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Original Research

Polymorphisms in *MCT1*, *CD147*, *PDK4*, and *DMRT3* genes in Arabian and Quarter Horses

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ABSTRACT

Monocarboxylate transporter isoform 1 (MCT1) and its ancillary protein, CD147 function to transport H^+ and lactate ions, retarding systemic acidosis and fatigue. The *PDK4* gene is involved in the adenosine triphosphate production, and the *DMRT3* gene is involved in the locomotor system. This study investigated polymorphisms of *MCT1*, *CD147*, *PDK4*, and *DMRT3* in Arabian and Quarter Horses to associate polymorphisms with performance. Arabian Horses were divided into high performance and untrained horses groups based on success in endurance competition, whereas Quarter Horses were separated by a speed index. Polymorphisms were analyzed by sequencing (*MCT1* and *CD147*), ARMS-PCR (*PDK4*), and PCR-RFLP (*DMRT3*). To compare the frequencies of SNPs, the Fisher's exact test was performed in the software R at 5% of significance. The *A* alleles from the polymorphisms Lys457Gln:1573A>C of *MCT1* and Ile51Val:168A>G of *CD147* were essentially fixed in both breeds. Only two Arabian from the high performance and one from the untrained horses group appeared *AC* in *MCT1*. For the *DMRT3* polymorphism (g.22999655C>A:ECA23), all the animals were *CC*, except one Arabian in the high and two Quarter Horses in the low performance group that were *AC*. For the *PDK4* polymorphism (g.38973231A>G:ECA4), Arabian showed a significantly greater frequency of the *G* allele than Quarter Horses ($P < .01$). Considering the kind of exercise practiced by each breed, it is likely that the *PDK4* polymorphism influences the pathway to energy production.

1. Introduction

Certain horses display an innate ability to develop athletic skills and can also perform in varying classes of intensity during the physical exercises of competition. A great athletic performance largely depends on the ability of the animal to produce energy. Adenosine triphosphate (ATP) is the direct source of energy for muscle contraction and high-intensity physical exercise requires substantial amounts of ATP. Rapid production of energy occurs via anaerobic respiration, releasing lactate and H^+ ions into the muscle. The accumulation of these ions leads to acidosis and fatigue [1,2], culminating in a decline in muscle performance [3].

Monocarboxylate transporter isoform 1 (MCT1) present in red blood cell (RBC) membranes is key in the transport of lactate and H^+ ions from blood plasma to red blood cells.

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The gradient generated between muscle and plasma aids the maintenance of the acid/base balance and delays muscle fatigue. The ancillary glycoprotein Cluster of Differentiation 147 (CD147) is required for the appropriate localization and function of MCT1 in the plasma membrane [4]. This ancillary protein is integrated into the plasma membrane and belongs to the immunoglobulin superfamily.

The study of polymorphisms in *MCT1* and *CD147* and their effects in the protein formation and transport of lactate and H^+ ions can provide insight for genetic technologies used for breeding selection of athletic equines. Reeben et al. [5] found polymorphisms in *MCT1* (Lys457 Gln—AY457175.1:c1573A>C) and *CD147* gene (Met125Val–EF564280.1:c389A>G) in Standardbred horses, but they did not observe correlation with the transport of lactate in red blood cells. Mykkänen et al. [6] studied the same polymorphisms in Standardbred and Finnhorses with myopathy, but they did not find an association with disease. Koho et al. [7] analyzed polymorphisms in Standardbred, Finnhorse, Warmblood, and Icelandic horses where they found two single-nucleotide polymorphisms (SNPs) in *MCT1*: Val432Ile:1498G>A and Lys457Gln:1573A>C; however, characterization of these SNPs could not account for variation in gene expression. In the *CD147* gene, they identified the SNPs Met125Val:389A>G and Ile51Val: 168A>G, in addition to two SNPs (888G>C and 990C>T) in the 3′ untranslated region of the gene, which is known to regulate mRNA stability. The authors concluded that the SNPs at nucleotide 389 and 990 have an effect on the expression of the CD147-MCT1 transport complex. This was also the first time that the Ile51Val polymorphism was reported, and the authors suggested that this SNP affected the formation of the MCT1-CD147 complex, and therefore, the activity of lactate transport across the RBC membranes.

