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Some inherited disorders in pacing horses in Turkey

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Abstract: This study was carried out to detect the presence of mutant alleles of polysaccharide storage myopathy (PSSM) and severe combined immunodeficiency (SCID) disorders in pacing horses raised in different regions of Turkey. Blood/hair samples from 264 (182 Indigenous, 31 Iranian, 24 Afghan, and 27 Bulgarian) pacing horses aged 4 and over were used. As a result of the study, a mutation-heterozygosis (GA) in the GYS1 gene related to PSSM disease was detected in one of the pacing horses (Bulgarian horse). No deletions were observed in the DNA-PKcs gene region in the pacing horses for SCID disease. This study has been carried out to identify the status of two inherited disorders in pacing horses in Turkey. It was determined that there are no mutant genes in indigenous pacing horses, in terms of PSSM and SCID disorders, which are the major hereditary disorders in horses.
Keywords: Horse, pacing, PSSM, SCID, Turkey.

Türkiye'deki Rahvan atlarında bazı kalıtsal bozukluklar

Özet: Bu çalışma, Türkiye'de farklı yörelerde halk elinde yetiştirilen ve Rahvan koşularına katılan atlarda polisakkarid depolama miyopatisi (PSSM) ve şiddetli bileşik immun yetersizlik (SCID) hastalıkları bakımından mutant allel varlığının araştırılması amacıyla yapılmıştır. Bunun için 4 yaş ve üzeri 264 (182 Yerli, 31 İran, 24 Afgan ve 27 Bulgar) rahvan atın kan/kıl örnekleri kullanılmıştır. Çalışma sonucunda, incelenen Rahvan atlarından bir tanesinde (Bulgar rahvan atında) PSSM hastalığı ile ilgili GYS1 geninde mutasyon-heterozigotluk (GA) tespit edilmiştir. SCID hastalığına ilişkin, çalışmada kullanılan rahvan atlarında DNA-PKcs gen bölgesinde delesyon gözlenmemiştir. Sonuç olarak bu çalışma, Türkiye'deki rahvan atlarında bu iki kalıtsal hastalığın durum tespitine yönelik olarak gerçekleştirilmiştir ve bu çalışmada, at yetiştiriciliğinde önemli kalıtsal bozukluklardan ikisi olan PSSM ve SCID hastalıkları açısından yerli rahvan atlarında zararlı genlerin bulunmadığı tespit edilmiştir.
Anahtar sözcükler: At, PSSM, rahvan, SCID, Türkiye.

Introduction

Horse breeding was mainly carried out to use horses as an energy source before mechanization. Nowadays, it is limited to breeding horses for riding and working. However, it has become more widespread in horse racing, show jumping, sports riding, pacing, and local sports such as javelin throwing. Horses that are successful in such competitions are generally used in breeding. Under these circumstances, it is possible that hereditary diseases are passed to the generations and the frequency of the relevant mutant gene also increases. Techniques developed in recent years can explain the inheritance of hereditary diseases, and the carriers can be identified and the eradication of the disease is facilitated. While some hereditary diseases are detected only in certain breeds, others can be found in many breeds. However, when a crossing is done, it is always possible that mutant alleles are passed to other breeds.

Genes that cause hereditary disorders and thus patient and carrier animals can be detected by molecular techniques and these diseases can be removed more easily from the herd (10, 21).

Hereditary diseases have been reported in horses such as Hyperkalemic Periodic Paralysis (HYPP), Polysaccharide Storage Myopathy (PSSM), Malignant Hyperthermia (MH), Glycogen Branching Enzyme Deficiency (GBED), Severe Combined Immunodeficiency (SCID), Junctional Epidermolysis Bullosa (JEB), Hereditary Equine Regional Dermal Asthenia (HERDA), Gray Horse Melanoma, Lavender Foal Syndrome (LFS) (6, 9).

PSSM is a muscle disease and autosomal dominant glycogen storage disorder that occurs when sugar is stored in the muscles in the form of polysaccharides, which is an abnormal form of sugar instead of glycogen. As a result of a mutation in the Glycogen synthase 1 gene (GYS1), the

clinical symptoms of PSSM appear and are seen in warm-blooded horses, especially in Quarter horses and other breeds (11, 15). The main clinical sign of the disease is cramps and involvement in the muscles. Muscle pain, stiffness, fatigue even at the lightest exercise, reluctance to move, muscle atrophy, high serum CK and AST levels are also observed. Muscle glycogen concentration is four times higher than the normal value (7, 31).

