Received: 7 August 2018 | Accepted: 11 January 2019
DOI: 10.1111/jvim.15425
Journal of Veterinary Internal Medicine Open Access
ACVIM American College of Veterinary Internal Medicine

STANDARD ARTICLE

Coding sequences of sarcoplasmic reticulum calcium ATPase regulatory peptides and expression of calcium regulatory genes in recurrent exertional rhabdomyolysis

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Funding information
Foundation for the National Institutes of Health, Grant/Award Number: R01HL129814, R37AG26160; Grayson Jockey Club Research Foundation; DDT NIH, Grant/Award Number: R01 HL129814, R37 AG26160; Morris Animal Foundation, Grant/Award Number: D16Eq004

Background: Sarcolipin (*SLN*), myoregulin (*MRLN*), and dwarf open reading frame (*DWORF*) are transmembrane regulators of the sarcoplasmic reticulum calcium transporting ATPase (SERCA) that we hypothesized played a role in recurrent exertional rhabdomyolysis (RER).

Objectives: Compare coding sequences of *SLN*, *MRLN*, *DWORF* across species and between RER and control horses. Compare expression of muscle Ca^{2+} regulatory genes between RER and control horses.

Animals: Twenty Thoroughbreds (TB), 5 Standardbreds (STD), 6 Quarter Horses (QH) with RER and 39 breed-matched controls.

Methods: Sanger sequencing of SERCA regulatory genes with comparison of amino acid (AA) sequences among control, RER horses, human, mouse, and rabbit reference genomes. In RER and control gluteal muscle, quantitative real-time polymerase chain reaction of SERCA regulatory peptides, the calcium release channel (*RYR1*), and its accessory proteins calsequestrin (*CASQ1*), and calstabin (*FKBP1A*).

Results: The *SLN* gene was the highest expressed horse SERCA regulatory gene with a uniquely truncated AA sequence (29 versus 31) versus other species. Coding sequences of *SLN*, *MRLN*, and *DWORF* were identical in RER and control horses. A sex-by-phenotype effect occurred with lower *CASQ1* expression in RER males versus control males ($P < .001$) and RER females ($P = .05$) and higher *FKBP1A* ($P = .01$) expression in RER males versus control males.

Conclusions and Clinical Importance: The *SLN* gene encodes a uniquely truncated peptide in the horse versus other species. Variants in the coding sequence of *SLN*, *MLRN*, or *DWORF* were not associated with RER. Males with RER have differential gene expression that could reflect adaptations to stabilize RYR1.

KEYWORDS
exercise, myopathy, RYR1, skeletal muscle, tying up

Abbreviations: AA, amino acid; ATP2A1, gene encoding sarcoplasmic reticulum calcium transporting ATPase; BLAT, BLAST-like alignment tool; *CASQ1*, calsequestrin; cDNA, complementary DNA; CK, creatine kinase; CT, cycle thresholds; *DWORF*, dwarf open reading frame; ER, exertional rhabdomyolysis; F, female; *FKBP1A*, calstabin; G, gelding; GAPDH, glyceraldehyde phosphate dehydrogenase; *GYS1*, glycogen synthase 1; *MRLN*, myoregulin; PAS, periodic acid-Schiff's; PCR, polymerase chain reaction; PLN, phospholamban; QH, Quarter Horses; qRT-PCR, quantitative real-time polymerase chain reaction; RER, recurrent exertional rhabdomyolysis; RYR1, calcium release channel; S, stallions; SERCA, sarcoplasmic reticulum calcium transporting ATPase; *SLN*, sarcolipin; SR, sarcoplasmic reticulum; STD, Standardbred; TB, Thoroughbred; UCSC, University of California, Santa Cruz.

The project was performed at Michigan State University.

1 | INTRODUCTION

Exertional rhabdomyolysis (ER) in horses is characterized by multiple episodes of stiffness, muscle cramping, reluctance to move, and muscle damage and can have many causes.[1] Exertional rhabdomyolysis affects 5%-7% of Thoroughbred (TB) and Standardbred (STD) racehorses, and recurrence can be so frequent that 17% of ER horses are unable to race again in the same season.[2–4]

The term recurrent exertional rhabdomyolysis (RER) has been used to describe a chronic form of ER in racehorses with a proposed underlying cause of abnormal myoplasmic calcium (Ca^{2+}) regulation.[5,6] This hypothesis was based on finding a lower threshold for inducing a contracture in isolated skeletal muscle bundles of RER versus control horses exposed to increasing concentrations of halothane, potassium, and caffeine, all of which induce Ca^{2+} release from the sarcoplasmic reticulum (SR).[5,6] In addition, higher caffeine-induced Ca^{2+} release was found in cultured myotubes from RER versus control horses, as detected by Fura-2 fluorescence imaging.[7] Further studies of isolated SR membranes and genetic linkage analysis have not identified an underlying cause for alterations of Ca^{2+} regulation in RER.[8,9]

