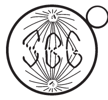

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Short Communication

Spi2 gene polymorphism is not associated with recurrent airway obstruction and inflammatory airway disease in thoroughbred horses

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Abstract

The aim was to detect the presence of polymorphisms at exons 1, 2, 3 and 4 of the Spi2 gene, and evaluate a possible association between them and recurrent airway obstruction (RAO) or inflammatory airway disease (IAD) in thoroughbred horses, through single-strand conformational-polymorphism (SSCP) screening. Although polymorphism was not detected in exons 1, 2 and 3, three alleles and six genotypes were identified in exon 4. The frequencies of allele A (0.6388) and genotype AA (0.3888) were higher in horses affected by RAO, although no association was found between polymorphism and horses with either RAO or IAD.

Key words: horse, respiratory disease, SSCP, alpha-1-antitrypsin, serpin.

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"Heaves", also known as chronic obstructive pulmonary disease (COPD), or more recently, recurrent airway obstruction (RAO), is one of the most frequently diagnosed pulmonary disorders in horses over six-years-old (Larson and Busch, 1985; Bracher *et al.*, 1991). Occurrence is by exposure to a dusty environment (hay, molds, pollen). Dust (allergens) induces the release of chemotactic factors that recruit and activate neutrophils (Franchini *et al.*, 1998). At the site of inflammation, neutrophils release granules containing potentially toxic products, such as proteases, elastases and collagenases, which promote tissue destruction. There are, however, proteins known as protease inhibitors, which, when bound to active sites on proteases, diminish or even impede their action.

The alpha-1-antitrypsin (AAT) enzyme, a plasma glycoprotein protease inhibitor of the serpin family (serin protease inhibitor), is primarily synthesized by the liver, and to a lesser extent by monocytes, bronco-alveolar macrophages and the mammary gland. Its main function is to inhibit neutrophil protease elastase, which induces tissue destruction.(Lai *et al.,* 1983). Mutations or altered transcription in its gene can lead to AAT deficiency, one of the causes of COPD in humans (De Meo and Silverman, 2004). Genetic susceptibility to RAO was first proposed by Schäper (1939), who discovered that 14 out of the 24 descendants of the stallion "Egmont", affected by RAO, followed suit. The hereditary basis of the disorder in horses was further demonstrated by Marti *et al.* (1991), in a clinical study with 90 German warm-blooded horses and 42 Lipizzaners. The risk of contracting RAO was found to be 3.2 times higher ($p < 0.05$) when one parent (dam or sire) had the disease and 4.6 times higher ($p < 0.05$) when both parents did. The AAT enzyme, more commonly known as protease inhibitor (PI) in horses, is a highly polymorphic biochemical system, with 25 alleles (haplotypes) characterized by two-dimensional electrophoresis. Equine AAT differs from the human form, in as much as it is controlled by a family of four linked loci (multigene), denominated serpines (Spi 1, 2, 3, 4), cytogenetically assigned to chromosome ECA24q15-16 (Lear *et al.*, 1999). PI enzyme concentration is higher in the broncho-alveolar lavage fluid of horses with clinical signs of RAO (Milne*,* 1994). Corbella *et al.* (1977) also found variations in AAT serum levels in horses with respiratory diseases, thereby implying the possible relevance of AAT in the clinical manifestation of RAO and inflammatory airway disease (IAD). IAD is also highly prevalent. It occurs in younger horses and affects performance. Despite similar clinical symptoms, it is still uncertain whether, IAD becomes RAO in young horses (Mair and Derksen, 2000).

The aim here was to screen for polymorphisms in the exon regions of the Spi2 gene, which is approximately 5 kb long and has 4 exons (Wade *et al.*, 2009), by PCR sin-

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gle-strand conformational polymorphism (SSCP), so as to check for any possible association with RAO or IAD, which could indicate susceptibility to these disorders.

