Short Communication

Molecular Screening of LFS and PSSM-I Diseases in Arabian Horse Population in Turkey [1,2]

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Abstract

Polysaccharide Storage Myopathy (PSSM) and Lavender Foal Syndrome (LFS) are inherited diseases in horse. PSSM-I, a point mutation in *Glycogen Synthase-1* gen (*GYS1*) causes increased activation of the enzyme and abnormal polysaccharides formation. LFS caused by one nucleotide deletion in *Myosin-Va (MYO5A)* leads frame shift mutation and premature termination of the protein. In the study, a total of 239 blood samples, taken from Anadolu (n=70), Karacabey (n=70) and Sultansuyu (n=99) stud farms were screened. Diseases related mutations were not detected in the sampled Arabian horse population.

Keywords: Arabian horses, LFS, PCR-RFLP, PSSM-I

Türkiye Arap Atı Popülasyonunda LFS ve PSSM-I Hastalıklarının Moleküler Taraması

Özet

Atların "Polisakkarit Depolama Hastalığı" (Polisaccarite Storage Myopathy, PSSM) ve "Lavanta Renkli Tay Sendromu" (Lavander Foal Syndrome, LFS) aktarılan kalıtsal hastalıklardır. PSSM-I'de, *Glikojen Sentaz-1* (GYS1) geninde meydana gelen bir nokta mutasyonuna bağlı olarak enzim etkinliğinde artış şekillenmekte ve anormal polisakkaritler oluşmaktadır. Lavanta renkli tay hastalığında ise Miyozin-Va (*MYO5A*) geninde çerçeve kayması mutasyonuna neden olan bir bazlık delesyon sonucunda proteinde prematüre sonlanma şekillenmektedir. Sunulan araştırmada, Anadolu (n=70), Karacabey (n=70) ve Sultansuyu (n=99) Tarım İşletmeleri'nde yetiştirilen damızlık Arap atlarından temin edilmiş 239 adet kan örneği kullanılmıştır. İncelenen popülasyonda LFS ve PSSM hastalıklarıyla ilişkilendirilen mutasyonlara rastlanmamıştır.

Anahtar sözcükler: Arap atı, LFS, PZR-RFLP, PSSM-I

INTRODUCTION

Pleiotropic effects of pigmentation genes are well described in horses [1]. Melanocytes are the key cells in pigmentation. Melanocytes derived from the neural crest, which arise dorsal edge of the neural plate during embryogenesis. Neural crest derived melanocytes are found several parts of the body, such as skin, hair, eye layers and also several types of neurons and glia [2]. Hence, mutations in the genes that affect melanocyte development cause pleiotropy on neurologic functions,

hearing, and sight. One of them causes Lavender Foal Syndrome (LFS) also known as Coat Color Dilution Lethal (CCDL) which only reported in Arabian horse foals [3-5] and is inherited in an autosomal recessive manner [3]. Brooks et al.[6] have determined the genetic mutation causing this syndrome by SNP-based whole genome association (WGA) study. According to this study, the mutation is a single base deletion of cytosine (c.4459delC) on *MYO5A* gene, located on ECA1 chromosome. This deletion causes frameshift resulting in a premature stop codon (p.Arg1487AlafsX13). Normally synthesized protein functions in organelle

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transport and membrane trafficking in brain and skin cells [7]. The protein also has a role in axonal and dendritic transport in neurons [8]. Thus newborn foals display diverse neurological abnormalities and have diluted coat colors like lavender, pewter and silver. Diluted coat color is the decisive symptom of LFS. LFS has no cure, and thus the foals with these conditions are euthanized after birth [6].

