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Para: Hugo Adrian Carignano
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Asunto: Animal Genetics - Decision on Manuscript ID AnGen-16-10-0318.R2

17-Mar-2017

Dear Dr. Carignano:

Thank you for the revision of Manuscript ID AnGen-16-10-0318.R2, entitled "<i>BoLA-DRB3</i> gene polymorphisms influence Bovine Leukaemia Virus infection levels in Holstein and Holstein x Jersey crossbreed dairy cattle.". Based on the changes you have made, I am pleased to accept the paper for publication in Animal Genetics. Your manuscript will now go forward for copy editing and production. You may be contacted if there are any problems or questions with the final copy of the paper.

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BoLA-DRB3 gene polymorphisms influence Bovine Leukaemia Virus infection levels in Holstein and Holstein x Jersey crossbreed dairy cattle.

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Summary

Bovine Leukemia Virus (BLV) infections, causing persistent lymphocytosis and lethal lymphosarcoma in cattle, have reached high endemicity in dairy farms. We observed extensive inter-individual variation in the level of infection (LI) by assessing differences in proviral load (PVL) in peripheral blood. This phenotypic variation appears to be determined by host genetics variants, especially those located in BoLA-DRB3 MHCII molecule. We performed an association study using sequencing-based typed *DRB3* alleles over 800 Holstein and Holstein x Jersey cows considering the LI *in vivo* and accounting for filial relationships. The *DBR3*0902* allele was associated with a low level of infection (LLI) (< 1% of circulating infected B-cell) while *DRB3*1001* and *DRB3*1201* alleles were related to a high level of infection (HLI). We found evidence that 13 polymorphic positions located in the pockets of the peptide-binding cleft (PBC) of the *DRB3* alleles were associated with LI. *DRB3*0902* had unique haplotypes for each of the pockets: Ser^{13}-Glu^{70}-Arg^{71}-Glu^{74} (Pocket 4) Ser^{11}-Ser^{30} (Pocket 6), Glu^{28}-Trp^{61}-Arg^{71} (Pocket 7) and Asn^{37}-Asp^{57} (Pocket 9) and all of them were significantly associated with a LLI. Conversely, Lys^{13}-Arg^{70}-Ala^{71}-Ala^{74} and Ser^{13}-Arg^{70}-Ala^{71}-Ala^{74} corresponding to the *DRB3*1001* and **1201* alleles, respectively, were associated with a HLI. We showed that the amino acids specific pattern in the *DRB3*0902* PBC might be related to the set-point of a very low PVL level in adult cows. Moreover, we identified two *BoLA-DRB3* alleles associated with a HLI, which is compatible with a highly contagious profile.

Keywords: BLV, *BoLA-DRB3* alleles, level of infection, genetic control

145 **Introduction**

146 Bovine Leukemia Virus (BLV, genus: deltaretrovirus) chronically infects cattle rapidly
147 spreading over herdmates mainly via ~~through~~ direct exposure to body fluids/blood
148 contaminated with infected lymphocytes. In American countries lacking a consistent
149 BLV eradication program (i.e. Argentina, AGREGAR), BLV has gradually reached
150 high levels of prevalence (EFSA, 2015). Worryingly, in heavily infected herds a drop in
151 milk production to either individual/herd levels is observed (Erskine *et al.*, 2012; Norby
152 *et al.*, 2015), followed by a premature culling and replacement of (Bartlett *et al.* 2013)
153 impacting on dairy farms profitability (Ott *et al.*, 2003). BLV tropism in the host is
154 primarily directed to B cells where it is integrated into the genome (provirus) (Gillet *et*
155 *al.*, 2007). Infected cells, surviving host immune response clearance, undergo a tight
156 mRNA BLV transcriptional silencing, promoting cellular transformation and BLV
157 persistence/replication through mitotic division (Barez *et al.*, 2015; Gillet *et al.*, 2016).
158 The set–point of proviral load (PVL) in peripheral blood, which is directly related to the
159 level of infection (LI) *in vivo* (Myrski *et al.*, 1996; Jimba *et al.*, 2010), is variable among
160 individuals (Juliarena *et al.*, 2007; Gutierrez *et al.*, 2012) and apparently stable for
161 extended periods of time (Florins *et al.*, 2007). From an epidemiological point of view,
162 this amount of circulating proviral DNA determines the risk of BLV transmission from
163 infected cows to healthy individuals (Mammerickx *et al.*, 1987). Host genetic factors,
164 particularly allelic variants in the Bovine Leukocyte Antigen (BoLA) class II *DRB3*
165 gene, could be affecting the proviral set–point variability (Juliarena *et al.*, 2008;
166 Miyasaka *et al.*, 2013). These allelic variants are located in the exon 2 encoding the
167 Peptide-Binding Cleft (PBC) of the surface molecule (Russell *et al.*, 1997). Conserved
168 and polymorphic amino acid side chains positions interacting with the antigenic peptide,
169 are grouped into "pockets" throughout the PBC (Stern *et al.*, 1994). These structures are

