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ASSOCIATION OF TLR4 POLYMORPHISMS WITH SUBCLINICAL MASTITIS IN BRAZILIAN HOLSTEINS

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ABSTRACT

The identification of dairy cows with greater or lower potential to develop mastits has been pursued for many years among different segments of the milk industry, including governmental organizations. Genomic studies have suggested that Single Nucleotide Polymorphisms (SNPs) within the pattern recognition receptors (PRR) could lead to different responses to pathogens, and consequently result in mastitis resistance or susceptibility. To investigate whether toll like receptor 4 (TLR4) gene is associated with subclinical mastitis in Holstein cows from a property in the state of Goiás, Brazil, TaqMan allelic discrimination and somatic cell count were performed. One hundred and fifty milk samples were analyzed for SCC and centesimal composition. Twenty percent of those samples with SCC above 200,000 (n=13) were screened for real-time PCR identification of microorganisms and blood samples were genotyped for TLR4 SNPs. There was a higher prevalence of Gram-positive bacteria in the analyzed samples (88.9%) and animals that had the combined genotypes AACCCC, GGTCGG and GACCGC presented the lowest somatic cell scores, and consequently those genotypes have the potential to be applied as molecular markers for assisted animal selection to improve milk quality.

Key words: TLR-4, single nucleotide polymorphism, mastitis, milk quality

INTRODUCTION

Mastitis has been considered as the disease with greatest impact in dairy herd worldwide, due to its high prevalence and to the economic loss caused by it (4). Additionally the disease has a profound negative impact on the productivity of dairy industries as a consequence of the milk quality as well as its lower industrial yield (1). The mammary gland tissue is protected by two defense systems of the immune system: innate or nonspecific immunity and the acquired or specific immunity. Both innate and acquired immunity interact in the attempt to provide protection against mastitis causing microorganisms (2).

The innate immune response is characterized by the quick activation of the antimicrobial defense mechanisms by the host, capable of responding to a great variety of pathogens, predominant in the initial stages of infection, and is constituted by the physical barrier of the teat's sphincters; macrophages, neutrophils, NK (natural killer) cells, and unspecific soluble factors. The start of the induced innate immune response relies

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on the recognition of the pathogen by the host cells through pattern recognition receptors (PRR). Macrophages as well as epithelial cells have receptors capable of discriminating microorganisms groups by means of interacting with molecular structures evolutionary conserved denominated pathogen associated molecular patterns (PAMPs). PRRs induce the transcription and secretion of cytokines and chemokines, responsible for the early neutrophil and monocytes blood recruitment to the infection site (12).

Some of these recognition receptors, denominated toll-like receptors (TLR), together with interleukin-1 (IL-1) receptor comprise the IL-1R family. These evolutionary conserved receptors recognize a great variety of PAMPs and consequently contribute directly to the inflammatory response (6). For example, TLR-4 is able to recognize Gram negative bacteria lipopolyssacharide (endotoxin), cell wall components of other important bacteria and fungi such as *Mycobacterium tuberculosis, Aspergillus fumigatus, Cryptococcus neoformans* e *Candida albicans,* as well as cellular stress components, such as heat shock proteins (HSP), fibrinogen, among others (8).

The TLR4 is polymorphic among bovine species and its expression has been shown to be associated with intramammary infection (12). This observation has raised the possibility of testing this receptor gene as a candidate of molecular marker for mastitis resistance and consequently to be used in an assisted selection of dairy herds. The aim of the present study was to identify Single Nucleotide Polymorphisms (SNPs) within TLR4 gene in Holstein dairy caws from a dairy farm in the state of Goias, Brazil, and to correlate their frequency with sub clinic mastitis, somatic cell counts (SCC) and milk quality.

MATERIALS AND METHODS

Farm selection and Sampling

Rural property selection was done considering the following criteria: a) milk production from pure Holstein breed animals; b) to have animal breeding records (birth date, animal birth order, and lactation period in days); and c) to have record of historical SCCs (somatic cell score) analyses from individual animal milk. The month of April of 2010 was selected for sample collection, due to some particular characteristics that provide great challenge to the animals such as maximum soil water retention (100 mm approximately), milk production decline of about 3% due to climate change, average temperatures from 15 to 31ºC, and relative humidity of 60 to 70% (5).

Milk samples from 150 cows were collected from a rural property in the State of Goiás, Brazil. Phenotypic data records, SCC and centesimal raw milk composition were evaluated due to lactation period and Holstein breed milk standards (15) to select animals for SNP analysis in blood samples, resulting in 69 samples.

Individual milk samples from the cows were representative of a 24 hour milking period. Milk samples were kept with Bronopol (Chemical Land®) and submitted to SCC and centesimal composition by flow cytometry and near infrared spectroscopy (9).

Blood collection for SNPs analysis was performed by puncture of the coccigean vein in vacuum tubes containing 10% EDTA. Immediately following blood collection, samples were homogenized and kept refrigerated and sent to the molecular biology lab from Centro de Pesquisa em Alimentos da Escola de Veterinária da Universidade Federal de Goiás.