Recent discoveries regarding fundamental modes of intracellular Ca^{2+} regulation have identified additional regulatory mechanisms for the SR Ca^{2+} transporting ATPase (SERCA) that may play a role in the genesis of RER in horses. After contraction, SERCA induces muscle relaxation by catalyzing the transport of 2 Ca^{2+} ions into the lumen of the SR using the free energy from hydrolysis of 1 ATP molecule. Phospholamban (PLN) inhibits SERCA activity and is primarily expressed in cardiac and slow twitch muscle fibers.[10,11] Sarcolipin (*SLN*), first discovered in 1974 as a peptide that copurifies with SERCA, was subsequently found to decrease the Ca^{2+} affinity of SERCA[12] and decrease the energetic coupling efficiency of SERCA (Ca^{2+}/ATP transport ratio <2), thereby decreasing SR luminal Ca^{2+} stores.[12–14] In addition, transcripts that previously were annotated as long noncoding RNAs recently have been found to encode small transmembrane peptides MRLN and dwarf open reading frame (DWORF) that also regulate the activity of SERCA in skeletal muscle (Figure 1).[15,16] Dwarf open reading frame has been shown to enhance SERCA activity in the mouse heart by displacing PLN and, in cell culture models, by displacing SLN and MRLN (Figure 1). A decrease in *SLN* and *MRLN* or increase in *DWORF* expression could increase SR Ca^{2+} stores by decreased SERCA inhibition (ie, decreased Ca^{2+} affinity), thereby acting to increase calcium release channel (RYR1) Ca^{2+} release and myoplasmic Ca^{2+} concentration during contraction and potentially leading to clinical manifestations of RER.

Our goal was to determine if RER is associated with variants in the coding sequences of *SLN*, *MRLN*, or *DWORF*, altered expression of Ca^{2+} regulatory genes involved in SR Ca^{2+} uptake and Ca^{2+} release or both. The first aim of our study was to compare the coding sequences of *SLN*, *MRLN*, and *DWORF* in the horse with other species. The second aim was to determine if the coding sequences for these genes differed between RER and control horses. The third aim was to determine if there was a difference in expression of skeletal muscle Ca^{2+} regulatory genes between horses with and without RER.

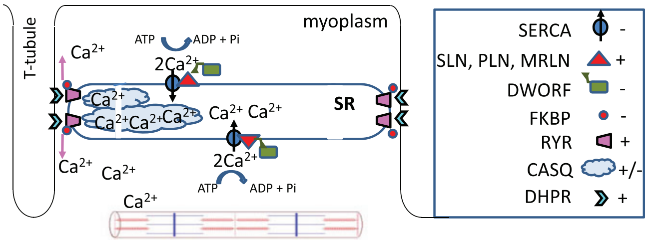

FIGURE 1 Schematic of key Ca^{2+} regulatory proteins in skeletal muscle sarcoplasmic reticulum (SR). Sarcoplasmic reticulum calcium transporting ATPase (SERCA) is the SR Ca^{2+} pump with isoform SERCA1 expressed in fast twitch type 2 fibers and SERCA2 expressed in slow twitch and cardiac muscle fibers. SERCA is inhibited by sarcolipin (SLN), phospholamban (PLN), or myoregulin (MRLN). Phospholamban primarily inhibits SERCA2, and SLN and MRLN inhibit SERCA1, depending upon species. Dwarf open reading frame (DWORF) displaces the SERCA inhibitors, PLN, SLN, and MRLN. FKBP (calstabin) modulates Ca^{2+} release through the Ca^{2+} release channel which has 2 isoforms: RYR2 (cardiac and slow twitch) and RYR1 (fast twitch muscle fibers). Calsequestrin (CASQ) is the luminal, high-capacity Ca^{2+} binding protein, which directly modulates Ca^{2+} release by RYR. DHPR, the dihydropyridine receptor, is a voltage-gated Ca^{2+} channel that triggers RYR to release Ca^{2+}. The legend (right) indicates the effect of each regulatory protein on myoplasmic Ca^{2+} concentration: increase (+) or decrease (–)

2 | METHODS

2.1 | Pilot study

To determine if SERCA1 (expressed in fast twitch type 2 fibers) or SERCA2 (expressed in cardiac and type 1 muscle fibers) was primarily expressed in equine gluteal muscle, we initially evaluated transcripts per million reads from RNA-seq data obtained from gluteal muscle of 6 healthy Arabian horses (NCBI's Gene Expression Omnibus GEO Series accession number GSE104388). Mean transcripts per million reads (SD) were 3.7 times higher for SERCA1 (562 ± 152 TPM) than SERCA2 (243 ± 145 TPM). When muscle fiber type composition was assessed for 6 of the TBs in our study, we found they had 50% fewer type 1 fibers than did the Arabian horses used in the RNA-seq analyses (TB: 8% ± 3% type 1, 92% ± 5% type 2: Arabian 17% ± 3% type 1, 83% ± 8% type 2). Thus, SERCA1 rather than SERCA2 seemed to be the primary isoform of interest when studying SERCA inhibitors in TB gluteal muscle.

The study was approved by the Institutional Animal Use and Care Committee of Michigan State University.

2.2 | Comparative amino acid sequences

The entire coding sequences for *SLN* and *MRLN* were identified from the annotated references genomes of horse (EquCab2; http://ncbi.nlm.nih.gov/genome/145). Horse coding sequences were verified by comparison to RNA-seq data for Arabian control horses (NCBI's Gene Expression Omnibus GEO Series accession number GSE104388) and comparison to Sanger sequencing described below. Coding sequences for *DWORF* were identified in the annotated human and mouse reference genomes and used as a BLAST-like alignment tool (BLAT) on EquCab2 (University of California, Santa Cruz [UCSC] genome browser; https://genome.ucsc.edu/). Reads for the equine *DWORF* coding sequence were present in the reference genome, but the reads ended abruptly without a stop codon, suggesting that EquCab2 is not well assembled in the 3′ region of this gene. The RNA-seq data from Arabian horses (GEO Series accession number GSE104388) was used to complete the derived equine *DWORF* sequence.