170 responsible for the differences in affinity for a range of antigenic peptides bound to the
171 class II molecules (Li *et al*., 2000; Liu *et al*., 2002; Siebold *et al*., 2004; Zavala-Ruiz *et*
172 *al*., 2004) and are therefore influencing the magnitude and extent of the adaptive
173 immune response. Specific amino acid variants in the *BoLA-DRB3* PBC have been
174 associated with resistance to the development of persistent lymphocytosis (PL) in BLV
175 infected animals (Xu *et al*., 1993) and relevant health traits, i.e. dermatophilosis
176 (Maillard *et al*., 1996), mastitis (Sharif *et al*., 2000, Yoshida *et al*., 2009) and foot and
177 mouth disease (Garcia-Briones *et al*., 2000; Baxter *et al*., 2009). In Argentina, the
178 utilization of specific *BoLA-DRB3* alleles has been suggested in breeding programs
179 aiming to limit the viral spread within herds (Juliarena *et al*., 2008, Esteban *et al*., 2009;
180 Juliarena *et al*., 2016). In this study we aim to determine whether genetic variation at
181 *BoLA-DRB3* gene assessed by allele sequencing-based typing is related to the risk of
182 BLV transmission measured by the level of BLV infection in a naturally infected
183 Holstein and Holstein x Jersey cattle.
184
185 **Materials and methods**
186
187 Study Population
188 Blood samples were collected from the coccygeal vein from 1800 Holstein and Holstein
189 x Jersey cows representing 25 half-sib families, belonging to 16 commercial dairy farms
190 located in the central region of Argentina. Standard operating procedures causing
191 minimal animal distress were followed according to INTA normative for Good
192 Practices for Animal Welfare. All animals were between 3 to 12 years of age. Plasma
193 and fresh blood were collected and frozen at -20°C until used. Genomic DNA was
194 extracted from whole blood samples using a commercial kit (Blood Genomic DNA

AxyPrep^{TM}, Axygen Biosciences, Union City, USA) and quality and concentration was assessed using a micro-volume spectrophotometer (NanoDrop^{TM} Technologies, Inc. Wilmington, USA). DNA samples were normalized to a minimum concentration of 10 ng/µl.

