

Characterization of gene polymorphisms related to immune system physiology in Mangalarga horses

[Caracterização de polimorfismos gênicos relacionados à fisiologia do sistema imune em equinos da raça Mangalarga]

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ABSTRACT

The objectives of this study were to standardize a PCR-RFLP genotyping method for the AY_731081:g.1900T>C SNP of the equine *CD14* gene, and to characterize this SNP and two other polymorphisms (AY_005808: c.1530A>G of the *TLR4* gene and AX_463789: g.133T>C of the *Cε* gene) in Mangalarga horses, in order to contribute to future studies investigating the association between DNA markers and traits related to immune system physiology in this breed. A total of 151 Mangalarga horses of both sexes and variable ages, representative of the population of São Paulo State, were used. PCR-RFLP was found to be adequate for genotyping of the AY_731081: g.1900T>C SNP of the equine *CD14* gene. However, this polymorphism is probably not present in Mangalarga horses, thus impairing association studies using this marker in the breed. The population genetic parameters obtained for the *TLR4* AY_005808:c.1530A>G and *Cε* AX_463789:g.133T>C polymorphisms suggest the use of these markers in association studies with immune system-related traits in Mangalarga horses.

Keywords: horse, *CD14*, *Cε*, SNP, *TLR4*

RESUMO

Os objetivos deste trabalho foram a padronização da metodologia PCR-RFLP para genotipagem do SNP AY_731081:g.1900T>C do gene *CD14* equino, bem como a caracterização em equinos da raça brasileira Mangalarga deste e de outros dois polimorfismos, o AY_005808: c.1530A>G do *TLR4* e o AX_463789: g.133T>C do *Cε*, a fim de promover o embasamento necessário para futuras pesquisas visando à associação entre marcadores de DNA e características relacionadas à fisiologia do sistema imune na raça. Para tanto, foram utilizados 151 animais Mangalarga, de ambos os sexos e de idades variadas, representativos da população do estado de São Paulo. O método de PCR-RFLP mostrou-se adequado para a genotipagem do SNP AY_731081: g.1900T>C do gene *CD14* equino. Entretanto, tal polimorfismo provavelmente não ocorre em equinos Mangalarga, impossibilitando estudos de associação com o marcador na raça. Os parâmetros genético-populacionais obtidos para os polimorfismos AY_005808:c.1530A>G do gene *TLR4* e o AX_463789:g.133T>C do gene *Cε* demonstraram a possibilidade de realização de pesquisas.

Palavras-chave: cavalo, *CD14*, *Cε*, SNP, *TLR4*

INTRODUCTION

The immune system is of vital importance for animals since it confers protection against foreign pathogens, including bacteria, viruses, parasites and toxins. In horses, immune system

disorders normally result in fatigue, frequent infections, loss of appetite, and weight loss, with consequent substantial economic losses for the production system.

The *CD14* gene product, a lipopolysaccharide receptor, plays a crucial role in the cellular

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recognition of bacterial lipopolysaccharides (Kitchens, 2000; Landmann *et al.*, 2000). The mechanism of lipopolysaccharide recognition also involves Toll-like receptors (Fujihara *et al.*, 2003), such as that encoded by the *TLR4* gene (Toll-like receptor 4). Equine immunoglobulin E (IgE), partially encoded by the *Cε* gene, resembles the immunoglobulins of other species (Navarro *et al.*, 1995) and its serum levels have been associated with specific allergic reactions (Curik *et al.*, 2003).

Vychondilova-Krenkova *et al.* (2005) identified 10 SNPs in four genes related to the immune response in horses, *CD14*, *TLR4*, *Cε* and *FcεR1 alpha*, and developed genotyping techniques to be used in association studies of gene polymorphisms with phenotypes of interest. Three non-synonymous polymorphisms are important: one in the *CD14* gene (AY_731081:g.1900T>C) genotyped by direct sequencing of the PCR products; one in the *TLR4* gene [AY_005808:c.1530A>G, identified as A1636G:AY005808 by Vychondilova-Krenkova *et al.* (2005)], and one in the *Cε* gene (AX_463789:g.133T>C). The last two were genotyped by PCR-RFLP.

In view of the above considerations, the objectives of the present study were to standardize a PCR-RFLP genotyping method for the AY_731081:g.1900T>C SNP of the equine *CD14* gene, and to characterize this SNP and two other polymorphisms (AY_005808: c.1530A>G

of the *TLR4* gene and AX_463789: g.133T>C of the *Cε* gene) in Mangalarga horses.

