21]	14	13	14.5 (12–16)
IVSs (mm)	10*	11*	13.93 ± 1.38	8*	8.6*	16.5 ± 1.180[21]	22*	14	16.5 (14–19)
LA (mm)	44*	48*	24.13 ± 4.06	40*	NR	24.8 ± 4.3[21]	57*	66*	33 (28–46)
AO (mm)	26*	30	29.18 ± 2.71	23*	NR	28.3 ± 2.9[21]	36*	26*	29.5 (28–34)
LA/AO	1,7*	1,6*	0.83 ± 0.15	1.7*	>1.6*	0.86[21]	1.6*	2.5*	1.1 (0.9–1.5)
FS (%)	9*	16*	24.47 ± 3.21	10*	14.46*	25[10]	11*	15*	25 (18–36)

*Value deviates from the breed-specific reference value.

LVIDd = Left ventricle internal dimension during diastole. LVIDs = Left ventricle internal dimension during systole. LVWd = Thickness of the wall of the left ventricle during diastole. LVWs = Thickness of the wall of the left ventricle during systole. IVSd = Thickness of the interventricular septum during diastole. IVSs = Thickness of the interventricular septum during systole. LA = Left atrium. AO = Aorta. FS = Fractional shortening. NA = Not applicable. NR = Not on record.

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Y00399) as a probe, nontranscribed canine *PLN* sequences and sequences upstream from the *PLN* promoter region were derived from the *Canis familiaris* trace archive of the **National Center for Biotechnology Information (NCBI)** GenBank via a BLAST search.[c] Obtained sequences were aligned with the canine cDNA by use of specialized software.[d] To screen the canine *PLN* for mutations, we amplified the canine promoter, the 5′UTR, part of intron 1 downstream from exon 1 and upstream from exon 2, the complete translated region, and part of the 3′UTR. Primers (PLN_1, PLN_2, and PLN_3) were designed by use of computer software (**Appendix**).[23,e] Product lengths were determined. For each **polymerase chain reaction (PCR)** procedure, 25 ng of genomic DNA was used as a template in a 15-µL reaction mixture consisting of 1X PCR reaction buffer,[f] 200µM dNTPs, 1.5mM $MgCl_2$, 0.6 units of platinum *Taq* polymerase,[g] and 0.33µM of each primer. Deoxyribonucleic acid was initially denatured at 94°C for 4 minutes and then subjected to 35 cycles consisting of 30 seconds at 94°C, 30 seconds at the annealing temperature, and 30 seconds at 72°C. The final extension was 10 minutes at 72°C. The reaction was diluted (15X dilution), and 1 µL was used in a 10-µL tercycle reaction using 1-µL cycle sequencing mix[h] with 0.32µM of the primer and 2mM $MgCl_2$. For sequencing of the PLN_1 and PLN_3 PCR products, PLN_1 and PLN_3 reverse primers were used; PLN_2 primers were M13 tailed, and M13 forward primer was used for sequencing. The tercycle program consisted of 25 cycles. Each cycle had 3 steps: 30 seconds at 96°C, 15 seconds at 55°C, and 2 minutes at 60°C. Tercycle products were purified by use of multiscreen 96-well filtration plates[i] with gel filtration media.[j] The high-quality sequences obtained were compared with *C familiaris* trace archive sequences and annotated canine cDNA Y00399 sequence by use of computer software.[d] Canine sequences were compared with their human counterparts by use of a software program.[k]

Results

Clinical evaluation—All 6 dogs with DCM had evidence of overt congestive heart failure. Five dogs (the 2 Doberman Pinschers, the 2 Newfoundlands, and 1 Great Dane [GD2]) had clinical signs of left-sided heart failure with pulmonary edema; 1 dog (GD1) had clinical signs of right-sided heart failure with ascites and pleural effusion. The ECG recordings were indicative of atrial fibrillation in the Newfoundlands and Great Danes; in the 2 Doberman Pinschers, the ECG recordings revealed widened QRS complexes and P waves, indicative of left ventricular and atrial enlargement. Echocardiographic 2-dimensional M-mode examination revealed marked left ventricular chamber dilatation with reduced shortening fraction and left atrial enlargement with increased LA:AO in all dogs (**Table 1**).