PSSM disease was first identified in Quarter horses in the USA (27), then it was detected in Ponies, Morgan, Arabian, Thoroughbred, Standardbred and Warm-Blooded horse breeds as well as many other horse breeds (17, 29).

In 8% of 94 horses slaughtered in a slaughterhouse in Britain, 22% of 46 horses presented to the clinic (19) and by histological examination of skeletal muscle biopsies of 1426 horses from different breeds 572 horses (40.1%) were diagnosed with PSSM (15). Horses (588 [34%] of 1714) from different breeds were found positive for GYS1 mutation and most of them were heterozygote (28). Haflinger horses (9 [18%] of 50) in Austria were heterozygote (25); 250 (62%) of 403 draft horses of 13 breeds in Belgium, France, Germany, Netherlands, Spain and Sweden carried the mutant allele (3). The prevalence of genetic susceptibility to Type 1 PSSM in Shire, Morgan, Appaloosa, Quarter, Paint, Exmoor Pony, Saxon-Thuringian Coldblood, South German Coldblood, Belgian, Rhenish German Coldblood and Percheron horses varied between 0.5 and 62.4%. Thoroughbred, Akhal-Teke, Connemara, Clydsdale, Norwegian Fjord, Welsh Pony, Icelandic, Schleswig Coldblood and Hanoverian horses were free for the mutation (18). The prevalence of GYS1 mutation has been reported to be high in Draft (87%) and Quarter (72%) horses and low in Warmblood (18%) and other light horse breeds (24%) (16).

SCID is an autosomal recessive disorder and was first reported in Arabian horses in 1973 (20). This disease is caused by the DNA-protein kinase (DNA-PKcs) catalytic subunit gene on the 9th chromosome which is a 5 base pair deletion (frameshift mutation) (2, 23). The DNA-PKcs enzyme is necessary for the gene that regulates antigen receptor on B and T lymphocytes (22).

Affected foals are normal at birth, but secondary infections develop immediately after. The number of B and T lymphocytes is insufficient, they can't mature and there is no antibody synthesis, thymus and peripheral lymphoid tissues are hypoplastic. The pathogenesis of this disease in horses is completely related to the deficiency of B and T cells. In this disease, sufficient antigen-specific immune response for protection from infectious diseases has not occurred. Therefore, foals that fail to produce enough antibodies after vaccination or infection are very susceptible to diseases and usually die within the first few months after birth (9, 12, 14, 32).

The frequency of SCID carriers in Arabian horses in the United States is 8.4% (4); this rate is 2.3% for the foals of Arabian breeding stock (24) while no carriers have been found among Iranian Arabian horses (26); 16 of 88 Arabian horses (18%) in Morocco have been reported as carriers (23); in the state farm in Turkey, no mutant allele has been encountered in 239 Arabian horses (13) while 44 of 508 Arabian horses (8.7%) in the USA were reported to be carriers of SCID (8).

The pacing horses bred in Turkey are local and imported horses from Iran, Afghanistan, and Bulgaria. Some of the local pacing horses may have been influenced by Arabian horses. Hereditary disorders that are reported only in Arabian horses can also be seen in indigenous horses. This study aimed to detect the presence of mutant alleles that determine the inherited diseases PSSM and SCID in pacing horses in Turkey.

Materials and Methods

This research was conducted within the scope of the decision of the ethics board dated 27/08/2010 and no. 2010-96-337 of Ankara University Animal Experiments Local Ethics Board. The present number is not known, as there is no registration system-based breeding of pacing horses. Animals used in the study are formed, in 7 different geographic regions of Turkey were kept under extensive conditions, pacing horses 4 years and older. The samples of the pacing horses were taken by going to the relevant city and towns on the dates of the pacing horseraces, and by reaching the place where the horse was found in places where the races were not (for example, Ankara, Antalya, Aydın, Bursa, Erzincan, Erzurum, Eskişehir, İzmir, Konya, Kütahya, Mardin, Samsun, Trabzon, etc.). The animal material of the research is formed by a total of 264 pacing horses (blood/hair samples [from mane] of them) some of which are indigenous (n=182) and imported [Iranian (n=3), Afghan (n=24), Bulgarian (n=27)]. Some samples in the projects no 110 0 824 supported by TUBITAK were also used as materials.

To make DNA isolation from the hair samples, the section where the root parts of these samples were cut for 0.5 cm and 4 or 6 hair samples were left to incubation at 56 ^{o}C for a night with Proteinase-K. Following the incubation, 200 µl was taken from these samples and DNA isolation was performed according to MagAttract DNA Blood Mini M48 Kit protocol by using the Qiagen BioRobot M48 device (1).