Abnormal polysaccharide and glycogen accumulation in striated muscles is called Polysaccharide Storage Myopathy (PSSM) and PSSM is distinct cause of Equine chronic exertional rhabdomyolysis. As a muscle disorder, rhabdomyolysis is disruption of striated muscles and causes of the disease could be associated with exercise (Exertional rhabdomyolysis) and unrelated to exercise [9]. Up to date there are two forms of PSSM in horses; PSSM-Type I (PSSM-I) and PSSM type II (PSSM-II). PSSM-I has a genetic base, which is a dominant mutation on *Glycogen Synthetize-1* (*GYS1*) gene. The mutation is a single base substitution (c.926G>A), causes an amino acid change (Arg309His). *GYS1* gene translates an enzyme called "glycogen synthase" [10]. The mutation increases the enzyme activity which results with production of amylase resistant polysaccharides. This type of PSSM is very common in several horses, especially in Quarter horses, Draft horses. PSSM-I is a rare genetic diseases for Arabian horses [10].

Restriction fragment length polymorphism method is based on whether a mutation forms specific enzyme cleavage site it has been widely used in diagnosis of mutations, polymorphisms which were previously associated with quantitative traits [11].

First aim of the presented study was to screen 30th exon of *MYO5A* gene and 6th exon of *GYS1* gene for the causative mutations for LFS and PSSM-I respectively by PCR-RFLP in the 239 Arabian horses, which bred in stud farms in Turkey. Second aim was to place the diagnosis of the diseases on routine laboratory work.

MATERIAL and METHODS

Animal Material

In the study, a total of 239 blood sample, taken from Anadolu (n=70), Karacabey (n=70) and Sultansuyu (n=99) stud farms of the Ministry of Agriculture was used for the further analyzes. The samples were collected in a previous study [12]. Also positive control samples carrying the mutations were sent by Dr. Brooks and Dr. McCue, for LSF and PSSM-I diseases respectively.

Total DNA Isolation

DNA extractions from samples were performed using the spin-column based commercial kits (DNeasy Blood& TissueKit, Qiagen GmbH, Hilden, Germany). DNAs were quantified by spectrophotometry at wavelengths of

260 nm and 280 nm. To determine degradation, Ethidium bromide (EtBr) stained 1% agarose gel electrophoresis were used.

PCR

PCR analysis for both regions was conducted in compliance with the method proposed by Brooks et al.[6] and McCue et al.[10]. The oligonucleotide sequences were shown in *Table 1*.

Electrophoresis of amplicons was conducted for 20 min at 120V in EtBr stained 2% agarose gel and visualized under UV light. The amplicons stored at -20°C until Restriction Fragment Length Polymorphism (RFLP) analysis.

RFLP Analysis

RFLP analysis is used to identify the mutations which cause differentiation in the enzyme cut sites. To detect the mutation causing LFS diseases, Faul (Smu) enzyme was used. This enzyme recognizes the patterns in the 5' strand "CCCGC(N)$_4$" and in the 3' strand "GGGCG(N)$_6$". The deletion mutation on the *MYO5A* gene causes a loss of enzyme cut region. To detect the mutation causing PSSM-I diseases, HpyCH4V enzyme was used. HpyCH4V recognizes the pattern TGCA, and cuts between G and C bases. The mutation on *GYS1* gene causes a gain of enzyme cut region. Thus the RFLP pattern in normal, heterozygote carrier and homozygous mutant individuals are different (*Table 2*).

RFLP analysis was carried out with 2U of each enzyme on 10 µl PCR products. Incubations were performed as instructed (New England Biolabs Inc.). Electrophoresis of RFLP results were conducted 20 min in EtBr stained 2% agarose gel with 100V, then results were visualized with UV light (Gel Logic 2000, Kodak).

Sequence Analysis

Positive control samples (n=2) and randomly selected samples (n=10 for each gene), additionally 40 individuals presenting abnormal band pattern for RFLP analysis of *MYO5A* were sequenced for further analyses. PCR stage was repeated with the same conditions and same oligonucleotides were used for sequencing. Results were aligned to ENSECAG00000021742 and ENSECAG00000021428 for *MYO5A* and *GYS1* regions respectively by using BioEdit software [13].