BLV-Phenotyping

Animals were screened by anti-p24 ELISA assay to discriminate BLV infected animals (Gutierrez *et al*., 2009). The % of Reactivity (% of R) obtained in the ELISA assay was used as a cost effective indirect measure of LI (Gutierrez *et al*., 2012). Two groups of contrasting phenotypes (samples with % of R between 25-100% and those >200%) were then confirmed by determining blood PVL. The PVL relative quantification was performed by real-time PCR using TaqMan technology as described in Gutierrez *et al*. (2009; 2011). Briefly, the primers BLVMGBF and BLVMGBR (Lew et al., 2004) were used to amplify a fragment of the BLV *pol* gene. A fragment of the *18S* gene was amplified simultaneously and used as reference. As internal control a cell line (FLK, Fetal Lamb Kidney cells) containing 4 copies/cell of BLV proviral DNA, at a concentration of 1% in peripheral blood mononuclear BLV-free cells (PBMCs). This proportion of BLV-infected cells corresponds to a very low level of natural infection (Gutierrez *et al*., 2009), considered to be 5% in aleukemic animals (Hopkins & DiGiacomo, 1997). The relative PVL was defined according to the Ratio (R) (Pfaffl, 2001) as "No Detectable" (ND) if a Cycle Threshold (Ct) value was not obtained (below the detection limit 1 cell infected/2000 cells uninfected), as "Low" (L) if R was less than 1 ($R < 1$) and "High" (H) if it was higher than one ($R > 1$). A total of 832 animals were categorized by LI as cases (n = 432, HLI) and controls (n = 400, ND and LLI) according to the aforementioned approach.

220

221 Genotyping

222 A fragment of 247 bp containing the hypervariable domain of the surface molecule
223 *DRB3* was amplified by PCR using primers forward HLO30 and reverse HLO32 as
224 described in Miltiadou *et al*. (2003). An aliquot of 0.6 µl of a 10 ng/µl genomic DNA
225 dilution was utilized in a final volume of reaction mixture of 15 µl (1X Buffer Taq
226 Platinum, 200 µM of each dNTP, 0.2 µM of each primer, 2 mM $MgCl_2$ and 1 U Taq
227 polymerase Platinum) (Invitrogen, USA). The thermal amplification cycle included a
228 denaturation step at 94 °C for 3 min; followed by 35 cycles of 94 °C for 20 sec, 60 °C
229 for 20 sec and 72 °C for 1 min; the final extension step was at 72 °C for 5 min.
230 Amplicons were purified with 0.5 U of *Shrimp* alkaline phosphatase and 5 U of
231 Exonuclease I (USB Corp, Cleveland, OH). The mix was incubated at 37 °C for 30 min
232 and then 15 min at 85 °C to inactivate the enzymes.
233 The sequencing of the amplified fragment was performed using the *BigDye* ® v3.1
234 chemistry (Applied Biosystems Inc., Foster City, CA), primed with HLO30 and HLO32
235 primers and analysed using an ABI 3730xl automatic sequencer (Applied Biosystems
236 Inc., Foster City, CA). The raw sequence reads in both directions were collected with
237 the "Sequencing Analysis" software (Applied Biosystems Inc., USA), aligned and
238 manually edited if necessary using the Staden Package (http://staden.sourceforge.net/).
239 Heterozygous positions were identified generating consensus sequences that were
240 subsequently compared to the 132 known alleles of the *BoLA-DRB3* gene
241 (www.ebi.ac.uk/ipd/mhc/bola) using Haplofinder
242 (www.bioinfortmatics.roslin.ac.uk/haplofinder/haplofinder.py) following Baxter *et al*.
243 (2008). In short, this program uses all known *DRB3* alleles to create a list of all possible
244 heterozygous combinations using IUPAC ambiguity codes. Haplofinder compares the

245 consensus sequence obtained with the list of all possible combinations. The output

246 showed the two possible homozygous or heterozygous alleles for the input sequence.

247

248 Statistical analysis

249 The association between a particular *BoLA-DRB3* allele and the LI was calculated as the

250 Odds Ratio (OR) as follows:

$$OR = \frac{P(HLI/P)/P(HLI/A)}{P(LLI/P)/P(LLI/A)} = \frac{P(HLI/P)\ x\ P(LLI/A)}{P(HLI/A)\ x\ P(LLI/P)}$$

251 where: P(HLI/P) is the probability that a defined *BoLA-DRB3* allele is present in an

252 animal having HLI, P(HLI/A) the probability that a defined *BoLA-DRB3* allele is absent

253 in an animal having HLI, P(LLI/P) is the probability that the allele is present in an

254 animal having LLI, and P(LLI/A) the same condition being the allele absent. A value of