Following DNA isolation, the relevant samples were subjected to PCR by using ABI 7500 thermal cycler, and PCR was performed according to the relevant kit protocol.

Determination of Genotypes: PSSM (GYS1) SNP genotyping; it was aimed to determine the Arg309His

mutation in the glycogen synthase (GYS1) gene (GenBank: NC_009153.2). Arginine amino acid (CGT) in the 309th position of the gene turns into histidine (CAT) amino acid as a result of the G>A mutation. The study was carried out according to the 5 'nuclease method and genotyping was done by working two PCR mixes, as wild-type and mutant for each sample. The primer sequences used to replicate the gene region and to detect the mutation were; for Wild-Type PCR mix, P1: 5' CCGAATCCAGGAGTTTGTGTG 3', P2: 5' CATTGTTCTGACGCTCAGGAAC 3', for Mutant PCR mix, P1: 5' CCCGAATCCAGGAGTTTGTGTA 3', P2: 5' CATTGTTCTGACGCTCAGGAAC 3'. In the study, 5 'FAM TATGGGTATGTGGGCCAGATACCCA BHQ 3' sequence was used as the TaqMan® probe sequence for both Wild-Type and Mutant PCR mix. 5 µl 10X buffer, 4 mM MgCl2, 1.2 pmol P1 and P2, 0.6 pmol Probe, 100 ng DNA, 0.3 µl HotStart Taq DNA Polymerase was used and PCR Grade Water was added to be 25 µl total volume of the mix used for the Wild-Type and Mutant PCR mixes used in the PCR. The PCR program was used to replicate the GYS1 gene region, consisting of 35 cycles including 10 min at 95 °C for initial denaturation, 15 sec of denaturation at 95 °C, and 1 min at 60 °C of bonding. The PCR process was performed with the ABI 7500 device and on which PCR mix is irradiated were determined by looking for fluorescent radiation revealed by the FAM dye in the probe.

SCID (DNA-PKcs) SNP Genotyping; it was aimed to detect 5 base pair deletions (TCTCA) in the DNA-Dependent Protein Kinase, catalytic subunit (DNA-PKcs) gene region (GenBank: AF448228.1). Frame-shift occurs as a result of the 5 base pair deletions in codon 9480 of the gene, and as a result, unstable protein is synthesized. The study was performed according to the 5 'nuclease method and genotyping was performed for each sample with Wild-Type (no deletion) and Mutant (with deletion) PCR mix. The primer sequences used to replicate the gene region and to detect the mutation were; for the Wild-Type PCR mix, P1: 5' ATAAGGAAACAAGGTAATTTATCA TCTCA 3', P2: 5' GAAACATCGATTTGTGATGATGT CATC 3', for the Mutant PCR mix, P1: 5' TAAGGAAACAAGGTAATTTATCAAATTCC 3', P2: 5' GAAACATCGATTTGTGATGATGTCATC 3'. In the study, 5 'FAM CTTCTAAAAACCTGGACAAACAG ATATCCGG BHQ 3' sequence was used as the TaqMan® probe sequence for both Wild-Type and Mutant PCR mixes. 5 µl 10X buffer, 4 mM $MgCl_2$, 1.8 pmol P1 and P2, 1.0 pmol Probe, 100 ng DNA, 0.3 µl HotStart Taq DNA Polymerase was used for Wild-Type and Mutant PCR mixes and PCR Grade Water was added to be 25 µl total volume of the mix. A PCR program consisting of 40 cycles including 10 min at 95 °C for initial denaturation, 15 sec of denaturation at 95 °C and 1 min at 60 °C of bonding was used to replicate the DNA-PKcs gene region. The PCR process was performed with the ABI 7500 device and on which PCR mix is irradiated were determined by looking for fluorescent radiation revealed by the FAM dye in the probe.

Results

The presence of the GYS1 mutant allele which determines to PSSM and DNA-PKcs mutant allele which determines to SCID disorder in pacing horses were studied in Turkey. This is the first study regarding inherited disorders in pacing horses in Turkey.

In this study, alleles were evaluated for PSSM and SCID diseases by looking for fluorescent radiation after PCR amplification.

