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Precision medicine using whole genome sequencing identifies a novel *dystrophin* (*DMD*) variant for X-linked muscular dystrophy in a cat

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Abstract

Background: Muscular dystrophies (MDs) are a large, heterogeneous group of degenerative muscle diseases. X-linked dystrophin-deficient MD in cats is the first genetically characterized cat model for a human disease and a few novel forms have been identified.

Hypothesis/Objectives: Muscular dystrophy was suspected in a young male domestic shorthair cat. Clinical, molecular, and genetic techniques could provide a definitive diagnosis.

Animals: A 1-year-old male domestic shorthair cat presented for progressive difficulty walking, macroglossia and dysphagia beginning at 6 months of age. The tongue was thickened, protruded with constant ptyalism, and thickening and rigidity of the neck and shoulders were observed.

Methods: A complete neurological examination, baseline laboratory evaluation and biopsies of the trapezius muscle were performed with owner consent. Indirect immunofluorescence staining of muscle cryosections was performed using several monoclonal and polyclonal antibodies against dystrophy-associated proteins. DNA was isolated for genomic analyses by whole genome sequencing and comparison to DNA variants in the 99 Lives Cat Genome Sequencing dataset.

Results and Clinical Importance: Aspartate aminotransferase (687 IU/L) and creatine kinase (24 830 IU/L) activities were increased and mild hypokalemia (3.7 mmol/L) was present. Biopsy samples from the trapezius muscle confirmed a degenerative and regenerative myopathy and protein alterations identified by immunohistochemistry resulted in a diagnosis of a in *dystrophin*-deficient form of X-linked MD. A stop gain variant (c.4849C>T; p.Gln1617Ter) *dystrophin* was identified by genome sequencing. Precision/genomic medicine efforts for the domestic cat and in

Abbreviations DNA deoxyribonucleic acid: CK, creatine kinase; DMD, dystrophin; FF, superfine; Gln, glutamic acid; LoF, loss of function; MD, muscular dystrophies; MU, University of Missouri; NCBI, National Center for Biotechnology Information; OMIA, online Mendelian inheritance in animals; TAE, tris(hydroxymethyl)aminomethane—acetic acid—ethylenediaminetetraacetic acid; Ter, termination amino acid; WES, whole exome sequencing; WGS, whole genome sequencing.

veterinary medicine support disease variant and animal model discovery and provide opportunities for targeted treatments for companion animals.

KEYWORDS
animal models, *Felis catus*, precision medicine, whole genome sequencing

1 | INTRODUCTION

Muscular dystrophies (MD) are a large and heterogeneous group of degenerative muscle diseases.[1] The most common form of MD in people is associated with deficiency of the subsarcolemmal membrane protein dystrophin, referred to as a dystrophinopathy, with many mutations in the *dystrophin* (*DMD*) gene having been identified.[2] The most severe form of the disease in people is referred to as Duchenne MD and the milder form as Becker MD.[3] In animals, a spontaneously-occurring dystrophin-deficient mouse (mdx) was described in 1984.[4] In dogs, a recent review identified dystrophin-deficient MD in 16 different dog breeds with 20 different variants in the *DMD* gene.[5] X-linked dystrophin-deficient MD in the Golden retriever,[6] Labrador retriever,[7,8] and CXMD dogs in Japan[9] among others[10] has been used as a large animal model of dystrophinopathy in pre-clinical trials.[11,12] In animals, dystrophin-deficient MD should be referred to as X-linked dystrophin-deficient MD and not Duchenne or Becker MD, which refer to the disease in humans.

In contrast, clinical and pathological descriptions of dystrophin-deficient MD in cats are rare and only three variants have been confirmed. The disease first was described in 1989.[13] In 1992, two 5-month-old male domestic shorthair littermates with muscle hypertrophy as a prominent clinical feature of the disease were described,[14] followed in 1994 by the first molecular description of a deletion in the *DMD* promotor in a male cat with similar clinical and histopathologic findings as the previous reports.[15] Recently, a nonsense variant was identified in a family of Maine coon cats[16] and a missense variant in a family of Maine coon crossbred cats.[17] Herein, we report a highly deleterious variant causing loss of function (LoF) and likely underlying dystrophin-deficient MD in a random bred cat from the United Kingdom.

Other forms of MD can occur in cats, including congenital MD associated with laminin α2-deficiency[18-20] and limb-girdle MD associated with β-sarcoglycan deficiency,[21,22] although the causal gene variants have not yet been identified. Clinical examination cannot distinguish among various myopathies including the MDs, and further diagnostic testing is necessary including evaluation of muscle biopsy samples by histopathology, histochemistry, immunohistochemistry, and measurement of serum creatine kinase (CK) activity. With the improvement of molecular techniques, such as short-read whole genome sequencing (WGS) and, to some extent, whole exome sequencing (WES), distinguishing among various myopathies is now possible by rapid identification of the underlying variant responsible for causing disease,[23,24] including MD in both dogs and cats.[17,25]

Increased availability of next-generation sequencing allows identification of new pathogenic genes and the wide spectrum of clinical phenotypes associated with known defective proteins. The deletion in the muscle promoter of *DMD* was 1 of the earliest genetic variants discovered in the cat.[9] Currently, approximately 185 causal DNA variants are suggested in the cat for a variety of diseases and traits,[26] including specific variants in *DMD*. As wider use of WGS and WES becomes available at more affordable prices and annotation and assembly of the reference genomes improve, the spectrum of genetic variants resulting in MDs in cats will expand.