MATERIAL AND METHODS

A total of 151 Mangalarga horses of both sexes and variable ages, originated from six farms located in the State of São Paulo, registered by the Brazilian Association of Mangalarga Horse Breeders (ABCCRM), were used. Whole blood samples (5mL) were collected from each animal by puncture of the left jugular vein into vacuum tubes containing EDTA in agreement with the Brazilian legislation for animal well-being (protocol No. 111/2008 approved by the Ethics Committee on Animal Experimentation – Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista, Botucatu, SP).

After the removal of red blood cells, DNA was extracted from leukocytes by a non-phenol method using digestion with proteinase K and precipitation with NaCl and alcohol (Sambrook 1989).

The polymorphisms AY_731081:g.1900T>C of the *CD14* gene, AY_005808:c.1530A>G of the *TLR4* gene, and AX_463789:g.133T>C of the *Cε* gene were genotyped by PCR-RFLP. The primers for amplification of the gene regions of interest, the restriction enzymes used for detection of the polymorphisms, and other important information are shown in Table 1.

Table 1. Candidate genes, sequence of the forward and reverse primers used for amplification of the regions of interest, PCR annealing temperature of the primers, restriction enzymes used for detection of the polymorphisms, GenBank accession number of the gene sequences, nucleotide change in the DNA sequences, amino acid substitution in the protein sequence, and location of the polymorphisms in Mangalarga horses

Gene	5'- 3' primer sequence	AT (°C)	Enzyme (manufacturer)	GenBank number	Nucleotide change	Amino acid substitution	Gene region
<i>CD14</i>	F - CCCAGCGCACTGAGTTCT R - CTGATATCCCCACGGTCAG	64	<i>AciI</i> (New England Biolabs, USA)	AY731081	g.1900T>C	V – A (conservative)	Exon 2
<i>TLR4</i>	F - GGCTCAACCATCTCTCCACCT R - CCACGGTTTACCATCCAGCAAG	64	<i>BssSI</i> (New England Biolabs, USA)	AY005808	c.1530A>G	M – V (conservative)	Exon 3
<i>Cε</i>	F - GTCTCCAAGCAAGCCCCATTA R - TTTACCAGGGTCTTTGGACACCTC	68	<i>Eco47I</i> (Fermentas, USA)	AX463789	g.133T>C	S – P (non-conservative)	Exon 1

F: forward; R: reverse; AT: annealing temperature.

For analysis of the AY_731081:g.1900T>C SNP of the *CD14* gene, a 259 base pairs (bp) fragment containing the sequence of exon 2 of the gene

was amplified using a primer pair generated with the Primer 3 program (Rozen and Skaletsky, 2000). Each reaction mixture contained 50ng

genomic DNA, 0.24 μ M of each primer, 10mM Tris-HCl, pH 8.0, 50mM KCl, 1.2mM MgCl₂, 0.32mM of each dNTP, and 0.75U *Taq* DNA polymerase (Invitrogen, USA) in a final volume of 25 μ L. After initial denaturation at 94°C for 5 min, amplification was carried out in 36 cycles at 94°C for 1min, 64°C for 30s and 72°C for 1min, followed by a final extension at 72°C for 10min. The amplified fragments were digested in reaction medium containing 7 μ L of the PCR product and 10U of the restriction enzyme *Aci*I (New England Biolabs, USA) at 37°C for 16h. For analysis of the AY_005808:c.1530A>G polymorphism of the *TLR4* gene and of the AX_463789:g.133T>C polymorphism of the *C ϵ* gene, a fragment of 2172 bp containing the sequence of exon 3 of the *TLR4* gene and a fragment of 1601 bp containing the sequence of exon 1 of the *C ϵ* gene were amplified as described by Vychondilova-Krenkova et al. (2005).

After digestion of the amplified products, the DNA fragments of the *CD14* gene were separated in 3% high-resolution agarose gels. The fragments resulting from digestion of the amplicons of the *TLR4* and *C ϵ* genes were separated by 3% conventional agarose gel electrophoresis. A 100-bp molecular weight standard was used in each gel to calculate the size of the amplified and digested fragments. The bands were visualized by staining with ethidium bromide under ultraviolet light. The genotypes of the individuals were determined for each polymorphism by analysis of the size of the fragments in bp.

