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Prevalence of the E321G MYH1 variant for immune-mediated myositis and nonexertional rhabdomyolysis in performance subgroups of American Quarter Horses

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Background: Immune-mediated myositis (IMM) in American Quarter Horses (QHs) causes acute muscle atrophy and lymphocytic infiltration of myofibers. Recently, an E321G mutation in a highly conserved region of the myosin heavy chain 1 (MYH1) gene was associated with susceptibility to IMM and nonexertional rhabdomyolysis.

Objectives: To estimate prevalence of the E321G MYH1 variant in the QH breed and performance subgroups.

Animals: Three-hundred seven elite performance QHs and 146 random registered QH controls.

Methods: Prospective genetic survey. Elite QHs from barrel racing, cutting, halter, racing, reining, Western Pleasure, and working cow disciplines and randomly selected registered QHs were genotyped for the E321G MYH1 variant and allele frequencies were calculated.

Results: The E321G MYH1 variant allele frequency was 0.034 ± 0.011 in the general QH population (6.8% of individuals in the breed) and the highest among the reining (0.135 ± 0.040; 24.3% of reiners), working cow (0.085 ± 0.031), and halter (0.080 ± 0.027) performance subgroups. The E321G MYH1 variant was present in cutting (0.044 ± 0.022) and Western Pleasure (0.021 ± 0.015) QHs at lower frequency and was not observed in barrel racing or racing QHs.

Conclusions and Clinical Importance: Knowing that reining and working cow QHs have the highest prevalence of the E321G MYH1 variant and that the variant is more prevalent than the alleles for hereditary equine regional dermal asthenia and hyperkalemic periodic paralysis in the general QH population will guide the use of genetic testing for diagnostic and breeding purposes.

KEYWORDS
equine, genetics, muscle, MYH1, myosin heavy chain 1

1 | INTRODUCTION

Immune-mediated myositis (IMM) in American Quarter Horses (QHs) causes recurrent episodes of acute epaxial and gluteal muscle atrophy.1 Susceptibility to IMM was recently attributed to an E321G missense mutation in the myosin heavy chain 1 (MYH1) gene encoding type IX myosin heavy chain that alters the amino acid composition of the globular head region, potentially impacting protein stability.2 In horses carrying this allele, exposure of the novel myosin isoform to the extracellular environment appears to initiate lymphocytic infiltration and destruction of predominantly type IX myofibers.2 More recently, this same genetic variant was associated with nonexertional rhabdomyolysis (non-ER) in QHs with or without atrophy and lymphocytic infiltrates.3

Abbreviations: AQHA, American Quarter Horse Association; ER, exertional rhabdomyolysis; GBED, glycogen-branching enzyme deficiency; HERDA, hereditary equine regional dermal asthenia; HYP, hyperkalemic periodic paralysis; IMM, immune-mediated myositis; MYH1, myosin heavy chain 1; MYHM, myosin heavy chain 1 myopathies; PCR, polymerase chain reaction; PSSM1, polysaccharide storage myopathy type 1; SE, standard error; QH, Quarter horse; VGL, Veterinary Genetics Laboratory; YOB, years of birth.
Many specialized performance equine subgroups have developed that utilize predominantly QHs selected for adeptness at sports ranging from mostly stationary halter horses to extremely fast barrel and racing animals. Selection for this range of athletic performance has led to marked population stratification and founder effects within this breed. Disproportionately high frequencies of certain disease alleles have therefore occurred in specific purpose-bred subpopulations. For example, there is an overwhelming prevalence of hyperkalemic periodic paralysis (HYPP) in horses bred for halter work and a high prevalence of lethal white foal syndrome in American Paint Horses, a QH-derived breed bred to have distinct spotted coats. Thus, it is possible that IMM, a disorder associated with a gene impacting the fastest contracting skeletal muscle fibers, could have a higher prevalence in certain performance QH subtypes.