2 | MATERIALS AND METHODS

2.1 | Animals

A 1-year-old male indoor domestic shorthair cat (random bred; Fcat-23286) was referred to a specialty veterinary hospital for progressive difficulty walking, macroglossia, and dysphagia. Because a congenital neuromuscular disease was suspected, a complete neurological examination, baseline laboratory evaluation and muscle biopsy were performed with owner consent. Approximately 5 mL of EDTA whole blood also was collected by venipuncture from an unaffected female littermate for DNA extraction to genotype candidate causal variants with informed consent from the owner.

2.2 | Histopathology and immunofluorescent staining

Muscle biopsy samples (unfixed chilled and fixed in neutral buffered formalin) were collected from the trapezius muscle under general inhalational anesthesia. Biopsy samples were shipped by courier service under refrigeration to the University of California, San Diego, Comparative Neuromuscular Laboratory. Upon receipt, the unfixed biopsy sample was flash frozen in isopentane precooled in liquid nitrogen and then stored at −80°C until further processing using a standard panel of histochemical stains and reactions.[27] Formalin-fixed biopsy samples were routinely processed into paraffin. Indirect immunofluorescence staining of muscle cryosections was performed using several monoclonal and polyclonal antibodies against dystrophy-associated proteins as previously described.[16] Immunostaining of the affected cat tissue was compared to archived frozen control feline limb muscle (vastus lateralis).

2.3 | Whole genome sequencing and data processing

Deoxyribonucleic acid was isolated from the remaining archived frozen muscle of the affected cat using the DNeasy Blood and Tissue kit (Qiagen, Redwood City, CA) following the manufacturer's protocol. Deoxyribonucleic acid then was submitted to the University of Missouri (MU), evaluated for quality and quantity by agarose gel (1.5% in tris(hydroxymethyl)aminomethane—acetic acid—ethylenediaminetetraacetic acid [TAE]) electrophoresis (80 V for 90 minutes) and visualized with ultra-violet light after ethidium bromide staining. Approximately 1 µg of high molecular weight DNA was submitted to the MU Genomics Technology Core to construct a 550 base-pair (bp) sequencing library using a TruSeq DNA PCR-Free kit (Illumina, San Diego, California). The barcoded case sample library was pooled with approximately 21 additional cat genomic sequencing libraries and loaded onto a single S4 flow cell on a NovaSeq 6000 (Illumina) according to the manufacturer's recommendations to produce approximately 30X sequencing coverage of 150 bp paired-end reads. Data were processed using a custom Nextflow workflow following best practices for the Genome Analysis toolkit (GATK) version 4.2.[28,29] Reads were mapped to Felis_Catus_9.0 (GCF_000181335.3) using Minimap version 2.[30] Duplicate reads were marked using Picard version 2.[31] Specific tools from GATK 4.2 for genotyping, variant database construction, and hard-filtering were completed as previously described.[32] The produced 22 cat dataset was combined with the previously produced 340 cat variant call file[24,32,33] and the 61 cat WES dataset for the 99 Lives Cat Genome Sequencing Initiative.[34-36] The National Center for Biotechnology Information (NCBI) RefSeq *Felis catus* annotation 104 and Ensembl Variant Effect Predictor[37] were used to characterize the variants. Exonic variants and 10 bp flanking each exon were filtered and visualized using VarSeq software (GoldenHelix, Bozeman, Montana). The WES and WGS data are available in the NCBI short read archive under project accession numbers PRJNA308208, PRJNA627536, PRJNA999287 and PRJNA844099 and others (File S1).[38] The case cat is accession SAMN36728792.