Detection of mastitis causing agents by Real Time PCR

Raw milk samples from 150 animals were submitted to SCC and twenty percent (n=13) of the analyzed raw milk samples that presented SCC above 200,000 cs/mL (n=65) were submitted to DNA extraction and identification of the most frequent mastitis causing microorganisms by real time PCR according to the Pathoproof™ Mastitis PCR Assay F-870-L (Finnzymes Diagnostics®) protocol.

SNPs genotyping

Blood DNA extraction was performed from the buffy coat

(13) using the High Pure PCR Template Preparation kit (Roche Diagnostics®).

Allelic discrimination was performed with TaqMan (Applied Biosystems®) assay based on three published SNPs deposited at the National Center for Biotechnology Information (NCBI). Among *Bos taurus taurus*, the locus 1 (Genbank accession number rs8193046) present the allelic variation A/G and is localized in intron 1 of the TLR4. Locus 2 (Genbank accessioon numer rs8193060) present allelic variation C/T and is localized in exon 3, coding for a serine amino acid. Locus 3 (Genbank accession number rs29017188) present allelic variation C/G and is localized in the 5'UTR region. Primers and probes used for genotyping were designed with the Primer Express software (Applied Biosystems®) based on the *Bos taurus* TLR4 reference sequence (GenBank accession number DQ839567.1) (Table 1).

Table 1. Sequence of primers and Major Groove Binding (MGB) probes used for allelic discrimination

Locus	Primer/Probe name	Sequence 5'→ 3'	Amplicon size	Gene position
1	TLR4 F1	GAGAGGAGAGTTGCTTGGAAGTCT	107bp	5039-5062
	TLR4 R1	GCTCCATGCACTGGTAACTAATGT		5145-5122
	TLR4 P1	FAM-CAGGAAGACACCGCA–MGB		5076-5090
	TLR4 P1.1	VIC-AAGACACCACATCTAA-MGB		5080-5095
2	TLR4 F3	CCACTCGCTCCGGATCCT	79 bp	9396-9413
	TLR4 R3	CCTTGGCAAATTCTGTAGTTCTTG		9474-9451
	TLR4 P3	FAM-ACTGCAGTTTCAACCGTATC-MGB		9416-9435
	TLR4 P3.1	VIC-ACTGCAGCTTCAACCGTA-MGB		9416-9433
3	TLR4 F4	CCAGCTTCCTCTTGTTGTTACTTCA	150 bp	173-197
	TLR4 R4	CGGGAGGAGAGGAAGTGAGA		322-303
	TLR4 P4	FAM-TATTTATCTCCTCTGCCACCGGA-MGB		265-243
	TLR4 P4.1	VIC-TTATCTCCTCTGCCACCCGAG-MGB		262-242

Statistical analysis

Allelic and genotypic frequencies for each locus and combined genotypes were calculated using the POPGENE software version 1.32 (17) and χ^2 test was used to detect Hardy-Weinberg equilibrium. SCC does not present normal distribution and in order to confer linearity to the results and to adjust the day sampling to the lactation period, they were transformed to somatic cell score (SCS) according to the formula: $SCS = \log_2[SCC/100000)+3]$; where the SCC value represent somatic cells per microliter (cs/μL) (16). Analysis of variance and Duncan test were applied to detect significance differences between SCS of SNP combined genotypes of the TLR4 gene using R software (10).

RESULTS AND DISCUSSION

Genotype patterns of different polymorphisms in Brazilian Holstein dairy cattle

The allele and genotypic frequencies and the Chi-square test to test for the *Hardy-Weinberg* equilibrium are presented in Table 2. The heterozygous genotype GA presented the most prevalent among the analyzed animals for locus 1 (47.8%), while the homozygous genotypes AA (30.4%) and GG (21.8%) where less prevalent. The frequencies observed for the allele A and G of this locus where 53.5% and 46.5%, respectively. The analysis of locus 2 revealed that the homozygous genotype CC where the most frequent (43.4%), followed by the heterozygous genotype TC (42.0%) and homozygous genotype TT (14.6%), with frequencies of 53.5% for allele C and 46.5% for allele T.

The heterozygous genotype GC was the most frequent observed (49.2%) in locus 3, whereas the homozygous genotypes CC and GG presented frequencies of 30.4% and 20.4%, respectively and the frequency for allele C for this

locus was 53.5% and for allele G was 46.5%.

Our results relative to allelic and genotypic frequencies observed for SNPs at locus 1 are similar to those described by Wang et al (14) that reported a frequency of 54% for allele A and 46% for allele G. Those authors also reported a frequency of 30% for genotype AA, 47% for genotype GA and 23% for genotype GG in Holstein cows in China. The statistical analyses by the non-parametric Chi-square test revealed that the population is in *Hardy-Weinberg* in all three loci (P>0.05), indicating that the allelic and genotypic frequencies will remain constant in the following generations.