PCR products from 10 animals were submitted to direct sequencing to confirm the genotyping efficiency of the equine *CD14* AY_731081:g.1900T>C polymorphism by PCR-RFLP.

Allele and genotype frequencies, observed and expected heterozygosity and the Hardy-Weinberg equilibrium at each polymorphic site analyzed were calculated for the population

studied using the PopGene 1.32 program (Yeh et al., 1999). Selective neutrality was evaluated by the Ewens-Watterson *F* test using the Popgene 1.32 and PyPop programs (Lancaster et al., 2007). Analysis with the Popgene 1.32 program established a confidence interval for *F* and the PyPop program was used to determine the significance of the normalized deviation between expected and observed *F* using Slatkin's exact test (Slatkin, 1996).

RESULTS AND DISCUSSION

Only the allele C of the AY_731081:g.1900T>C polymorphism of the equine *CD14* gene was detected in the sample of animals studied. Thus, only individuals carrying genotype CC were found (Table 2). Genotype CC was characterized by the presence of three fragments of 116, 105, and 38bp (Figure 1). According to the restriction map of the amplified fragment, genotype TT would be characterized by the presence of two fragments of 143 and 116 bp and the heterozygous genotype (CT) by the presence of four fragments corresponding to the combination of the two homozygous patterns. The fixation of the allele C of the equine *CD14* gene polymorphism in the sample studied disagrees with the findings of Vychodilova-Krenkova et al. (2005) who observed a low frequency of allele T (0.14) in a sample of seven horses of unspecified breed. This difference between results might be explained by the probable absence of the *CD14* gene polymorphism in Mangalarga horses. One possible explanation is that the founding populations of this breed were homozygous at this locus, or fixation of allele C may have occurred later as a result of indirect selection. However, the possibility of a rare occurrence of allele T in the breed cannot be ruled out and studies involving larger samples are necessary to confirm this possibility. In view of the absence of the *CD14* polymorphism in the sample studied, it was not possible to estimate population genetic parameters as proposed in the section Material and Methods.

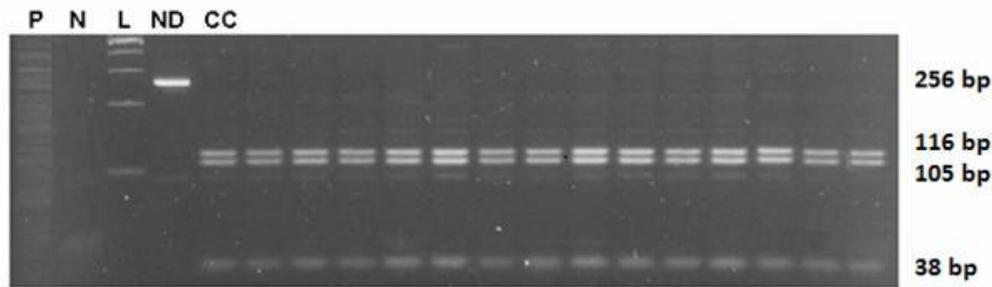


Figure 1. Mangalarga horse. Band pattern of the AY_731081:g.1900T>C polymorphism of the equine *CD14* gene obtained by PCR-RFLP and 3% high-resolution agarose gel electrophoresis. Lane P: digestion of viral DNA with *AciI*; lane N: negative control; lane L: 100-bp molecular weight standard; lane ND: amplified DNA not digested with *AciI* (259 bp); lane CC: genotype resulting from digestion of the amplified products with *AciI*. The numbers on the right indicate the size of the DNA fragments in base pairs.

In humans, the C159T SNP of the *CD14* gene has been associated with asthma and IgE levels in populations from northern and northwestern India (Sharma *et al.*, 2004). Recently, Faria *et al.* (2009) investigated the association of this polymorphism with the severity of lung disease in cystic fibrosis. The authors reported that genotype TT does not affect the severity of the disease but seems to be a risk factor for pulmonary symptoms. According to Vychodilova-Krenkova *et al.* (2005), the AY_731081:g.1900T>C SNP of the equine *CD14* gene is potentially relevant for physiological changes in the immune system, since it is a non-synonymous polymorphism that gives rise to a valine-to-alanine substitution in the protein sequence encoded by exon 2 of the gene. However, the probable absence of the *CD14* SNP in Mangalarga horses does not permit association studies of the marker with immune system-related traits in the breed. Thus, the study of the segregation of other polymorphisms described for this gene or the identification of new polymorphisms in the DNA of the breed is necessary. In this respect, it is known that polymorphisms that segregate in certain breeds do not segregate in others. This fact supports the need for and importance of studies investigating allele and genotype frequencies of DNA polymorphisms in different breeds of species of interest, including horses, prior to association studies of molecular markers with important traits, in order to prevent wasting resources by analyzing phenotypes that cannot be compared with specific DNA variations.

