Scientific Papers. Series D. Animal Science. Vol. LXVII, No. 2, 2024
ISSN 2285-5750; ISSN CD-ROM 2285-5769; ISSN Online 2393-2260; ISSN-L 2285-5750

BLOOD PARASITE DETECTION AND BoLA-DQA1 GENETIC DIVERSITY IN CATTLE FROM TUNISIA

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Abstract

*Blood parasites, particularly those of the Anaplasma marginale, Babesia and Theileria spp., present a challenge to successful livestock farming. In the present work, PCR analysis was carried out to detect possible infection by the above-mentioned parasites in three cattle populations from northern Tunisia. We also sequenced exon 2 of the BoLA-DQA1 gene in 17 Holstein cattle. Our results showed a low level of infection by the screened parasites, with prevalences of 8.8%, 5.9% and 0.0% for Theileria spp., Anaplasma mariginale and Babesia ssp., respectively. On the other hand, a total of 11 alleles were observed in the BoLA-DQA1 gene in the analysed samples. Six alleles were detected for the first time. BoLA-DQA1*10011 and *0101 alleles were the most frequent. These two alleles were also the most frequent in all Holstein cattle populations so far studied. Finally, four and eight amino acid positions were under positive selection by DATAMONKEY and PAML, respectively. Such selection, associated with high polymorphism observed in the BoLA-DQA1 gene, might suggest an important qualitative and quantitative parasite pressure that would favour distinct allele types.*

Key words*: Anaplasma, Babesia, BoLA-DQA1, cattle, Theileria.*

INTRODUCTION

The livestock production sector plays an important role in Tunisia's economy with a contribution of approximately 40% of the value of agricultural products (Belguesmi, 2023). However, this sector in general and that of cattle farming in particular is facing several constraints with diseases (parasites, viruses, and bacteria) constitute one of the biggest problems. Among these diseases, those transmitted by ticks limit the growth of animal farming sector and affect health and productivity of animals in various regions of the world (de Castro, 1997). Bovine theileriosis, babesiosis and anaplasmosis are considered among the most economically important diseases. Additionally, animals that recover from an acute infection may become long-term carriers without the infection being detected microscopically (Brown, 1990). Indeed, blood smear microscopy is often the preferred diagnostic method for these parasites (Bono et al., 2008). To overcome this problem, conventional PCR assays can be used effectively for the specific detection of several species of piroplasms and *A. marginale* (Almeria et al., 2001).

On the other hand, as the diversity of the MHC loci reflects adaptive and non-adaptive evolutionary processes within and between populations, it is of great interest to a wide range of scientists, including breeders, population geneticists, and evolutionary biologists (Goszczynski et al., 2014; Takeshima et al., 2014). One of the most significant factors affecting genetic diversity is the domestication bottleneck experienced by most domesticated animals (Zhang et al., 2013). This bottleneck reduces genetic diversity compared to their wild ancestors and alters the distribution of genetic variation among loci (Buckler et al., 2001). However, for MHC genes, while reduced variability might be the outcome of population bottlenecks, a high level of diversity could could result from balancing selection driven by pathogens or other mechanisms despite extreme population bottlenecks (see Bohórquez et al. (2020) for an overview).

The MHC in cattle (also known as bovine leukocyte antigen-BoLA) is located on

chromosome 23 and is similar to other mammals' MHC (Takeshima & Aida, 2006). Previous studies of MHC genes in cattle have detected a significant association of the genetic diversity of these genes with certain diseases, such as mastitis (Takeshima et al., 2008; Yoshida et al., 2012), leukemia (Zanotti et al., 1990), ketosis (Mejdell et al., 1994) and infection by ectoparasites (Martinez et al., 2006). Other studies have also associated BoLA gene polymorphism with protein composition and milk fat content (Nascimento et al., 2006) as well as milk production (Rupp et al., 2007).

