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Asociación de los alelos BoLA-DRB3 y TLR4 con mastitis subclínica en vacas de Colombia

Associação de Bola-DRB3 alelos e TLR4 com mastite subclínica em vacas da Colômbia

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Summary

Background: molecular markers for genetic resistance can be used to control mastitis in dairy cattle. The Major Histocompatibility Complex and the Toll-like receptor 4 (*TLR4*) are two promising genes that warrant investigation. **Objective:** to identify associations between genotypes of BoLA-DRB3 locus and T4CRBR2 fragment and subclinical mastitis (SM). **Methods:** 996 lactating cows from 32 herds comprising Holstein (80%), Holstein x Jersey cross (12.5%), and other crosses (7.5%) were evaluated monthly during two years, diagnosed for SM and genotyped for the second exon of BoLA DRB3 and the *TLR4* coreceptor-binding region 2 (T4CRBR2) using a Polymerase chain reaction-restriction fragment length polymorphism technique (PCR-RFLP). The association between candidate alleles and subclinical mastitis was measured by logistic regression. **Results:** the most frequently observed alleles for BoLA-DRB3 were *DRB3.2 *8*, **22*, **24*, **16*, **10*, **23*, **gba*, **11*, **2*, **mbb*, **jba*, **3*, and **15*, accounting for 58.9% of the population. Frequencies for T4CRBR2 alleles *A* and *B* were 0.352 and 0.647, respectively. Based on 57,408 observations during the period, the mean SM prevalence was 16.2% (95% CI 13.0 and 19.4) per udder quarter and 37.6% (95% CI 32.1 and 43.2) per cow. The predominant microorganisms isolated from SM quarters were *Streptococcus agalactiae* and Coagulase-Negative Staphylococci (CNS). Allele *DRB3.2 *23* was associated with SM occurrence and CNS infection. No alleles were associated with *Streptococcus agalactiae* infection. Allele **mbb* was associated with occurrence of CNS infection and alleles **jba* and **15* were associated with resistance to CNS infection.

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No significant relationship between T4CRBR2 and SM was observed. **Conclusion:** DRB3.2 gen may play an important role in the occurrence of SM and certain alleles may confer resistance to specific pathogens.

Key words: *genetic markers, mammary gland, marker-assisted selection.*

Resumen

Antecedentes: los marcadores moleculares genéticos de resistencia para mastitis bovina son una herramienta para el control de la enfermedad en rebaños lecheros. Los genes del Complejo Mayor de Histocompatibilidad y el Receptor tipo Toll 4 (*TLR4*) son dos genes candidatos promisorios que justifica investigar. **Objetivo:** identificar asociaciones entre los genotipos del locus BoLA-DRB3 y del fragmento T4CRBR2 con la ocurrencia de mastitis subclínica. **Métodos:** 996 vacas lactantes de 32 hatos de las razas Holstein (80%), Holstein x Jersey (12,5%) y otros cruces (7,5%), fueron visitadas mensualmente por dos años, diagnosticadas para mastitis subclínica y genotipificadas para el segundo exón del DRB3 y para la región 2 de unión al correceptor del TLR4 (T4CRBR2) por medio de las técnicas de Reacción en cadena de la polimerasa y de Longitud del polimorfismo del fragmento de restricción (PCR-RFLP). La asociación entre los alelos candidatos y la mastitis subclínica se midió por regresión logística. **Resultados:** los alelos más frecuentes para el DRB3.2 fueron **8*, **22*, **24*, **16*, **10*, **23*, **gba*, **11*, **2*, **mbb*, **jba*, **3* y **15*, que suman el 58,9% del total en la población. Las frecuencias para los alelos A y B del T4CRBR2 fueron de 0,352 y 0,647, respectivamente. Basados en 57.408 observaciones, la prevalencia de MS a nivel de cuarto fue 16,2% (95% IC 13,0 y 19,4) y a nivel de vaca fue de 37,6% (95% IC 32,1 y 43,2). Los microorganismos más frecuentes fueron *Streptococcus agalactiae* y Estafilococo Coagulasa Negativo (ECN). El alelo DRB3.2 **23* fue el más asociado con la ocurrencia de MS y con la infección por ECN. No se hallaron alelos asociados a infección con mastitis por *Streptococcus agalactiae*. Con respecto a la infección por ECN, el **mbb* se asoció con la ocurrencia y los alelos **jba* y **15* se asociaron con resistencia. No se observó asociación entre T4CRBR2 y MS. **Conclusión:** el gen DRB3.2 puede jugar un papel importante en la presencia de MS y ciertos alelos pueden conferir resistencia a patógenos específicos.

