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Association between BoLA-DRB3 and somatic cell count in Holstein cattle from Argentina

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Abstract Different studies have proved that the resistance/susceptibility to mastitis is genetically determined. The major histocompatibility complex in cows is known as bovine lymphocyte antigen (BoLA). Genes from the BoLA have been associated with the occurrence of infectious diseases such as mastitis and leukosis, especially the BoLA-DRB gene. The object of the present study was to detect associations between BoLA-DRB3 alleles and somatic cell count (SCC), as an indicator of resistance/susceptibility to mastitis in Holstein cattle ($N = 123$) from La Pampa, Argentina. Fisher's exact test and Woolf–Haldane odds ratio were applied to study the association between SCC and BoLA-DRB3 allele frequencies. Significant association was noted between BoLA-DRB3.2*23 and *27 alleles ($p < 0.05$) and protective or susceptibility effects, respectively. In addition, alleles BoLA-DRB3.2*20 and *25 exhibit suggestive association with high SCC ($p < 0.1$). These results were partially in agreement with data reported from Japanese Holstein cattle, but differed from those published by other authors. A possible explanation for the contrasting results could be that the mastitis is a multifactor disease caused by different pathogens. Moreover, most of the studies were carried out using PCR-RFLP method, which has less resolution than PCR-SBT because PCR-RFLP defined alleles included more than one sequenced alleles.

Keywords BoLA-DRB3 · Mastitis · Resistance/susceptibility · Somatic cell count

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Introduction

Mastitis is the inflammation of the mammary gland caused by microorganisms and is one of the most frequent infectious diseases in dairy cattle with important economic losses. Mastitis is a complex disease that involves three major factors: the microorganisms as the causative agent, the cow as host, and the environment which can influence both the cow and the microorganisms. Among the major microorganisms responsible for the development of mastitis can be mentioned *Streptococcus agalactiae*, *Staphylococcus aureus* and *Streptococcus dysgalactiae*. The somatic cell count (SCC) is an indication of subclinical and clinical mastitis; an elevated SCC indicates inflammation in the udder (more than 200,000 leukocytes or white blood cells per milliliter). The somatic cell score (SCS) is the $\log^2$ transformation of SCC and is optimal for the requirements of statistical procedures. The SCS and SCC presented a high correlation (ranged from 0.30 to 0.98 with a mean value of 0.70) with the development of subclinical and clinical mastitis, suggesting that both traits were partially determined by the same genes and common mechanisms of host defense [1, 2]. Usually, dairy farmers get higher milk price from dairy industry when SCC value

is lower than 250,000. Different studies have proved that the resistance/susceptibility to this condition is genetically determined [3]. Therefore, there exists a considerable interest in defining genetic and immunologic markers that can be advantageously used against the disease. In addition, lack of information concerning genetic factors related to disease resistance/susceptibility has limited the development of effective vaccines against mastitis [4].

Genetic population studies on polymorphisms from major histocompatibility complex (MHC) in human, domestic and laboratory species led to identify associations with autoimmune and infectious diseases [3, 5, 6]. In bovine MHC (named bovine lymphocyte antigen, BoLA) some polymorphisms were associated with resistance/susceptibility to infectious diseases such as mastitis [4, 7, 8], enzootic bovine leukemia [9], dermatophilosis [10], vermination [11, 12] and ticks [12–14] as well as with immune response traits [15, 16]. The general structure of BoLA is similar to the MHC of others mammalian species and is made up of three regions: Class I, Class II and Class III, which fulfill different roles. The class II genes encode proteins that presented processed peptides derived from extracellular antigens to CD4 cells, like DRA, DRB, DQA and DQB [3, 17]. In the bovine DR subregion, there are at least three BoLA-DRB loci but only the BoLA-DBR3 gene in functional. This gene is the highly polymorphic, since more than 103 alleles have been reported thus far (http://www.ebi.ac.uk/ipd/mhc/bola/index.html; accession date May 13, 2011).