Although it is likely that athletic performance in horses is influenced by a multitude of genes, until recently, only a few genetic variants were related to performance traits. Rapid energy production and the development of high speed are fundamental to great performance. *PDK4* and *DMRT3* are candidate genes for performance in horses and have been studied as markers for athletic potential. The *pyruvate dehydrogenase kinase, isozyme 4* (*PDK4*) [8,9] located on equine chromosome four controls the oxidation of fatty acids, a highly effective mechanism for generating ATP in skeletal muscle [10]. Eivers et al. [11] identified a significant increase in mRNA expression of *PDK4* in the skeletal muscles of horses during recovery from exercise. Hill et al. [9] investigated the possibility of associations between SNPs identified in *PDK4* and running performance in Thoroughbreds. They found the SNP g.38973231A>G strongly associated with the elite racing trait, where horses with the *AA* and *AG* genotypes showed better performance compared to the *GG* genotype.

The *doublesex-related and mab-3-related transcription factor 3* (*DMRT3*) located on equine chromosome 23 encodes an important transcription factor in the setting of spinal cord circuits controlling movement in vertebrates. A nonsense mutation (DMRT3_Ser301STOP) at SNP g.22999655C>A of the *DMRT3* gene has been shown to be significantly associated with gait performance in horses, especially in the performance of harness racing horses as well as the pace and tölt of Icelandic horses [12]. Promerová et al. [13] tested horses from 141 horse breeds and found the *A* allele of the g.22999655C>A SNP highly prevalent (frequency>0.90) among breeds used for harness racing or selected for performance of four-beat gaits at high speeds. Thus, the *A* allele of the mutation of *DMRT3* gene might be disadvantageous to racing breeds because gaited horses are less efficient in reaching high speed [12].

The effects of DNA polymorphisms in phenotype are intrinsic parameters of each breed in a certain environment. Each gene described in the physiology of skeletal muscle, and the locomotor system is a candidate marker for athletic performance. The objective of this study was to analyze polymorphisms in the *MCT1* (AY457175.1: c1573A>C), *CD147* (EF564280.1:c168A>G), *PDK4* (g.389732 31A>G:ECA4), and *DMRT3* (g.22999655C>A:ECA23) genes in Arabian and Quarter Horses and to associate the polymorphisms in each breed with performance. Our subject was to check whether the polymorphism of each gene could influence the levels of performances in each breed.

2. Material and Methods

2.1. Animals

Arabian horses, of both genders, were selected according to their performance and separated in two groups: 33 untrained horses (UT), which were pasture grazed and not in training at the time, but could have had some training during their lifetime, and 20 animals of high performance (HP), which were athletic horses that participated in endurance competitions of 160 km and had won at least once in an official competition of the Fédération Equestre Internationale (FEI***).

Two hundred ninety six animals, of both genders, composed the Quarter Horses base population. Quarter Horses, born between 1982 and 2011, registered with the Brazilian Quarter Horse Association (ABQM), were also divided into two groups, differentiated by the ABQM Speed Index (SI) in horse races. This measure is a merit registration on race and was created to allow animal performance comparisons under different conditions such as race track, track type, country, climate, and distance. Based on the distribution curve from the SIs of the base population animals (n = 296), phenotypes were adjusted for the systematic effects of environment (fixed effects), gender, interaction between race track and distance (228, 275, 301, 320, 365, 402 and 502 meters), between the year of the race (1988–2013) and the age of the animal at race (2, 3, and 4 years). Later, 20 horses of the highest performance index (HI) and 20 horses of the lowest performance index (LI) were selected. The age, sex, and number of animals considered in each performance group are described in Table 1.