Palabras clave: *glándula mamaria, marcadores genéticos, selección asistida por marcadores.*

Resumo

Antecedentes: o uso de marcadores moleculares de resistência para mastites permite controlar esta doença em rebanhos leiteiros. Os genes Toll Like Receptor 4 (TLR4) e o Complexo Mayor de Histocompatibilidade são dois genes candidatos promissórios que justifica pesquisar. Objetivo: identificar associações entre genótipos do locus BoLA-DRB3 e do fragmento T4CRBR2 com a ocorrência de mastite subclínica. **Método:** 996 vacas em lactação de 32 rebanhos da raça Holandesa (80%), Holandesa x Jersey (12,5%) e outras cruzas (7,5%) foram visitadas mensalmente por dois anos, diagnosticadas para mastites subclínica e genotipadas para o exon segunda BoLA DRB3 e região 2 da ligação co-receptor TLR4 (T4CRBR2) através das técnicas de Reação em Cadeia da Polimerase e do Polimorfismo de Comprimento do Fragmento de Restrição (PCR-RFLP). A associação entre alelos candidatos e mastite subclínica foi realizada por meio de regressão logística. **Resultados:** os alelos mais frequentes **8, *22, *24, *16, *10, *23, *gba, *11, *2, *mbb, *jba*, **3 e *15*, com um 58,9% do total da população. As frequências dos alelos *A* e *B* do T4CRBR2 foram 0,352 e 0,647, respectivamente. Com base em 57.408 observações, a prevalência da SM em quartos mamários foi de 16,2% ((IC 95% 13,0 e 19,4) e ao nível de vaca foi de 37,6% (IC 95% 32,1 e 43,2). Os microrganismos mais comuns foram: *Streptococcus agalactiae* e Estafilococos Coagulase-negativo, ECN. O alelo DRB3.2 **23* foi o mais associado com a ocorrência de SM e com a infecção por ECN. Não foram encontrados alelos associados à infecção por *Streptococcus agalactiae.* Em relação à infecção por ECN, o **mbb* esteve associado com ocorrência e os alelos **jba* e **15* estiveram associados com resistência. Não existiu associação entre MS e os alelos do T4CRBR2. **Conclusão:** o gene DRB3.2 bovino pode desempenhar um papel importante na presencia de MS e alguns alelos podem conferir resistência à patógenos específicos.

Palavras chave: *glândula mamaria, marcadores genéticos, seleção assistida por marcadores.*

Introduction

Despite efforts to control and prevent mastitis through udder health programs, mastitis remains the dairy industry's costliest disease (Francoz *et al*., 2012). Reducing mastitis losses is necessary to keep milk producers economically competitive and maintain costs of dairy products within reasonable limits. Current methods to reduce mastitis incidence, such as therapeutic and prophylactic measures, are not totally effective. Another approach could be to improve cows' natural genetic-resistance to udder pathogens (Detilleux, 2002).

Interest in genetic markers for disease resistance has increased, particularly in the Major Histocompatility Complex (MHC) genes, also known as bovine leukocyte antigen (BoLA), which has been widely studied. This complex has been mapped to chromosome 23 and consists of classes I, II and III, which span about 2.5 Mbp (Andersson and Davies, 1994). Most studies associating the occurrence of infectious diseases in cattle with gene candidates have focused on gene DRB3 of the BoLA IIa region which encodes for the antigen binding groove of the molecule and is highly polymorphic (Xu *et al*., 1993; Dietz *et al*., 1997a; Dietz *et al*., 1997b; Duangjinda *et al*., 2009). Although breeding programs would benefit from selecting for alleles associated with resistance (or host defense mechanisms) to multiple pathogens, conflicting results have been reported on whether specific alleles offer protection or susceptibility to mastitis. Consequently, further studies are warranted to clarify the role of DRB3 alleles in SM.