Analysis of the canine PLN promoter, 5′UTR (exon 1), and part of intron 1 region—The genomic structure of canine phospholamban is known (**Figure 1**).[18,19,24] Two high-quality sequences covering regions upstream and downstream from *PLN* exon 1 were selected from the *C familiaris* trace archive (GenBank accession No. TI261908801 and TI298985450). The canine *PLN* promoter region sequence was determined on the basis of previously assessed sequence conservation among 5 mammalian *PLN* promoter regions.[19,24] In the dog, the *PLN* regulatory elements were identified in the sequence 200 bp upstream of the canine *PLN* exon 1.[19] The exon 1-intron 1 splice site was based on the canine cDNA sequence Y00399 and the presence of the typical splice donor site (GT) in intron 1. A DNA fragment containing 200 bp of the canine *PLN* promoter and 83 bp of exon 1 had 83% identity with the human counterpart (GenBank accession No. AF177763). Sequencing of the canine *PLN* promoter, exon 1, and 155 bp of intron 1 with the PLN_1 reverse primer (Appendix; **Figure 2**) resulted in a high-quality sequence in the 6 dogs with DCM. The DNA sequence was deposited in the NCBI GenBank (accession No. AY576871). We compared the DNA sequences obtained from dogs with DCM with corresponding *C familiaris* trace file sequences and exon 1 with the annotated canine cDNA sequence (Y00399) and found no variations.

Analysis of the canine *PLN* intron 1, 5′UTR (exon 2), complete translated region, and the 3′UTR—From the NCBI GenBank *C familiaris* trace archive, the DNA

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  1  AGCAACAGCA GCGACAATAC AATGAAAGTT ATTTGACTAA GCCAATAATA TTCTCACTCA
 61  TTAAAATTCC ATATATCTAT TTTAGTTCTT CAACATTATC ACCATCAGTA CACAATTATT
121  TCTAAGCCTG AAGATTCATG AATCTTTAAA GGGAGCTTTC TACCACCCCA AACTTTTTTT
181  TTTTTTCATT TATCTCCCAT ACACTTTTAA AAATTACAAG CAAAAAAATG TGGCACAAAG
241  TGTTAATGAC CTTTCCATAC TCAAAGTAAG ATAAGTGACA TTCTAAAAGG TTTGGTTGTG
301  ATAAGACTAT GGCTAACCAA TCATAACTTC AGAATTCTTG TATGACATCA TAAGACCTCC
361  CTAGAACACT TTTCCTCCTA CACCTACTTC AATTGTTGCC ATAAGTCTGG TAACAGAGTC
421  AGAAAACTTT CTAACTAAAC ACCGATAAGA CTTCATACAA CTCACAATAC TTTATATTGT
481  AATCATCACA AGAGCCAAGG TAAGAAATAG ATTTCATTAA TTTTGGTAAT TCTTGTTTTT
541  CAGAATTCAA AATTACTAAA CTTTTTCAAG AGTTAACCAT GTTTTGTAAA TTTCAGTTCA
601  AAGTTAATAA AATGTGTGAT ATGACAAAAA CTTTAATTTC TAAATAAATG CCTGTTTGGT
661  GACAGTAAGA GCAAGACTTA ACTTTGAAAT AATAAGGGTC GCATCATCTT TGAC
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Figure 2—Sequence of the canine *PLN* promoter, exon 1, and part of intron 1. Six annotated *PLN* promoter elements are boxed.[19] Exon 1 sequence is indicted in bold letters; PLN_1 primers used to amplify promoter, exon 1, and part of intron 1 are underlined.