Here, we first used PCR technique to detect and identify blood parasite infections in cattle breeds in North Tunisia. Updating the prevalence of these parasites will allow to evaluate and to take appropriate measures to eradicate them. Second, the polymorphism of the BoLA-DQA1 gene was studied in seventeen Tunisian Holstein cattle. We used several tests to evaluate the effect of selection - as an evolutionary process - shaping DQA1 sequence diversity.

MATERIALS AND METHODS

Samples collection

Blood samples were collected randomly from 68 dairy cattle from North Tunisia between April and June 2023. The studied animals were from intensive (29 samples from Bousalem) and extensive (23 from Fernana, 16 de Menzel Bourguiba) farming. The studied cattle population belong mainly to the Holstein breed with the age of animals ranging between 1 and 10 years. The blood samples collected in 15 ml tubes containing a few drops of EDTA were immediately stored at -20°C.

DNA extraction and PCR-based blood parasite detection

DNA from whole-blood samples was extracted using the «FavorPrepTM Tissue Genomic DNA Extraction» Kit for DNA purification. All samples were controlled for successful DNA extraction using PCR amplification with the primer pairs PCO3/PCO4 that amplify the bovine β-globin gene (Konnai et al., 2006).

The detection of *Theileria* ssp., *Babesia* ssp., and *A. marginale* was performed using PCR as previously described by (Adaszek & Winiarczyk, 2008; Lew et al., 2002).

PCR and sequencing of BoLA-DQA1

Amplification of 374 bp long sequences including the whole Exon 2 of the BoLA-DQA1 gene was performed for 17 unrelated cows of Tunisian Holstein breed [Fernana (n = 6), Menzel Bourguiba (n = 6) Bousalem (n = 5)] using the primers described by Kulaj et al. (2015). The PCR products were then purified with ExoSAP enzymes and both strands were sequenced using an ABI 3130xl DNA Analyzer.

Statistical analyses of BoLA-DQA1 sequences

The obtained sequences were aligned and edited using the BioEdit v.7 program (Hall, 1999). Alleles of our DQA sequences were reconstructed with Phase 2.1.1 (Stephens et al., 2001) using five replicate runs of 1000 generations after 1000 generations of burn-in.

DnaSP program (Librado & Rozas, 2009) was used to calculated genetic diversity parameters, nucleotide diversity (π), haplotype diversity (Hd), and mean number of pairwise differences (k). The same program was also used to test for deviation from neutral evolution of BoLA-DQA1 locus by D* and F* tests of Fu and Li (1993), and Tajima's D test.

To detect positive selection on the coding BoLA-DQA1 exon 2 sequences (240 bp), we used CODEML (PAML 4 package, Yang (2007)) and the DATAMONKEY web server (http//www.datamonkey.org/) (Pond & Frost, 2005). For CODEML, we have compared model M7 (beta) against M8 (beta plus omega) using the likelihood ratio test (LRT) and used the BEB to detect codons under positive selection with a posterior probability above 95% (Yang et al., 2000). For the DATAMONKEY web server (http//www.datamonkey.org/; Pond & Frost, 2005) we used four different tests to infer codons under positive selection, Single Likelihood Ancestral Counting (SLAC), Fixed Effects Likelihood (FEL), Fast Unconstrained Bayesian AppRoximation (FUBAR) and Mixed Effects Model of Evolution (MEME) (Murrell et al., 2012, 2013).

The phylogenetic neighbour-joining tree of the currently detected BoLA-DQA1 alleles was constructed using MEGA 6.0 software (Tamura et al., 2013), including all DQA1 alleles from GenBank database that have similar length with our sequences. We used the *Ovis aries*

sequences (LN827890, OK626230) as outgroups.

Finally, we used the cited above dataset to construct a median-joining (MJ) network (Bandelt et al., 1999) using the software Network 4.2.0.1 (available at http//www.fluxus-technology.com/sharenet.htm).