Other candidate genes with key roles in immune cells are the Toll-like receptor (TLR) family, which mediate recognition of numerous microbial components (Taro and Akira, 2005). Multiple TLRs detect several features of a microbe simultaneously and in particular, TLR4 has been characterized for detecting the structure of the Gram-negative bacterial lipopolysaccharide (Underhill and Ozinsky, 2002). The TLR4 gene encodes for a protein of 841 amino acids, of which two domains are for the putative co-receptor-binding regions 1 and 2 (T4CRBR1 and T4CRBR2). The genetic polymorphism of these regions has been characterized by PCR-Single-strand conformation polymorphism and PCR-RFLP (Wang *et al.*, 2007). Interestingly, mastitis in cows strongly increases mRNA expression of TRL4 (Goldammer *et al*., 2004), but whether that improves pathogen perception and signaling still remains to be elucidated.

The objective of this study was to identify associations between the most frequent genotypes of BoLA-DRB3 locus and fragment T4CRBR2 in cows with the occurrence of subclinical mastitis.

Materials and methods

Ethical considerations

This study was approved by the Ethics Committee for Animal Experimentation of the University of Antioquia (Act number 48, December 12, 2008).

Herd selection

A convenience sample of 32 herds was selected from the 3,049 registered dairy farms from the six municipalities that comprise the specialized dairy production area in the high plains of northern Antioquia, Colombia (Entrerrios, Belmira, Santa Rosa de Osos, San Jose de la Montaña, San Pedro de los Milagros, and Donmatias). Within each municipality, herds were selected based on the following typical management conditions in the region, including herd size, type of milking system, nutrition program, and breed. Other aspects taken in to account when selecting the herds were easy road access, on-farm cooling tanks, proper cow identification, and owner's willingness to collaborate with sample collection. All cows in each selected herd were included in the study.

Visit protocol and sample collection

Farms were visited each month for 2 years (January 2009 to December 2010) for collection of milk samples during the evening milking. A California Mastitis Test (CMT) was performed on all milking cows that were more than 4 days postpartum. Udder and teats were prepared by the farmer using the normal routine. Subsequently,

the first streams of milk were discarded before collection. CMT was performed as instructed by the manufacturer. When a positive or suspect CMT resulted, the teat ends were disinfected with cotton swabs drenched in 70% alcohol, and duplicate samples (approximately 5 ml each) were collected from every quarter and later submitted for a somatic cell count (SCC) using a DeLaval Cell Counter (DCC) (DeLaval Stockholm, Sweden). Any sample containing SCC≥200,000 cells/mL was submitted for bacterial culture. The SCC test and bacterial cultures were performed at the Microbiology Laboratory of the Veterinary Medicine School of the University of Antioquia.

Bacteriological culture

Samples were analyzed using standard laboratory methods for microbiological analysis (MacFaddin, 2000; Winn *et al*., 2006). Growth of three or more bacterial species was considered contaminated and discarded from the analysis (Parker *et al*., 2008). Performed tests were intended to identify the following microorganisms: *Staphylococcus aureus,* Coagulase-negative *Staphylococci* (CNS)*, Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae, Streptococcus pyogenes, Corynebacterium spp., Escherichia coli, Enterobacter aerogenes, Klebsiella spp., Pseudomonas spp*., and yeast (*Candida spp*.).

Case definition

An udder quarter was considered affected by SM if it had a trace CMT score or higher and a subsequent SCC ≥200,000 cells/mL. A cow was classified as having SM if one or more quarters were affected by SM.

For pathogen prevalence, a quarter was considered infected if it was diagnosed as having SM and either one or two pathogens were isolated from the milk sample. Quarters with negative CMT score were not sampled and hence were considered as non-infected in the analysis. A cow was considered infected if one or more quarters were infected. Only *Streptococcus agalactiae* and CNS infections were analyzed in detail.

Blood collection and DNA extraction

EDTA vacutainer tubes were filled with 10 mL blood collected from the tail veins. Genomic DNA was isolated from white blood cells using the salting-out method as previously described (Miller et al., 1988).

Amplification of gene DRB3.2

Amplification of gen RB3.2 (GenBank accession No. 282530) was performed by Nested polymerase chain reaction (PCR) with two sequential reactions, the first round using primers HLO30 (5´-ATC CTC TCT CTG CAG CAC ATT TCC-3´) and HL031 (5´TTT AAA TTC GCG CTC ACC TCG CCG CT 3´), followed by a second reaction using primers HLO30 (5´-ATC CTC TCT CTG CAG CAC ATT TCC-3´) and HLO32 (5´-TCG CCG CTG CAC AGT GAA ACT CTC-3´), as suggested by Gilliespie *et al.* (1999).