The amino acid (AA) sequences of *PLN*, *SLN*, and *MRLN* were compared to the human and mouse sequences because these species have well-established references genomes and to rabbit because we currently are performing comparative biochemical assays on SR preparation from horse and rabbit. The 2016 Ensembl (https://useast.ensembl.org/index.html) was used to evaluate the genome of rabbit (OrynCun; https://www.ncbi.nlm.nih.gov/assembly/GCF_000003625.3), mouse (http://www.informatics.jax.org/), and human (GRCh38.p12version). For rabbit, coding sequences for *DWORF* from the annotated human and mouse reference genomes were used to BLAT the respective reference genomes (UCSC genome browser; https://genome.ucsc.edu/).

We also performed comparative sequence analysis for *SLN* on species closely related to the horse including ass (NCBI accession NW_014638236.1, Genebank ERX607030, ERX607036, ERX607001,) Przewalski's horse (databases NC_007675973.1, Genebank ATBW010 57363.1), and zebra as well as another Perisodactyl, the Southern white rhinoceros (http://rohsdb.cmb.usc.edu; LOC101603223). To obtain *SLN* sequence for the zebra (unknown genus), we Sanger sequenced muscle tissue archived in the Neuromuscular Diagnostic Laboratory in the same blinded fashion as described for RER and control horses.

2.3 | Gene sequencing

2.3.1 | Horses

The *SLN* sequence was determined for 17 TB (11 females [F], 3 geldings [G], 3 stallions [S]), 5 STD (2 F, 2 G, 1 S), and 6 Quarter Horses (QH; 3 F, 3 G) that had experienced repeated episodes of ER and had muscle biopsy specimens archived in the Neuromuscular Diagnostic Laboratory (Supplemental Table 1). Control horses included 13 TB (3 F, 10 G), 6 STD (1 F, 5 G), and 3 QH (1 F, 2 G) with no known history of RER and samples archived in the Neuromuscular Diagnostic Laboratory. The *MRLN* and *DWORF* sequences were determined in a subset consisting of 3 TB, 3 STD, and 3 QH with RER and 4 TB, 4 STD, and 3 QH controls (Supplemental Table 1). The RER criteria included a history of repeated episodes of ER reported by the referring veterinarian and muscle biopsy specimens with normal periodic acid-Schiff's (PAS) staining for glycogen. Preference for study inclusion was given to horses with numerous episodes of ER, documented increases in serum creatine kinase (CK) activity and centrally displaced nuclei on histological examination (Supplemental Table 1). All QH examined were negative for the glycogen synthase 1 (*GYS1*) mutation responsible for type 1 polysaccharide storage myopathy.[17] Control horses had no known history of ER and, for TB and QH, no evidence of muscle histopathology. Hair samples rather than muscle biopsy specimens were available from local STD racehorse controls.

2.3.2 | DNA isolation and sequencing

Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland) was used to isolate genomic DNA from hair roots, buffy coat, or frozen muscle samples according to the manufacturer's protocol.

2.3.3 | Primers

Primers were designed using Primer3Plus software[18] to cover the predicted protein coding regions of horse *SLN*, *MRLN*, and *DWORF* as well as the 5′ upstream sequence, likely containing the 5′ untranslated region of the 3 horse genes based on similarities across species. For *SLN*, 8 primers were designed to cover the possible noncoding exon 1 (2148 bp) and predicted coding exon 2 (2092 bp; Supplemental Table 2). The regions sequenced for *SLN* comprised 6332 bp. For *MLRN*, 4 primers were designed to cover noncoding exon 1, possible noncoding exon 2, noncoding exon 3, and coding exon 4 (Supplemental Table 2). The region sequenced for *MRLN* comprised 2747 bp. For *DWORF*, based on the open reading frame that begins in exon 1 and encodes the first 4 AA of the protein with the remaining protein being encoded in exon 2, 2 primers were designed to cover 500 bp upstream of the coding sequence, the first 4 AA in exon 1, and exon 2 and the 3′ untranslated region.[15] The region sequenced for *DWORF* comprised 1300 bp. Primers are listed in Supplemental Table 2.

2.3.4 | PCR and sequence analysis

Each primer pair was used to amplify intervening genomic DNA using polymerase chain reaction (PCR) in 25 μL reactions that included 2.0 μL sample DNA, 12.5 μL Hot Start PCR 2× Master Mix Taq Polymerase (Thermo Fisher Waltham, MA), 0.5 μL of 20 μM forward and reverse primers (Invitrogen), and 9.5 μL molecular biology grade water. The PCR reactions started with 15 minutes at 95°C, then 35 cycles of 30 seconds at 94°C, 30 seconds at the primer-specific annealing temperature, and 30 seconds at 72°C, followed by a final extension of 10 minutes at 72°C. The PCR products were resolved on 1% agarose gels. The PCR products then were purified using ExoSAP-IT Product Cleanup (Affymetrix, Santa Clara, California) and sequenced by the Michigan State University Research Technology Support Facility Genomics Core using the 96-capillary electrophoretic ABI 2730xl platform (Sanger sequencing). Sequences were aligned to Equcab2.0 (http://ncbi.nlm.nih.gov/genome/145) and analyzed using Sequencher software (version 5.4.5; Gene Codes Corporation).