A thorough understanding of any disease must include characterization of the at-risk population. For genetic disorders, this involves identifying environmental risk factors, constructing pedigrees, and determining disease allele frequencies. For IMM, a recent infectious respiratory disease, vaccination against certain respiratory pathogens and rhabdomyolysis appear to be environmental triggers. Pedigree analysis identified 4 QH stallions that were overrepresented in the lineage of affected horses. A genetic test for the E321G MYH1 variant is now available, allowing veterinarians to diagnose IMM and non-ER with ease and providing horse owners with a tool to make informed breeding decisions and avoid producing affected foals. To best utilize genetic testing, the prevalence of the E321G MYH1 variant in the QH population and specific performance subgroups must be elucidated. Based on clinical severity or high breed prevalence, genetic testing for some diseases (eg, hereditary equine regional dermal asthenia [HERDA], HYPP) is mandated by the American Quarter Horse Association (AQHA) for every breeding animal.

The objective of our study was to estimate the E321G MYH1 variant frequency in the general QH population and in 7 distinct subpopulations of QHs used for barrel racing, cutting, halter, racing, reining, Western Pleasure, and working cow horse competitions.

2 | MATERIALS AND METHODS

2.1 | Study design

This was a prospective, genetic study of AQHA-registered horses in which the authors were blinded to the identity of individual horses.

2.2 | Samples

With permission from the AQHA, the association’s animal registration records were examined to select the study population described herein. Each horse was assigned a random study ID number by personnel uninvolved in the genotyping for this project to maintain animal anonymity. Mane hair samples from each horse were provided by the Veterinary Genetics Laboratory (VGL), University of California, Davis, which houses the AQHA’s repository of hair samples from all registered animals.

2.2.1 | Selection criteria for whole-population controls

A random sampling scheme was used to select control QHs for estimating the whole-breed frequency of the E321G MYH1 variant. Between March 2013 and September 2017, a single week day per month was randomly selected, and on that date, every 10th QH hair sample submitted by the AQHA to the VGL for DNA testing was selected until 40 samples with sufficient hair follicles for subsampling were available for each year in the sampling period. In total, 200 controls were sampled, and anonymized hair samples were provided.

2.2.2 | Selection criteria for performance horses

The eligibility criteria for elite horses in the 7 performance subgroups of interest (barrel racing, cutting, halter, racing, reining, Western Pleasure, and working cow) were placement in the list of 75 AQHA-registered horses that had earned the most competition points (halter discipline) or sport money (all other disciplines) in the United States between January 1, 2014, and June 15, 2017. Records of sport money and competition point earnings were obtained from an online records company for all disciplines except racing, for which records were obtained directly from the AQHA.

2.3 | Genotyping

Genomic DNA was isolated from each horse’s hair sample using the Gentra Puregene Tissue Kit (QIAGEN, Germantown, Maryland).

Genotyping was performed by pyrosequencing. Forward, reverse, and sequencing primers were designed using Pyrosequencing Assay Design Software V1.0 (Biotage AB, Uppsala, Sweden). Reverse biotin-labeled and regular forward primers were ordered through Integrated DNA Technologies, Inc. (Skokie, Illinois). Forward (5′-TAAAAAAGCTG CATGTGTA-3′) and reverse (5′-AAACACATACCTGAAT-3′) primers were used to amplify a 159-bp region (ECA11: 52993805-52993946) containing the site (ECA11: 52993878) of the E321G MYH1 variant. The resulting amplicon was annealed with the sequencing primer (5′-TGCTGGGGACTGTGA-3′), and the relative expression of each allele was determined by pyrosequencing following manufacturer protocol using the PyroMark Gold Q96 Kit (QIAGEN). All sample plates were analyzed using a positive control and no template control, with the resulting data collated and genotypes inferred and tabulated for further analysis.