The first reaction was carried in 10 µL total volume containing 50 ng genomic DNA, 1X PCR buffer (10 mM Tris-HCL pH 9.0; 50 mM KCl; 0.1% Triton® X-100), $MgCl_2$ (2.5 mM), dNTPs (0.2 mM), 0.5 mM of each primer and 1.5 units of *Taq* DNA polymerase (Fermentas®, Thermo Scientific, Waltham, MA, USA). For the second reaction, the composition in a total of 40 µL was: 4X buffer (10 mM Tris-HCL pH 9.0; 50 mM KCl; 0.1% Triton® X-100), $MgCl_2$ (1.75 mM), dNTPs (0.25 mM), 0.5 mM for each primer and 2.0 units of *Taq* DNA Polymerase (Fermentas®, Thermo Scientific, Waltham, MA, USA), and 2 µl of the first reaction product. The reactions were performed in a thermal cycler (Bio-Rad C-1000®, Bio-Rad Laboratories, Hercules, California, USA) using the following cycling profile: initial denaturation at 94 °C for 5 min; 35 cycles at 94 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min; and a final extension at 72 °C for 7 min. The final product was a 284 bp segment that was digested separately with 3 restriction endonucleases: *RsaI*, *HaeIII* and *BstyI,* as described by Van Eijk *et al*. (1992). Digestions were performed as prescribed by the manufacturer (FastDigest®, Fermentas®, Thermo Scientific, Waltham, MA, USA). The resulting fragments were resolved in a 8% denaturating polyacrylamide gel by running at 1600/1900 V,

25/60 mA for 2.5 hours and then stained with nitric acid as described by Budowle *et al.,* (1991). The designation into different alleles was made using a reference ladder (Low Range DNA Ladder, O'gene ruler™, Fermentas®, Thermo Scientific, Waltham, MA, USA) according to the allelic nomenclature described by Van Eijk *et al.* (1992).

Amplification of gene TLR4 **(***GenBank accession No. DQ839566)*

The coreceptor-binding region 2 of the gen was amplified using the following primers: forward 5'-AGACAGCATTTCACTCCCTC-3′ and reverse -5′-ACCACCGACACACTGATGAT-3′. The PCR reactions were carried out in 20 µL total volume containing 50 ng genomic DNA, 1X buffer (10 mM Tris-HCL pH 9.0; 50 mM KCl; 0.1% Triton® X-100), $MgCl_2$ (2.0 mM), dNTPs (0.2 mM), each of the primers (0.25 µM) and 0.5 U *Taq* DNA Polimerase (Fermentas®, Thermo Scientific, Waltham, MA, USA). The reactions were conducted in a thermal cycler (Bio-Rad C-1000®, Bio-Rad Laboratories, Hercules, California, USA) using the following protocol: initial denaturation at 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 61.5 °C for 30 s, 72° C for 50 s; and a final extension at 72 °C for 6 min. The final product was a 382 pb amplicon digested with *AluI* FastDigest® enzyme as prescribed by the manufacturer (Fermentas®, Thermo Scientific, Waltham, MA, USA). The sample was then electrophoresed in agarose gel and DNA band patterns were observed under a transiluminator (UV Transilluminator UVP) and genotypes identified based on an allelic ladder (Low Range DNA Ladder, O'gene ruler™, Fermentas®, Thermo Scientific, Waltham, MA, USA).

Statistical analysis

Analysis of allele frequencies. The frequencies of each restriction product from *RsaI*, *HaeIII* y *BstYI* (*PsuI*) enzymes were calculated using PopGen32 (Yeh *et al.*, 2000). Due to the highly polymorphic nature of BoLA–DRB3.2, only alleles with >2% frequency were used for the analysis and considered as independent variables. The dependent variables were the disease state: SM, infection by *Streptococcus agalactiae,* and CNS infection. The polymorphism for the T4CRBR2 region of TLR4 gene was assessed by calculating the frequencies for the A and B alleles.

Logistic regression analysis. Data was examined for biologically implausible entries and any erroneous entries were removed. Descriptive statistics were computed for all variables of interest using standard methods. Observations were stratified by municipality and sampling weights were computed as the inverse of the probability of the herd being selected in the municipality (i.e., # of herds in municipality divided by # of herds selected in municipality). The clustering and repeated measures structure of the collected data (multiple observations from quarters clustered within cows, clustered within herds) were taken into account by using variance linearization estimation procedures with herds identified as the primary clustering unit (this is equivalent to using robust standard errors which are allowed to vary across clusters of herds) using the survey package of Stata™ (StataCorp 2011, College Station, TX, USA). The model response variables were SM, *Streptococcus agalactiae,* and CNS infection (presence or abscense). The effect of cluster was at random and each one of the risk factors (breed, month in lactation, parity, and the DRB3.2 and TLR4 alleles) were fixed effects.