2.4 | Gene expression

2.4.1 | Horses

Muscle samples were obtained prospectively from 14 fit TB RER racehorses (9 F, 2 G, 3 S; age 5.2 ± 2.4 years) with median serum CK activity of 251 U/L and mean (SD) CK activity of 970 ± 2166 U/L. Samples also were obtained from 20 fit control TB racehorses (9 F, 6 G, 5 S; age 3.4 ± 1.6 years) with median CK activity of 250 U/L and mean CK activity of 267 ± 86 U/L. All horses were housed either at the same race training center or a nearby racetrack in Lexington, Kentucky (Supplemental Table 1). The RER horses had a history of episodes of ER documented by a veterinarian and had not exhibited clinical signs within 48 hours of muscle biopsy. Control horses were in training and had no history of ER. Twelve of the horses used for gene expression studies also were Sanger sequenced for *SLN* as described above.

2.4.2 | Primers

Primers for *GAPDH*, *RYR1*, *SERCA1*, *CASQ1*, *DWORF*, *PLN*, *MRLN*, *SLN*, and *FKBP1A* were designed to cross exon-exon boundaries using NCBI (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and referencing NCBI EquCab 2.0 (Supplemental Table 3). The glyceraldehyde phosphate dehydrogenase (GAPDH) gene was used as a housekeeping control because it showed minimal variability and is less variable across age in skeletal muscle.[19]

2.4.3 | Muscle biopsies

Gluteus medius muscle biopsy specimens were obtained in the morning 1-4 hours after jogging or light galloping exercise from a standardized site using a modified Bergstrom biopsy needle as previously described.[20] A portion of the sample was flash-frozen in liquid nitrogen and stored at −80°C. A second portion was oriented in cross-section and frozen within 12 hours of sampling in isopentane that was suspended in liquid nitrogen.

2.4.4 | Muscle histopathology

Cryostat sections (7-μm thick) were stained with hematoxylin and eosin and PAS and evaluated for the presence of centrally located nuclei, degenerating myofibers, or macrophages.[21]

2.4.5 | RNA extraction

Total muscle RNA was isolated from flash frozen samples using TRIzol/chloroform extraction after tissue homogenization with a biopulverizer (BioSpec Products, Inc, Fartlesville, Oklahoma) as previously described.[22] Treatments with DNase were performed on columns (Direct-zol RNA MiniPrep Plus, Zymo, Irvine, California) with DNase I (RNase-free; New England BioLabs, Inc Ipswich, MA) according to manufacturer's instructions.

2.4.6 | Complementary DNA synthesis

Complementary DNA (cDNA) was made using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific Waltham, MA). Each 20 μL reaction contained 2 μL of 10× RT Buffer, 0.8 μL of 100 mM dNTPs, 2 μL of RT Random Primers, 1 μL of RT Enzyme, approximately 1200 ng of sample RNA, and the remaining volume made up of sterile nuclease-free distilled water. The reactions then were run in a ProFlex PCR system (Applied Biosystems, Life Technologies Waltham, MA) under the following conditions: 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5 minutes, and 4°C until recovery. All reactions then were diluted with sterile nuclease-free distilled water to reach a total volume of 100 μL.

2.4.7 | Quantitative real-time PCR

Genes selected included the Ca^{2+} release channel (*RYR1*), calstabin (*FKPB1A*), which stabilizes Ca^{2+} leak from RYR1, and calsequestrin (*CASQ1*), a luminal high-capacity Ca^{2+} binding protein that modulates RYR1 Ca^{2+} release. Expression of gene encoding SERCA (*ATP2A1*), *PLN*, *SLN*, *MRLN*, and *DWORF* also was determined. Thermocycling for quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using EvaGreen dye (Biotium, Inc, Fremont, California), ROX Reference Dye (Invitrogen, Life Technologies Carlsbad, CA), and Hot Start *taq* DNA Polymerase (New England BioLabs, Inc Ipswich, MA), using the QuantStudio 3 Real-Time PCR System (ThermoFisher Scientific Waltham, MA). The PCR reactions were run in duplicate (20 μL volume reactions). Each reaction contained 2 μL of sample cDNA, 2 μL of 2.5 mM dNTPs, 2 μL of 10× PCR buffer, 1 μL of EvaGreen dye, 1.5 μL of 1:10 ROX reference dye dilution, 0.125 μL of Hot Start *taq* DNA Polymerase, 2 μL of 1.6 μM forward primer, 2 μL of 1600 μM reverse primer, and 7.4 μL of sterile nuclease-free distilled water. Reactions were run for 40 cycles under the following conditions: denaturation at 95°C for 10 minutes, annealing at 60°C for 1 minute; melt curve stages at 95°C for 15 seconds, 60°C for 1 minute, and 95°C for 15 seconds. Cycle thresholds (CT) were automatically calculated by the QuantStudio 3 Real-Time PCR System. For each gene of interest, 100% geometric efficiency was established. Nontemplate controls run for each gene showed no amplification.

