

FREQUENCY ANALYSIS OF THE ARG309HIS MUTATION IN THE GYS1 GENE IN COLD-BLOODED AND HUCUL HORSE POPULATIONS

Monika Komorowska¹, Jakub Zipper², Adrianna Musiał³, Bogusława Długosz⁴,
Katarzyna Olczak⁵, Aleksandra Błaszczak⁴, Monika Stefaniuk-Szmukier⁶,
Katarzyna Ropka-Molik⁷

¹University of Warmia and Mazury in Olsztyn, Faculty of Biology and Biotechnology, M. Oczapowskiego 1A, 10-719 Olsztyn, Poland

²University of Agriculture in Krakow, Faculty of Animal Science and Biology, Al. Mickiewicza 21, 31-120 Krakow, Poland

^{3,6,7}National Research Institute of Animal Production, Department of Molecular Biology, 32-083 Balice near Krakow, Poland

⁴University of Agriculture in Krakow, Department of Animal Reproduction, Anatomy and Genomics, Al. Mickiewicza 21, 31-120 Krakow, Poland

⁵National Research Institute of Animal Production, Department of Horse Breeding, 32-083 Balice near Krakow, Poland

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Abstract

The incidence of genetically based diseases is a major breeding problem involving economic losses due to the costs of mating and maintaining pregnant mares, treatment, and mortality of affected offspring. Polysaccharide storage myopathy is a genetically determined disease inherited in an autosomal dominant manner and caused by a mutation in the GYS1 gene. The aim of the study was to estimate the frequency of the Arg309His mutation in the GYS1 gene in populations of cold-blooded and Hucul horses. The research material consisted of hair roots collected from 99 Hucul horses and 121 cold-blooded horses. Genotyping was performed using PCR-RFLP and Sanger sequencing. The results demonstrated the presence of the mutation in both cold-blooded and Hucul horses, in both heterozygous and homozygous forms for the unfavorable allele.

Keywords: horse, PCR-RFLP, PSSM1, polysaccharide storage myopathy, recurrent exertional rhabdomyolysis, polysaccharide storage myopathy

Introduction

Civilizational progress has been closely associated with animal domestication and their use for various purposes. The use of equids increased mobility and improved warfare, while products obtained from them also provided food for human populations at that time. Recent reports indicate that modern horses derive from a population originating in the lower Volga and Don regions, which spread across Eurasia around 2000 BC together with the expansion of the Sintashta culture (Librado et al., 2021). Horse domestication also initiated precise, directional selection aimed at creating specialized and phenotypically diverse horse

populations adapted to specific types of use. Breeding practices based on the use of a limited number of stallions substantially reduced genetic diversity in horses. From the 17th century onward, selection practices increased the proportion of mutations determining the occurrence of genetic diseases to approximately 4% across the genome (Orlando and Librado, 2019). The development of next-generation sequencing technologies enabled the identification of precise genomic locations associated with inherited diseases of breeding importance (Finno et al., 2009; Wade et al., 2009). These include polysaccharide storage myopathy (PSSM), first described in 1992 by Dr Stephanie Valberg (Valberg et al., 1992). Exertional rhabdomyolysis observed in this syndrome includes a range of symptoms related to damage to striated muscles, influenced by disturbances in glycogen metabolism and storage. The first description of PSSM in a subgroup of animals affected by exertional rhabdomyolysis concerned draft horses. Clinical signs developed after the horses returned to work following several days of rest (McCue et al., 2008b). Therefore, PSSM was also referred to as Monday disease or holiday disease, because symptoms appeared after a prolonged period of rest during which the animals were fed a grain-rich diet (Valberg et al., 2011).

Type I PSSM is inherited in an autosomal dominant manner. The disease is caused by the c.926G>A (Arg309His) mutation in exon 6 of the *GYS1* gene, resulting in substitution of arginine (CGT) with histidine (CAT). The *GYS1* gene encodes glycogen synthase, which enables glycogen synthesis from glucose molecules. This mutation contributes to the accumulation of amylase-resistant glycogen in striated muscles. Its structure is substantially less branched than that of a normal glycogen molecule, making it unavailable to the body (McCue et al., 2008b). PSSM most strongly affects the gluteal, abdominal and back muscles as well as the forelimb muscles (Valberg et al., 2011). Type II PSSM is also distinguished; it is not associated with a mutation in the *GYS1* gene, although its genetic basis has not yet been elucidated (Valberg et al., 2021; Valberg et al., 2023).