255 OR > 1 implies that those animals carrying the allele are more likely to develop HLI,

256 while a value of OR < 1 implies the opposite. To prevent spurious results due to

257 population stratification we carried out two different statistic association test. First, we

258 controlled for the presence of defined groups by means of the Cochran-Mantel-Haenszel

259 test (CMH) (Mantel & Haenszel, 1959). The groups were formed by clustering animals

260 according to the family to which animal belonged. On the other hand, the association

261 was performed using logistic regression, evaluating the significance of the logistic

262 regression coefficient through the t-statistic. This methodology allowed incorporating

263 principal components (PC) as covariates (Price *et al*., 2006) calculated for the

264 population in the study. All the analyses were performed using the software PLINK

265 v1.04 (Purcell *et al*., 2007a) on genotyped animals for *BoLA-DRB3*. Confidence

266 intervals of the 99% for the OR were calculated. Finally, the Max(T) permutation

267 procedure as implemented in PLINK was performed with 10000 permutations to correct

268 for multiple tests and to adjust the p-values. Empirical p-values corrected were
269 generated. Association test having a p-value < 0.05 were considered as significant.
270 A haplotype-specific association test was performed between polymorphic peptide
271 binding pockets in the BoLA-DRB3 molecule and the LI utilizing WHAP v2.09
272 (Purcell at al., 2009b) with default parameters.
273
274 **Results**
275
276 Assigning of genotypes
277 *BoLA-DRB3* SBT genotyping was successful in 809 cows with PVL data and in 23 bulls
278 used as parents (19 Holsteins and 4 Jersey). The genotypic consistency in the pedigrees
279 was corroborated in all families by segregation analyses based on SBT *DRB3* alleles. A
280 total of 118 cows (14.5%) had Mendelian errors in the genotype assigned and were
281 excluded from further analysis. Additional SNP typing data confirmed inaccuracies
282 derived from the pedigree rather than in the DRB3 allele assignment.
283
284 *BOLA-DRB3* allelic frequencies
285 A total of 25 alleles were identified. Table 1 shows their absolute frequencies in the
286 population and after grouping the animals according to their proportion of Holstein.
287 Alleles with the highest frequencies were *DRB3*0101*, **1101*, **1501* and **1201* with
288 percentages of 17.5, 17.2, 13.2 y 11.6 % respectively. Eight alleles (*DRB3*0201,*
289 **0801, *0902, *1001, *1601, *2502, *2703** and **4501)* had frequencies ranging from
290 1.5 to 10%, and the remaining 13 alleles had allelic frequencies below 1.5%
291 (*DRB3*0301, *0601, *0701, *0901, *1002, *1103, *14011, *1602, *1701, *1801,*
292 **20011, *20012,* and **2201*).

293 Considering animals with 75-100% Holstein (n = 552 cows) we found the *DRB3*0101*
294 (20.6%), *DRB3*1101* (18.6%) and *DRB3*1501*(15.3%) alleles as the most abundant,
295 whereas in animals with 0-50% Holstein (n = 114 cows) the most represented alleles
296 were *DRB3*4501* (20.9%), *DRB3*2703* (18.7%) and *DRB3*1101* (11.5%). The
297 *DRB3*0101* (20.6%) and *DRB3*1501* (15.3%) alleles had a prevalence above 10% in
298 the animals between 75-100% Holstein compared to 0-50% group (5.0% for both
299 alleles). In contrast, *DRB3*2502* (9.0%), *DRB3*2703* (18.7%) and *DRB3*4501* (20.9%)
300 alleles were prevalent in the 0-50% group relative to the 75-100% group (0.3%, 5.7%
301 and 0.3%, respectively) (Table 1).
302
303 Case-control association analysis between *BoLA-DRB3* alleles and the LI
304 After correction for Mendelian inconsistencies, 338 cows (51.4%) showed high LI
305 (HLI) and 320 of them (48.6%) had low LI (LLI, controls) being considered cases and
306 controls, respectively. The percentage of animals with LLI per family was between 20%
307 and 92% (Table S1). Small families and alleles with frequencies below 1.5% were
308 excluded from the association study. To reduce the chance for spurious associations we
309 have taken into account the population structure by two methods, CMH test and PC
310 correction. The *DRB3*0902* allele was significantly associated with the LLI profile
311 ($p_{CMH} < 0.0001$ and $p_{PC} < 0.0001$), while the *DRB3*1001* ($p_{CMH} = 0.024$ and $p_{PC} =$
312 0.001) and *DRB3*1201* ($p_{CMH} < 0.001$ and $p_{PC} < 0.001$) alleles were significantly
313 associated with a HLI profile in both correction methods (Tables 2 and 3). Notably,
314 17.6% (19/108) of the animals carrying the *DRB3*0902* allele had a high level of PVL.
315 On the other tail of the phenotype, the 20.9% (18/86) of cows carrying the *DBR3*1001*
316 allele and a 29.1% (49/168) with the *DRB3*1201* allele showed low levels of infection
317 (LLI).