The object of the presented work was to determine the association between polymorphisms from exon 2 in BoLA-DRB3 gene and SCC, as an indicator of resistance/susceptibility to mastitis in Holstein cattle from La Pampa, Argentina.

Materials and methods

Population studied

Blood samples were collected from 123 Holstein cattle belonging to a commercial dairy farm in the Province of La Pampa. Pedigree, yield milk and SCC data were recorded monthly from each animal during lactation, from July 2006 until August 2010. Regarding their SCC, animals were grouped in: control (SCC $< 250{,}000$; $N = 64$) and Case (SCC $> 250{,}000$; $N = 59$). This limit was choose because a SCC reference value of 250,000 appears to be a useful standard indicating that milk from individual cows is either mastitic (250,000 or above) or physiologically normal (less than 250,000) (NMC Global Milk Quality http://www.nmconline.org). The studied animals did not share common sire or dam.

DNA extraction and genotyping

The genomic DNA was extracted from blood samples taken from 123 animals by means of DNazol technique (Invitrogen, Carlsbad, CA, USA).

Initially, BoLA-DRB3 alleles were genotyped using PCR-RFLP technique described by van Eijk et al. [18]. Briefly, PCR products were digested with *Rsa*I, *Bst*Y and *Hae*III enzymes, and restriction fragments were resolved by 8%—1× TBE PAGE electrophoresis. The allelic variants were assigned using the nomenclature described by van Eijk et al. [18].

Samples with misunderstanding alleles or genotypes were confirmed by PCR-sequence-based typing (SBT) method described by Takeshima et al. [19]. Briefly, this technique is based on amplify exon 2 of the BoLA-DRB3 gene by single-step PCR with DRB3FRW and DRB3REV primers [20] and by analyzing the sequence data with Assign 400ATF software (Conexio Genomics, Fremantle, Australia) [21].

Statistical analysis

Gene and genotypic frequencies were estimated by direct counting. Deviations from Hardy–Weinberg (HW) equilibrium were estimated by the F_{IS} parameter [22]. The significance of this statistic was measured by the Markov Chain method [23]. The observed heterozygosity (h_o) and the unbiased expected heterozygosity (h_e) were computed according to Nei [24].

The association between polymorphisms from exon 2 in BoLA-DRB3 gene and SCC was evaluated using a classical case–control study design. Fisher's exact test and Woolf–Haldane odds ratio (OR) were applied when comparing polymorphisms from BoLA-DRB3.2 allele frequencies (in number of alleles) between cases and controls. For this purpose, the population studied was divided, based on long-term SCC data of more than one consecutive lactation, into two groups: (i) Case group composed by individuals with SCC higher to 250,000 per milliliter of milk (SCC $\geq$ 250,000). This group included animals which had at least twice events of mastitis. (ii) Control group included animals with SCC lower to 250,000 per milliliter of milk (SCC $<$ 250,000). Bonferroni adjusted p-values less than 0.05 were considered significant. Statistical analysis was performed using R statistical computing software and GENOPOP 4.0 software [25].

Results and discussion

Allele frequencies are presented in Table 1. Even though, the gene frequencies comparison did not showed significant

Table 1 Gene frequencies, observed (h_o) and expected (h_e) heterozygosities and Hardy–Weinberg equilibrium (HWE) estimated through F_{IS} index (p value) for BoLA-DRB3 gene for both groups analyzed: control (SCC < 250,000) and case (SCC ≥ 250,000)

Group	*N*	Allele frequencies															
		7	8	9	10	11	13	15	16	17	18	20	22	23	24	25	26
Control (SCC < 250,000)	128	3.13	2.34	0.78	3.13	7.03	0.78	0.78	10.16	0.78	2.34	3.13	8.59	14.84	11.72	3.13	7.03
Case (SCC ≥ 250,000)	118		1.69	0.85	5.93	6.78		1.69	7.63	0.85	0.85	7.63	11.86	8.47	6.78	8.47	3.39

