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Genetic Monitoring for Severe Combined Immunodeficiency Disease (SCID) Carriers in Arabian Horses of Iran

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ABSTRACT

SCID is a lethal genetic autosomal recessive disorder that has been observed in humans, dogs, mice, and horses. Affected animals are incapable of generating specific antigens for immune responses needed to protect them from infectious diseases. The frequency of affected recessive allele varies in different regions so that the outcome of normal breeding with carriers of recessive alleles is differently distributed. Little information is available for SCID carriers in Iranian horses to prevent carriers breeding. In this study, the occurrence of the SCID alleles was tested in representative samples of Persian Arabian (or Asil) horses. Blood samples were collected from 244 Arabian horses in eight provinces of Iran. The ARMS-PCRs were used for the first time to identify SCID carriers, based on three distinguishing primer pairs. Each sample was used in two separate PCRs with a common forward primer. The two reverse primers differed in their 3′ end: one reverse primer could pick the wild-type allele while the other could pick the mutant allele with a 3′ end deletion. An internal control (HMS02 locus) was used in both reactions to verify whether the amplifications worked correctly. The results showed a mutated allele frequency of 0.8% in the Arabian horse population of Iran. This is the first report identifying SCID carriers' frequency among Arabian horse population in Iran.

Keywords: Arabian Horses, ARMS-PCR, DNA-PK$_{CS}$, SCID.

INTRODUCTION

Severe Combined Immunodeficiency Disease (SCID) is a lethal genetic disorder in which the affected animals are incapable of generating specific antigens for immune responses needed to protect them from infectious diseases. SCID is a genetic disorder that has been observed in humans, dogs, mice, and horses. (Perryman, 2004; Bosma *et al*., 1983; McGuire and Poppie, 1973). In 1983, SCID was described in C.B-17 BALB/c mice in which the disease was inherited as an autosomal recessive disorder (Bosma *et al*., 1983). SCID was subsequently described in three breeds of dogs: Basset Hound (X-linked inheritance), Cardigan Welsh Corgi, and Jack Russell Terriers (autosomal recessive inheritance) (Perryman, 2004). The spontaneously occurring molecular detections in mice, horses, and dogs have been defined and offer clues to the disease patterns observed in the affected animals (Perryman, 2004). SCID was first reported in Arabian foals in Australia (McGuire and Poppie, 1973). The mode of SCID inheritance was verified through test breeding (Poppie and McGuire, 1977). Perryman and Torbeck (1980) showed that SCID in Arabian or cross-bred Arabian horses was inherited as an autosomal recessive

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condition (Georgescu *et al.*, 2006). Heterozygous animals appear normal and healthy but could transfer mutations to their offspring. In homozygous offspring, SCID is 100 percent deadly within the first six months of life due to immunodeficiency (Zavrtanik *et al.*, 2005; Perryman, 2004).

Shin *et al*. (1997a) defined the molecular basis of SCID in horses. They showed that affected animals had a deficiency in both the number and function of B and T lymphocytes, resulting in an incompetent immune system (Zavrtanik *et al*., 2005). Bailey *et al*. (1997) demonstrated with fluorescence *in situ* hybridization (FISH) that the mutated gene responsible for SCID was located on ECA9p12 equine chromosome. Shin *et al*. (1997a) showed that SCID was caused by a deletion in five base pair of the coding region of the catalytic subunit of DNA-protein kinase (DNA-PK_{CS}), which is responsible for the formation of key immune defense molecules. This kinase is usually involved in the mechanisms of V(D)J recombination during early lymphoid differentiation where it is required for the repair of DNA double-strand breaks. The deletion causes a frame-shift mutation at codon of amino acid 3155, resulting in deletion of the following 967 amino acids from the C-terminal end that includes the entire phosphatidylinositol 3-kinase domain and, thus, has been found to lack the DNA-PK_{CS} (Wiler *et al*., 1995).