Table 1. *Oligonucleotide sequences for GYS1 and MYO5A regions*

Oligonucleotide Name	Oligonucleotide Sequences 5'-3'	References
MYO5A_F	CAG GGC CTT TGA GAA CTT TG	[6]
MYO5A_R	CAG CCA TGA AAG ATG GGT TT	
GYS1_F	TGA AAC ATG GGA CCT TCT CC	[10]
GYS1_R	AGC TGT CCC CTC CCT TAG AC	

Table 2. *Expected patterns of MYO5A and GYS1 regions after restriction with Faul and HpyCH4V enzymes, respectively*

Enzyme	Homozygous Mutant	Homozygous Normal	Heterozygote Carrier	References
Faul	287bp, 482bp	96bp, 287bp, 396bp	96bp, 287bp, 396bp, 482bp	[6]
HpyCH4V	54bp, 80bp, 97bp	80bp, 152bp	54bp, 80bp, 97bp, 152bp	[10]
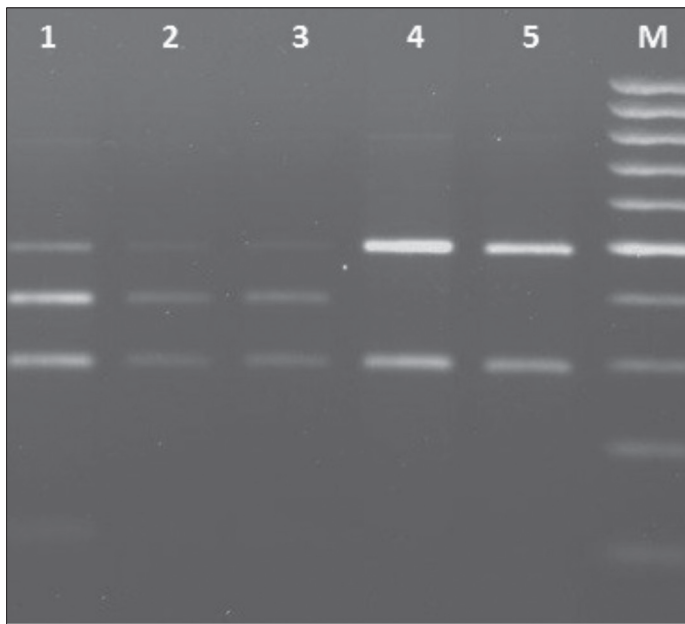
Fig 1. Observed band patterns of *MYO5A* region after restriction with Faul enzyme. Lane 1 heterozygous positive control sample. Lanes 2 and 3 are analysed samples from the present study. Lanes 4 and 5 are homozygous positive control samples. Lane M, molecular size marker (100 bp DNA ladder)
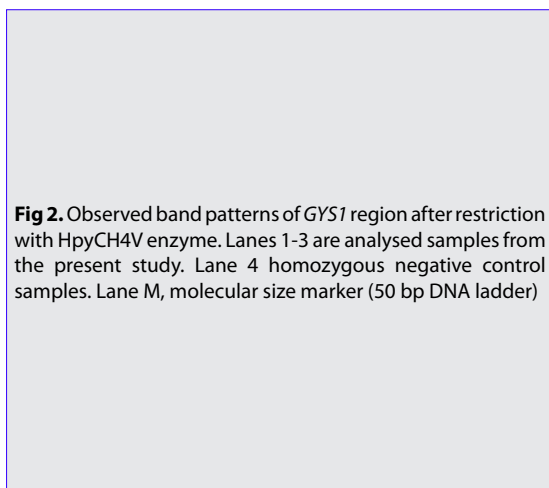
Fig 2. Observed band patterns of *GYS1* region after restriction with HpyCH4V enzyme. Lanes 1-3 are analysed samples from the present study. Lane 4 homozygous negative control samples. Lane M, molecular size marker (50 bp DNA ladder)
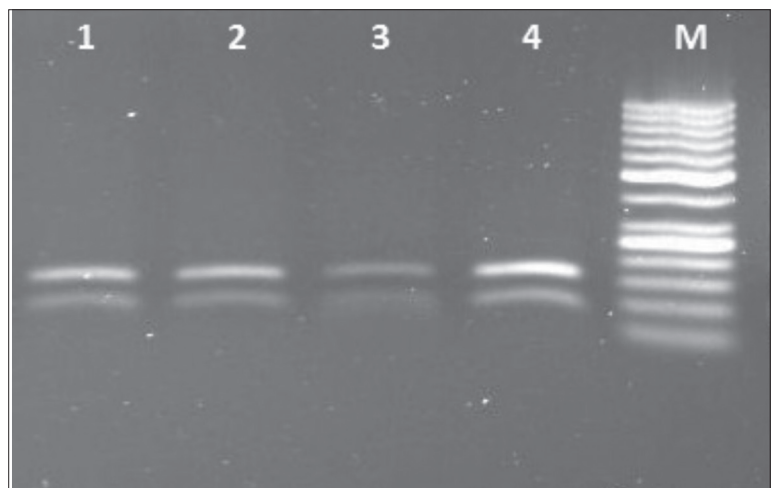
RESULTS