The survey logistic model took the general form:

$$(Y) \sim \text{binary outcome (probability } \pi),$$

$$\text{Logit } (\pi) = \text{intercept} + ß_1 b + ß_{2\text{-}14}\text{DRB3.2} + ß_{15}\text{TLR4} + ß_{16}\, lm + ß_{17}\, p + e$$

Where Y is the outcome variable; π is the fitted probability of the outcome; $ß_1$ to $ß_{17}$ are the coefficients associated with each covariate: b is breed covariate, DRB3.2 is DRB3.2 alleles *(*8, *24, *22, *15, *3, *jba, *mbb, *11, *2, *gba, *23, *10, *16*) covariate, TLR4 is the covariate TLR4 allele (**T4CRBR2*), p is parity covariate, lm is lactation month covariate, and e is the random residual effect.

Unconditional logistic analyses between each risk factor and the outcomes were computed, and associations with $p<0.15$ were retained for consideration in multivariable models.

Multivariable logistic regression models were constructed with variables remaining in the model if they had a Wald test p≤0.05. The potential confounding effect of breed was evaluated by refitting the final models with breed omitted to see if the coefficients for other predictors changed substantially. Results from the final models are presented as odds ratios (ORs) along with their 95% confidence intervals. If OR was greater than 1, the factor was considered as a risk factor for causation of the disease. When OR was less than 1, the factor should be viewed as a sparing or a resistant factor (Martin *et al*., 1987). Model adequacy was assessed by the Hosmer-Lemeshow goodness of fit test (Hosmer and Lemeshow, 2000) for regression analysis (Vittinghoff *et al*., 2012). It should be highlighted that the Hosmer Lemeshow goodness of fit test was performed for all regression models, observing a proper adjustment for each. Analysis was done using the Stata's survey command of Stata® (StataCorp 2011, College Station, TX, USA).

Results

A total of 57,408 observations were made at the quarter level, accounting for 996 cows in 32 herds, 80.0% of which were Holsteins, 12.5% Holstein x Jersey, and 7.5% other crossbreds. The mean (±SD) of the population demographic and production parameters is shown in table 1. Mastitis prevalence at the quarter and cow levels were 16.2% (95% CI 13.0 and 19.4) and 37.6% (95% CI, 32.1 and 43.2), respectively. The most frequently isolated bacteria were *Streptococcus agalactiae* and CNS in 32.4% and 17.3% of the cases, respectively (data not shown).

Table 1. Demographic and production values for the bovine population (n = 996).

Variable	Mean (±SD)	Minimum	Maximum
Days in lactation	187.3 (127)	4	966
Parity	3.4 (2.1)	1	15
Animals per herd	34.3 (23.7)	6	136
Production (Liters/day)	18.1 (6.3)	4	50
Concentrate (Kg/day)	4.92 (2.1)	1	13

Allele frequencies of BoLA DRB3.2 genes digested with RsaI, HaeIII and BstYI

The PCR product of the *DRB3.2* locus was a 284 bp amplicon. Digestion with *RsaI* resulted in 92 genotypes and 19 alleles identified as *a-o*, *r*, *s*, *u,* and *w,* with frequencies ranging from 0.0005 for allele *r*, to 0.1898 for allele *f*. The effective allele number was 7.9 and the heterozygosity value was 0.8243.

HaeIII enzyme resulted in 26 genotypes and 8 alleles identified as *a-f, h* and *i*. Frequencies varied from very low for allele *c* (0.0040) to high for allele *a* (0.5371), with 2.74 effective number of alleles and 0.6345 heterozygocity value.

The *BstY* (*PsuI*) restriction enzyme yielded 8 genotypes with 4 of the 5 alleles (*a, b, d* and *e*). The lowest frequent was allele *d* (0.0040) and the most frequent was allele *b* (0.6662). The effective number of alleles was 1.95 and the heterozygosity value was 0.4679.

Allele frequencies of TLR4 (T4CRBR2 region)

By digestion with *AluI* enzyme, a segment of 382 bp was amplified from locus *T4CRBR2* by PCR. Two alleles *(*A* y **B)* were obtained with frequencies of 0.3522 and 0.6478, respectively. The effective number of alleles was 1.83 and heterozygosity was 0.5515. Genotypic frequencies of the *T4CRBR2* locus for each breed examined are shown in table 2.