All horses were in good health and the procedures involving the animals were performed in accordance with the Brazilian standards of animal welfare issued by the Ethics Committee on Animal Use (n° 019314/13).

Table 1
Number of animals and sex of Arabian and Quarter Horses divided into performance groups.

Group	N	Female	Male
AR UT	33	14	19
AR HP	20	6	14
QH LI	20	15	5
QH HI	20	16	4

Abbreviations: AR, Arabian; QH, Quarter Horses; UT, untrained horses group; HP, high performance group; LI, low Speedy Index group; HI, high Speedy Index group; N, number of animals.

2.2. Analysis of the Polymorphisms

2.2.1. Blood Samples and DNA Extraction

Blood samples of each animal were collected in duplicate by venipuncture of the left jugular in the neck region, using vacuum tubes containing 7.5 mg of EDTA. The DNA extraction procedure was accomplished by Illustra Blood GenomicPrep Mini Spin Kit (GE Healthcare), according to the manufacturer's instructions.

2.2.2. Genotyping

The DNA regions to be amplified for analysis of single-nucleotide polymorphisms in *MCT1*, *CD147*, *PDK4*, and *DMRT3* genes were generated based on the sequences reported in the literature [5–7,9,12]. The primers used for genotyping (Supplemental Table 1) of *MCT1* and *CD147* (Sequencing), *PDK4* (ARMS-PCR), and *DMRT3* (PCR-RFLP) were designed through available programs: PRIMER1 [14], Primer3Plus [15], and OligoAnalyzer 3.1 (PrimerQuest program, IDT).

PCR products of 342 bp (*MCT1*) and 278 bp (*CD147*) were sequenced to genotype randomly 20 Arabians (10 of each performance group: high performance/untrained horses [HP/UT]) and 24 Quarter Horses (12 of each performance group: high/low Speed Index groups [HI/LI]) for *MCT1* (AY457175.1:c1573A>C–Lys457Gln) polymorphism and 18 Arabians (HP = 7; UT = 11) and 23 Quarter Horses (HI = 12; LI = 11) for *CD147* (EF564280.1:c168A>G Ile51Val) polymorphism. The PCR was performed in a final volume of 18 μL, and the reaction for amplification consisted of 100 ng of genomic DNA, 0.15 mM of each primer, and 10 μL of AmpliTaq Gold Fast PCR Master Mix (Applied Biosystems). The temperatures of annealing of primers were 56°C for MCT1 and 58°C for CD147. Sanger sequencing was accomplished by Eurofins Genomics (Louisville). Results were analyzed with Chromas Lite software (Technelysium Pty Ltd, Australia).

Genotyping of the *PDK4* gene SNP (g.38973231A>G:ECA4) was performed by ARMS-PCR technique [14], with amplification of 235 bp for *A* allele and 177 bp for *G* allele. The ARMS-PCR reaction for *PDK4* gene, in a final volume of 15 μL, consisted of 50 ng of genomic DNA; 0.2 μM of each outer primer; 0.7 μM of each inner primer; 10 mM of Tris-HCl, pH 8.0; 50 mM of KCl; 1.7 mM of $MgCl_2$; 0.26 mM of each dNTP; and 0.75 U of Taq DNA polymerase (Fermentas). The temperature of annealing of primers was 63°C.

The *C* and *A* alleles of the *DMRT3* gene (g.22999655C>A:ECA23) were determined by PCR-RFLP with amplified fragments of 470 bp and digested by the enzyme *Dde*I (New England BioLabs). The PCR reaction for the SNP g.22999655C>A of the *DMRT3* gene in a final volume of 25 μL was composed of 50 ng of genomic DNA, 0.25 mM of each primer, 0.4 mM of each dNTP, 2.5 mM of $MgCl_2$, 1X PCR buffer (20 mM of Tris-HCl, pH 8.4; 50 mM of KCl), and 0.75 U of Taq polymerase (Fermentas). The temperature of annealing of primers was 59°C. For restriction fragment length polymorphism (RFLP) analysis, 10 μL of the PCR product was mixed with 10X enzyme buffer and 4 units of *Dde*I (New England Biolabs Inc.).