2.4 | Candidate variant validation

The candidate variant was validated in the case using fluorescence-based Sanger sequencing. Cat reference assembly F.catus_Fca126_mat1.0 (GCF-018350175.1; BioProject: PRJNA684600; BioSample: SAMN19729387) and RefSeq Annotation 105 were used to design PCR primers for the *DMD* stop gain (XM_023249210.1, ENSFCAT00000068370:c.4849C>T; p.Gln1617Ter) using Primer3Plus.[39] The PCR forward primer was 5′-CACACGGTCATTTCAAAAGC-3′ and the reverse primer was 5′-TACGGCACAGACATGGAAAG-3′. The PCR conditions were optimized for annealing temperature (range 55-61°C) and $MgCl_2$ concentration (1.5 mM or 2.0 mM; Figure S1A) using 1 U ChoiceTaq DNA polymerase (Denville Scientific, Inc., Metuchen, New Jersey) in 25 µL reactions with 1X PCR buffer supplied by the manufacturer, 0.4 µM each primer, 0.2 mM each nucleotide and 10-20 ng template to produce a single PCR product with an expected size of 422 bp. The optimized 25 µL PCR conditions were 1.5 mM $MgCl_2$ and had an initial denaturation at 94°C for 3 minutes followed by 35 cycles of 94°C for 30 seconds denaturation, 55°C for 30 seconds annealing, 72°C for 1 minute extension with a final extension at 72°C for 10 minutes. The PCR was conducted on a Veriti thermal cycler (Applied Biosystems, Waltham, Massachusetts). The *DMD* amplicon was amplified for both the affected male and the unaffected female sibling (Fcat—24202 [326979-20]). The PCR products were verified by agarose gel (1.5% in TAE) electrophoresis by adding 5 µL PCR product to 1 µL of 6X tracking dye (30% [w/v] glycerol, 0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol superfine [FF]) and size separation of the products at 100 V for 90 minutes. The fragments were visualized by ethidium bromide staining and recorded using an AlphaImager (Bio-Rad Laboratories, Inc., Hercules, California). Amplified fragments (Figure S1B) from the remaining PCR product (approximately 20 µL) of the case, the unaffected sibling, and two control cats (laboratory male control Fcat-4406 and V9.0 cat genome female control Fcat-12682) were purified using QIAquick PCR Cleanup Kit (Qiagen). Approximately 45 ng was submitted to the MU Genomics Technology Core for Sanger sequencing using a BigDye Terminator v3.1 Cycle sequencing kit (Applied Biosystems) and an ABI 3730XL DNA Analyzer (Applied Biosystems). The generated sequences were aligned to the cat reference genome assembly F.catus_Fca126_mat1.0 and visually inspected using Sequencher 5.1 (GeneCodes, Ann Arbor, Michigan) to confirm amplification of *DMD* and presence of the variant in the case cat.

2.5 | In *silico* analyses

Genomic tools and resources at the NCBI website,[40] including ClinVar,[41,42] the Genome Data Viewer,[43] the Basic Local Alignment Search Tool (BLAST),[44,45] and the Genome Aggregation Database (*gnomAD*)[46] were used to correlate the variant in the cat to *DMD* variants in humans. The MutPred-LOF web application was used to evaluate the effect of the LoF variant in both cats and humans.[47] Multi-species sequence alignment of the exon containing the variant was performed using CLC Sequence Viewer 8 (QIAGEN).

3 | RESULTS

3.1 | Clinical history

Clinical signs of progressive difficulty walking, a stilted, stiff gait, and macroglossia were apparent beginning at 6 months of age. The tongue was thickened and protruded with constant ptyalism. Thickening and rigidity of the neck and shoulders also were observed (Figure 1). Routine blood test results at the time of presentation showed abnormalities limited to increased aspartate aminotransferase (AST) activity (687 IU/L; reference range, 10-50 IU/L), markedly increased CK activity (24 830 IU/L; reference range 50-200 IU/L) and mild hypokalemia (3.7 mmol/L; reference range, 3.8-5.3 mmol/L). Based on the young age of presentation and markedly increased CK activity, a neuromuscular disease was suspected, and muscle biopsies performed.

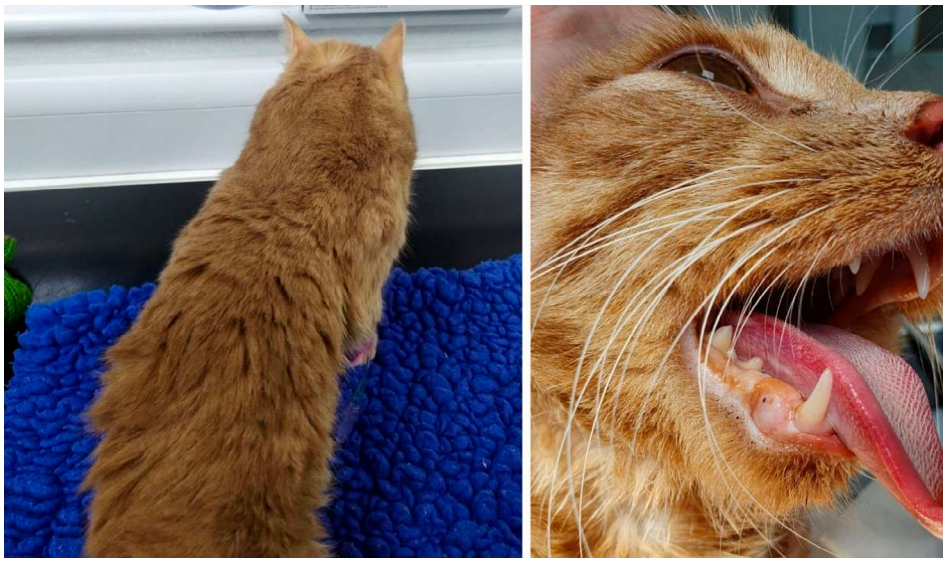

FIGURE 1 A 1-year-old male domestic shorthaired cat with suspected muscular dystrophy. The cat had a 6-month history of difficulty walking, prominent hypertrophy of the neck muscles (left), progressive dysphagia and a thickened tongue (right). The cat also could not close its mouth.