Group	*N*	Allele frequencies													h_O	h_e	HWE (p value)
		27	28	31	32	34	35	36	37	39	43	45	46	47	51			
Control (SCC < 250,000)	128	0.78	7.81	0.78			0.78	1.56			2.34	1.56	0.78			0.95	0.93	<0.01
Case (SCC ≥ 250,000)	118	6.78	4.24		0.85	2.54		1.69	2.54	0.85	0.85			0.85	0.85	0.88	0.94	<0.01

N number of analyzed chromosomes genotyped within each group

differences between case and control groups ($p = 0.476$), some alleles exhibit differential distributions. In example, alleles *7, *13, *31, *35, *45 and *46 were only present in control samples, while variants *32, *34, *37, *39, *43, *47 and *51 were privatives of the case group. The others detected alleles were present in both groups (*8, *9, *10, *11, *15, *16, *17, *18, *20, *22, *23, *24, *25, *26, *27, *28, *36 and *43).

The values obtained for ho, he and HW disequilibrium *p*-values are summarized in Table 1. Several studies have shown that heterozygosity confers a selective advantage against infectious diseases (heterozygote advantage or homozygote disadvantage). For example, human leukocyte antigen (HLA) class II heterozygosity was shown to be associated with resistance to infections with HBV and HCV [26, 27]. Furthermore, deviation from the HW proportion, with regard to BoLA-DQA1, was observed in cows with mastitis caused by *Escherichia* or *Streptococci* bacteria [28]. Despite both groups were in disequilibrium ($p < 0.01$), the obtained results did not show an increment of homozygote in case group compared with control one.

Fisher's exact test and Woolf–Haldane OR were applied to study the association between SCC and BoLA-DRB3.2 allele frequencies (Table 2). Table 1 shows those alleles that appear to be more or less common in control vs case group. In the studied population, significant association was noted with allele BoLA-DRB3.2*23 and *27 ($p < 0.05$) with protective and susceptibility effect respectively. In addition, alleles BoLA-DRB3.2*20 and *25 exhibited suggestive association with high SCC ($p < 0.1$).

Several authors have studied the association between BoLA-DRB3 alleles, using RFLP or SBT genotyping methods, and different variables related with mastitis (SCC, presence-absence of clinical mastitis, mastitis pathogens) in Holstein cattle [3, 6]. These studies showed discordant results among them and with the results obtained in the present study. For example, using PCR-RFLP method, the DRB3*23 allele was found in the resistant population with $p < 0.05$ in this analysis, opposed to data reported by Sharif et al. [29] and Rupp et al. [8], who associated this variant with severe mastitis cases and with SCC > 250,000. Moreover, Dietz et al. [30] and Kelm et al. [31] found BoLA-DRB3*16 associated with high SCS while Sharif et al. [29] associated it with low SCS. Rupp et al. [8] found no association between DRB3*16 allele and SCS. In the other hand, DRB3*22 allele was found in greater percentage in cows with SCC ≥ 250,000 and in those susceptible to mastitis in accordance with that reported by Kullberg et al. [32] for first lactation and by Rupp et al. [8]. Dietz et al. [30] associated DRB3*22 allele to a decrease in SCC during second lactation. DRB3*24 allele seemed to promote resistance to mastitis as well as low SCS in accordance with that reported by Rupp et al. [8]. On the other hand, Starkenburg et al. [33] and Sharif et al. [29] found *24 allele associated with udder diseases. Starkenburg et al. [33] reported association of the BoLA-DRB3*7 allele with high SCS. Also, using PCR-RFLP method, Chu et al. [2] detected that *Bst*YI AA was the most favorable genotype for mastitis resistance, while *Bst*YI BB was the most unfavorable genotype for the same trait.