318

319 ~~Haplotype-specific association analysis of polymorphic peptide binding pockets of the~~

320 ~~BoLA-DRB3 molecule with LI.~~

321 In DRβ–antigen complex expressed in the cell surface, amino acid (AA) positions

322 constitute specific pockets in the antigen binding groove (i.e. DRβ 9, 11, 13, 26, 28, 30,

323 37, 47, 56, 57, 60, 61, 67, 70, 71, 74, 78, 85 and 86) (Nielsen *et al*., 2008; Zhang *et al*.,

324 2012; Shen *et al*., 2013; Karosiene *et al*., 2013). Table 4 shows the amino acid residues

325 in the peptide binding pockets in each *BoLA-DRB3* allele identified in this population.

326 Positions DRβ 11 (*Ala, Cys, His, Ser, Thr, Tyr*) and 37 (*Phe, Leu, Asn, Arg, Thr, Tyr*)

327 were the most polymorphic with six alternate AA. Subsequently, cows were grouped

328 according to the AA sequence in the binding pockets in line with the distribution

329 proposed by Bondinas *et al*. (2007). Positions DRβ 9, 26, 47, 60, 67 and 86 were not

330 included in the analysis because were not significantly associated with the LI (Table 4).

331 Associations between the haplotypes in Pockets 4 (Table 5), 6 (Table 6), 7 (Table 7), 9

332 (Table 8) and 10 (Table 9) and the profiles of HLI/LLI were evaluated utilizing WHAP

333 (Purcell *et al*., 2007b). As covariates we considered 8 PCs to correct for population

334 stratification. The amino acid sequence Ser^{13}-Glu^{70}-Arg^{71}-Glu^{74} conforming Pocket 4

335 was significantly associated with LLI (OR = 0.14; p = 1.76 x 10^{-16}). The haplotypes

336 containing Lys^{13}-Arg^{70}-Ala^{71}-Ala^{74} and Ser^{13}-Arg^{70}-Ala^{71}-Ala^{74} in the same pocket were

337 significantly associated with the HLI profile (OR = 3.10, p = 1.41 x 10^{-7} and OR = 3.96,

338 p = 2.07 x 10^{-5}, respectively) (Table 5).

339 Six haplotypes were identified when animals are grouped according to the amino acid

340 sequence at Pocket 6. Motifs Ser^{11}-Tyr^{30} (OR = 1.34, p = 3.26 x 10^{-3}) and Thr^{11}-Tyr^{30}

341 (OR = 2.57, p = 2.71 x 10^{-8}) were found significantly associated with a high PVL.

342 Conversely, Ser^{11}-Ser^{30} and Tyr^{11}-Cys^{30} were associated with a LLI profile (OR = 0.15;

343 p = 1.38 x 10^{-15} and OR = 0.54; p = 2.00 x 10^{-5}, respectively) (Table 6).