Table 2. Genotypic frequencies for T4CRBR2 among 996 cows sampled.

Locus	Breed	Genotypic frequency						Total	%
		AA		*AB*		*BB*			
		n	%	n	%	n	%	n	%
T4CRBR2	Holstein	60	7.5	446	55.6	296	36.9	802	80.5
	Holstein x Jersey	8	6.9	64	55.2	44	37.9	116	11.6
	Crossbreed	9	11.5	37	47.4	32	41.0	78	7.8
	Total	77	7.7	547	54.9	372	37.3	996	100.0

Allele frequencies of BoLA DRB3.2

A total of 149 alleles were found from 996 cows sampled; those with frequencies below 0.02 are not reported. When alleles with frequencies below 2% were removed from the analysis of association with SM, only 381 cows remained in the regression

analysis. The distribution of those alleles with frequencies > 2% in 996 cows ranged from 0.048 to 0.123 (Table 3). In order of decreasing frequencies, the most common alleles were **8* (0.123), **22* (0.095), and **24* (0.070).

Table 3. Allelic frequency of BoLA *DRB3.2* among 996 cows sampled (only includes alleles with frequencies >2%).

Frequency range	DRB3.2 Allele (individual frequency)
>0.10	*8 (0.123)
>0.05 a 0.10	*24 (0.070), *22 (0.095).
> 0.02 a 0.05	*15 (0.022), *3 (0.025), *jba (0.027), *mbb (0.028), *11 (0.028), *2 (0.028), *gba (0.031), *23 (0.031), *10 (0.033), *16 (0.048)
<0.02	Others (136 possible alleles)

Logistic regression analysis

An initial logistic regression analysis of DRB3.2 and TLR4 (T4CRBR2) alleles and some factors that included data from all 381 cows (without the breed variable) showed no changes in OR or standard errors for genetic variables. No association between SM and breed in the final model was observed (p>0.05). Regarding months in lactation, a significant (p<0.01) linear association occurred between time in lactation and SM risk, which was significant in the third month and beyond. Similarly, parity was significantly (p<0.01) associated with increased risk of suffering SM from third parity and beyond (p<0.01). Regarding T4CRBR2 alleles, no association was found with SM, *St. agalactiae*, or CNS infection. For the *DRB3.2* alleles, only allele **23* behaved as a risk factor for SM and showed significant (p<0.05) OR of 1.46 (Table 4).

No association was found between DRB3.2 alleles and *Streptococcus agalactiae* infection in the mammary gland. The analysis of DRB3.2 alleles and some cow factors associated to the infection by CNS in the mammary gland are shown in table 5. Breed was not a significant risk factor for the occurrence of such infection (p>0.05). A significantly high infection risk was observed for parities 3 (p = 0.049), 5 (p<0.01), and 6 (p = 0.04). Months of lactation, when referenced with time between birth and first month of lactation, presented an increased risk of CNS infection at 5 or more months (p<0.05). For CNS infection the DRB3.2 genes, alleles **jba* and **15* behaved as protective factors with 0.32 (p = 0.04) and 0.48 (p<0.01) OR, respectively, and alleles **23* and **mbb* behaved as a risk factor with 2.17 (p = 0.013) and 2.43 (p = 0.016) OR, respectively.

Table 4. Logistic regression model output determining the probability of udder quarters with subclinical mastitis for selected cow variables (n = 20,909).

Variable	OR	SE	P value	95% CI
Month in lactation			<0.01[a]	
1	Referent			
2	1.15	0.11	0.16	[0.94 – 1.42]
3	1.54	0.21	<0.01	[1.17 – 2.04]
4	1.75	0.18	<0.01	[1.41 – 2.16]
5	2.08	0.28	<0.01	[1.57 – 2.74]
≥ 6	2.94	0.32	<0.01	[2.34 – 3.69]
Parity			<0.01[a]	
1	Referent			
2	1.36	0.28	0.14	0.89 – 2.08
3	2.11	0.56	<0.01	1.23 – 3.63
4	2.67	0.58	<0.01	1.70 – 4.18
5	3.94	0.82	<0.01	2.57 – 6.04
6	3.68	0.92	<0.01	2.20 – 6.16
7	3.53	0.90	<0.01	2.10 – 5.96
≥ 8	3.30	1.07	<0.01	1.69 – 6.43
Allele				
*23	1.46	0.25	0.04	1.01 – 2.09
Cons	0.035	0.06	<0.01	0.02 - 0.05

OR: odds ratio compared to referent; SE: Standard error; P value: level of significance; 95% CI: 95% Confidence interval; *Cons*: Intercept; [a]Overall P-value for categorical variable.