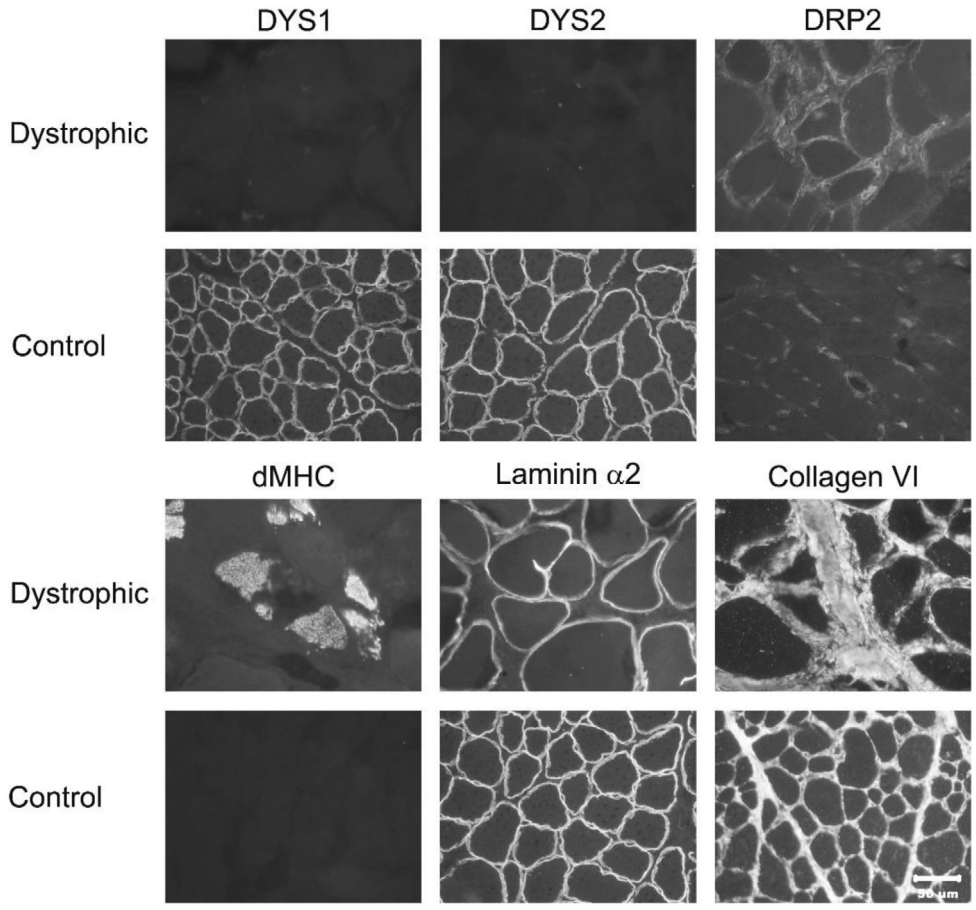

FIGURE 2 Immunofluorescent staining of cryosections from the trapezius muscle of the dystrophic cat and archived control vastus lateralis muscle was performed for localization of dystrophy-associated proteins. Antibodies against dystrophy-associated proteins included those against the rod (DYS1) and carboxy terminus (DYS2) of dystrophin, utrophin (DRP2), developmental myosin heavy chain (dMHC) to demonstrate regenerating fibers, and against laminin α2 and collagen 6. Protein localization for both DYS1 and DYS2 could not be detected and utrophin was increased compared to archived control muscle. Regenerating fibers were highlighted by staining for dMHC. Staining for both laminin α2 and collagen 6 was like control tissue. Bar in lower right image equals 50 µm for all images.

3.2 | Histopathology and immunofluorescent staining

Biopsy samples from the trapezius muscle confirmed a degenerative and regenerative myopathy consistent with a form of MD. To further characterize the form of MD, immunofluorescent staining was performed using various monoclonal and polyclonal antibodies to determine the presence or absence of dystrophy-associated proteins (Figure 2). Compared to control muscle, sarcolemmal staining in the dystrophic muscle was not detected using antibodies against the rod (DYS1) and C-terminus (DYS2) of dystrophin. Sarcolemmal staining for utrophin protein (DRP2) was increased in the dystrophic muscle. Regenerating fibers were highlighted by staining for developmental myosin heavy chain (dMHC). Staining intensity for laminin α2 was like

the intensity in the control muscle. Staining for collagen 6 highlighted endomysial fibrosis. Based on these findings, a dystrophin-deficient form of X-linked MD was diagnosed.