The number of SCID affected foals produced and carrier frequencies of SCID differ between countries. From the 1970's through 1997, SCID carrier estimates from research studies have ranged from 8 to 28 percent with SCID affected foal estimates ranging from 0.18 to 3 percent (the 28 percent carrier rate calculated in the 1970's is thought to have been greatly overestimated) (Perryman and Torbeck, 1980). From the start of genetic testing from 1998 through 2011, VetGen Lab. tested 10,687 horses, with 15.73 percent of the tested animals as SCID carriers and 0.30 percent of them (32 foals, totally) as SCID affected. Although this is not a truly random sampling, it does provide a snapshot of the prevalence of SCID in the Arabian horses gene pool (Arabian Horses Association, 2012, unpublished data).

The frequency of the mutated allele differs from population to population so that the outcomes of normal breeding with carriers of recessive alleles in different countries are different. In the past, the only way to identify heterozygous horses was by use of progeny testing. However, identification of mutation enabled development of genetic testing that make identification of carriers easier (Zavrtanik *et al.*, 2005). Although the first paper on SCID was published in 1973 and the mode of inheritance was determined in 1980, a direct DNA test did not become commercially available until 1997. The use of this test now allows breeders to make informed choices and avoid producing affected foals. An accurate diagnosis of SCID is not only important because of the grave prognosis but it also provides information that both parents must be carriers and should not be used for further breeding. (Zavrtanik *et al.*, 2005)

Persian Arabian (or Asil) horses are kept mostly in Khozestan, Lorestan, Ilam, Kerman, Isfahan, Yazd, Tehran, Khorasan, and Fars Provinces of Iran. In addition, Persian Arabian horses are mated with other Iranian horse breeds to produce cross-bred horses. Therefore, it is possible to introduce this mutation to gene pools of other Iranian horse breeds. Little information is available for SCID carriers in Iranian horses to prevent carriers breeding. Seyedabadi *et al*. (2011) screened 120 samples of Persian Arabian horses from Khozestan and Tehran, but did not find any carriers or affected samples. In this study, the occurrence of the SCID alleles was tested in representative samples of Persian Arabian horses.

MATERIALS AND METHODS

Samples

Blood samples were collected from 244 non-related Arabian horses that had been selected randomly in eight provinces in north (Guilan), northwest (Eastern

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Azerbaijan), west (Kermanshah and Hamedan), southwest (Khozestan) and center (Yazd, Kerman and Alborz) of Iran. All samples were collected with the consent of horse owners and their prior information about the aim of the study.

DNA Purification and ARMS-PCRs

Total genomic DNA was purified from whole blood using AccuPrep® Genomic DNA Extraction Kit (Bioneer, Republic Korea) according to the manufacturer's instructions. The quality of all extracted genomic DNA was assessed by running on agarose gel. The quantity of samples was controlled by nanodrop spectrophotometer (Thermo Fisher Scientific, USA).

The Amplification Refractory Mutation System-Polymerase Chain Reactions (ARMS-PCRs) were used to identify SCID carriers, based on three distinguishing primer pairs designed by Bernoco and Bailey (1998). Briefly, each sample was used in two separated PCRs for which forward primers *5′-TGTTGCAAAAGGAGACAGAAT-3′* were common. The two reverse primers however, differed at their 3′ end: (1) one reverse primer could pick the wild-type allele *5′-AGAGTTTAAGGGGAATTCTCTG-3′*, (2) while the other was complementary to mutant allele that had a five base pair deletion at its 3′ end *5′-TAGTTTAAGGGGAATTCTCTGAA-3′*. The homozygote genotypes (wild-type or affected with mutant allele) were amplified in only one reaction but heterozygote genotypes were amplified in both reactions. The fragment length was 169 and 166 bp for normal and mutant alleles, respectively. An internal control was used in both reactions to verify whether the amplifications worked correctly. The internal control was HMS02, which amplified a fragment with 218-238 bp length and its primers were *5′-ACGGTGGCAACTGCCAAGGAAG-3′* and *5′-CTTGCAGTCGAATGTGTATTAAATG-3′* (Bradley *et al*., 2004).