2.2.3. Allele and Genotype Frequencies of the Target SNPs

Allele and genotype frequencies for each of the polymorphisms genotyped were calculated according to Weir [16]. For comparing the frequencies of the groups, the Fisher exact test was performed in the software R [17], with significance level of 5%.

3. Results and Discussion

Among the Arabian Horses sequenced for *MCT1* (AY457175.1:c1573A>C—Lys457Gln), 17 were homozygous *AA*, whereas two Arabians of the high performance group and one of the untrained horses group were shown to be heterozygous *AC* for the polymorphism. All the 24 Quarter Horses were homozygous for the *A* allele.

A new polymorphism (AY457175.1:c1498G>A), that has not been reported previously in any other study, was found in the *MCT1* gene sequence. Among the Arabian Horses, 18 were homozygous *G* for *MCT1* (AY457175.1:c1498G>A), whereas two were heterozygotes, *AG*, one in the HP group and one in the UT group. Among the Quarter Horses, 20 were homozygous *G* and four (two of each performance group) were heterozygotes, *AG*.

As in our study, only three horses proved to be heterozygous *AC* for the *MCT1* polymorphism, it was not possible to determine the influence of the alleles on the athletic performance of the studied horse breeds. This result is concurrent with what has been described in the literature for this polymorphism. Reeben et al. [5] studied the Lys457Gln SNP in *MCT1* gene in healthy Standardbred horses and those with myopathy where they found this polymorphism in a single horse within the control group. Therefore, they could not conclude any association of the polymorphism with signs of myopathy induced by intense exercise, as we could not associate group performance with the polymorphisms. Mykkänen et al. [6] also observed the same polymorphisms in *MCT1* gene in one Finnhorse with signs of myopathy. Taking into consideration, the results of our study and the studies of Mykkänen et al. [6] and Reeben et al. [5], the polymorphism AY457175.1:c1573A>C for the *MCT1* gene is not breed specific. Koho et al. [7] found three Finnhorses that were homozygous for this polymorphism but did not observe an association with the expression of the MCT1 protein. Thus, the results described so far for the Lys457Gln polymorphism in *MCT1* gene do not indicate an association with breed or athletic performance. It is likely that with a higher number of animals in future studies, some association can be found between the polymorphism and performance traits in athletic horses.

All the sequenced horses for the polymorphism Ile51Val of *CD147* gene were homozygous *AA*, and it was not possible to determine the influence of this polymorphism on the athletic performance of Arabian and Quarter Horses. The Ile51Val polymorphism of *CD147* gene had been reported previously only by Koho et al. [7] in a single Warmblood horse. Taking into consideration that the polymorphism is in a genomic region that might be involved in homo-oligomerization of the CD147 protein [18] and that low amounts of MCT1 and CD147 were found in the single horse that showed the polymorphism, it is believed that the SNP may affect the correct formation of the CD147 protein. However, the polymorphism we studied did not show such an association.

For the *DMRT3* polymorphism, the 441 bp fragment (allele *C*) and a fragment of 221 bp (allele *A*) were obtained (Supplemental Table 2). Almost all the horses were *CC*, with the exceptions of one Arabian of the high performance group and two Quarter Horses of the low performance index group that were *AC*. The genotype *AA* was not detected for any animals of all breeds.