2.5 | Statistical analysis

2.5.1 | Quantitative real-time PCR

Relative quantitation of gene expression was calculated by the comparative threshold cycle method (2-ΔΔCT) using the CT of GAPDH

TABLE 1 Mean (SD) gene expression relative to GAPDH (ΔCT)

	RER females N = 9	Control females N = 9	RER males N = 5	Control males N = 11
RYR1	5.57 ± 0.64[a]	5.64 ± 0.82[a]	5.55 ± 0.65[a]	5.81 ± 0.66[a]
FKBP1A	5.19 ± 2.76[a]	6.36 ± 1.32[a]	4.31 ± 1.49[ab]	6.77 ± 0.67[a]
CASQ1	−2.31 ± 2.14[acd]	−3.32 ± 1.83[ad]	−0.23 ± 1.42[b]	−1.64 ± 1.43[bc]
ATP2A1	7.98 ± 1.03[a]	8.06 ± 0.55 [a]	7.02 ± 1.99[a]	8.72 ± 1.51[a]
SLN	−4.67 ± 1.74[a]	−5.31 ± 1.68[a]	−3.23 ± 1.24[a]	−4.18 ± 2.34 [a]
MRLN	6.91 ± 0.69[a]	7.55 ± 1.20[a]	7.52 ± 0.91[a]	6.21 ± 1.16[a]
PLN	8.27 ± 1.48[a]	8.12 ± 1.61[a]	8.42 ± 1.68[a]	8.53 ± 1.34[a]
DWORF	7.95 ± 1.41[a]	7.94 ± 1.07[a]	7.64 ± 1.14[a]	8.64 ± 1.61[a]

Genes include *RYR1* and its regulators *FKBP1A* and *CASQ1* as well as SERCA (*ATP2A1*) and its inhibitors *SLN*, *PLN*, and *MRLN*, plus *DWORF* which displaces the SERCA inhibitors. Different letters indicate differences between rows. $P \leq .05$.

Abbreviations: *ATP2A1*, gene encoding sarcoplasmic reticulum calcium transporting ATPase; *CASQ1*, calsequestrin; CT, cycle thresholds; DWORF, dwarf open reading frame; *FKBP1A*, calstabin; GAPDH, glyceraldehyde phosphate dehydrogenase; MRLN, myoregulin; PLN, phospholamban; RER, recurrent exertional rhabdomyolysis; *RYR1*, calcium release channel; SERCA, sarcoplasmic reticulum calcium transporting ATPase; SLN, sarcolipin.

(Table 1). Data was tested for normality using the Shapiro Wilks test. A 2-way analysis of variance and Tukey post hoc test were performed to examine differences in ΔCT values for RER and control horses stratified by sex using GraphPad Prism 7 (Graphpad Software, La Jolla, California).

3 | RESULTS

3.1 | Comparison of AA sequences

3.1.1 | Sarcolipin

The coding sequence of *SLN* in RER and control horses was truncated at 29 versus 31 AA relative to the human, mouse, rabbit, and Southern white rhinoceros sequences. The zebra, ass, and Przewalski's horse had the same truncated sequence as did the horse (Supplemental Table 4). Homology of the horse SLN AA sequence was 77% to rabbit (24/31), 77% to human (24/31), and 81% to mouse (25/31). Most importantly, the *SLN* AA sequence was missing putative regulatory sites Ser4, Thr5, Cys9, and Tyr31 (Figure 2).[32–35]

3.1.2 | Myoregulin

The myoregulin (*MRLN*) AA sequence was similar in length across species at 46 AA. Sequence identity of horse MLRN was 78% with rabbit (36/46), 85% with human (39/46), and 74% with mouse (34/46). Most AA differences occurred in the cytoplasmic domain with only 1 AA impacting charge, horse neutral Thr15 versus human and rabbit basic Lys15.

3.1.3 | Dwarf open reading frame

The DWORF peptide was similar in length between horse and human at 35 AA and was 34 AA in mouse with 3 AA that could not be deduced from the rabbit reference genome (Figure 2). Sequence homology of horse DWORF was 86% with human (30/35) and 69% with mouse (24/35). Amino acid substitutions that altered AA charge were not identified when comparing AA across horse, human, and mouse.

3.2 | Coding sequence of *SLN*, *MRLN*, *DWORF* in RER and control horses

No differences were detected in coding sequences of *SLN*, *MRLN*, and *DWORF* between RER and control horses.

3.3 | Gene expression

Sarcolipin was the most highly expressed SERCA regulatory gene (Table 1). No significant difference was found in the expression level of Ca^{2+} regulatory genes between control females and control males or between RER females and control females (Figure 3). An impact of sex and phenotype was observed in which RER males had significantly higher expression of *FKBP1A* (P = .01) than did control males (Figure 3). The RER males had lower expression of *CASQ1* than did control females (P < .001) and lower expression than RER females (P = .05; Figure 3).