PCR amplifications were performed in reactions each containing 50 ng genomic DNA, 1X PCR buffer (Fermentas, Canada), 0.2 mM of each dNTP (Roche Applied Science, Germany), 2 mM $MgCl_2$ (Fermentas, Canada), 0.6 µm of both common forward and one of reverse ARMS (mutant or wild type) primers (Metabion, Germany), 0.12 µM of both forward and reverse HMS02 primers (Metabion, Germany), and 0.5 U Taq DNA polymerase (Fermentas, Canada). PCR reactions were performed on GeneAmp® PCR System 9700 (Applied Biosystems, USA). PCR conditions were as follows: an initial denaturation for 3 minutes at 95ºC, followed by 33 cycles of 30 seconds at 94ºC, 40 seconds at 61ºC, 40 seconds at 75ºC and a final extension of 10 minutes at 72ºC. Genotyping was performed using electrophoresis on 2% agarose gel (Invitrogene, USA) with pUC Mix Marker 8 (Fermentas, Canada) ladder. In addition, 25 samples that were not carrier of SCID based on the results of ARMS PCR, and all positive samples were sequenced by using BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystem, USA). Sequences were determined using an Applied Biosystems 3130 Genetic Analyzer based on capillary electrophoresis. Sequences were aligned using MEGA5 (Kumar *et al*., 2008).

RESULTS

The number of samples used according to their origin and sex were as follow: 228 Persian Arabian horses (113 stallions and 11 mares) and 16 Arabian horses imported from other countries (13 stallions and 3 mares). The results showed that three of the Persian Arabian horses (two male and one female) and one of the imported Arabian stallions were carriers for SCID and the rest of samples were clear (Table 1 and Figure 1). These results demonstrated that frequency of the mutated allele was as low as 0.008 in this population of horses. This is the first report on SCID carrier frequency among

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Table 1. Number (and percentage) of the SCID carriers among Arabian horses in Iran.

Samples	Carrier		Total
	Negative	Positive	
Persian Arabian horses	225 (98.7%)	3 (1.3%)	228
Imported Arabian horses	15 (93.75%)	1 (6.25%)	16
Total	240 (98.4%)	4 (1.6%)	244

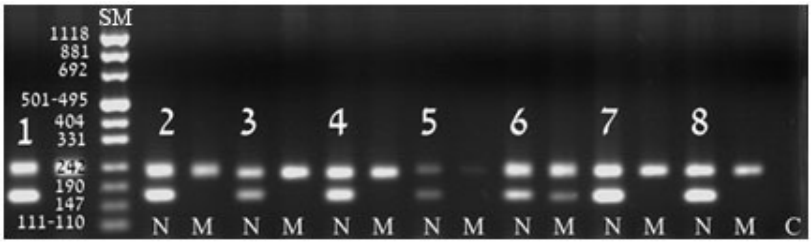

Figure 1. The ARMS-PCRs and internal control products that were electrophoresed on 2% agarose gel. From left to right, any two adjacent lanes with subtitle N and M belonged to one sample. Sample IDs were mentioned above lanes N. Samples in lanes N and M were amplified with wild type primers and mutated primers, respectively. Sample 6 was a carrier horse, but others were clear. Lane C contained no DNA (negative control of PCR). Sample 1 is a negative control (wild type). Lane SM was pUC Mix Markr 8 as size marker. All reactions included the internal control (HMS02) which amplified a 218-238 bp fragment.

Arabian horse population in Iran. To verify the results, all carriers and a subpopulation of clears were sequenced. The sequencing results confirmed the genotypes obtained by ARMS-PCRs.

DISCUSSION

The first genetic test based on ARMS-PCRs for detection of SCID carriers in the Arabian horses was introduced by Bernoco and Bailey (1998). In the current study, we used the same PCR design. Because the expected results of the PCR in non-carriers were absence of the PCR product and to ensure accuracy of the PCR reactions, amplification of another region of genomic DNA (i.e. HMS02 locus) (Bradley *et al*., 2004) was used as internal control, simultaneously. This locus was amplified under the same PCR conditions for the DNA-PK_{CS} locus, but the fragments resulting from HMS02 had different lengths, and were separated on agarose gel.