Table 5. Logistic regression model determining the probability for quarters to have Coagulase Negative Staphylococci infection for selected cow variables (n = 21,064).

Variable	OR	SE	P value	95% CI
Month in lactation			<0.01[a]	
1	Referent			
2	0.97	0.27	0.92	[0.54 – 1.75]
3	1.69	0.63	0.18	[0.77 – 3.67]
4	1.29	0.47	0.49	[0.61 – 2.73]
5	2.19	0.77	0.034	[1.06 – 4.51]
≥6	2.79	0.66	<0.01	[1.71 – 4.52]
Parity			<0.05[a]	
1	Referent			
2	1.01	0.36	0.97	0.48 – 2.12
3	2.07	0.73	0.049	1.00 – 4.27
4	1.76	0.56	0.08	0.91 – 3.37
5	2.66	0.76	<0.01	1.47 – 4.81
6	2.76	1.29	0.04	1.05 – 7.26
7	1.82	1.06	0.30	0.55 – 6.04
8 or more	2.68	1.80	0.15	0.67 – 10.7
Alelle				
**23*	2.17	0.63	0.013	1.19 – 3.95
**mbb*	2.43	0.84	0.016	1.19 – 4.96
**jba*	0.32	0.16	0.037	0.11 – 0.93
**15*	0.48	0.11	<0.01	0.30 – 0.79
Cons	0.00	0.00	<0.01	0.00 - 0.01

OR: odds ratio compared to referent; SE: Standard error; P value: level of significance; 95% CI: 95% Confidence interval; *Cons*: Intercept; [a]Overall P-value for categorical variable.

Discussion

This study analyzed the potential association of *TLR4* (T4CRBR2) gene and DRB3.2 alleles with the occurrence of SM, *Streptococcus agalactiae,* and CNS related infections. In terms of allelic frequencies, the results of digestion with 3 restriction endonucleases showed that *DRB3.2* locus was highly polymorphic. Results of digestion with *RsaI* endonuclease coincided with those previously assigned to letters *a*-*o*, *r*, *s*, (Van Eijk *et al.*, 1992), *u* (Gelhaus *et al.*, 1995) and *w* (Maillard *et al.*, 1999). Digestion with *HaeIII* enzyme coincided with pattern *a* to *f* described by Van Eijk *et al*. (1992), *h* (Gelhaus *et al*., 1995), and *i* (Roslin, 2002). Similar to the fragments attained with *RsaI, HaeIII* enzyme showed high heterozygosity value, implying a high degree of genetic variation in this locus. The alleles resulting from digestion with *BstY* (*PsuI*) endonuclease coincided with those reported by Van Eijk *et al*. (1992).

This study found 149 distinct DRB3.2 alleles, though frequencies below 2% were considered irrelevant for the analysis of association with SM, *S. agalactiae* and CNS infection, and therefore excluded to avoid confounding results. Demonstration of the existence of new DRB3.2 alleles should be accompanied by proper sequencing, which was beyond the scope of this study.

The number of alleles was high, but according to Lewin and Van Eijk (1994), when all restriction fragment patterns for each enzyme (RsaI, BstY1, HaeIII) are considered, a total of 2048 combination of sites are possible, and twice that number of alleles is possible if a 3 base pair deletion is included as a source of variation (Lewin and Van Eijk, 1994). The high polymorphism of *DRB3.2* found was probably accentuated by the large number of animals in the sample (n = 996) and also by the different breeds and crosses represented. The sample was mainly composed by Holstein and Holstein x Jersey crossbreds; the category "other breeds or crosses", despite being the group with the lowest number of animals, was represented by a great diversity that included Ayrshire, Swedish Red, Swiss Brown, Jersey, and several crosses of Holstein with other breeds such as Ayrshire, Angus, Blanco Orejinegro (BON), Brahman and Gir.