RESULTS AND DISCUSSIONS

Detection of blood parasites with PCR

Among the 68 cattle samples analyzed by PCR to detect possible infection by blood parasites, only ten (14.7%) were positive, each for only one type of hemoparasite. Indeed, six samples (8.8%) were infected with *Theileria spp.* and four (5.9%) by *A. marginale*. These samples presented the specific bands of the 18s rRNA genes of the genus *Theileria* (370 bp) and msp1α of A. marginal (603 bp). The presence of parasites of the genus *Babesia* was not detected in any of the analyzed samples.

During the current study, a low prevalence was observed for blood parasites of the genus Theileria and Anaplasma in cattle from Tunisia. In addition, no infection with parasites of the genus Babesia was detected. On the contrary, previous studies have shown greater infection in various regions around the world. Indeed, the microscopic study of 278 blood samples belonging to different bovine breeds in Tunisia (M'ghirbi et al., 2008), showed that 104 samples (37.4%) were positive for different species of piroplasms. Similarly, PCR analysis of 405 cattle samples in Egypt, showing clinical signs for blood parasites, indicated that 12.66% and 24.05% were positive for Babesia and Theileria spp., respectively (Nayel et al., 2012). On the other hand, Moumouni et al. (2015) observed that 71% of the samples analyzed were positive for hemoparasites. From a methodological point of view, the use of PCR for the detection of blood parasites has already shown its effectiveness. Indeed, using microscopic analysis, fluorescent antibody testing and PCR, Nayel et al. (2012) showed the absence of significant differences in the detection power of these three methods. Additionally, Almeria et al. (2001) suggested that the use of PCR was significantly more effective in the detection of *Theileria* ssp. and *Babesia* ssp. compared to microscopic observation. The low prevalence observed during the current study might indicated good management of breeding conditions which would limit the spread of the disease or that of ticks as a vector of the studied parasites. On the other hand, the use of different treatments could effectively reduce blood parasites. However, the observed prevalences cannot be generalized for the Tunisian cattle herd; they are rather an estimate of the infection rates in the studied population.

BoLA-DQA1 polymorphism

The total size of the sequences obtained in this study is 374 bp with 28 variable positions (7.49%) of which 4 (1.1%) are singletons. The average nucleotide composition across all sequences is 31.4% T, 26.1% C, 23.7% A, and 18.8% G. This composition is observed for all individuals with rare variations of around 0.1%. The values of haplotypic diversity (h = 0.863), nucleotide diversity (π = 0.01643) and mean pairwise differences (k = 6.144) were relatively high. A total of 11 different alleles were revealed in the 17 Tunisian cow samples. Among these alleles, five have been already detected in other cattle breeds (Table 1). We identified 11 alleles in 17 cattle from Tunisia belonging to the Holstein breed, of which six alleles were detected for the first time. Although this number of alleles seems very high compared to those detected by other studies, such allelic diversity is characteristic of MHC genes (Klein et al., 1993). Indeed, while only three DQA1 alleles were observed in a population of 34 cattle in Iraq (Al-Waith et al., 2018), 15 alleles were identified in 51 samples belonging to 8 cattle breeds studied by Takeshima et al. (2007). Similarly, Kulaj et al. (2015) identified 14 alleles, including three for the first time, by analyzing 71 cattle from the Polish Holstein-Friesian breed.

On the other hand, the BoLA-DQA1*0101 allele having a frequency of 0.32 in Tunisia, showed a frequency of 0.2606 in a Holstein-Frisian population (Kulaj et al., 2015) and was also the most frequent among all alleles detected in the cattle breeds studied by Takeshima et al. (2007). The second most frequent allele in Tunisian cattle was BoLA-DQA1*10011 with a frequency of 11.8%. This allele presented a frequency of 0.3592 in the Holstein-Friesian breed (Kulaj et al., 2015) and was also the most

frequent allele in the Danish black pied breed (45%; Takeshima et al., 2007). Other studies carried out by different research teams have shown that the BoLA-DQA1*10011 and *0101 alleles are the most frequent in Holstein cattle (Takeshima et al., 2008; Miyasaka et al., 2011; Schwab et al., 2009).