344 Pocket 7 is composed by the AA side chains positions 28, 61 and 71. From 8 identified

345 haplotypes, 4 were significantly associated to LI, two of them with a LLI Glu^{28}-Trp^{61}-

346 Arg^{71} (OR = 0.13; p = 2.71 x 10^{-16} and Asp^{28}-Trp^{61}-Ala^{71} OR = 0.40; p = 7.75 x 10^{-5}),

347 and two haplotypes with a HLI profile (Asn^{28}-Trp^{61}-Ala^{71} OR = 3.08; p = 6.87 x 10^{-7}

348 and Asp^{28}-Cys^{61}-Ala^{71} OR = 3.79; p = 6.19 x 10^{-7}) (Table 7).

349 The haplotype Asn^{37}-Asp^{57} (OR = 0.15, p = 1.62 x 10^{-15}) and the corresponding to Tyr^{37}-

350 Asp^{57} (OR = 0.71, p = 0.02) within Pocket 9 were significantly associated with LLI. The

351 opposite effect, a significant association with a HLI profile was found for the

352 Haplotypes Phe^{37}-Asp^{57} (OR = 1.98, p = 7.6 x 10^{-5}) and Tyr^{37}-Val^{57} (OR = 3.34, p =

353 2.71 x 10^{-5}) (Table 8).

354

355 **Discussion**

356

357 In this work the *DRB3* allelic diversity was assessed in more than 800 Holstein and

358 Holstein x Jersey dairy cows by PCR-sequencing typing (SBT) (Miltiadou *et al*., 2003;

359 Baxter *et al*., 2008). Early studies surveyed the genetic diversity at the bovine *DRB3*

360 *locus* in South American creole cattle (Giovambattista *et al*., 1996; Miretti *et al*., 2001;

361 Ripoli *et al*., 2004) and European breeds (van Eijk *et al*., 1992; Gelhaus *et al*., 1995;

362 Maillard *et al*., 1999; Gilliespie *et al*., 1999). Most of these studies used PCR-RFLP

363 genotyping resulting in ambiguities and poorly resolving actual alleles. SBT empowered

364 cattle diversity and disease association studies increasing sample processivity and

365 genotyping accuracy (Takeshima *et al*., 2015a; 2015b; Giovambattista *et al*., 2013).

In 832 animals we identified 25 alleles, most of them in low frequency (<5%), and only 7 alleles above 5% (*DRB3*0101, *0902, *1001, *1101, *1201, *1501* and **2703*) accounting for 82.3% of the total. These results are similar to those reported in American and Canadian Holstein herds (Dietz *et al*. 1997, Sharif *et al*. 1998) identifying 27 variants, 7 of them totalling 88.7% of frequency in the Canadian Holstein (*DRB3*0101, *0901/02, *1001, *1101/03, *1201, *1501/02, and *2701/03/04/05/06/07/08/09/10*). Similarly, the *DRB3*0101, DRB3*1101* and *DRB3*1501* variants were highly abundant in Holstein Japanese herds (>10%) (Miyasaka *et al*., 2011; Takeshima *et al*., 2014). In Holstein x Jersey animals (0-50% Holstein) 5 alleles reached >-5% frequency (*DRB3*0101, *1101, *1201, *1501* and **2703*), the same alleles had high frequencies in the 75-100% Holstein group as well (Table 1). The *BoLA-DRB3* frequencies found in the Argentinean Holstein are in concordance with other Holstein and Jersey herds reported in the literature.

In particular, we were interested in assessing whether predominant *DRB3* alleles in our population affect susceptibility/resistance to BLV infection. The population studied was categorized according to the level of infection by proviral load (PVL) quantification. The case-control association study showed that *DRB3*0902* allele was significantly associated with low level of infections (LLI), whereas *DRB3*1001* and *DRB3*1201* were associated with high level of infections (HLI). Xu *et al*. (1993) found that *DRB3* alleles associated with resistance to develop PL shared a motif with 2 polar amino acids –Glu^{70}-Arg^{71}– in BoLA DRβ domain 1 (*DRB3*0901/02,*2701/02/03/04/05/06/07/08/09/10* and **0701/02/03*). In contrast, *DRB3*1201* and *DRB3*1501/02* alleles shared the tetra-aminoacidic motif Val^{75}-Asp^{76}-Thr^{77}-Tyr^{78} associated with susceptibility to PL. Mirsky *et al*. (1998) proposed that PL resistance allele (*DRB3*0901/02*) was involved in controlling the number of infected B-