Following physical examination of the respiratory tract, blood samples from 51 thoroughbred horses were collected in tubes containing EDTA. The horses were stabled at various Brazilian racetracks and farms located in Paraná, São Paulo and Rio Grande do Sul. The diagnosis of healthy horses (controls, n = 10) and those with RAO (n = 18) or IAD (n = 23) was based on clinical signs, bronco-alveolar cytology (Hoffman, 1999) and ventigraphy. After collection, the tubes were centrifuged, to separate the buffy coat for subsequent freezing at -80 °C until DNA extraction, all according to Plante *et al.* (1992).The primer pairs were designed to amplify no more than 400 base pairs (bp), using the Clone 7 Manager program, and according to the sequence deposited at GenBank (accession number AF034077). Primer sequences used were: Exon 1-5'TCTTGCAGGACAATGCCATC3'5' (forward) and 5'GGTTGGTTGTGCAACCTT AC3' (reverse); Exon 2-5'TAGACCTTTTCCCACCCTG3' (forward) and 5'CTGTG GCATCTCAAGGTT3' (reverse); Exon 3-5'GTGGGCAGGGGCATAGGG3' (forward) and 5'CCACGGACGCAGGGACAGAC3' (reverse), and Exon 4-5'CCCGACCCTG CTCAGAAC3' (forward) and 5'GAGAGCTTTGCCCGTCACACTC3' (reverse). Amplified fragment sizes were 687, 346, 271, and 342 bp respectively. Exon 1 was cleaved using the *Nru*I restriction enzyme, thereby resulting in one fragment of 269 and another of 422 bp. Samples were kept for 5 min at 94 °C, and then submitted to 30 cycles of 30 s at 94 °C, 30 s at 60 °C and 1 min at 72 °C, followed by a final extension of 7 min at 72 °C. The PCR products were mixed with a loading buffer (formamide 95%, 0.5 M EDTA and bromophenol blue with 0.5% glycerol) and left at 95 °C during 10 min for denaturation. It was then cooled on ice and loaded onto an 8% polyacrylamide gel. Electrophoresis was run using 1x TAE buffer at 80V for approximately 48 h at room temperature. Gels were stained by silver nitrate (Sanguinetti *et al.*,1994) evaluated on transilluminator. Allelic and genotypic frequencies were defined by direct count, whereupon the association between alleles and genotypes and RAO and IAD was assessed by χ^2 test ($p < 0,05$). The project was approved by the ethical committee of UFSM under protocol number # 23081.000917/2008-12.

PCR-SSCP analysis of exons 1, 2 and 3 of the Spi2 gene indicated no polymorphism. On exon 4, three different band patterns could be identified (Figure 1). They were called A, B and C. The allelic and genotypic frequencies observed in healthy horses and horses with RAO and IAD appear in Table 1. No association was detected between exon 4 alleles and genotypes of the Spi2 gene and RAO or IAD. Although Matthews (1979) observed a higher frequency of certain PI alleles in cross-bred horses with RAO, Vinocur *et al.* (2005) found no like association in thoroughbred horses. The drawback in SSCP screening is the lack of sensitivity. As also observed by Sheffield *et al.* (1993), as the amplified fragments of exons 1, 2 and 3 presented more than 200 bp, which, makes the method less sensitive. The amplified fragments had a GC content of approximately 50%. According to Nataraj *et al.* (1999), 100-300 bp fragments are easily detected by SSCP analysis, when they have a 60% GC, but are not detected when they possess 40% GC. This is attributed to the hydrogen bridges that influence the complexity of the tertiary structure formed by the DNA tape (Cuzcano *et al.*, 2005). Thus, there are possibly polymorphisms on exons 1, 2 and 3 which have not been detected due to their size. It is important to remember that the other serpins (Spi1, Spi3 and Spi4), as well as various other genes, may be involved in the pathogenesis of this disease, and thus there are many other candidate genes. Recent information from equine and human medicine, while revealing major differences between human COPD and equine RAO, has shown greater similarity between RAO and human asthma. Thus, studies of equine RAO based on human medicine currently tend to be guided by human asthma, and not just COPD.

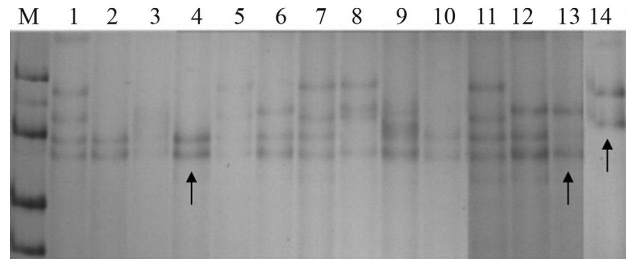

Figure 1 - Electrophoretic profile of the polymorphisms identified on exon 4 of the Spi2 gene characterized by SSCP on 8% polyacrylamide gel. Lanes 2, 4 and 10 show genotype AA, lanes 1,5,7 and 11 genotype AB, lanes 6, 12 -AC, lane 8 - BC; lane 13 - CC and lane 14 - BB. M - DNA Molecular Weight Ladder (100 bp). The alleles A, B and C are indicated by arrows on lanes 4, 13 and 14, respectively.

Table 1 - Allelic and genotypic frequencies of the polymorphism identified in exon 4 of the Spi2 gene in healthy horses and horses with RAO and IAD.