Consistent with previous studies, the most common *DMRT3* allele found among Arabian and Quarter Horses was that not associated with gaited horses, specifically the *C* allele [12,13]. Because English horses, such as Thoroughbreds, were in the formation of the Quarter Horse breed, we did not expect to find the gait allele (*A* allele). However, Iberian wild horses were also used in the formation of the breed and could have introduced the *A* allele of *DMRT3* in Quarter Horses. According to Andersson et al. [12], the ability to pace in Icelandic horses is altered only when the homozygosis *A* allele appears. The *A* allele in the low performance group for two Quarter Horses here may be detrimental for achieving higher velocities. This fact could be contradicted as the *A* allele appeared in the high performance group for the Arabian horse; however, the *DMRT3* mutation shows that different genotypes are optimal for different disciplines in different breeds. For Standardbreds, the *A* allele was shown to be practically fixed [13], and the effect of the mutation was very strong affecting all racing performance traits [19]. Andersson et al. [12] saw AA homozygous Standardbreds showing pace and trot. So, they deduced that the *DMRT3* mutation may give the animal the ability to pace or trot at certain speeds and genetic alterations determine the gait that is optimal for the horse. The *DRMT3* genotype was associated with the batida/picada gait type in Mangalarga Marchador horses with a prominance of the *CC* polymorphism in the batida gait and excess of AA in the picada gait type [20]. Because both batida and picada gait horses can perform lateral gaits, the *DMRT3* mutation may not be exclusively responsible for controlling the lateral gait pattern in this breed. Thus, as most of the animals were *CC* for the *DMRT3* polymorphism, we can confirm that Arabian and Quarter Horses are not gaited breeds, and we could not conclude anything about the influence of the A allele in the performance of these breeds.

For the *DPK4* polymorphism, the 235 bp and 356 bp fragments (allele *A*) were detected, in addition to fragment of 177 bp (allele *G*, Supplemental Table 2). Arabian and Quarter Horses showed variation for the *PDK4* SNP, and the frequency differences were significant (Table 2). The frequency for the *A* allele was higher for Quarter Horses than for Arabian horses. However, there was no significant difference in the individual performance groups of both breeds for the *PDK4* polymorphism (Tables 3 and 4).

Table 2
Comparison of genotype and allelic frequencies observed (and expected) of the g.38973231A>G polymorphism of the PDK4 gene between Arabian and Quarter Horses.

PDK4	Arabians (n = 53)		Quarter Horses (n = 296)		Fisher Test
	n	Frequencies	n	Frequencies	*P* value
Genotypes					.001[a]
GG	38 (35.7)	0.71 (0.672)	141 (149.2)	0.48 (0.504)	
GA	11 (15.6)	0.21 (0.295)	137 (121.9)	0.46 (0.412)	
AA	4 (1.7)	0.08 (0.033)	18 (24.9)	0.06 (0.084)	
Alleles					.018[b]
G	87	0.82	419	0.71	
A	19	0.18	173	0.29	

[a] Significantly different at $P < .01$.
[b] Significantly different at $P < .05$.

Quarter Horses presented more of the *A* allele and more *AG* genotype than Arabians for the *PDK4* polymorphism. However, in our study, we have two populations, the difference of allele frequencies may be more explainable by aspects other (genetic drift and/or founder effects) than selection for performance. According to Hill et al. [9], Thoroughbreds with an *A* allele in their genotype had an advantage over *GG* horses, in terms of elite race performance. The regulation of glucose/carbohydrate metabolism is tightly controlled by pyruvate dehydrogenase kinase (PDK) via phosphorylation of pyruvate dehydrogenase complex (PDC). The PDK isoforms alternatively regulate carbohydrate metabolism depending on the duration and intensity of exercise. As Thoroughbreds perform short and intense exercise during competitions, oxidation of glucose is used to produce energy because it is the fastest pathway. The *G* allele of the *PDK4* SNP is the responsible for blocking the formation of the PDC, resulting in the beta-oxidation of fatty acids to acetyl-CoA that is then used as the substrate for oxidative phosphorylation. Oxidation of fatty acids occurs in the mitochondria and is slower than glucose oxidation. Thus, the oxidation of fatty acids is important for equines that perform long and submaximal intensity exercises, like Arabian horses, owing to endurance demands

Table 3
Comparison of genotype and allelic frequencies of the g.38973231A>G polymorphism of the PDK4 gene between Arabians of untrained group and high performance group.