Direct sequencing of the amplified fragment of the *CD14* gene from 10 animals confirmed the

genotypes detected by PCR-RFLP. All individuals sequenced presented the same sequence in the region studied, i.e., a cytosine (C) at position 1900, considering the GenBank sequence AY731081 as a reference. In this respect, the PCR-RFLP method proposed in this study was found to be robust, of low cost and appropriate for laboratories with a basic structure of equipment and reagents, thus permitting further analysis of this polymorphism in the breeds in which it occurs.

Regarding the AY_005808:c.1530A>G polymorphism of the equine *TLR4* gene, alleles A and G were detected in the sample studied, with allele G predominating over allele A. Genotypes AG and GG presented similar frequencies and were more prevalent than genotype AA (Table 2). The allele frequencies of the equine *TLR4* gene polymorphism obtained for the sample studied were different from those reported by Vychodilova-Krenkova *et al.* (2005) for horses of unspecified breed (0.44 for allele G and 0.56 for allele A). Similarly, the genotype frequencies differed from those reported by these authors (0.375 for genotype AA, 0.375 for genotype AG, and 0.25 for genotype GG). These differences in allele and genotype frequencies might be due to the small sample size (eight animals) and the different breed studied by Vychodilova-Krenkova *et al.* (2005). On the other hand, discrepant allele frequencies for polymorphisms of the candidate gene may indicate that these polymorphisms are related to differences in phenotypes between breeds. In contrast to indirect selection, it may only represent effects of genetic drift.

Table 2. Allele and genotype frequencies of the AY_731081:g.1900T>C polymorphism of the *CD14* gene, AY_005808:c.1530A>G polymorphism of the *TLR4* gene and AX_463789:g.133T>C polymorphism of the *Cε* gene in a sample representative of the Mangalarga horse population from the state of São Paulo

Polymorphism	Allele frequency		Genotype frequency		
	C	T	CC	CT	TT
AY_731081:g.1900T>C (<i>CD14</i>)	1.00	0.00	1.00	0.00	0.00
AY_005808:c.1530A>G (<i>TLR4</i>)	0.31	0.69	0.09	0.44	0.47
AX_463789:g.133T>C (<i>Cε</i>)	0.47	0.53	0.28	0.38	0.34

The observed heterozygosity (0.44) of the equine *TLR4* gene SNP was close to the expected value (0.43). The similarly observed heterozygosity found for the *TLR4* polymorphism compared to the expected heterozygosity may indicate the absence of selection favoring the homozygous genotypes over the heterozygous genotype. With respect to Hardy-Weinberg equilibrium, a test comparing observed and expected genotype frequencies, the calculated chi-square value (0.029) was lower than the tabulated value (3.84), with no significance at the 5% level, showing that the Mangalarga horse population studied was in equilibrium for the locus containing the *TLR4* gene polymorphism. The

result of the Ewens-Watterson test and Slatkin's test of selective neutrality obtained for the *TLR4* gene polymorphism was not significant ($p = 0.126$) at the 5% level (Table 3), indicating the lack of preferential mating or selection favoring one of the alleles. However, the values observed were borderline significant. In this respect, although the Mangalarga horse population studied is in equilibrium for the *TLR4* locus, the fact that the population genetic parameters obtained were close to significance does not definitively rule out some potential application of this marker in association studies with important traits in this breed.

Table 3. Ewens-Watterson F test^a of selective neutrality and Slatkin significance for polymorphic sites of the *TLR4* and *Cε* genes in a sample representative of the Mangalarga horse population from the state of São Paulo.