High polymorphisms in *DRB3.2* from cattle in other Colombian studies were reported by Zambrano *et al*. (2011), who identified 23 and 18 alleles in 66 pure Holstein and 25 BON cows, respectively. Martinez *et al*. (2005) found 35 and 11 alleles in 140 BON and 22 Brahman cows, respectively. Hernandez, (2010) reported 41 alleles in 360 cows from different breeds, mostly creole Colombian cattle; Giovambattista *et al*. (2013) reported 24 (22 reported and two new) in Hartón del Valle breed. In Mexico, high polymorphism was also reported by Fernandez *et al.* (2008) who identified 52 *DRB3.2* alleles in 98 cattle, and in Thailand Duangjinda *et al*. (2009) reported 40 different alleles in 409 Holstein x Zebu cows. In particular, the high frequency observed for allele

**23* was also reported by Zambrano *et al*. (2011) in Holstein and BON x Holstein cows from Colombia. In this study, the high frequencies of alleles *DRB3.2 *8*, **22* and **24* in Holstein cows was similar to that reported by other authors (Dietz *et al*., 1997a; Sharif *et al*., 1998; Rupp *et al.*, 2007) and by Starkenburg *et al*. (1997) who reported that alleles **8, *22,*23, *24* and **27* accounted for 66.8% of all alleles. Van Eijk *et al*. (1992) and Gelhaus *et al*. (1995) reported high frequencies for alleles **8* and **11* in Holstein cows. With regards to T4CRBR2, the predominant allele was *B* in the three cattle populations, genotypic frequency for AB was the highest in both loci, with AA being the lowest, in agreement with the findings by Wang *et al.* (2007).

Relationship between bovine subclinical mastitis, S. agalactiae and CNS infection and the polymorphisms of TLR4 (T4CRBR2) and DRB3.2 genes

In the logistic regression analysis, breed was not associated with SM, *S. agalactiae* or CNS infection. An increased risk for SM was observed as the number of months in lactation and parities increased. Number of births and stage of lactation have also been previously associated with SM, and is likely attributed to the normal tissue deterioration from manipulation and aging (Breen *et al*., 2009). No association was observed between genotypes of *T4CRBR2* and SM, *Streptococcus agalactiae*, or CNS infection in the logistic regression model. This results agree with those of Wang *et al*. (2007), who found no significant association between genotypes of locus T4CRBR2 and Somatic Cell Score, as an index for mastitis. No literature reports could be found on possible associations between *T4CRBR2* and *Streptococcus agalactiae* or CNS infection. However, the identification of TLR-4 on milk fat globule membranes suggest a direct role for the mammary gland parenchyma in pathogen detection (Reinhardt and Lippolis, 2006). It has also been proposed that TLR4 is likely involved in the signal transduction pathway that mediates the pathogenesis of *E. coli* mastitis (De Schepper *et al.*, 2008).

In this study, allele **23* was linked to SM susceptibility, which concurs with other findings that report the association of this allele to increased prevalence of SM caused by *Streptococcus dysgalactiae* (Hameed *et al*., 2008), higher SCC in cows in their third or higher parity (Dietz *et al*., 1997a) and occurrence of severe mastitis (Sharif *et al.*, 1998). By contrast, Baltian *et al.* (2012) found this allele to have a protective effect against mastitis diagnosed by SCC. The diverging results in the literature linking different *DRB3.2* alleles to resistance or susceptibility towards SM may be attributed to multiple reasons such as: differences in pathogens, genetic background, environmental factors, interactions between the former variables, and/or criteria established to diagnose mastitis (Sharif *et al*., 1998).

When regression analysis was restricted to cows infected with CNS, alleles **jba* and **15* were linked to resistance from CNS; yet, no literature reports linking these alleles to either pathogen could be found. However, Duangjinda *et al*. (2009) reported allele **15* is associated to mastitis resistance in Holstein x Zebu dairy cows. Alleles **23* and **mbb* were associated with susceptibility to CNS infection in the mammary gland. There is molecular evidence regarding the potential association between allele **23* and the occurrence of CNS-induced clinical mastitis. A significant association between the presence of glutamic acid at position β74 and occurrence of mastitis caused by *Staphylococcus spp.* with a relative risk of 11 was detected. (Sharif *et al*., 2000). No literature reports could be found linking the **mbb* allele to any mastitis causing pathogen.

We found an association between allele *DRB3.2 *23* and the risk of suffering SM. Alleles *DRB3.2 *23 and *mbb* were related to susceptibility to infection caused by CNS, and **jba* and **15* were related to resistance. DRB3.2 gen may play an important role in the occurrence of SM and certain alleles may confer resistance to specific pathogens.

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