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  1  TGAGAGAAGG AGGCAAAAGA TTGAAGTTAT TACACTCCAA ACACAAGATA GATAAGACCT
     PLN2_forward
 61  CATGGTGACC CTCATCAAAA AGGAATACAG AGATAATTTG ATGTCACAAA TACAAATAAT
121  ATTAAGGAAG ATGAATTAAT ACAAATTGTG TTTTTACCTG TTCAGAGAAT AGGTTACACA
181  CATGATCCTA ACCCAGTCAT TATTTTTATA TTCCAGGCTA CCTAAAAGAA GAGAGTGGTT
                                                           PLN3_forward
241  GAGCTCACAT TTGGCCGCCA GCTTTTTACC TTTCTCTTCA CCATTTAAAA CTTGAGACTT
301  CCTGCTTTCC TGGGGTCATG GATAAAGTCC AATACCTCAC TCGCTCTGCT ATTAGAAGAG
                         M   D  K  V   Q  Y  L   T  R  S  A   I  R  R
361  CTTCAACCAT TGAAATGCCT CAACAAGCAC GTCAAAATCT TCAGAACCTA TTTATAAATT
      A  S  T   I  E  M  P   Q  Q  A   R  Q  N   L  Q  N  L   F  I  N
421  TCTGTCTCAT TTTAATATGT CTCTTGTTGA TCTGCATCAT TGTGATGCTT CTCTGAAGTT
      F  C  L   I  L  I  C   L  L  L   I  C  I   I  V  M  L   L  -
481  CTGCTGCAAT CTCCAGTGAT GCAACTTGTC ACCATCAACT TAATATCTGC CATCCCATGA
                                                 PLN2_reverse
541  AGAGGGGAAA ATAATACTAT ATAACAGACC ACTTCTAAGT AGAAGATTTT ACTTGTGAAA
601  AGGTCAAGAT TCAGAACAAA AGAAATTATT AACAAATGTC TTCATCTGTG GGATTTTGTA
661  AACATGAAAA GAGCTTTATT TTCAAAAATT AACTTCAAAA TGACTATAGG TGCGCATAAT
721  GTAATTGCTG AATTCCTCAA CAAAGCTTGT AAAAGTTTCT ATGCCAAATT TTTTCTGAGG
781  GTAAAGTAGG AGTTTAGTTT TAAAACTGCT CTGCTAACCA GTTCACTTC
                                  PLN3_reverse
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Figure 3—Sequence of part of intron 1 and part of exon 2 of the canine *PLN*. The PLN_2 primer (used to amplify the sequence upstream from exon 2 and 5′UTR of exon 2) and PLN_3 primer (used to amplify translated and 3′UTR of exon 2) are underlined. The exon 2 sequence is indicated in bold letters; the coding sequence starts in exon 2 at position 318 with an ATG coding for methionine and ends at position 476 with a TGA stop codon. The 52 amino acids are presented below the nucleotide sequence.

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sequences TI322443892 and TI257556674 spanning the region upstream from exon 2 and part of exon 2 were selected and aligned with the canine cDNA Y00399 sequence. The beginning of exon 2 was based on the Y00399 DNA sequence and the presence of the universal splice acceptor site (AG) in intron 1. The translated region has been determined by Uyeda et al.[24] Comparison of the 5′UTR region and the translated region of exon 2 to the same regions in humans (NM_002667) revealed 84% and 92% similarity, respectively.

The DNA fragments amplified with the PLN_2 and PLN_3 PCR primers were sequenced. High-quality DNA sequences from the 6 dogs covered 130 bp of intron 1, 101 bp of the 5′UTR (exon 2), the complete 159-bp translated region (exon 2), and 279 bp of 3′UTR (Appendix; **Figure 3**). No nucleotide changes were found in these sequences, compared with the trace archive and Y00399 sequences. The sequence covering the region starting 130 bp prior to exon 2 and ending 279 bp downstream from the stop codon in exon 2 was deposited in the NCBI GenBank (accession No. AY576872).