3.3 | DNA variant identification

This case presentation was expected to be rare, and both dominant and recessive modes of inheritance were considered during the variant filtering with no *a priori* consideration of a candidate gene. The 99 Lives dataset has 2 289 566 variants from 413 additional cats, the unique variants for the case cat are presented in Table 1 and File S2A,B. Considering recessive/hemizygous and unique variants with a passing Variant Quality Score Recalibration (VQSR) Tranche score[32] and suspected to alter the gene coding sequences, 71 variants were identified, including a nonsense stop gain in *DMD*, and 2 missense variants. The *DMD* stop gain variant (XM_023249210.1, ENSFCAT00000068370:c.4849C>T; p.Gln1617Ter) is at chromosome position X:28201541 in Felis_catus_9.0 and chromosome position X:27949145 in assembly Fca126 (Table 1, File S2A), which is at the beginning of exon 35 of a 13 533 transcript (4511 amino acids) with 78 exons. The variant is suggested to cause LoF, because approximately 65% of the DMD protein is truncated. The variant had 12X sequencing reads for the case, suggesting good sequencing coverage of a region on the X chromosome in a male cat. Considering dominant inheritance and the case as heterozygous, 735 variants were identified including 16 LoF variants, and 132 missense variants (Table 1, File S2B). All variants detected in the 99 Lives dataset for *DMD* are presented in (File S2C), including the variant recently published in a family of cats[16] and a variant identified in Japan,[48] which are MD cats known to be in the 99 Lives cohort.

For variant validation, a single PCR product of the expected size, 422 bp, was produced (Figure S1A,B). Both control cats, Fcat_4406 (male) and Fcat_12682 (female), were hemizygous/homozygous for the wildtype allele and the affected male cat, Fcat_23286, was hemizygous for the nonsense LoF variant. The normal female sibling did not have the *DMD* variant (Figure 3). This variant was unique to 414 cats within the 99 Lives dataset. *DMD* (ENSFCAG00000022242.4) is at position 26 990 371-29 004 949 on the X chromosome (NC_018741.3) in Felis catus_9.0 reference assembly using Ensembl annotation 104 and at position chromosome X (NC_058386.1):26741260–29120869 in the F._catus_Fca126 assembly using NCBI annotation 105.

TABLE 1 DNA variants in a cat with a muscular dystrophy.

		Case—unique variants[a]	
Variant	**414 cat variants[b]**	**Hemi-/homozygous**	**Heterozygous**
3′ UTR	779 313	4	59
5′ UTR premature start codon gain	14 709	0	1
5′ UTR	253 248	2	19
Disruptive inframe deletion	3232	0	0
Disruptive inframe insertion	7348	0	0
Downstream gene	11 812	4	22
Frameshift	122 878	0	14
Inframe deletion	12 416	0	6
Inframe insertion	17 437	0	2
Initiator codon	1320	0	2
Intergenic	24	34	180
Intron	312 834	10	113
Missense	404 240	2	132
Non-coding exon	363 539	1	9
Splice acceptor	6348	0	0
Splice donor	5763	0	0
Splice region	99 728	2	21
Stop gained	12 325	1 (*DMD*)	0
Stop lost	1019	0	0
Stop retained	444	0	0
Synonymous	692 633	4	121
Upstream gene variant	11 315	7	33
Total variants	3 133 975	71	735

[a]Exon with flanking 10 bp and passing Tranche scores.
[b]Data from 362 cats with WGS and 62 cats with WES. Includes variants with lower Tranche scores.