In addition, pyrosequencing results were validated in a subset of horses using a polymerase chain reaction (PCR) assay and Sanger sequencing protocol previously described for evaluating the E321G MYH1 variant in QHs. Control and performance horses from our study were pooled after genotyping by pyrosequencing, and 5 horses from each MYH1 genotype group (ie, homozygous wild type, heterozygous, and homozygous alternative) were selected by computer randomization to have their samples used for this validation step.

2.4 | Allele frequency and standard error calculations

Spreadsheet software was used to calculate a basic allele frequency for the general QH population and each of the 7 performance subgroups according to the formula...
where \( M_y \) represents the E321G MYH1 variant, \( N \) represents the wild-type MYH1 allele, \( H_x \) represents the number of horses with genotype \( X \), and \( n \) is the total number of horses in the study population for which allele frequency is being calculated. Because multinomial sampling schemes were used to select the study populations, standard error (SE) associated with each allele frequency calculation was also estimated according to the formula

\[
SE_{f_M} = \sqrt{\frac{f_M \times (1 - f_M)}{2n}}.
\]

Chi-square contingency tests were performed using the same software to determine if the frequency of the E321G MYH1 variant in each of the performance subgroups was statistically significantly different when compared to the control group.

3 | RESULTS

3.1 | Whole-breed allele frequency

The years of birth (YOB) of control QHs selected to represent the general population of this breed ranged from 1994 to 2017 (median YOB = 2013), and the distribution of sexes was slightly female-skewed (39% male versus 61% female) in this study group. The sampling scheme utilized was assumed to provide fair coverage of the entire geographic region from which AQHA-registered animals typically originate (the United States and Canada); however, these data were not evaluated. Of the 200 control equine hair samples obtained, a total of 146 high-quality DNA samples were successfully isolated and genotyped by pyrosequencing. The E321G MYH1 variant frequency (estimated value \( \pm SE \)) in the general QH population was 0.034 \( \pm 0.011 \) (Table 1), with 10 individuals (ie, 6.8% of the population) genotyping as heterozygous for the E321G MYH1 variant and no homozygotes identified (Figure 1).

3.2 | Allele frequency in performance subgroups

The elite performance QHs utilized in our study had YOB ranging from 1998 to 2015 (median YOB = 2011). The distribution of elite males and females within each discipline was approximately equal from 1998 to 2015 (median YOB = 2011). The distribution of elite performance QHs utilized in our study had YOB ranging from 1994 to 2017 (median YOB = 2013), and the distribution of sexes was slightly female-skewed (39% male versus 61% female) in this study group. The sampling scheme utilized was assumed to provide fair coverage of the entire geographic region from which AQHA-registered animals typically originate (the United States and Canada); however, these data were not evaluated. Of the 200 control equine hair samples obtained, a total of 146 high-quality DNA samples were successfully isolated and genotyped by pyrosequencing. The E321G MYH1 variant frequency (estimated value \( \pm SE \)) in the general QH population was 0.034 \( \pm 0.011 \) (Table 1), with 10 individuals (ie, 6.8% of the population) genotyping as heterozygous for the E321G MYH1 variant and no homozygotes identified (Figure 1).

![Figure 1](image)

**FIGURE 1** The distribution of MYH1 genotypes within the reining (n = 37), working cow (n = 41), halter (n = 50), cutting (n = 45), control (n = 146), Western Pleasure (n = 48), barrel racing (n = 42), and racing (n = 44) QH study groups. Abbreviations: My, E321G variant; N, wild-type allele; QH, Quarter Horse

53%-63% male versus 37%-47% female) except for the working cow and reining disciplines, in which males were overrepresented (76%-77% male versus 23%-24% female). Because top-performing horses in a given discipline often share common ancestors from a limited number of genetic lines with breeding stock residing on a limited number of farms, potential geographic bias in these samples was considered irrelevant.