Table 1. List of currently detected alleles (grey shaded) and their frequency as well as alleles retrieved from GenBank and used in our study. Breeds where alleles were detected according to Takeshima et al. (2007)

GenBank Accession N°	Allele	Frequency	Breed[1]
PP335540	BoLA-DQA1*008,04	0.088	-
PP335541	BoLA-DQA1*036,01	0.029	-
PP335542	BoLA-DQA1*001,07	0.029	-
PP335543	BoLA-DQA1*001,01,01	0.147	-
PP335544	BoLA-DQA1*001,01,02	0.088	-
PP335545	BoLA-DQA1*010,01,03	0.059	-
AB257101	BoLA-DQA1*1203	-	DR
AB257102	BoLA-DQA1*0801	-	AY x LM, JE
AB257103	BoLA-DQA1*0101	0.324	BF, DB, HF
AB257104	BoLA-DQA1*0203-1	-	BF, JE, DB
AB257105	BoLA-DQA1*0204	-	BF x AY, BF, JE,
AB257106	BoLA-DQA1*0301	-	DR, JE
AB257107	BoLA-DQA1*0401	-	BF x HE, BF
AB257108	BoLA-DQA1*0103	-	DR, JE
AB257109	BoLA-DQA1*10011	0.088	BF, DB
AB257110	BoLA-DQA1*12011	0.029	DB, BF
AB257111	BoLA-DQA1*12012	-	-
AB257112	BoLA-DQA1*1401	-	DR
AB257113	BoLA-DQA1*12021	-	BF, DR, DB, JE
AB259566	BoLA-DQA1*10012	-	BF, DR, DB
AB259567	BoLA-DQA1*0102	-	BF
AB267074	BoLA-DQA1*0203-2	0.059	BF, DB
AB362375	BoLA-DQA1*1402	0.059	HF
AB362376	BoLA-DQA1*1002	-	JB
AB362377	BoLA-DQA1*12013	-	JE

[1]AY, Ayrshire; BF, British Friesian; DB, Danish Black Pied; DR, Danish Red; HE, Hereford; HF, Holstein Friesian; JB, Japanese black; JE, Jersey; LM, Limousin.

BoLA-DQA1 selection and phylogeny

The 240-coding nucleotide of the 11 detected alleles were translated into eight amino acid sequences. Among them, only BoLA-DQA1*008,04, *036,01 and *001,07 are translated to new amino acid sequences (Figure 1). Overall, neutrality tests showed that the studied sequences were selectively neutral. In fact, the Tajima test was negative (D= -0.5746) and not significant (p>0.1). The results of the Fu and Li tests were also non-significant (D* = 0.82646; F* = 0.42724) (P > 0.1). However, Positive selection by the Datamonkey web server was observed at one (site 13), two (50, 64), and three (50, 64, 71) codons by MEME, FEL, and FUBAR, respectively (Figure 1). No positive selection was suggested with SLAC. In addition, eight codons (13, 42, 50, 51, 63, 64, 70, 71) were reported under positive selection by CODEML (Figure 1). Purifying negative selection was observed at position 4 by SLAC, FEL and FUBAR and at position 65 only by FEL.

The neighbor joining (NJ) phylogenetic tree (Figure 2) indicated that the currently detected alleles in Tunisian cattle with those downloaded from GenBank were paraphyletic and were belonging mainly to two different groups. The first one encompasses 10 of the Tunisian alleles that were divided in two subgroups. The second groups contain only allele DQA1*0203-2 and other alleles from different cattle breeds.