At sequences level, Yoshida et al. [34] studied the presence of amino acid substitutions at the 9, 11, 13, and 30 positions. DRB3*0101 (correspond to DRB3.2*24) and DRB3*1501 (DRB3.2*16) had amino acid motifs of Glu^{9}, Ser^{11}, Ser^{13}, and Tyr^{30}, and they were considered to have susceptibility to all 4 mastitis pathogens. In contrast, DRB3*1101(DRB3.2*22) and DRB3*1401 (DRB3.2*27) had amino acid motifs of Gln^{9}, His^{11}, Gly^{13}, and His^{30} in these positions, and they also had Val^{86}, so these alleles were considered to have resistance to Streptococcal and coagulase-negative Staphylococcal mastitis. However, in the case of *Escherichia coli* mastitis, amino acid substitutions at the 9, 11, 13, and 30 positions had little effect, but rather substitutions at the 47, 67 positions of pocket 7, and at the 71, 74 positions of pocket 4, Tyr^{47}, Ile^{67}, Ala^{71}, and Ala^{74}, were associated with resistance. This motif was present in DRB3*1201 (DRB3.2*8). On this way, susceptibility or resistance to the mastitis-causing pathogens was thought to vary by the presence of amino acid substitutions at the 9, 11, 13, 30, 47, 67, 71 and 74 positions.

In the same way, Yoshida et al. [35] evidenced that DRB3*0101 allele might be associated with susceptibility to coagulase-negative *Staphylococci* and *Escherichia coli*, and DRB3*1501 might be associated with susceptibility to *Escherichia coli*. However, DRB3*1101 might be associated with resistance to *Streptococci* and coagulase-negative

Table 2 Association of BoLA-DRB3 alleles with somatic cell count in Argentine Holstein farm studied

Allele	Fisher's exact test	OR	95% CI
BoLA-DRB3.2*10	0.225 (5.93–3.13)	2	0.56–6.86
BoLA-DRB3.2*11	0.197 (6.78–7.03)	0.9616	0.36–2.58
BoLA-DRB3.2*16	0.140 (7.63–10.16)	0.766	0.31–1.86
BoLA-DRB3.2*20	0.098 (7.63–3.13)	2.683	0.80–8.96
BoLA-DRB3.2*22	0.262 (11.86–8.59)	1.504	0.65–3.46
BoLA-DRB3.2*23	**0.049 (8.47–14.84)**	0.557	0.25–1.25
BoLA-DRB3.2*24	0.074 (6.78–11.72)	0.574	0.23–1.41
BoLA-DRB3.2*25	0.062 (8.47–3.13)	3.010	0.92–9.88
BoLA-DRB3.2*26	0.104 (3.39–7.03)	0.485	0.14–1.62
BoLA-DRB3.2*27	**0.013 (6.78–0.78)**	9.236	1.14–75.01
BoLA-DRB3.2*28	0.110 (4.24–7.81)	0.522	0.17–1.57

In brackets, gene frequencies (in percentage) estimated for cases and control

OR odds ratio; *CI* confidence interval

Staphylococci, and DRB3*1201, with resistance to *Streptococci*, *Escherichia coli*, and *Staphylococcus aureus*.

SBT analysis showed that in the study population BoLA-DRB3.2*27 and *23 alleles corresponded to BoLA-DRB3*14011, BoLA-DRB3**2707 and BoLA-DRB3*2703 respectively. Even thought BoLA-DRB3*14011 allele had all amino acid motifs (Gln^{9}, His^{11}, Gly^{13}, and His^{30}) correlated with resistance to mastitis by Yoshida et al. [34], this allele was associated with high SCS in our population. Furthermore, BoLA-DRB3* *2707 and *2703, associated herein with low SCS, did not have any of amino acid motifs correlated with resistance to mastitis reported by Yoshida, but were partially in agreement with data reported from Japanese Holstein cattle by Takeshima et al. [28] which associated BoLA-DRB3*2703 with resistance with mastitis.

A possible explanation for the contrasting results could be that the mastitis is a multifactor disease caused by different pathogens. As showed by Yoshida et al. [34, 35] resistance-susceptibility could be varying according to the pathogen that the animals were exposed to. Moreover, most of the studies were carried out using PCR-RFLP method, which has less resolution than PCR-SBT because PCR-RFLP defined alleles included more than one sequenced alleles, and only few studies considered BoLA-haplotypes in the analysis which could explain the resistance/susceptibility better. Finally, we cannot exclude that observed inconsistence among studies might be consequence of false positive or negative results due to used experimental designs.

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