Polysaccharide Storage Myopathy: In terms of PSSM disease, if radiation was observed only in the Wild-Type PCR mix, the sample was considered Wild-Type (GG), if radiation was observed in both Wild-Type and Mutant PCR mixes, the sample was considered heterozygote (GA) and if radiation was observed only in the Mutant PCR mix, the sample was considered homozygote mutant (AA). The GYS1-GG homozygote of a normal individual in Figure 1, the GYS1-GA heterozygote of a mutant carrier individual in Figure 2, and the SNP images of all investigated horses for GYS1 are given in Figure 3. A GYS1 mutant allele in a horse of Bulgarian origin was detected as a heterozygote. The GYS1 gene of all pacing horses (Indigenous, Iranian, and Afghan origins) was found to be homozygote for Wild-Type (Table 1).

Severe Combined Immunodeficiency: In terms of SCID disease, if radiation was observed only in the Wild-Type PCR mix, the sample was considered Wild-Type (no deletion in both alleles), if radiation was observed in both the Wild-Type and Mutant PCR mixes, the sample was considered heterozygote (deletion in only one allele) if radiation was observed only in the Mutant PCR mix, the sample was considered as a homozygote mutant (deletion in both alleles). In terms of DNA-PKcs allele, there were images of the homozygote Wild-Type allele and Wild-Type genotypes of all individuals (Figures 4 and 5). All samples of pacing horses have been homozygote Wild-Type in Turkey. No mutant allele or heterozygote genotypes were detected in the horses studied (Table 1).

While the frequency of the GYS-1 mutant allele was 3.71% in Bulgarian horses, this rate was 0.38% in all horses. In terms of the GYS-1 allele, 263 horses were homozygote, only 1 horse was a heterozygote. The DNA-PKcs wild-type allele frequency was found at 100%. Therefore, all genotypes were wild-type homozygote.

Table 1. Distribution of GYS-1 (PSSM) and DNA-PKcs (SCID) allele frequencies (%) and genotypes in pacing horses from different origins.

Origin	n	GYS-1 genes frequencies		DNA-PKcs genes frequencies		GYS-1		DNA-PKcs	
		Wild-Type	Mutant	Wild-Type	Mutant	Homozygote Wild	Heterozygote Genotypes	Homozygote Wild	Heterozygote Genotypes
Indigenous	182	100.00	0.00	100.00	0.00	182	-	182	-
Iranian	31	100.00	0.00	100.00	0.00	31	-	31	-
Afghan	24	100.00	0.00	100.00	0.00	24	-	24	-
Bulgarian	27	96.29	3.71	100.00	0.00	26	1	27	-
Total	264	99.62	0.38	100.00	0.00	263	1	264	-

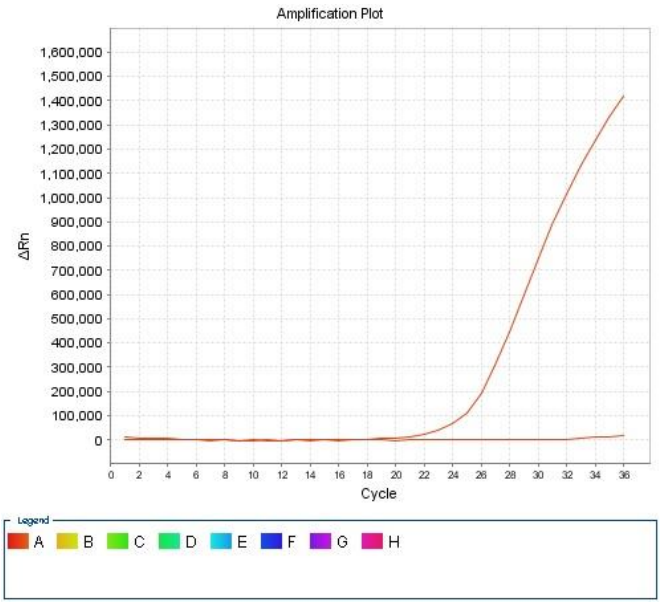

Figure 1. GYS1-GG (normal-wild type homozygote).