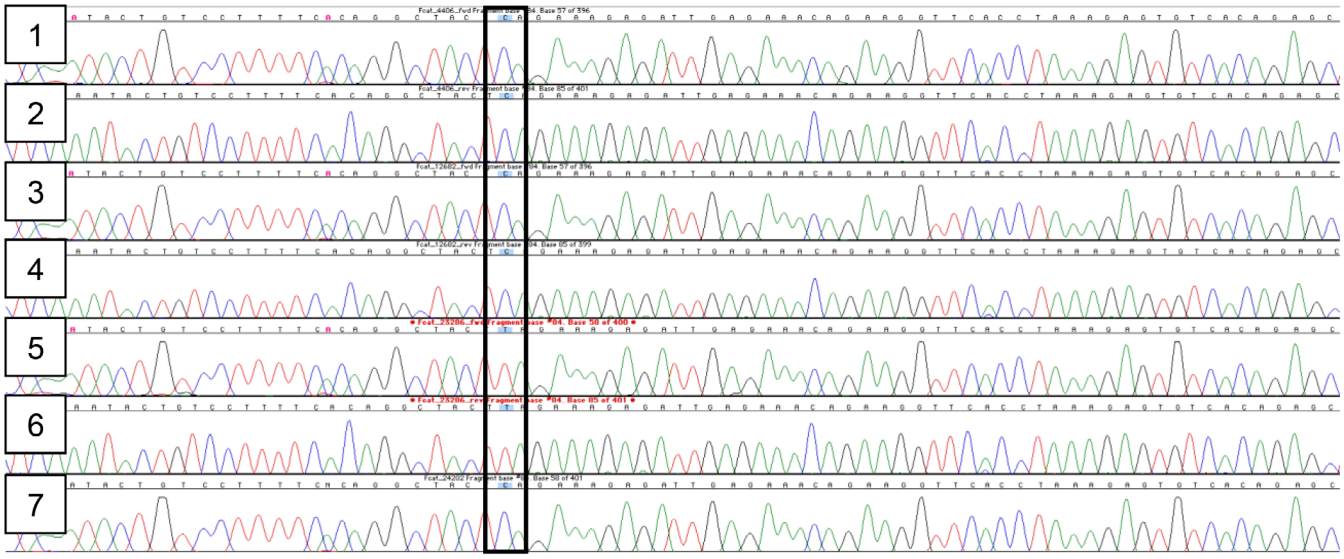

FIGURE 3 Electropherograms for *DMD* Sanger sequencing. 1: Control cat—Fcat_4406 coding strand; 2: Control cat—Fcat_4406 complementary strand; 3: Control cat—Fcat_12682 coding strand; 4: Control cat—Fcat_12682 complementary strand; 5: Case cat—Fcat_23286 coding strand; 6: Case cat—Fcat_23286 complementary strand; 7: Sibling cat—Fcat_24202 coding strand. Box indicates region where the case cat is hemizygous for the c.4849C>T variant. Cytosine (C) are blue peaks and thymine (T) are red peaks.

3.4 | In *silico* analyses

The NCBI cat reference assembly F.catus_Fca126 is annotated with 8 identified *DMD* transcripts in the domestic cat, which code for 78 exons and approximately 4511 amino acids. The cat variant is positioned in exon 35, which has over 93% homology with approximately 13 human *DMD* transcripts containing exon 35.

The ClinVar (accessed March 27, 2023) database of human deoxyribonucleic acid (DNA) variants (v2.1.1 data set [GRCh37/hg19]) was used to provide comparative genetic support for the function of the cat variant.[41,42] The cat *DMD* c.4849C>T; p.Gln1617Ter variant is analogous to the human p.Gln1618 (NM_004006.3(*DMD*):c.4852C>T (p.Gln1618Ter)), which is at position X:32365193 in the human genome assembly. In humans, this premature translational stop signal has been observed in familial cases of DMD and is considered pathogenic.[49] Like the cat, the human variant is also a cytosine to thymine transition and changes the codon from CAA to TAA, which is an ochre termination codon. This variant is not present in gnomAD population database,[46] thus additional frequency information in humans was not available.

MutPred-LOF web application was used to predict the effect of the LoF variant in both cats and humans. The complete cat predicted mRNA (XM_023249210.1) was compared to the predicted mutant mRNA with the stop termination (Q1617X). The predicted score from MutPred_LOF was 0.46141 as compared to the Q1618X variant introduced into human *DMD* isoform X1 XP_006724531.1, which produced a score of 0.45563. Scores > 0.7 are considered the most deleterious by this prediction application. Multiple species alignments of the region (exon 35) across diverse mammals and zebrafish indicated the region is highly conserved for both nucleotides and amino acids (Figure 4).

4 | DISCUSSION

Duchene muscular dystrophy (DMD) is an X-linked inherited neuromuscular disease in people that is a commonly recognized heritable condition. The gene causing DMD, *dystrophin* (*DMD*), is 1 of the larger genes in the mammalian genome and the pooled global DMD birth prevalence has been estimated as 19.8 per 100 000 live male births.[50] The ClinVar database has documented 480 variants as likely pathogenic and 2503 variants as pathogenic for DMD in humans, causing the different forms, severities, and presentations of the disease.[41,42] Precise definition of the causal variant is not only vital to the prognosis and counseling for DMD patients and their families, but also defines amenable treatments. Read-through compounds, such as ataluren, for patients with nonsense (stop codon) mutations, represent exon skipping treatments for DMD.[51-53] The targeted treatments have increased the lifespan of DMD patients by decades. Recently, delandistrogene moxeparvovec, which is an adeno-associated virus (AAV) vector-based gene therapy designed to deliver a gene encoding a micro-dystrophin protein, has been approved in the United States for the treatment of ambulatory DMD pediatric patients and a defined variant in *DMD*.[54] The ongoing preclinical trials in canine models are supporting the treatment developments and options[55-57] and substantial advances have been made in a variety of treatments.[58-64] Defining the feline *DMD* variants supports the use of cats as a biomedical model in preclinical trials, like that of canine models.