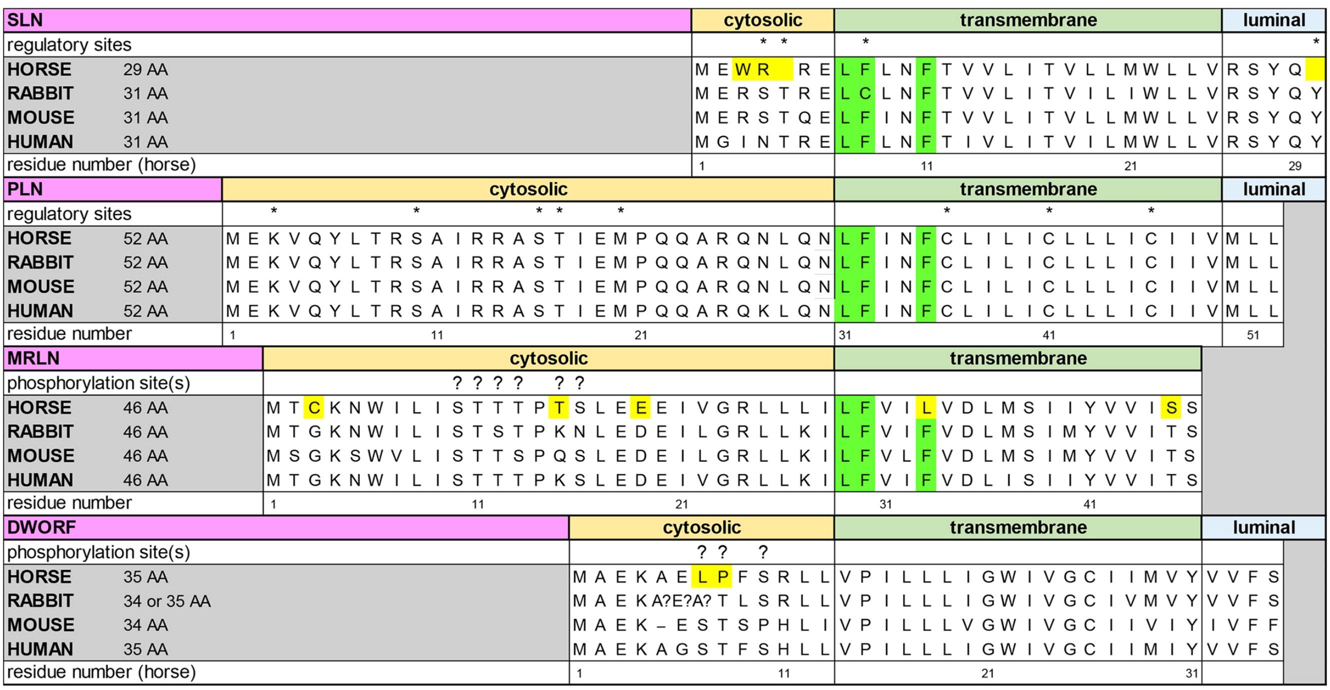

FIGURE 2 Amino acid sequence (AA) of SLN, PLN, MRLN, and DWORF derived from mouse, human, and rabbit reference genomes, and Sanger sequencing of horse genes in the present study. Yellow highlight indicates AA substitution or deletion unique to horse. Green highlight indicates highly conserved residues near the myoplasmic-membrane interface of SERCA inhibitory peptides.[16,23,24] Asterisk (*) indicates regulatory site residues.[10,25–31] Question mark (?) indicates putative phosphorylation sites or lack of consensus sequence for rabbit DWORF from the reference genome. DWORF, dwarf open reading frame; MRLN, myoregulin; PLN, phospholamban; SERCA, sarcoplasmic reticulum calcium transporting ATPase; SLN, sarcolipin

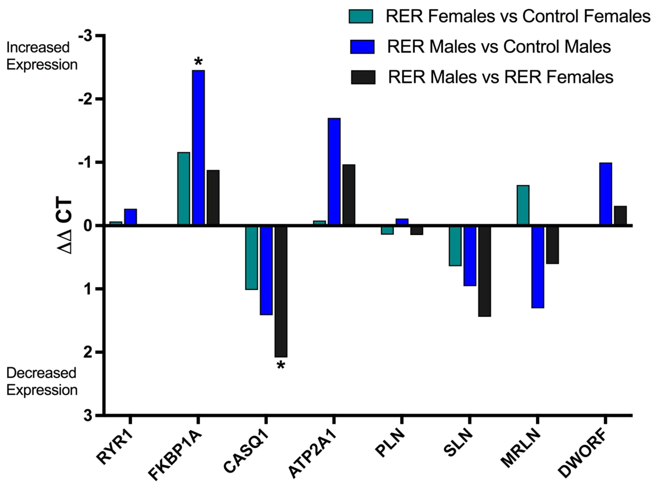

FIGURE 3 Quantitative real-time polymerase chain reaction for genes involved in myoplasmic Ca^{2+} regulation in RER and control horses stratified by sex and expressed as relative abundance of transcripts compared to GAPDH and their respective control group (ΔΔCT). Genes include *RYR1* and its regulators *FKBP1A* and *CASQ1* as well as SERCA (*ATP2A1*) and its inhibitors *SLN*, *PLN*, and *MRLN*, plus *DWORF* which displaces the SERCA inhibitors. Asterisks indicate difference for *FKBP1A* at *P* = .01 and *CASQ1* at *P* = .05. *ATP2A1*, gene encoding sarcoplasmic reticulum calcium transporting ATPase; *CASQ1*, calsequestrin; DWORF, dwarf open reading frame; *FKBP1A*, calstabin; GAPDH, glyceraldehyde phosphate dehydrogenase; MRLN, myoregulin; PLN, phospholamban; RER, recurrent exertional rhabdomyolysis; *RYR1*, calcium release channel; SERCA, sarcoplasmic reticulum calcium transporting ATPase; SLN, sarcolipin