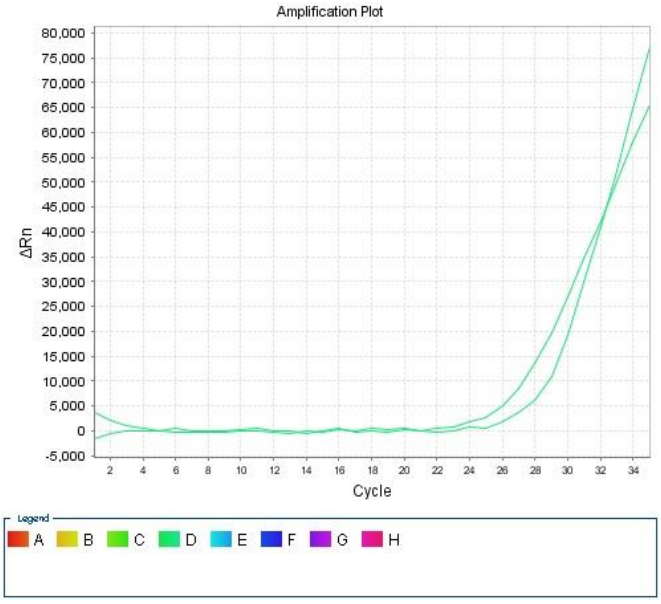

Figure 2. GYS1-GA (wild type heterozygote).

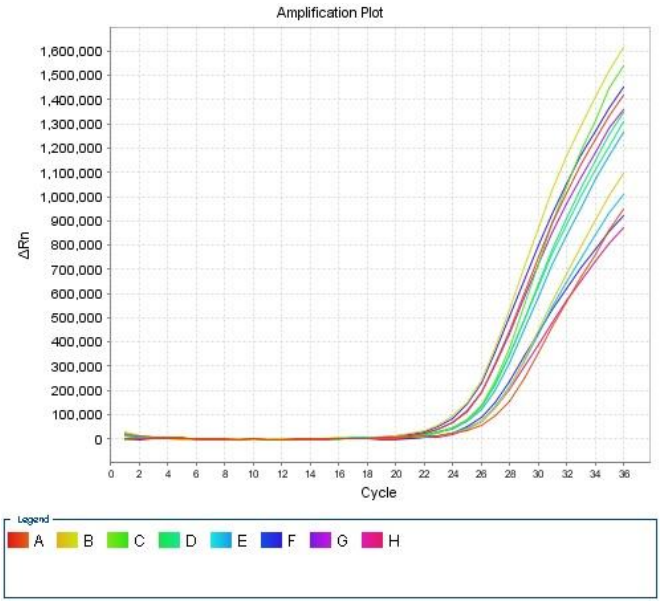

Figure 3. Peak image for GYS1 in all horses.

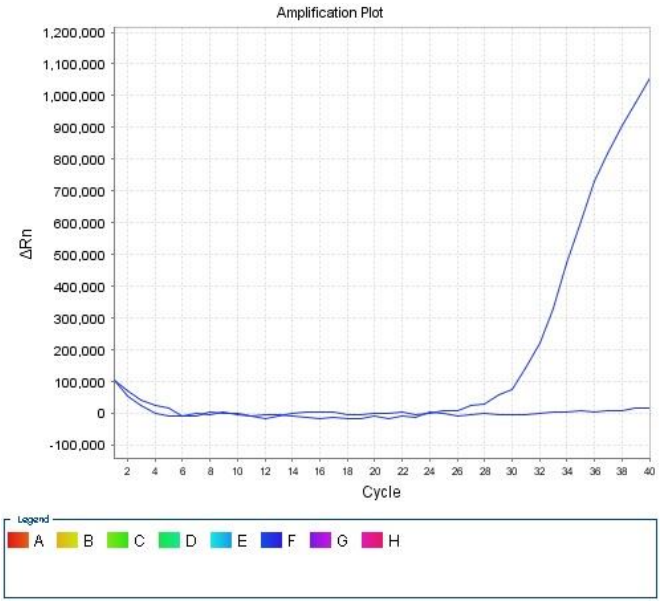

Figure 4. DNA-PKcs (wild type homozygote-normal).

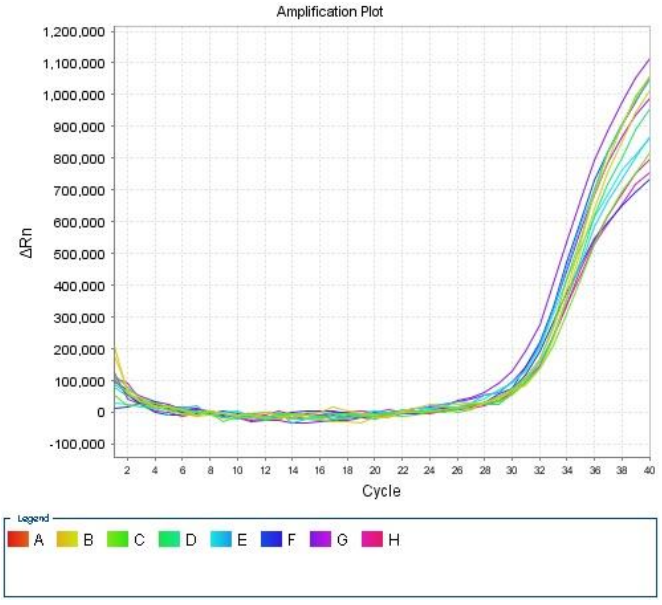

Figure 5. Peak image for DNA-PKcs in all horses.