Table 2. Genotypic and allelic frequencies (%) and X^2 test value to determine *Hardy-Weinberg* equilibrium

Locus	Genotype	Nº of animals	Genotypic frequency	Allele	Allelic frequency	X^2 (P)
1 (rs8193946 A>G)	AA	21	30.4	A	53.5	0.24 (0.48)
	GA	33	47.8	G	46.5	
	GG	15	21.8			
2 (rs8193060 C>T)	CC	30	43.4	C	53.5	0.24 (0.61)
	TC	29	42.0	T	46.5	
	TT	10	14.6			
3 (rs29017188 C>G)	CC	21	30.4	C	53.5	0.24 (0.61)
	GC	34	49.2	G	46.5	
	GG	14	20.4			

Association of single SNP and somatic cell score

As shown in Table 3, genotype AA at locus 1, CC at locus 3 and CC at locus 2 had the lowest SCS mean, suggesting that the alleles A and C could be associated with genotypes of lower SCS. On the other hand the genotypes GG at locus 1, TT at locus 2 and GG at locus 3 had the highest SCS mean, what could be related to alleles T and G be associated with highest SCS, and consequently to a higher susceptibility to mastitis.

Similar to our findings, Wang et al. (14) reported the presence of the polymorphism at intron 1 of TLR4 (A/G) using PCR as well as Single-Strand Conformational Polymorphism (SSCP) techniques. Additionally, that group also observed an association between homozygous genotype AA with a lower SCS, relative to homozygous animals with allele G. Those results reinforce the idea that allele A could be strongly associated with mastitis resistance.

Table 3. Somatic cell score (SCS) mean among different genotype of Holstein cows of Goiás, Brazil (n=69)

Locus	Genotype	SCS mean
1 (rs8193946 A>G)	AA	2.4
	GA	2.7
	GG	3.6
2 (rs8193060 C>T)	CC	2.6
	TC	2.8
	TT	3.5
3 (rs29017188 C>G)	CC	2.4
	GC	2.8
	GG	3.5

Association between combined genotypes of SNPs and somatic cell

Analysis of the combined genotypes allowed us to further investigate the possible association of SNPs with SCS. Nine kinds of combined genotypes of three SNPs combination (rs8193946 A>G, rs8193060 C>T and rs29017188 C>G) were observed in 69 cows (Table 4).

As shown in Table 4, there was a significant association

between the combined genotypes 5 and 6 with higher SCS ($P<0.05$). The combined genotypes 2, 1 and 8 where more associated with lower SCS, which lead us to suggest that those genotypes have a potential to be used as genetic marker in assisted selection of animals with lower SCS and consequently higher milk quality. Although we should not dissociate this analysis from phenotypic characteristics that are result of a sum of genetic and environmental factors that determine the phenotypic variability of the dairy herd (3).

Table 4. Effect of combinations of three SNPs (rs8193946 A>G, rs8193060 C>T and rs29017188 C>G) on SCS of Brazilian Holstein dairy cattle (n=69) according to Duncan test*

Order of genotype	Genotype combination	Number of combinations	SCS mean	Standard error of the means
5	GGCCGC	1	5.3[a]	-
6	GGCCGG	1	5.0[ab]	-
4	GATCGG	1	3.9[abc]	-
7	GGTCGC	1	3.7[abc]	-
9	GGTTGG	10	3.5[abc]	0.51
3	GATCGC	25	2.72[bc]	0.21
2	AACCCC	21	2.39[c]	0.15
8	GACCGC	7	2.57[c]	0.21
1	GGTCGG	2	2.5[c]	0.70

* Mean scores followed by the same letter do not differ statistically ($P < 0.05$).

Identification of the most prevalent mastitis causing microorganisms among the animals with higher SCS

Sixty-five milk samples (43.3%) presented more than 200.000 cells/mL and twenty percent (n=13) were submitted quantitative real time PCR for the presence of the most common mastitis causing bacteria. It was observed a higher frequency of Gram-positive bacteria (88,9%): *Streptococcus uberis* (18,52%) *Streprotoccus dysgalactiae* (14,51%), *Staphylococcus* spp. (14,51%,), *Staphylococcus aureus* (11,11%), *Arcanobacterium pyogenes* (11,11%), *Enterococcus* sp. (7,41%), *Corynebacterium bovis* (7,41%) and *Streptococcus agalactiae* (3,7%). Among the Gram-negative, it was present DNA from *Escherichia coli* (3,7%), *Klebsiella oxytoca/K.pneumoniae* (3,7%) and *Serratia marcescens* (3,7%). *Staphylococcus* spp. (14,51%) and *Staphylococcus aureus* (11,11%) were the most prevalent Gram positive bacteria and a direct association between this coccos and bovine contagious or chronic mastitis was established previously (7), and sometimes the presence of *Staphylococcus spp* on the crude milk is enough to diagnostic intra-mammary infection. However, a clear association between *Staphylococcus* spp or other bacteria and SNP on the TLR gene is not known. Further study is needed to clarify the role of the genetic variants of TLR4 gene, and to analyze the mRNA expression levels of TLR4 gene.

In conclusion it was showed that animals that had the combined genotypes AACCCC, GGTCGG and GACCGC presented the lowest somatic cell scores, and consequently have the potential to be applied as molecular markers for assisted animal selection to improve milk quality.

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