Discussion

Because of the striking phenotypic similarity of DCM in humans and dogs, the contribution of genetic mutations to the etiology of DCM in humans has been recognized in research efforts regarding DCM in dogs. It is now clear that DCM is a genetic disease at least in some breeds of dog, although no causal mutation has yet been discovered.

Each breed is a genetic isolate; therefore, it is not surprising that the clinical manifestations of DCM are breed specific. Consequently, the disease could be genetically homogenous within a breed and genetically heterogeneous among different breeds. However, 2 DCM phenotypes have been identified in Doberman Pinschers: sudden death and development of congestive heart failure. Different mutations in the same gene or even different genes might cause these 2 variable disease phenotypes.

In humans, most DCM mutations were found in genes coding for proteins of the cell cytoskeleton and DCM was described as a cytoskeletalopathy.[25] The discovery of the PLN^{R9C} mutation as a cause of DCM in humans suggested another pathway to the development of DCM—a pathway in which Ca^{2+} kinetics are disrupted. It is likely that the pathophysiologic mechanisms involved in the development of DCM in dogs are the same as those in humans. Two characteristics of DCM in Doberman Pinschers make *PLN* an especially attractive candidate gene—ventricular arrhythmias and sudden death as a result of ventricular tachycardia and fibrillation.[10,26] Mutations in genes that result in disturbed ion transport in cells have been identified as causes of arrhythmias and sudden death in humans.[27] Therefore, genes coding for ion channels could play a role in the development of DCM in Doberman Pinschers and are also good candidate genes

An important resource which became recently available from the canine genome project is the 7X redundant dog sequence deposited in the NCBI GenBank trace archive. Together with the canine phospholamban cDNA sequence, it enabled rapid reconstruction and evaluation of the *PLN* in dogs.[24] The sequence of the canine *PLN* promoter and exon 1 that has been annotated in the NCBI GenBank (accession No. AF037348) had several nucleotide differences, compared with the sequence determined in the present study or the trace archive sequences. In addition, the intron-exon borders in the AF037348 sequence could not be matched with the canine phospholamban cDNA sequence Y00399. This was probably because of artifacts in the AF037348 sequence. Therefore, we deposited our sequence of the promoter, exon 1, and part of intron 1 in the GenBank under accession No. AY576871.

Another valuable tool in canine genetics is the canine radiation hybrid map.[28] Several genetic diseases in dogs have been mapped by use of microsatellite markers and linkage analysis.[29] The disadvantage of linkage analysis is the requirement of fairly complete family material and informative markers. Because *PLN* is a small gene, direct sequencing of affected dogs is a more efficient way of evaluation. In the present investigation, the promoter; the complete 5′UTR; the exon 1-intron 1 and the intron 1-exon 2 splice sites; the complete coding region (159 bp); and part of 3′UTR of the *PLN* in Doberman Pinschers, Newfoundlands, and Great Danes with DCM were sequenced. Our data have indicated that there were no mutations in those sequences. Therefore, in our opinion, the *PLN* is excluded with a high level of confidence as a cause of DCM in these 3 breeds of dog.

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Appendix

Polymerase chain reaction primers used to amplify regions of the canine phospholamban gene.

Primer	Forward primer 5′-3′	Reverse primer 5′-3′	Annealing temperature (°C)	Product length (bp)
PLN_1	AGCAACAGCAGCGACAATAC	GTCAAAGATGATGCGACCCT	58	714
PLN_2	TGAGAGAAGGAGGCAAAAGA*	CTCTTCATGGGATGGCAGAT*	58	544
PLN_3	GAGTGGTTGAGCTCACATTTG	GAAGTGAACTGGTTAGCAGAG	62	597

*Primers were M13 tailed: M13 forward, 5′-GTTTTCCCAGTCACGAC-3′ and M13 reverse, 5′-CAGGAAACAGCTATGAC-3′.

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