In other studies that have been conducted to detect the carriers of SCID, amplification was performed in one PCR reaction using a primer pair on both sides of the mutated region to amplify a part of the gene which included the deletion region. Hence, two equal-sized fragments would be obtained for normal animals, and two fragments with a 5 bp difference would be obtained for carriers (Seyedabadi *et al*., 2011; Georgescu *et al*., 2006; Zavrtanik *et al*., 2005; Swinburne *et al*., 1999). To detect the normal and mutated alleles, PCR products were electrophoresed either on 8% polyacrylamide gel (Seyedabadi *et al*., 2011), or on 4% agarose gel (Zavrtanik *et al*., 2005), or all the samples were sequenced (Piro *et al*., 2008; Georgescu *et al*., 2006; Swinburne *et al*., 1999). The advantages of using a 2% agarose gel are its ease and speed of preparation, high resolution, and low cost.

In 1997, a research was conducted on 257 foals from 19 different states in the USA. This study was based on clinical observations and calculation of the proportion of affected to normal foals. The

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frequency of carriers was estimated as 25 percent (Poppi and McGuier, 1977). However, upon introducing the genetic test by Bernoco and Bailey (1998), the frequency of carriers was reported 8.4 percent, with random sampling of 250 Arabian horses in the US. Upon investigating the pedigree, it was demonstrated that this disease had been distributed among the population by a popular stallion imported from Poland (Bernoco and Bailey, 1998). Poland is one of the important producers and exporters of Arabian horses. However, a study conducted on 271 non-related Arabian horses, reported no carriers (Terry, 1999). Table 2 shows the results of studies on SCID in different countries since the genetic test was created. Following the USA (Bernoco and Bailey 1998), the highest frequency was reported in Morocco; and upon investigating the pedigree it was realized that the mutated gene had been distributed among the population by three imported Arabian horses (Piro *et al*., 2008).

In the current study, the frequency of carriers was 1.6%, which was lower in comparison with Piro *et al*. (2008), Swinburne *et al*. (1999), and Bernoco and Bailey (1998). In the past, the only way for detecting carriers was through the birth of affected foals and investigating the parentage test results. Therefore, before the creation of genetic test by Bernoco and Bailey (1998), its frequency was estimated between 8 to 25 percent. Ever since the genetic test was created in 1998, the VetGen Lab has tested 10,600 horses through 2011, and detected 16.5 percent carriers, and 0.30 percent affected horses (Allelic frequency: 0.172). Although this sampling was not random, it may be considered as a snapshot of the prevalence of SCID in the gene pool of Arabian horses (Arabian Horses Association, 2012, unpublished data). However, since the frequency of this allele is low, its transmission to further generations can be prevented by preventing breeding between carriers in the populations.

Seyedabadi *et al*. (2011) tested 120 Arabian horses from two regions of Iran (Khuzestan and Kordan). All horses were reported to be homozygous for the wild-type allele. In the current study, 244 Persian Arabian and foreign Arabian horses were sampled from Khuzestan, Kermanshah, Yazd, Hamedan, Eastern Azerbaijan, Tehran, Kerman, and Guilan Provinces of Iran and four carriers were detected among them. The greater number of samples in the current study increases the chance of carrier detection. In the current study, three carriers (two males and one female) were detected among 228 Persian Arabian horses, and one

Table 2. Frequency of SCID carriers in some countries.

Country	No. of tested horses	No. of carriers (percentage)	Reference
USA	Prior to genetic test	- (25.8%)	Poppi and McGuire (1977)
Australia	Prior to genetic test	- (30%)	Studdert (1978)
USA	250	21 (8.4%)	Bernoco and Bailey (1998)
UK	106	3 (2.8%)	Swinburne *et al*. (1999)
Poland	271	None	Terry *et al*. (1999)
Brazil	205	3 (1.5%)	Teixeira *et al*. (2001)
Slovenian	128	None	Zavrtanik *et al*. (2005)
Romania	60	None	Georgescu *et al*. (2006)
Morocco	377	21 (5.5%)	Piro *et al*. (2008)
Iran	120	None	Seyedabadi *et al*. (2011)
South Africa (2004)	800	51 (6.4%)	Tarr *et al*. (2014)
(2009)	699	24 (3.4%)	
Turkey	239	None	Cinar Kul *et al*. (2014)

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carrier (male) was detected among 16 foreign Arabian horses imported to Iran. However, we do not have comprehensive and complete information on the horses' pedigree; hence we cannot comment on the precise source of SCID in Iran. Based on our results, the allelic frequency of SCID is very low in Iran.