Feline X-linked MD was the first genetically characterized cat model for a human disease (OMIA:001081-9685).[15] The deletion in the muscle promoter of *DMD* was characterized by Southern blot analyses, thus the exact extent and positioning of the variant has not been determined.[15] A second, larger deletion involving the promoter region of *DMD* also has been described in cats, but also not precisely

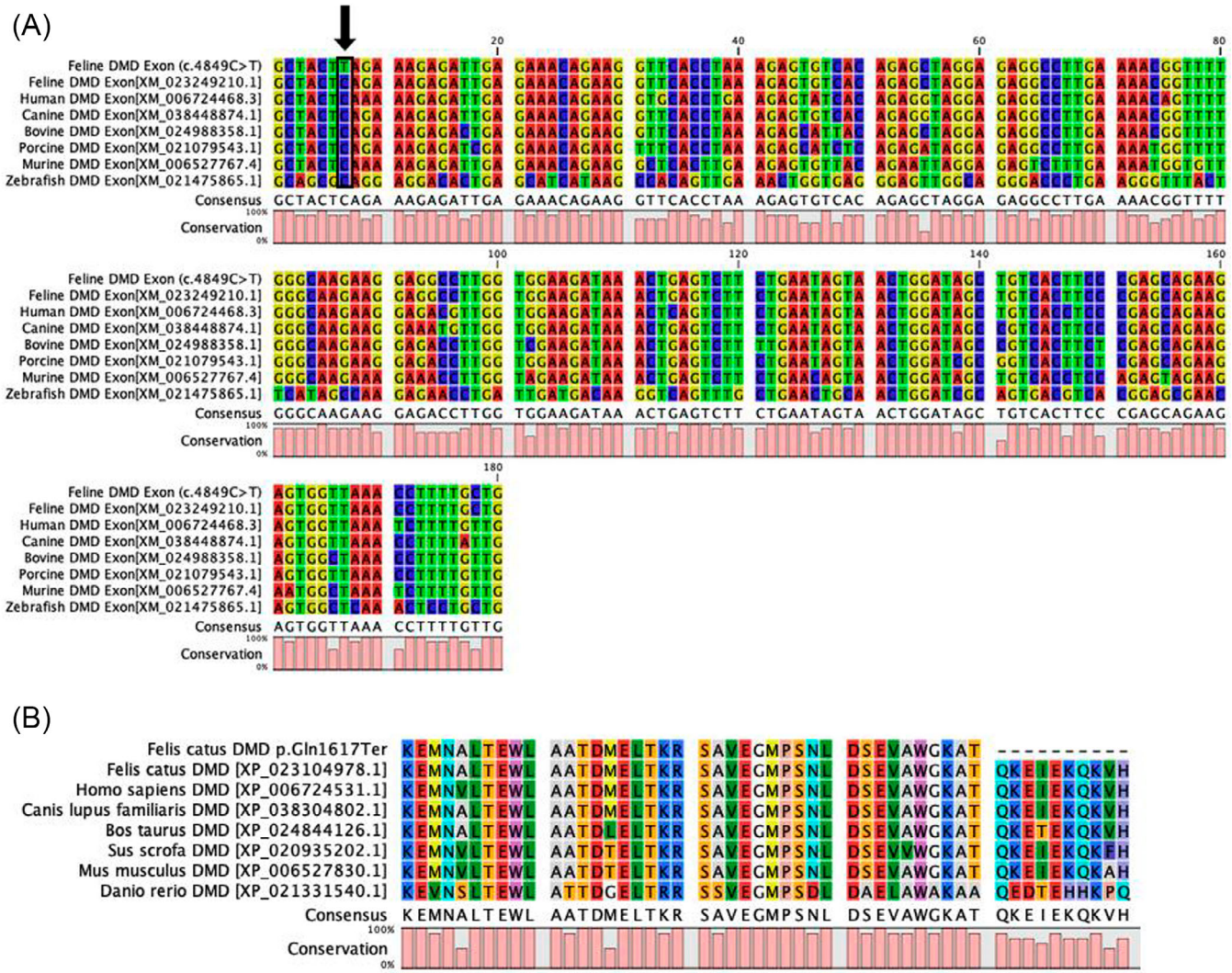

FIGURE 4 Multispecies alignment of *Felis catus DMD* exon 35. The alignments indicate the position of the *DMD* stop gain LoF variant (XM_023249210.1, ENSFCAT00000068370:c.4849C>T; p.Gln1617Ter) at chromosome position X:28201541 Felis_catus_9.0 at the beginning of exon 35. (A) Nucleotide alignment for diverse species (DMD affected feline case, normal feline, human, canine, bovine, porcine, murine, and zebrafish) indicating the 100% conservation of the nucleotide and the high conservation of the exon 35, lower histogram. Nucleotides are represented by the different colors. (B) Protein alignments for the same species depicting the termination in exon 35 for the affected cat. The alanine that is 2 amino acids upstream of the amber termination codon is the 5′ start of exon 35. The mammals have nearly 100% amino acid conservation within this region.