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                  1       10        20        30        40        50        60        70        80
BoLA-DQA1*10011   DHIGTYGISI YHTYGPSGYY THEFDGDEEF YVDLEKRETV WRLPVFSKFT SFDPQGALRN IAIVKHNLEI VIQRSNSTAA
BoLA-DQA1*0101    ....A...NV .......... .......... .......... .......... .......... ..TA.....V L.........
BoLA-DQA1*008:04  ....A...NV ..S....... .......... .......... .........A .......... ..TA.....V L.........
BoLA-DQA1*036:01  ....A...NV ..S......F .......... .......... .........A .......... ..TA.....V L.........
BoLA-DQA1*001:07  .......... ..S....... .......... .......... .......... .......... ..TA.....V L.........
BoLA-DQA1*1402    ....A...NV ..S....... .......... .......... .........A .......... ..VG.RT..V M.R.......
BoLA-DQA1*12011   .......... .......... .......... .......... .........A T......... ..TT...... ..........
BoLA-DQA1*0203-2  ....A...NV .......... .......... .......... .N..L....R R......... ..T....... ..........
------------------------------------------------------------------------------------------------------------
SLAC
FUBAR                                                           *                   *         *
FEL                                                             *                   *
MEME                        *
PAML                        *                         *       * *                 **        **
```

Figure 1. BoLA-DQA1 exon 2 alleles of Tunisian Holstein cattle. Asterisks at the bottom of the table indicate signals of positive selection as obtained from the different tests

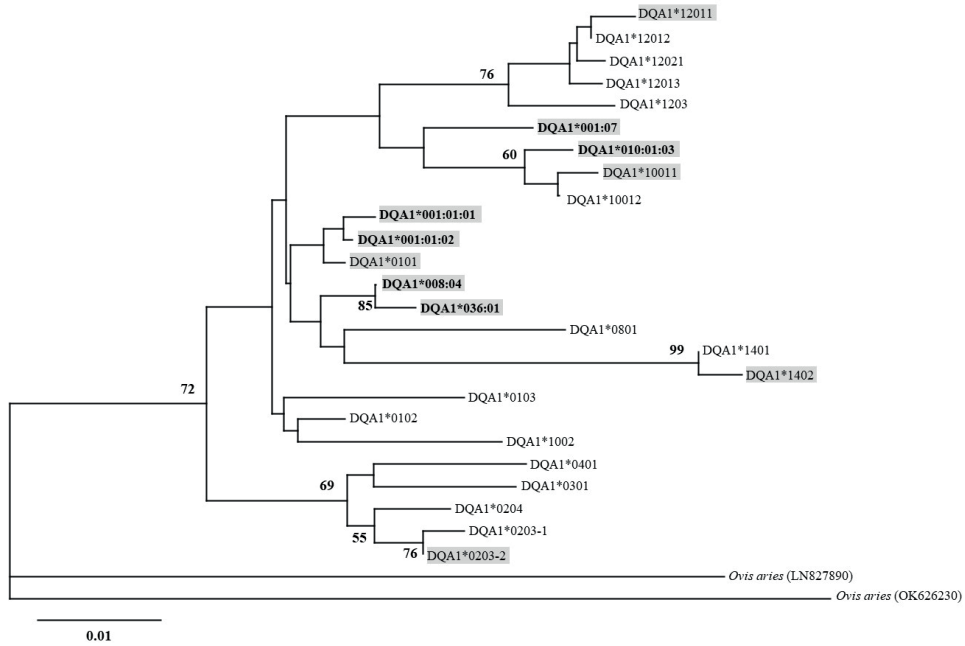

Figure 2. Neighbour-joining tree of BoLA-DQA1 allele. Numbers in the node indicate the bootstrap values if > 50%. Alleles observed in this study are grey shaded and newly identified alleles are marked in bold

The haplotype (allele) network (Figure 3) shows a central group of alleles allele that could be at the origin of the various other groups. In this central group, we find the BoLA-DQA1*0101 allele, the most frequent in Tunisia and which has also been detected in other cattle breeds. In addition, several other Tunisian alleles have a terminal position in the allele network which could indicate their relatively recent evolutionary status compared to the other central ones.