Allele	Healthy (n = 20)	RAO (n = 36)	IAD (n = 46)	Total (n = 102)
A	0.5500	0.6388	0.500	0.5588
B	0.2000	0.2777	0.3478	0.2941
C	0.2500	0.0833	0.1521	0.1470
Genotype	(n = 10)	(n = 18)	(n = 23)	(n = 51)
AA	0.2000	0.3888	0.2608	0.2941
BB	0	0.0555	0.0869	0.0588
CC	0.1000	0	0.0434	0.0392
AB	0.4000	0.3888	0.3913	0.3921
AC	0.3000	0.1111	0.0869	0.1372
BC	0	0.0555	0.1304	0.0784

In conclusion, polymorphism was not detected in exons 1, 2 and 3 of the Spi2 gene, although three alleles and six genotypes were identified in exon 4. However, the allelic and genotypic frequencies of this polymorphism were not associated with the incidence of RAO or IAD

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References

Bracher V, Fellenberg R and Windwe CN (1991) An investigation of the incidence of chronic obstructive pulmonary disease (COPD) in random populations of Swiss horses. Equine Vet J 23:136-141.

Corbella E, Ottonello S and Ubaldi A (1977) Serum antiproteases and respiratory diseases of the horse. Folia Vet Lat 7:258-272.

Cuzcano AE, Sandoval J, Guevara-Fujita ML and Fujita R (2005) Uso de la técnica SSCP para detectar mutaciones puntuales del ADN mitocondrial humano. Rev Peru Biol 12:349-358.

De Meo DL and Silverman EK (2004) Alpha1-antitrypsin deficiency: 2 genetic aspects of alpha(1)-antitrypsin deficiency: phenotypes and genetic modifiers of emphysema risk. Thorax 59:259-264.

Franchini M, Gilli U, Akens MK, Fellenberg RV and Bracher V (1998) The role of neutrophil chemotactic cytokines in the pathogenesis of equine chronic obstructive pulmonary disease (COPD). Vet Immunol Immunopathol 66:53-64.

Hoffman AM (1999) Bronchoalveolar lavage technique and cytological diagnosis of small airway inflammatory disease. Equine Vet J 11:330-336.

Lai EC, Kao FT, Law ML and Woo SL (1983) Assignment of the alpha-1 AT gene and sequence related gene to human chromossome 14 by molecular hybridization. Am J Hum Genet 35:385-392.

Larson VL and Busch RH (1985) Equine tracheobronchial lavage: Comparison of lavage cytologic features in horses with chronic obstructive pulmonary disease. Am J Vet Res 46:144-146.

Lear TL, Brandon R, Masel A, Bell K and Bailey E (1999) Horse alpha-1-antitrypsin, beta-lactoglobulins 1 and 2, and transferring map posicions 24q15-q16, 28q18-qter, 28q18-qter and 16q23, respectively. Chromosome Res 7:667.

Mair TS and Derksen FJ (2000) Chronic obstructive pulmonary disease: A review. Equine Vet J 1:35-44.

Marti E, Gerber H, Essich G, Oulehla J and Lazary S (1991) The genetic basis of equine allergic diseases 1. Chronic hypersensitivity bronchitis. Equine Vet J 23:457-460.

Matthews AG (1979) Identification and characterisation of the major antiproteases in equine serum and an investigation of their role in the onset of chronic obstructive pulmonary disease (COPD). Equine Vet J 11:177-182.

Milne EM (1994) Quantitation of alpha-1 proteinase inhibitor in the pulmonary epithelial lining fluid of horses with chronic obstructive pulmonary disease. Res Vet Sci 52:262-264.

Nataraj A, Olivos-Glander I, Kusukawa N and Highsmith E (1999) Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection. Electrophoresis 20:1117-1185.

Plante Y, Shmutz SM and Lang KDM (1992) Restriction fragment length polymorphism in the mitochondrial DNA of cloned cattle. Theriogenology 38:897-904.

Sanguinetti CJ, Dias NE and Simpson AJG (1994) Rapid silver staining and recovered of PCR products separated on polyacrylamide gels. Biotechniques 17:915-919.

Schäper W (1939) Untersuchungen über die Erblichkeit und das Wesen des Lungendampfes beim Pferd. Tierärztl Rundsch 31:595-599.

Sheffield VC, Beck JS, Kwitec AE, Sandstrom DW and Stone EM (1993) The sensitivity of single strand conformation polymorphism analysis for detection of single base substitutions. Genomics 16:325-332.

Vinocur ME, Brass KE, Caetano AR, Silva LF, Silva AC and Silva CAM (2005) Equine protease inhibitor system as a marker for the diagnosis of chronic obstructive pulmonary disease (COPD). Genet Mol Biol 28:382-385.

Wade CM, Giulotto E, Sigurdsson S, Zoli M, Gnerre S, Imsland F, Lear TL, Adelson DL, Bailey R, Bellone LL, *et al.* (2009) Genome sequence, comparative analysis, and population genetics of the domestic horse. Science 366:865-867.

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