defined because of the limitations of the techniques of discovery.[65] Two additional *DMD* variants in cats were characterized recently, both in families of Maine coon cats.[16,17] A previous report described a nonsense variant in exon 11 of the feline *DMD* gene, NC_058386.1 (XM_045050794.1): c.1180C>T (p.Arg394*), which was supported by mRNA sequencing and demonstrated the loss of most of the dystrophin protein.[16] These Maine coon cats are part of the 99 Lives cohort, thus the c.1180C>T variant was not present in the current case. Another study described a milder form of MD (Becker-like; OMIA:001081-9685), which was a missense variant (c.4186C>T; p.His1396Tyr) also showing familial inheritance in Maine coon cats.[17] This variant was not identified in the 99 Lives cohort of cats, further suggesting its rarity, and supporting causality for disease.

The *DMD* variant in the present case, c.4849C>T; p.Gln1617Ter, is a LoF variant caused by a nonsense mutation in exon 35 of the 78 exons annotated for cat *DMD* and likely truncates approximately 65% of the protein. This variant was unique to the case in the 99 Lives cohort, supporting its rarity. The present case is a random bred cat and only 1 sibling could be evaluated. The female sibling did not have the variant, suggesting the LoF variant was transmitted by the queen as a likely heterozygous carrier, or the case cat represented a de novo mutation. ClinVar contains an entry for the human counterpart variant (Q1618X; Variation ID: 1322710)—HGMD CM022951[66] that had the same predicted effect and segregates within a human family with DMD, supporting the cat variant as also pathogenic based on comparative genetic criteria. Loss of function variants are considered 1 of the

most pathogenic mutations, but many also can be well tolerated.[47,67] Each human genome may contain many stop variants with no observable impact upon phenotype.[68,69] However, the noise from poor annotation and poor sequencing techniques is considered part of the false identification of LoF variants that may not actually be present in the genome. In the case of the cat in our report, the variant was suggested as valid by high coverage and high quality of reads at the variant site and further proven by direct Sanger sequencing. Although the genome datasets are less substantial for cats than for humans, the accuracy of the current cat dataset has benefitted from the use of robust technologies and techniques to produce the sequencing data, consistent methods of variant identification based on the tools developed by the Human Genome Project[70] and more robust genome assemblies and annotations.[32] The high conservation of both the nucleotide and amino acid sequences within the region across diverse mammalian species also supports the determination of pathogenicity for the variant. The variant analyses had no a priori consideration of candidate genes. The recessive/hemizygous model identified 71 additional unique variants that would equally qualify for candidates for the disease based on the model selection alone. However, only 2 were within coding regions of genes, both missense variants in the gene *Sodium/Potassium-Transporting ATPase Subunit Beta-3 (ATPB3)* and an uncharacterized gene (LOC101082653). The remaining 16 genes, 10 uncharacterized genes and 34 intergenic variants were in regulatory or uncharacterized regions that would require extensive experimentation and comparative studies to define causality. Similarly, most of the 735 variants identified when considering the dominant model are non-coding. Most of the LoF frameshift variants are in uncharacterized genes. The gene *Titin* (*TTN*) has been associated with MDs,[71] which is a p.Ile31673Val missense variant in the cat case, however the clinical, pathological, and histological data supports a dystrophy deficiency and further investigation of other genes is not warranted.

The young age of this male cat, the markedly increased CK activity, the presence of the dystrophic phenotype in the muscle biopsy sample, and the non-detectable staining for the rod and carboxy-terminus of dystrophin with increased utrophin expression in muscle cryosections is consistent with the diagnosis of X-linked MD. Muscle hypertrophy is a consistent finding in X-linked dystrophin-deficient cats, but this finding is not limited to the feline species.[72] Hypertrophy of the gastrocnemius muscle (calf hypertrophy) occurs in Duchenne MD in people and cranial sartorius muscle hypertrophy occurs in the Golden retriever model.[72] Previous studies have determined this finding is true hypertrophy and not pseudohypertropy related to fatty and connective tissue infiltration or to hyperplasia.[72]

The expected LoF variant effect, population data, genetic data compared to humans and amino acid conservation within the exon support the identified variant as pathogenic and causing the clinical signs of X-linked MD in the cat of our report.[73] Precision/genomic medicine efforts for the domestic cat and in veterinary medicine increasingly support disease variant and animal model discovery and provide opportunities for targeted treatments for companion animals.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

The affected cat in this study was privately owned and examined with the consent of the owner. The work involved the use of a nonexperimental animal only. Established internationally recognized high standards ("best practice") of individual veterinary patient care were followed. The control cat tissues were obtained post-mortem and did not require IACUC approval. Dr. Lyons has an exemption ACUC protocol 9178 at the University of Missouri for the receiving of samples for genomic applications.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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