Symptoms of polysaccharide storage myopathy include muscle stiffness, spasms, excessive sweating, increased respiratory rate and difficulty moving. Increased serum creatine kinase activity and, following intense exercise, myoglobinuria have also been diagnosed (Parzyszek and Gruszczyńska, 2018). Asymptomatic cases of PSSM have also been reported. Most disease characteristics become apparent in animals at approximately 6 years of age. An episode usually occurs about 20 minutes after exercise (Valberg et al., 2011).

Diagnosis is based on molecular testing for mutations in the *GYS1* gene and on muscle biopsy followed by histological examination, which enables detection of amylase-resistant glycogen within skeletal muscle fibers. Molecular testing, however, is less invasive, does not require biopsy material, and unambiguously indicates the possible course of rhabdomyolysis associated with a particular allele combination (Naylor et al., 2012; McCue et al., 2008b). Although PSSM is inherited in an autosomal dominant manner, the occurrence of clinical signs is strongly influenced by external factors such as appropriate diet and exercise (Valberg, 2018).

To date, there are no literature data on the frequency of the *GYS1* gene mutation in Hucul horses. Data on the frequency of this mutation in cold-blooded horses will broaden knowledge of PSSM1. Therefore, the aim of the present study was to estimate the frequency of the mutant *GYS1* allele in populations of cold-blooded and Hucul horses.

Material and methods

Experimental material and DNA isolation

The research material consisted of horse hair roots stored in the biological material bank of the National Research Institute of Animal Production, Department of Animal Molecular Biology in Balice. Samples were obtained from Hucul horses originating from four studs and from Polish Coldblood horses from a state stud and private breeders in the Podkarpackie and Małopolskie regions. DNA was isolated from hair roots collected from 99 Hucul horses and 121

Polish Coldblood horses using the commercial Sherlock AX DNA extraction kit (A&A Biotechnology). DNA samples were stored at +4°C until analysis.

Genotyping of the *GYS1* gene mutation

The isolated DNA was used to genotype the rs1150416011 mutation using the PCR-RFLP method (polymerase chain reaction-restriction fragment length polymorphism). Primers for the analyzed fragment of the *GYS1* gene (F: 5' AGTGAAACATGGGACCTTCTCC, R: 5' CTAAACGGGGATCTCCTGCA) were designed using the publicly available Primer3Plus (3.3.0) software (Untergasser et al., 2012).

The PCR procedure was carried out according to the protocol using AmpliTaq Gold™ 360 Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). An annealing temperature of 57°C was used. The endonuclease was selected using the NebCutter program. Within the amplified fragment, HpyCH4V recognizes the TGCA sequence and cleaves it, producing two banding patterns: G – 202 and 80 bp, and A – 148, 80, 54 and 19 bp. Digestion was carried out for 8 h at 37°C. The resulting endonuclease-digested DNA fragments were then separated electrophoretically in a 5% agarose gel (Bioshop Canada Ltd.).

To confirm the presence of the c.926G>A mutation, amplicons from 72 randomly selected individuals were subjected to Sanger sequencing using BigDye™ Terminator v3.1 Cycle (Thermo Fisher Scientific) and a 3500XL capillary sequencer (Applied Biosystems, Foster, CA, USA; Thermo Fisher Scientific). The percentage frequencies of individual *GYS1* alleles in the analyzed horse population were then calculated using Excel (Microsoft Office).

Results

Sanger sequencing of 72 randomly selected individuals confirmed the presence of the c.926G>A mutation. A total of 220 Hucul and cold-blooded horses were genotyped. The results demonstrated the presence of the mutant A allele in both analyzed horse populations. The frequency of AA homozygotes in the two populations was 0.07, that of GG homozygotes was 0.56, and that of AG heterozygotes was 0.37. Heterozygotes accounted for 36.82% of both populations, AA homozygotes for 6.82%, and GG homozygotes for 56.36% (Table 1). The frequency of allele A was 0.25 and that of allele G was 0.75 (Table 2).

Among Hucul horses (n=99), 36 heterozygotes were identified, representing 36.36% of all horses of this breed; 55 individuals had the GG genotype, representing 56.56% of the study population; and 8 individuals had the AA genotype, corresponding to 8.08% of the study population (Table 1). The frequency of AA homozygotes was 0.08, that of recessive homozygotes was 0.57, and that of heterozygotes was 0.36. The frequency of allele A was 0.26, whereas that of allele G was 0.74 (Table 2).