391 cells in peripheral blood of infected cows. The BLV set−point PVL is a more
392 informative measure than PL as PVL is: 1) strongly related to disease progression
393 caused by the BLV infection (Mirsky *et al*., 1996; Jimba *et al*., 2010), 2) associated
394 with the infective capacity of BLV measured by syncytia formation (Jimba *et al*., 2010)
395 and 3) linked to the potential of viral spreading to healthy animals from infected cows
396 (Mammerickx *et al*., 1987). Interestingly, animals that do not show PL may equally
397 have high levels of circulating provirus (Mirsky *et al*., 1996; Juliarena *et al*., 2007). In
398 subsequent population studies, the *DRB3*0901/02* alleles were also related to low PVL
399 in Holstein breeds (Juliarena *et al*., 2008) and Japanese Black (Miyasaka *et al*., 2013).
400 These studies, however, did not consider the relationship between the individuals and
401 others variants, e.g. *DRB3*1701/*02/ DRB3*3201/02/03* in Holstein and *DRB3*1101* in
402 native Japanese cows were also associated with a low PVL.
403 *DRB3*0902* has been associated with a low PVL in different breeds. By contrast, alleles
404 associated with a high PVL were different and not consistent among studies, i.e.,
405 *DRB3*1001* and *DRB3*1201* in our population, *DRB3*1501/02* in another Argentinean
406 Holstein population (Juliarena *et al*., 2008) and *DRB3*1601* in a Japanese Black herd
407 (Miyasaka *et al*., 2013).
408 It has been suggested that the motif −ER− at positions 70−71 of the amino acidic DRβ
409 chain could be responsible for inhibiting the progression of pathogenesis produced by
410 BLV (Xu *et al*., 1993). Polymorphism in the antigen binding pockets causes differences
411 in the shape, electrostatic charge and three-dimensional conformation of the binding
412 groove, playing a central role in antigen presentation to T cells. There is evidence of
413 high positive selection in the antigen binding pockets in the BoLA-DRB3 molecule
414 (Takeshima *et al*., 2009) and in human MHC class II molecule (Hughes & Nei, 1989).
415 The binding affinity between the peptide and the MHC class II molecule is allele

416 specific (Rammensee *et al*., 1995) ultimately conditioning the immune response elicited

417 (Kumar *et al*., 1995; Murray, 1998). In this context, we evaluated the relationship

418 between 19 polymorphic positions shaping the antigen binding pockets and the level of

419 infection caused by BLV. According to the crystallographic structure of MHCII-

420 Antigen complexes (http://www.wwpdb.org), these are "contact" residues at the core of

421 the antigen-binding groove (Nielsen *et al*., 2007; Zhang *et al*., 2009). Most (13/19) of

422 the DRβ positions resulted associated with LI in the infected cows (Table 4).

423 *DRB3*0902* had unique haplotypes in each of the pockets analysed. For example, *Ser*^{11}-

424 *Ser*^{30} (Pocket 6), *Glu*^{28}-*Trp*^{61}-*Arg*^{71} (Pocket 7) and *Asn*^{37}-*Asp*^{57} (Pocket 9) were all

425 significantly associated with a LLI (Tables 5, 6, 7 and 8). It has been proposed that each

426 pocket contributes independently to the overall binding affinity for the antigenic peptide

427 (Sturniolo *et al*., 1999), however, positive and negative cooperative effects among

428 pockets has been detected (Zavala-Ruiz *et al*., 2003; Cárdenas *et al*., 2005; Ferrante

429 and Gorski, 2007; James *et al*., 2009). If the variant configuration in the Pockets 4, 6, 7

430 and 9 of *DRB3*0902* restrains the proviral set-point level in BLV infected animals, and

431 hence disease progression, this effect is not complete as 17.6% (19/108) of animals

432 carrying *DRB3*0902* allele had high level of PVL. This trend was also observed in

433 other Holstein and Japanese black populations (Juliarena *et al*., 2008; Miyasaka *et al*.,

434 2013).