4 | DISCUSSION

In resting striated muscle across all species, Ca^{2+} is tightly regulated to maintain 10 000-fold lower myoplasmic Ca^{2+} than SR luminal Ca^{2+} concentration because of the activity of SERCA.[36] The activity of SERCA is regulated by myoplasmic Ca^{2+} and by the inhibitory peptides, PLN and SLN.[37] More recently, MRLN in mouse skeletal muscle and sarcolamban in fly heart have been reported to be important SERCA inhibitors that have highly conserved protein sequences and molecular structure across species.[16,38,39] Such conservation for over 550 million years suggests an important, conserved system for the regulation of Ca^{2+} uptake by SERCA.[38] In keeping with this conservation, the transmembrane domains of SLN, PLN, and MRLN are similar within genes across species, with only a few conservative AA substitutions in this region in equine MRLN (Leu33 versus Phe33, Ser45 versus Thr 45) compared to mouse, human, or rabbit. Remarkably, however, equine SLN is distinct from 67 other species evaluated in our and other studies in that it is truncated at 29 AA, missing a terminal Tyr residue that has been shown to be directly involved in the inhibition of SERCA.[40] Other unique aspects of SLN included missing potential regulatory residues in the cytoplasmic domain including Ser4 (serine/threonine-protein kinase 16 phosphorylation), Thr5 (calmodulin-dependent protein kinase II phosphorylation), and Cys9 (acylation).[11,32,41] These changes do not prove a functional effect and potentially would be a fruitful area for future research in horses. In other species, deletion of Thr5 in SLN has been shown in cardiac muscle to selectively downregulate SR Ca^{2+} handling proteins and decrease SR Ca^{2+} uptake.[34] In addition, the luminal tail of SLN in the horse was missing the terminal Tyr, which is proposed to functionally interact with luminal residues in SERCA and to target SLN to the SR and endoplasmic reticulum.[39,42,43] Notably, the 29 AA equine *SLN* sequence is also found in zebra and ass, (ERX607036 and ERX607001) and Przewalski's horse but not in another Perisodactyl, the Southern white rhinoceros. Thus, the distinct *SLN* sequence of *Equus sp.* has been present for millions of years, before the divergence of *Equus caballus*, *Equus przewalski*, *Equus grevi*, and *Equus asinus*, and suggests a unique mechanism for myoplasmic Ca^{2+} regulation in the horse.[44]

A selection advantage for RER is suggested by the fact that STD horses with RER have faster racing times from a standing start than do STD without RER and by the high prevalence of RER in STD and TB at 5%-7%.[2,3] Small increases in myoplasmic Ca^{2+} concentration during muscle relaxation induced by the unique protein sequence of equine SLN could provide a selection advantage to horses with their superior athletic capacity by facilitating Ca^{2+} entry into mitochondria (which activates ATP production and metabolic processes), activating Ca^{2+}-dependent signaling pathways important for programming an oxidative muscle phenotype, and increasing the power of muscle contraction by initial enhancement of actomyosin force production.[45] Whereas slight increases in myoplasmic Ca^{2+} could enhance speed and endurance, excessive increases in myoplasmic Ca^{2+} lead to persistent myofiber contracture, enhanced reactive oxygen production, activation of proteases, and myodegeneration.[46] Thus, in horses, it is possible that slight alterations in the regulation of myoplasmic Ca^{2+} result in a fine balance between enhanced speed on the 1 hand and RER on the other.

The distinctive sequence of equine *SLN* made it a potential candidate gene for RER. However, no differences in *SLN* coding sequence were detected among 18 TB, 5 STD, and 6 QH with RER versus control horses. Coding sequences of *MRLN* and its inhibitor *DWORF* also were evaluated in a small number of horses, but no mutation associated with RER was identified. Additional resources were not directed to sequence more horses for *MRLN* and *DWORF*, because those RER horses that were sequenced had repeated episodes of ER of sufficient concern to submit muscle biopsy samples but no mutations were found. Multiple causes for RER may exist, some of which may be breed specific, and individual horses may have mutations in genes that were not evaluated in our study. However, based on our results, we propose that there is no high-frequency coding mutation in SERCA inhibitors *SLN* or *MRLN* or the dominant-negative SERCA activator *DWORF* in TB, STD, and QH with RER. Sequencing of *PLN* was not performed on the horses in our study because *PLN* had much lower mRNA expression in skeletal muscle than did *SLN* and the reference genomes across species had identical *PLN* AA sequences. Our results did not eliminate a *PLN* mutation as a basis for RER. Previous studies of RER have failed to identify a single genome-wide significant candidate locus for RER, suggesting that multiple genes, strong environmental influences, or both are at play.[47,48] The heritability of RER in TB and STD horses has been estimated at approximately 0.40.[49] Environmental factors such as sex, diet, fitness, and stress also have been shown to play important roles in expression of RER.[3,49]

A previous study of gene expression of RER in gluteal muscle of 4 female and 1 male French STD and 6 male and 4 female control

STD horses was performed using a mouse-equine microarray.[50] In contrast to the present study, muscle biopsy specimens were taken within 24 hours of an episode of ER. The previous study found that gene transcripts involved in muscle fiber Ca^{2+} homeostasis were modulated in a way that could increase myoplasmic Ca^{2+} concentration with down-regulation of *ATP2A1*, *RYR1*, and several other genes impacting myoplasmic, SR, or mitochondrial Ca^{2+} load (*SLC8A1*, *UCP2*, *ANXA6*). The *SLN* and *CASQ1* genes were not included in the array, and *ATP2A1* was found to be upregulated in the same samples using qRT-PCR. These results are difficult to compare with our study especially because they were not stratified according to sex and because the 2 studies sampled RER muscle at different times: between episodes versus within 1 day of an ER episode.