The complete elimination of a genetic abnormality may not be economically wise. By eliminating the carriers of a specific disease, other valuable traits may also be eliminated from that population or breed. Desired traits can be preserved in future generations if carriers with those traits are bred with non-carriers. The birth of affected foals can be prevented by detecting the disease-causing gene(s) and performing genetic tests. If the carrier possesses desired and valuable traits, and its elimination is not cost-effective in the breeding program, it can be bred to non-carriers. Their progenies must then be tested genetically to ensure that they are not affected.

The population of Arabian horses in Iran are currently classified into three groups: Persian Arabian, imported Arabian, and cross-bred Arabian horses (the result of breeding Persian Arabian and imported Arabian horses). The imported Arabian horses are more appealing (particularly in their head). This factor has led to their increased use in breeding (specifically in the paternal line) and, subsequently, to the increase in the number of Arabian cross-bred horses (Rafeie, 2011). The detection of a carrier stallion among 16 imported Arabian horses in this study shows that the prevalence of the SCID allele is higher in this group (carrier frequency 6.25%) than the Persian Arabian horses (carrier frequency 1.3%); however, there were only 16 imported Arabian horses tested as compared to 228 Persian Arabian ones. Further studies are required to verify this finding; however, implementing genetic testing in imported Arabian horses may prevent the propagation of SCID-carriers within the gene pool of the Persian Arabian horses.

Persian Arabian horses usually are not tested for SCID because of horse producers' low awareness of this disease, the low number of genetic testing centers, and the relatively high cost of the test. Nevertheless, the cost of this test is much less than the economic loss of birth of an affected foal. Discovering a practical way to complete elimination of SCID from the population of Arabian horses in the world while saving the valuable traits they carry is the final goal in the future. Performing genetic tests to detect and gradually eliminate this disease from the population are the first steps toward this goal.

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آزمون ژنتیکی برای شناسایی حاملین بیماری نقص ایمنی شدید مرکب (SCID) در اسب های عرب ایران

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چکیده

SCID یک بیماری ژنتیکی کشنده با توارث مغلوب اتوزومی است که در انسان، سگ، موش و اسب مشاهده می‌شود. مبتلایان قادر به تولید آنتی‌ژن‌های خاصی که برای پاسخگویی به بیماری‌های عفونی ضروری هستند، نمی‌باشند. فراوانی آلل مغلوب ایجاد کننده این بیماری در مناطق مختلف متفاوت بوده و در نتیجه احتمال آمیزش افراد حامل آلل مغلوب با افراد سالم متفاوت است. اطلاعات اندکی از حاملین SCID در اسب‌های ایرانی در دسترس بوده و در نتیجه نمی‌توان از آمیزش آنها در جمعیت‌های اسب ایران جلوگیری به‌عمل آورد. در این مطالعه، فراوانی آلل SCID در ۲۲۴ نمونه از اسب‌های عرب ایران (اصیل) از ۸ استان کشور مورد بررسی قرار گرفته و نخستین بار از روش ARMS-PCR با کمک ۳ آغازگر استفاده شد. برای هر نمونه دو PCR جداگانه در نظر گرفته شد که آغازگرهای پیش‌رو آنها یکسان بوده ولی از دو آغازگر پس‌رو که انتهای '۳ متفاوتی برای شناسایی جایگاه جهش حذفی داشتند، بهره گرفته شد. علاوه بر این از یک کنترل داخلی (HMS02) نیز جهت اطمینان از صحت تکثیر در هر دو نوع واکنش استفاده شد. نتایج نشان داد که فراوانی آلل جهش یافته در جمعیت اسب‌های عرب ایران حدود ۰/۸ درصد بوده است. این نخستین گزارش در زمینه شناسایی حاملین آلل ایجاد کننده بیماری SCID در جمعیت اسب‌های عرب ایران است.

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