Of the 350 performance equine hair samples obtained, a total of 307 high-quality DNA samples were successfully isolated and genotyped by pyrosequencing. The frequency of the E321G MYH1 variant was the highest in QHs from the reining (0.135 \( \pm 0.040 \), n = 37), working cow (0.085 \( \pm 0.031 \), n = 41), and halter (0.080 \( \pm 0.027 \), n = 50) performance subgroups. At \( \alpha = 0.05 \), the frequency of the E321G MYH1 variant in reining horses was statistically significantly different (\( P \) value = 6.5 \( \times 10^{-4} \)) from the frequency in the control group, and the difference in variant frequency between controls and working cow (\( P \) value = 5.0 \( \times 10^{-2} \)) and halter (\( P \) value = 6.0 \( \times 10^{-2} \)) horses approached significance. Cutting (0.044 \( \pm 0.023 \), n = 45) and Western Pleasure (0.021 \( \pm 0.015 \), n = 48) QHs also carried the E321G MYH1 variant; however, the difference in variant frequency in these groups as compared to controls was clearly statistically insignificant. The E321G MYH1 variant was not observed in either the barrel

**TABLE 1** The individual MYH1 genotype counts (N = wild-type allele, My = E321G variant), E321G MYH1 variant frequencies (estimated value \( \pm SE \)), and \( P \) values from chi-square testing in the control group and performance subgroups of QHs

| Study group       | Sample size | Genotype | Frequency | P-value  |
|-------------------|-------------|----------|-----------|----------|
|                   |             | N/N      | My/N      | My/My    |          |
| Control           | 146         | 136      | 10        | 0        | 0.034 \( \pm 0.011 \) |
| Reining           | 37          | 28       | 8         | 1        | 0.135 \( \pm 0.040 \) |
| Working cow       | 41          | 34       | 7         | 0        | 0.085 \( \pm 0.031 \) |
| Halter            | 50          | 42       | 8         | 0        | 0.080 \( \pm 0.027 \) |
| Cutting           | 45          | 41       | 4         | 0        | 0.044 \( \pm 0.023 \) |
| Western Pleasure  | 48          | 46       | 2         | 0        | 0.021 \( \pm 0.015 \) |
| Barrel racing     | 42          | 42       | 0         | 0        | NO |
| Racing            | 44          | 44       | 0         | 0        | NO |

Abbreviations: NO, not observed in this study group; QH, Quarter Horse; SE, standard error.
racing (n = 42) or racing (n = 44) QHs in this survey. Allele frequencies and individual genotype counts in the performance subgroups are summarized in Table 1. In the most affected performance subgroups, approximately 24.3% of reiners, 17.1% of working cow horses, and 16.0% of halter horses possessed at least 1 copy of the E321G MYH1 variant, which was present in the homozygous condition in only 1 individual from the reining discipline (Figure 1).

3.3 Validation of genotyping by pyrosequencing

A total of 453 horses were genotyped for the MYH1 variant by pyrosequencing in our study, which identified 413 homozygous wild-type individuals, 39 heterozygotes, and 1 homozygous alternate individual. Five individuals from each of the pools of homozygous wild-type and heterozygous individuals were randomly selected, in addition to the 1 individual homozygous for the E321G MYH1 variant, for genotyping by PCR and Sanger sequencing to validate the results obtained from pyrosequencing. There was perfect agreement between the results obtained by both methods of genotyping in this subset of 11 horses tested.