The analyses also demonstrated the presence of the mutant A allele in the cold-blooded horse population (n=121). Forty-five heterozygotes were identified, representing 37.2% of all horses of this breed examined. Interestingly, 5.8% of the horses had the AA genotype, indicating the presence of both alleles associated with PSSM1. In the cold-blooded horse population, 69 individuals had the GG genotype, representing 57% of the study population (Table 1). Overall, the frequency of allele A was 0.24 and that of allele G was 0.76 (Table 2). The results confirmed that the obtained data were consistent with Hardy-Weinberg equilibrium for the Hucul horse population ($0.07+0.38+0.55=1$), the cold-blooded horse population ($0.06+0.36+0.58=1$), and the two populations combined ($0.06+0.38+0.56=1$) (Table 2).

Table 1. Genotype frequencies in the studied populations of Hucul and cold-blooded horses

Genotype	Hucul horses		Cold-blooded horses		Total Hucul and cold-blooded horses	
	n	%	n	%	n	%
dominant homozygotes	8	8,08	7	5,8	15	6,82
heterozygotes	36	36,36	45	37,2	81	36,82
recessive homozygotes	55	56,56	69	57	124	56,36

Table 2. Allele frequencies in the studied populations of Hucul and cold-blooded horses

Gene	Hucul horses	Cold-blooded horses	Total Hucul and cold-blooded horses
Allele A frequency	0,26	0,24	0,25
Allele G frequency	0,74	0,76	0,75
Hardy-Weinberg equilibrium	0,07+0,38+0,55=1	0,06+ 0,36+ 0,58=1	0,06+0,38+0,56=1

Discussion

Polysaccharide storage myopathy is inherited in an autosomal dominant manner, and its principal cause is the Arg309His mutation in the *GYS1* gene. The aim of this study was to determine the frequency of this mutation in populations of Polish cold-blooded and Hucul horses. Knowledge of mutation frequency in these populations provides valuable information for breeders monitoring horse health and performance.

The *GYS1* gene mutation responsible for PSSM1 has so far been described in 30 horse breeds and occurs at varying frequencies. In Belgian horses, which belong to the cold-blooded group, the frequency of allele A was 0.242 among 149 animals examined (McCue et al., 2010). A high frequency of the mutant A allele of the *GYS1* gene, amounting to 0.346, was also found in North American Percheron draft horses (McCue et al., 2010). The frequency of allele A in Belgian cold-blooded horses corresponds to the results obtained in the present study, in which the frequency of allele A in cold-blooded horses was 0.24. By contrast, the frequency of the mutant A allele in Percherons is higher than the 0.24 observed in the cold-blooded horses analyzed here.

The mutant A allele of the *GYS1* gene and homozygosity for this mutation are common in North American draft horses and German cold-blooded horses. This is consistent with the present results, which showed a mutant allele frequency of 0.24 in Polish cold-blooded horses. The *GYS1* mutation was not found in Thoroughbreds, Welsh ponies or Norwegian Fjord horses (McCue et al., 2010).

Another study found 11% of PSSM-affected Percheron and Belgian horses to be homozygous for the *GYS1* mutation (McCue et al., 2008a). Among 403 genotyped European draft-type horses, 62% were found to carry the *GYS1* gene mutation (Baird et al., 2010). In the present study, the Hucul horse population (n=99) included 36.36% heterozygotes and 8.08% dominant homozygotes, whereas the cold-blooded horse population (n=121) included 37.2% heterozygotes and 5.8% dominant homozygotes.

The Arg309His mutation in the *GYS1* gene is estimated to have arisen approximately 1200–1500 years ago. This suggests that it may formerly have been favored by natural selection and advantageous for animals feeding irregularly and living under harsh conditions (McCue et al., 2008b). Old draft breeds are considered to have a high proportion of *GYS1* mutations, possibly as a result of selection practiced by breeders after the Second World War. The mutation may have been advantageous in hard-working animals consuming diets low in sugars. Consequently, as a result of a population bottleneck, the *GYS1* mutation occurs at high frequency in these horses (McCue et al., 2008a).