Polymorphism	N	No. of alleles	CI of F		F_{obs}	F_{exp}	F_{nd}^b	p
			Lower	Upper				
AY_005808: c.1530A>G (<i>TLR4</i>)	302	2	0.503	0.993	0.571	0.842 ± 0.167	-1.622	0.126
AX_463789: g.133T>C (<i>Cε</i>)	302	2	0.503	0.993	0.501	0.842 ± 0.167	-2.040	0.016

^a 95% confidence interval (CI) of F obtained after 1,000,000 simulations. ^b Normalized deviation between observed (F_{obs}) and expected F (F_{exp}).

Alterations in TLR signaling have been associated with the occurrence of different diseases in humans and SNPs identified in the TLR gene family are associated with these conditions (Santini *et al.*, 2008). Moura *et al.* (2008) reported an association between the A896G SNP of the human *TLR4* gene and *Helicobacter pylori* infection, and Santini *et al.* (2008) associated the T1196C polymorphism of the same gene with the development of gastric

cancer. In horses, the AY_005808:c.1530A>G polymorphism of the *TLR4* gene might play an important physiological role since it causes the substitution of methionine with valine in the protein sequence encoded by exon 3 of the gene (Vychodilova-Krenkova *et al.* 2005). Although conservative, this non-synonymous amino acid substitution may lead to changes in the mechanism of lipopolysaccharide recognition and consequently alter the immune response.

With respect to the AX_463789:g.133T>C polymorphism of the *Cε* gene, alleles C and T were detected in the sample studied, with similar frequencies. The same was observed for genotype distribution despite a slight predominance of the heterozygous genotype over the homozygous genotypes (Table 2). The allele and genotype frequencies of the equine *Cε* gene polymorphism observed for the Mangalarga population studied differed from those reported by Vychodilova-Krenkova *et al.* (2005) for horses of unspecified breed (0.13 for allele C and 0.87 for allele T; 0.26 for genotype CT and 0.74 for genotype TT). As discussed for the *TLR4* polymorphism, these divergences in allele and genotype distributions might be due to the small sample size (19 animals) and the different breed studied by Vychodilova-Krenkova *et al.* (2005). However, indirect selection for different alleles in populations or breeds used for variable purposes, as well as a founder effect or effect of genetic drift, cannot be ruled out.

The observed heterozygosity (0.38) of the equine *Cε* gene polymorphism was lower than the expected value (0.50). The lower observed heterozygosity compared to the expected heterozygosity may indicate the occurrence of selection favoring the homozygous genotypes over the heterozygous genotype. The calculated chi-square value (9.28) for deviation from Hardy-Weinberg equilibrium was significant at the 1% level, showing that the population studied was not in equilibrium for this locus. Thus, the alleles of the *Cε* polymorphism are not distributed as expected in populations that do not suffer selective pressures. The results of the Ewens-Watterson test and Slatkin's test of selective neutrality obtained for the *Cε* gene polymorphisms shown statistical significance ($P = 0.126$) at the 5% level (Table 3), suggesting that the alleles of the *Cε* locus are under selective pressure. Taken together, the population genetic parameters estimated for the polymorphism of the equine *Cε* gene demonstrate the applicability of this marker in association studies with traits of interest in Mangalarga horses.

Studies have demonstrated that alterations in the gene encoding the heavy chains of IgE may modify its function. According to Pan *et al.* (1996), polymorphisms in the human *Cε* gene can affect the expression of functional IgE in cases of chronic urticaria. Wagner *et al.* (2001)

detected polymorphisms in the equine *Cε* gene and suggested that different IgE allotypes may influence its binding to the Fcε receptors in immune system cells. Also in horses, serum IgE levels have been associated with specific allergic reactions (Curik *et al.*, 2003). The AX_463789:g.133T>C polymorphism of the equine *Cε* gene identified by Vychodilova-Krenkova *et al.* (2005) shows a high potential of physiological changes in the immune system of horses since it results in a non-conservative amino acid substitution (serine to proline) in domain I of the heavy chain of IgE.

CONCLUSIONS

The present results allow to conclude that: 1) PCR-RFLP was adequate for genotyping of the AY_731081: g.1900T>C SNP of the equine *CD14* gene. However, this polymorphism does not seem to be present in Mangalarga horses, thus impairing association studies using this marker in the breed. 2) The population genetic parameters obtained for the *TLR4* AY_005808:c.1530A>G and *Cε* AX_463789:g.133T>C polymorphisms in the sample of animals studied suggest the use of these markers in association studies with immune-system related traits in Mangalarga horses.

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