DNA concentrations results vary between 100-300 ng/µl. To optimize PCR protocol all DNA samples were diluted in to approximately 40 ng/µl concentration. By using PCR 769bp and 229 bp fragments were successfully amplified for *MYO5A* and *GYS1* genes, respectively. These amplicons were digested with Faul and HpyCH4v restriction enzymes to detect point mutations for each. Examples of gel images showing the digestion results of the amplified products cut by restriction enzyme were shown in *Fig. 1* and *Fig. 2*.

The sequences obtained from the samples showing abnormal band patterns were evaluated by comparison with the reference sequences and also mutation positive

controls samples for each gene regions. All of the analysed samples resulted with homozygous normal genotype.

DISCUSSION

LFS and PSSM-I diseases are genetic disorders. LFS is only observed in Arabian horses with the history of Egyptian originated or related Arabian horses [6]. The mutation causing LFS is recessive, thus heterozygous Arabian horses appear healthy. However when two carriers bred, the resulting offspring has 25% possibility of being affected with LFS. Brooks et al.[6] analyzed Egyptian Arabian Horses and the frequency of the carriers was found in 10.3% of the population consisting of 58 individuals. In the same study, another population, which does not have Egyptian origin, consisting 56 individuals the frequency of the disease determined to be 1.8% [6]. Another study conducted by Gabreski et al.[14] included the examination of 215 Arabian Horses, which has Egyptian blood line connection, the frequency of LFS found 1.62%. Tarr et al.[15] scanned Arabian horse population for severe combined immunodeficiency (SCID), LFS and cerebellar abiotrophy (CA) mutations, in 2004 and 2009. For LFS, the determined heterozygosity proportion was 13.3% (n=203) and 11.7% (n=197) in 2004 and 2009 respectively. In the presented study, we have examined 239 individuals from the Arabian horse population, the mutation causing LFS is not determined.

Abnormal activation of the enzymes in the glyco-genolytic or glycolytic pathway cause different types of glycogen storage myopathies [9] and *GYS1* is one of them. *GYS1* mutation is determined in many horse populations including Belgian draught horses, Quarter Horses and Cob Normand horses [10]. In the study we analysed Arabian horses and only homozygous normal band patterns were observed for *GYS1*, 6th exon region as expected.

In this study we used PCR-RFLP method to scan two mutations in population conducted by 239 Arabian horses. Abnormal band patterns observed for *MYO5A* mutation in 40 of the studied samples. It has seen that none of the samples were carry the mutation or any other base change in the sequence. The unexpected band patterns were decided as "star activity" of Faul enzyme.

The study conducted by the authors of this publication are the most comprehensive LSF and PSSM-I mutation scan studies in the Arabian horses bred in Turkey.

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