The present study is the first to analyze the *GYS1* gene mutation in Hucul horses. The frequency of the mutant A allele in this breed was 26%, which was higher than the 24% estimated for Polish cold-blooded horses. Hucul horses originate from the Hutsul region and descend from several horse breeds, including Tatar and Turkish horses. They historically lived in harsh, cold conditions with limited food availability. As interest in the breed increased in the mid-19th century, targeted breeding began (Bordzoł and Jackowski, 2008). The animals then had constant access to feed and lived under stable conditions. This change may have favored the manifestation of PSSM symptoms that had previously helped their ancestors survive. A high-fat, low-starch diet in horses heterozygous for the *GYS1* mutation has been shown to improve exercise tolerance and reduce PSSM symptoms (Ribeiro et al., 2004). Mutation carriers accounted for 37.2% of Polish cold-blooded horses and 36.36% of Hucul horses. These similar values may indicate a comparable proportion of heterozygous individuals in the two breeds.

Other studies identified 22.8% heterozygotes and 1.2% dominant homozygotes among 167 genotyped Quarter Horses (McCue et al., 2008a). It is estimated that approximately 6–12% of Quarter Horses in the United States are affected by PSSM (McCue and Valberg, 2007). Studies were also conducted in Norman Cob horses descended from 10 founder stallions and not exceeding the seventh generation. Among 52 animals examined, 19 were affected by PSSM. The authors indicated that the *GYS1* mutation was probably present in the founder stallions and was transmitted to their offspring (Herszberg et al., 2009). Highly intensive, directional breeding of both Quarter Horses and Norman Cobs favored the occurrence of the disease. A similar phenomenon may have occurred in Hucul horses, as intensive breeding may have contributed to propagation of the undesirable PSSM1-causing mutation. In addition, McCoy et al. (2014), using extended haplotype homozygosity (EHH) analysis, demonstrated that the wild-type G allele is surrounded by more diverse haplotypes than the A allele, which has a markedly extended core haplotype. Haplotype cores with high EHH values and high population frequencies indicate mutations whose frequencies increase more rapidly under selection. This means that haplotype changes do not result solely from demographic processes but are also influenced by the manner in which selection is conducted in Belgian and Quarter Horse breeding populations (McCoy et al., 2014).

Studies in Quarter Horses have shown that the severity of PSSM symptoms is also influenced by the *RYR1* Arg2454 mutation in exon 46 of the ryanodine receptor gene. The presence of mutations in both *GYS1* and *RYR1* in the genome contributes to more severe clinical signs of PSSM (McCue et al., 2009). Other studies have shown that the frequency of the *GYS1* mutation is also affected by factors such as mating strategies and inbreeding, i.e. mating related individuals. In Austrian Noriker horses, the highest percentage of animals carrying two variants of the *GYS1* mutation occurred among chestnut horses, accounting for 54% of mares and 62% of stallions (Druml et al., 2017). These studies indicate that many factors influence the occurrence of the *GYS1* mutation and PSSM clinical signs. Accordingly, numerous factors potentially contributing to the high frequency of the mutant *GYS1* gene in the analyzed cold-blooded and Hucul horse populations should be considered.

Interestingly, studies have shown significant differences in the severity of PSSM symptoms between heterozygous and homozygous horses with respect to the *GYS1* mutation. Dominant homozygotes were found to have a higher degree of subsarcolemmal vacuolization and a higher content of amylase-resistant polysaccharides in skeletal muscles than heterozygous individuals. This emphasizes the importance of genetic testing in affected horses to select appropriate treatment (Naylor et al., 2012).

The results of the present study indicate a high proportion of individuals carrying the mutant *GYS1* allele in both Polish cold-blooded and Hucul horses. The cited studies indicate a considerable influence of intensive breeding and inbreeding on the occurrence of the *GYS1* mutation, as well as effects of nutrition and environmental factors on the severity of PSSM

symptoms in horses. Genetic testing is an important tool for selecting appropriate therapy and for monitoring changes occurring within breeds.

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SUMMARY

The incidence of genetically based diseases is a major breeding problem involving economic losses due to the costs of mating and keeping in-foal mares, the treatment and the mortality of the offspring affected. Polysaccharide storage myopathy is a hereditary (autosomal dominant) disease caused by a mutation in the GYS1 gene. The aim of this study was to estimate the prevalence of the Arg309His mutation in the GYS1 gene for cold-blooded and Hucul horse populations. The study material consisted of hair roots collected from 99 Hucul horses and 121 cold-blooded horses. Genotyping was performed by PCR-RFLP and Sanger sequencing. The results showed the presence of mutations in both cold-blooded and Hucul horses, both heterozygous and homozygous for the unfavourable allele.

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