A sex bias exists for the expression of RER with males being less prone to RER than females.[3,4] It is noteworthy that sex-specific differences in Ca^{2+} regulatory gene expression were found in RER horses in our study. For example, RER males had lower expression of *CASQ1* than did RER females (P = .05) and control females (P < .001). A sex effect of *CASQ1* expression and muscle disease is seen in murine models in which male *CASQ1*-null mice developed fatal stress-induced malignant hyperthermic-like reactions, whereas female *CASQ1*-null mice were protected.[51,52] Calsequestrin is integral in regulating RYR1 Ca^{2+} release and is the principal Ca^{2+} binding protein in the SR (50-80 Ca^{2+} ions bound per molecule *CASQ1*) during the contraction/relaxation cycle when Ca^{2+}concentrations are ≥1 mM.[53]

The gene *FKBP1A* encodes calstabin which, when bound to RYR1, stabilizes the channel and in resting muscle decreases Ca^{2+} leak. The RER males had significantly higher expression of *FKBP1* than control males. If gene expression translates to protein expression, our results suggest that an adaptation toward lower myoplasmic Ca^{2+} concentration exists in RER males, whereby Ca^{2+} efflux through RYR1 is decreased by increased *FKBP1A*. The finding of altered *FKBP1A* and *CASQ1* expression in RER horses in our study is novel and clinically important because of the major roles FKBP1A and CASQ1 play in regulating RYR1 and excitation-contraction coupling.[51,53]

Altered expression of genes or proteins involved in myoplasmic Ca^{2+} regulation do not necessarily imply that these genes or proteins have a primary role in causing RER. Abnormal increases in myoplasmic Ca^{2+} can be a result both of primary defects in intramuscular Ca^{2+} regulation as well as secondary consequences of a loss of myofiber structural integrity from varied causes.[46] For example, diverse diseases such as dysferlinopathies, α-tocopherol deficiency, myotonic dystrophy, and dynamin-dependent centronuclear myopathy all result in increased expression of *SLN* in skeletal muscle.[54–56] In addition, mice with dystrophin-deficient muscular dystrophy (*mdx* model) have increased *CASQ1* expression in quadriceps muscle and abnormally high expression of SLN protein that correlates with decreased maximum velocity of SR Ca^{2+} uptake.[57] Thus, altered *CASQ1* or *FKBP1A* expression in RER could support either an adaptation induced by a primary defect in Ca^{2+} regulation or a secondary role of myoplasmic Ca^{2+} in generating RER.

It was not possible in our study to standardize the time interval between an episode of ER and when the muscle biopsy specimen was obtained for gene expression analysis. We therefore selected horses with modest increases in CK activity (median, 251 U/L; maximum, 8453 U/L) to prevent muscle cell damage from being the primary driver of altered gene expression. A weakness of our study was that *SLN* gene expression was evaluated without measurement of SLN protein expression. Examination of SLN protein content presented challenges because commercially available antibodies to SLN did not recognize the unique sequence of equine SLN (personal observation). Further experiments using a customized anti-horse-SLN antibody would be necessary to correlate SLN gene and protein expression. The notable RER sex-specific difference in *CASQ1* and *FKBP1A* expression was an unexpected finding in our study, and further experiments are needed to examine their impact on RER in males and females.

In conclusion, our results show that the *Equus* species has a novel SLN AA sequence with the potential for unique regulation of the Ca^{2+} affinity of SERCA. In the horses studied, mutations in the coding sequences of *SLN*, *MRLN*, or *DWORF* were not identified in TB, STD, or QH horses with RER. Differential expression of RYR1 regulators *FKPB1A* and *CASQ1* in RER males suggests that genesis of RER could be impacted by a sex-specific alteration in myoplasmic Ca^{2+} regulation.

ACKNOWLEDGMENTS

Funded by Morris Animal Foundation D16Eq004 and for DDT NIH grants R01 HL129814, R37 AG26160. The funding sources did not contribute to study design; in the collection, analysis, and interpretation of data; in the writing of the report; and in the decision to submit the article for publication. The assistance of Dr. James Slaughter, Dr. Susannah Lewis, referring veterinarians and Kentucky Equine Research is gratefully acknowledged.

CONFLICT OF INTEREST DECLARATION

Dr. Valberg is 1 of the owners of the patent for the PSSM genetic test and receives sales income from its use. Her financial interest has been reviewed and managed by the University in accordance with its conflict of interest policies.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Michigan State University IACUC approved protocol to collect muscle biopsies, blood, and hair from horses.

HUMAN ETHICS APPROVAL DECLARATION

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

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How to cite this article: Valberg SJ, Soave K, Williams ZJ, et al. Coding sequences of sarcoplasmic reticulum calcium ATPase regulatory peptides and expression of calcium regulatory genes in recurrent exertional rhabdomyolysis. *J Vet Intern Med*. 2019;33:933–941. https://doi.org/10.1111/jvim.15425