Discussion and Conclusion

The existence of mutant alleles leading to the occurrence of polysaccharide storage myopathy and severe combined immunodeficiency which are hereditary diseases has been investigated in pacing horses from different origins.

Polysaccharide storage myopathy disease has occurred as a result of a point mutation in the GYS-1 gene, which converts the amino acid of arginine to histidine (17). This mutation increases the enzymatic activity, leading to the accumulation of polysaccharides such as glycogen in the skeletal muscles. The inheritance of the mutation is autosomal dominant (11). This mutation is formed as a result of a 10 base pair substitution in the GYS-1 gene located on the 10th chromosome (16).

The frequency of the GYS-1 mutant allele was found to be 3.71% in the pacing horses of Bulgarian origin, and the frequency of the GYS-1 mutant allele was 0.38% in all horses in the study. These frequencies are much lower than the reported mutant allele frequencies (45-66%; 8-22%; 40.1%; 34%; 18%; 62%) in some studies (3, 15, 19, 25, 28, 30). In these studies, in which the mutant allele frequencies are reported higher in terms of PSSM may be due to different horse breeds and different geographies, and also PSSM is mostly seen in Quarter, Draft horses and related breeds. In this study, only one out of 27 horses of Bulgarian origin was found with the GA genotype for PSSM disorders, while all other (n=263) horses were determined with the GG genotype (wild-type). Analyses were repeated to ensure that of heterozygote GA genotype was not caused by a false reading, however, no errors were detected in the repeated analyses. Other researchers (5, 18) have reported that horse breeds such as Thoroughbred, Arabian, Akhal Teke, Icelandic Clydesdale are wild-type like all the Indigenous, Iranian and Afghan pacing horses. On the other hand, Bulgarian pacing horses are larger than the other pacing horses and have been derived through crossbreeding. Therefore, it is thought that the PSSM mutant allele may have been transferred to Bulgarian pacers from heavy horse breeds.

Severe combined immunodeficiency is an autosomal-simple recessive inherited that is generally seen in Arabian horses and their crosses. Heterozygote animals appear healthy, but homozygote recessive foals die shortly after birth as a result of secondary infections. If the herds' genetic status is not known, the mutant gene can subsist in the herd for centuries without the disorder being seen. Therefore, the disease can be eradicated and animal losses can be prevented, especially by which are carriers predetermined in breeding animals (4, 20, 26).

In this study, all samples (n= 264), were determined as wild type. The findings obtained in this study differed from the results of some research (4, 8, 23, 24) on SCID disorder. The horse breed in these reported studies is Arabian horses, and the mutant carrier rates have been reported to be high 2.3%, 8.4%, 8.7% and 18%, respectively in the above literature. However, 120 Iranian Arabian horses (26) and 239 Turkish Arabian horses (13) are reported to be not carriers. The fact that all of the wild types sampled Arabian horses from Iran and Turkey suggests that there was no mixture from outside especially the West of Iran and Turkey. The absence of mutant allele in pacing horses, even if they received blood from Arabian horses could be attributed to the fact that lacking the SCID mutant gene in Arabian horses in Turkey.

The existence of mutant alleles causing SCID and PSSM hereditary disorders in pacing horses in Turkey (Indigenous, Iranian, Afghan, Bulgarian) has been investigated in this study. While no mutant allele was detected for SCID, only one horse was detected as heterozygote with a mutant allele for PSSM. Except for one pacing horse originating from Bulgaria, genes that cause PSSM and SCID hereditary disorders were not found in the other horses. It is evident that pacing horses in Turkey are clean for these disorders and that hereditary disorders are also transferred through imports such as infectious diseases. Therefore, it is necessary to check for hereditary disorders as well as infectious diseases when animals are imported. It is suggestible that both pacing horses as well other horse breeds whose immune system has collapsed and which has died at an early age are checked for carrying the mutant allele for SCID and those that get tired quickly and have frequently stiffness of muscle are checked for carrying the mutant allele for PSSM.

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Ethical Statement

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (2010-96-337).

Conflict of Interest

The authors are declared that there is no conflict of interest.

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