4 DISCUSSION

Immune-mediated myositis in American QHs is characterized by autoimmunity directed at type IIX myofibers, resulting in episodes of marked muscle atrophy.1,2,15 A missense E321G MYH1 mutation in the gene that encodes the MYH1 protein found in type IIX myofibers was found to be responsible for IMM as well as for a codominantly inherited form of severe acute non-ER in QH-related breeds.2,3 These 2 myopathies have been grouped under the heading of myosin heavy chain 1 myopathies (MYHM) based on this newly recognized etiology.3 The major finding in the present study was the relatively common occurrence of the E321G MYH1 mutation in QHs. The whole-breed frequency of the E321G MYH1 variant was 0.034 with a prevalence in the general population of 6.8% horses. The frequency of the E321G MYH1 variant in the general QH population (0.034) is slightly higher than the whole-breed estimates of disease allele frequencies previously reported for HYPP (0.008) and HERDA (0.021) but lower than those for type 1 polysaccharide storage myopathy (PSSM1) (0.055) and glycogen-branching enzyme deficiency (GBED) (0.054).4 In essence, a random breeding of registered QHs in the population would produce a foal at greater risk of having the E321G MYH1 variant and potentially developing MYHM than 2 other important genetic diseases of this breed (HYPP, HERDA) for which testing is already mandated by the AQHA. Thus, it will be important to educate veterinarians, owners, and breeders of QHs about the genetic basis of MYHM and the relatively common occurrence of the E321G MYH1 mutations in QHs.

The American QH is a prolific breed, with over 2.8 million AQHA-registered animals alive worldwide in 2017, and is the most popular type of horse used in over 14 recognized equestrian sports in the United States.16 Individual QHs must possess specific and often disparate characteristics to excel in their respective disciplines (eg, a compact and heavily muscled body type is ideal for horses used to work livestock, whereas longer-legged and leaner animals are preferred for racing and hunter classes). Thus, although American QHs retain a remarkable level of genetic diversity when considered as a single population, the differential selective pressures required to succeed in various disciplines has led to marked population stratification within the breed. This has been demonstrated in recent analyses of genetic relatedness among performance subgroups of QHs, including 6 groups (not including barrel racing) examined in the present study of E321G MYH1 variant frequency.17 Our investigation supports previous suggestions that it is incorrect to assume without a confirmatory genetic survey that the frequency of a disease allele in 1 performance subgroup of QHs is similar to that in a different subgroup or the whole breed.4 On average, the prevalence of the E321G MYH1 variant was 54% different in magnitude among the 5 performance subgroups in this survey.

Within the performance subtypes examined in our study, the E321G MYH1 variant frequency was the highest in reining (0.135), working cow (0.085), and halter (0.080) horses, making up approximately 8%-14% of the total copies of MYH1 in those subpopulations. The frequency of the E321G MYH1 variant was statistically significantly higher in the reining horses than in the general population of QHs, approaching a significant difference in the working cow and halter horses. Cutting (0.44) horses and Western Pleasure (0.021) horses did not differ significantly in the frequency of the E321G MYH1 variant compared to the general population. The E321G MYH1 variant was not observed in the barrel racing or racing horses in this survey. The absence of the E321G MYH1 variant in barrel racing and racing QHs in this survey complements previous studies that reported the lowest frequency of disease alleles associated with HYPP, GBED, HERDA, and PSSM1 in barrel racing QHs versus all other performance subgroups except racing QHs, which were free of these disease alleles.4 Horses in these performance subgroups likely have relatively fewer deleterious alleles because of the larger influence of Thoroughbred bloodlines and thus greater heterozygosity at important disease loci.4,17 The combination of performance groups with the highest frequencies of the MYH1 mutation is unique compared to the other genetic disorders of QHs: GBED, HERDA, HYPP, and PSSM1 have the highest prevalence in halter, Western Pleasure, and cutting horse populations.4

Our study establishes MYHM as an important genetic disease of the American QH with a risk allele frequency in the general population higher than several other genetic diseases in this breed (HYPP and HERDA). The high frequency of the E321G MYH1 variant in reining QHs compared to the general population (3.6-fold greater) warrants a focus on screening breeding stock in this subpopulation for the E321G MYH1 variant.

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CONFLICT OF INTEREST DECLARATION
A patent exists for this genetic test in the horse (UC Davis/Michigan State University) WO2017165733A1. A percentage of the funds from this genetic test contribute to the research programs of Drs C. J. Finno and S. J. Valberg.

OFF-LABEL ANTIMICROBIAL DECLARATION
Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION
Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION
Authors declare human ethics approval was not needed for this study.

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