435 Regarding high level of infection, *DRB3*1001* showed unique haplotypes at Pockets 4

436 (*Ser*^{13}-*Arg*^{70}-*Ala*^{71}-*Ala*^{74}), 7 (*Asp*^{28}-*Cys*^{61}-*Ala*^{71}) and 9 (*Tyr*^{37}-*Val*^{57}) associated to a HLI,

437 sharing the haplotype at Pocket 6 with others alleles (Table 6). The *DRB3*1201* allele

438 exposed a unique AA sequence at Pocket 4 (*Lys*^{13}-*Arg*^{70}-*Ala*^{71}-*Ala*^{74}) and 7 (*Asn*^{28}-

439 *Trp*^{61}-*Ala*^{71}), being both of them significantly associated with a profile of HLI.

440

441 Amino acid sequence at Pocket 4 is unique for each *BoLA-DRB3* allele with frequencies
442 above 1.5% in the population studied (Table 5). Pocket 4 plays a key role in loading the
443 antigen peptide affecting the CD4+ T cells activation (Fu *et al*., 1995). Polymorphisms
444 in the Pocket 4 have also been associated with the pathogenesis caused by bacteria
445 (Raychaudhuri *et al*., 2012; Hu *et al*., 2015). DRB molecule positions have been
446 associated with clinical mastitis (β13, β71 and β74, Sharif *et al*., 2000) and with
447 differences in the antibody response in cows after experimental challenge with peptides
448 from the capsid protein of foot-and-mouth disease virus (β70, Baxter *et al*., 2009).
449 The *DRB3*0902* allele, associated with a LLI in this study, presents the Ser^{13}-Glu^{70}-
450 Arg^{71}-Glu^{74} haplotype at Pocket 4. However, *DRB3*2703* presenting the motif Arg^{13}-
451 Glu^{70}-Arg^{71}-Glu^{74} was not significantly associated with the LLI profile after correcting
452 for population stratification (data not shown). Conversely, haplotypes at Pocket 4
453 associated with a HLI were Lys^{13}-Arg^{70}-Ala^{71}-Ala^{74} and Ser^{13}-Arg^{70}-Ala^{71}-Ala^{74}
454 corresponding to the *DRB3*1001* and **1201* alleles, respectively.
455
456 The presence of other polymorphic genes within the MHC, acting independently, or in
457 linkage disequilibrium with *DRB3*, could be underlying the differences in BLV PVL.
458 Polymorphism in *TNF-α* gene is related to lymphosarcoma in cattle after BLV infection
459 (Konnai *et al*., 2006), and *DRB3-DQA1* haplotypes have been associated with extreme
460 PVL profiles in a Japanese Black population (Miyasaka *et al*., 2013). Genetic analysis
461 of extended MHC haplotypes associated with disease in humans indicated that prevalent
462 ancestral *DR-DQ* blocks have been maintained by recombination and positive selection
463 (Traherne *et al*. 2006), being peptide binding sites key players in disease resistance.
464 Population genetic studies to elucidate the structure of haplotype blocks and

465 recombination hot spots in the bovine MHC are necessary to identify other
466 chromosomal segments in the BoLA region potentially related to disease resistance.
467 The high gene density and the highly polymorphic nature of the MHC, along with the
468 intricate structure of linkage disequilibrium present in the region (Stewart *et al*., 2004;
469 Miretti *et al*., 2005; de Bakker *et al*., 2006; Traherne *et al*., 2006) highlights the need to
470 evaluate a large number of SNPs throughout the bovine genome (Larkin *et al*., 2012;
471 Daetwyler *et al*., 2013) in pursuit of accurately identifying genes responsible for the
472 phenotypic variation observed in animals infected with BLV.
473
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481
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