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\textsubscript{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-PKCS), 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-PKCS (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, Khurasan, 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
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’-ACGTTGGCAACTGCAAAGGAAG-3’ and 5’-CTTGCAGTCTGAATGTATTAAATG-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 95°C, followed by 33 cycles of 30 seconds at 94°C, 40 seconds at 61°C, 40 seconds at 75°C and a final extension of 10 minutes at 72°C. Genotyping was performed using electrophoresis on 2% agarose gel (Invitrogen, 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
Table 1. Number (and percentage) of the SCID carriers among Arabian horses in Iran.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Carrier</th>
<th></th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Persian Arabian horses</td>
<td>225</td>
<td>222 (98.7%)</td>
<td>228</td>
</tr>
<tr>
<td>Imported Arabian horses</td>
<td>16</td>
<td>15 (93.75%)</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>244</td>
<td>240 (98.4%)</td>
<td>244</td>
</tr>
</tbody>
</table>

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-PKcs 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
frequency of carriers was estimated as 25 percent (Poppi and McGuire, 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.

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

Organization of United Nations (FAO), Roma, Italy.
آزمون زننده برای شناسایی حاملین بیماری نقش ایمنی شدید مرکب (SCID) در اسب های عرب ایران

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چکیده

یک بیماری زننده کشنده با نوار مغلوب انوزومی است که در انسان، سگ، موش و SCID اسب مشاهده می‌شود. مبتلا‌اند قادرون به تولید آنتی‌ژن‌های خاصی که برای پاسخگویی به بیماری‌های عفونی ضروری هستند. نمایان نمایان، فراوانی آلل مغلوب ایجاد کننده این بیماری در مناطق مختلف متفاوت بوده و در نتیجه اختلال آمیت 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 آمیت آنها در جمعیت‌های اسب ایران گنجینه‌ای به‌عمل آورد. در این مطالعه، فراوانی آلل SCID اسب‌های عرب ایران (اصیل) از 8 استان کشور مورد بررسی قرار گرفته و نخستین بار از روش PCR با کمک 3 آلگاگر استفاده شد. برای هر نمونه دو جدایگانه در نظر گرفته شد که آلگاگرهای پیشرو آنها یکسان بوده ولی از دو آلگاگر پس رو به انتهای 3 متفاوتی برد. شناسایی جایگاه جهش حذفی داشتنی به‌هیک افراد که بر این یک کنتل داخلی (HMS02) نیز جهت اطمینان از صحبت نکشی در هر دو نوع واکنش استفاده شد. نتایج نشان داد که فراوانی آلل جهش یافته در جمعیت اسب‌های عرب ایران حدود 8/0 درصد بوده است. این نخستین گزارش در زمینه شناسایی حاملین آلل ایجاد کننده بیماری SCID در جمعیت اسب‌های عرب ایران است.