PDK4	Arabian—High Performance (n = 20)		Arabian—Untrained Horses (n = 33)		Fisher Test
	n	Frequencies	n	Frequencies	*P* Value
Genotypes					.335[ns]
GG	13	0.65	25	0.76	
GA	4	0.20	7	0.21	
AA	3	0.15	1	0.03	
Alleles					.191[ns]
G	30	0.75	57	0.86	
A	10	0.25	9	0.14	

Abbreviation: ns, not significant.

Table 4
Comparison of genotype and allelic frequencies of the g.38973231A>G polymorphism of the PDK4 gene between Quarter Horses of low and high performance.

PDK4	Quarter Horses—High Performance (n = 20)		Quarter Horses—Low Performance (n = 20)		Fisher Test
	n	Frequencies	n	Frequencies	*P* Value
Genotypes					.433[ns]
GG	11	0.55	10	0.50	
GA	6	0.30	9	0.45	
AA	3	0.15	1	0.15	
Alleles					1.000[ns]
G	28	0.70	29	0.725	
A	12	0.30	11	0.275	

Abbreviation: ns, not significant.

to produce energy for an extended period of time. It is not an advantage for the *PDK4* to block the PDC in racing breeds of short distances such as Thoroughbreds and racing Quarter Horses because these animals perform short and intense exercises and need fast production of energy.

4. Conclusion

The *A* alleles, corresponding to the respective polymorphisms AY457175.1:c1573A>C-Lys547Gln of *MCT1* and EF564280.1:c168A>G-Ile51Val of *CD147* genes, have shown to be fixed or nearly fixed in Arabian and Quarter Horses. It was not possible to determine the influence of the polymorphisms in *MCT1* and *CD147* genes in the athletic performance of Arabian and Quarter Horses.

The *C* allele of *DMRT3* gene was also shown to be virtually fixed for Arabian and Quarter Horses. As they are not gaited breeds, they do not show the *A* allele in high frequency and we could not determine its influence in the performance of either breed.

There was a significant difference in the frequencies of the g.38973231A>G *PDK4* SNP between Arabians and Quarter Horses. It is likely that the *PDK4* polymorphism directly influences the control of the pathway for energy production because these two horse breeds are selected for different athletic traits.

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Table S1
Description of primers used for genotyping of polymorphisms in *MCT1*, *CD147*, *PDK4* and *DMRT3* genes in Arabian and Quarter Horses.

Gene	Primer 5′—3′
MCT1—forward	GGGAAAGGAGATAGTTGTTTGCT
MCT1—reverse	GTCCACTCTGAACCCATGGG
CD147—forward	TCACCCCAGAGAGCAGGAT
CD147—reverse	TATCTGAGAGCCCGCTGACT
PDK4—forward inner	GCAGCAGTAAAGACTATGGATTGACTG
PDK4—reverse inner	CCATTAAACAATGACAATCTGAAACAAAT
PDK4—forward outer	GATGCAACTTTAACCCTCAACTTTCTAA
PDK4—reverse outer	CAGATTTTCAGAGAATAGAGCCAGGATA
DMRT3—forward	GGGAACAGAATCACCTCCTG
DMRT3—reverse	CGACTGGTTTCTTGCCAAAG

Table S2
Description of the produced standard bands for the different genotypes of the polymorphisms of *PDK4* and *DMRT3* genes.

Gene (SNP)	Genotype	Produced Standard Bands
PDK4	AA	235 and 356 bp
(g.38973231A>G:ECA4)	AG	177, 235 and 356 bp
	GG	177 and 356 bp
DMRT3	CC	441 and 29 bp
(g.22999655C>A:ECA23)	AC	441, 221, 221 and 29 bp
	AA	221, 221 and 29 bp

Abbreviation: SNP, single-nucleotide polymorphism.