Adaptation to different pathogens is often studied by the analysis of major histocompatibility complex (MHC) class I and class II genes which are characterized by significant polymorphism in the different species analyzed so far (Klein, 1986; Weber et al., 2004; Awadi et al., 2018; Balasubramaniam et al., 2017). Our analysis of the DQA1 locus identified several codons under positive selection as evidenced from the used tests. Selection might suggest that shared alleles between different breeds can result from locally similar pathogens leading to similar natural and artificial selection pressures. Indeed, Bohórquez et al. (2020) suggested that the observed polymorphism for BoLA-DRB3 was very similar for all breeds they have studied.

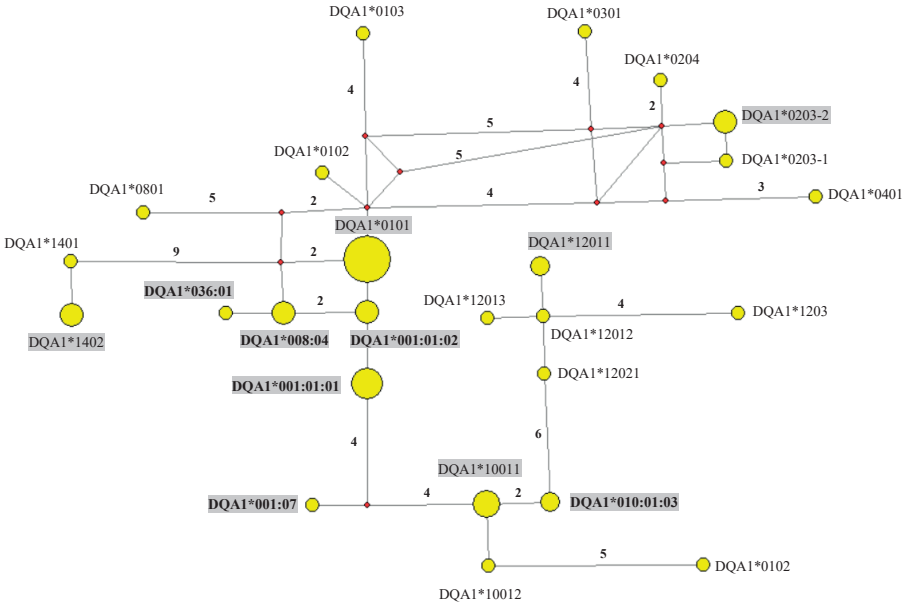

Figure 3. Median-joining network showing the relationships among BoLA-DQA1 alleles. Relative allele frequencies correspond to haplotype circle size (see Table 1). Numbers on lines connecting haplotypes indicate number of total mutation changes. Small red circle indicates inferred haplotype. Alleles observed in this study are grey shaded and newly identified alleles are marked in bold

In addition, the significant polymorphism observed in the Holstein breed in Tunisia could be linked to a significant diversity of pathogens. Indeed, the presence of seven specific alleles that have not been previously detected in different cattle populations around the world could suggest *in situ* evolution influenced by pathogens and environmental conditions. Such high polymorphism indicated that diversity in functionally important gene might persist even in the case of bottleneck events such those resulting from domestication and breeding (Bohórquez et al., 2020) In such situation, potential quantitative and qualitative differences of pathogens in varied habitats would result in high level of diversity. However, in the current study, introgression by alleles from other breeds could not be excluded.

CONCLUSIONS

The results of detection of blood parasites suggest relatively low infection rates by these parasites in the studied populations. On the contrary, the high polymorphism of the BoLA-DQA1 gene as well as the positive selection acting at several codons suggests a significant diversity of pathogens in Tunisia. On the other hand, the occurrence of a significant number of specific alleles of the Holstein breed in Tunisia might indicate an important potential of adaptation to local pathogens.

ACKNOWLEDGEMENTS

We thank A. Haiden (Research Institute of Wildlife Ecology, Vienna) for supporting with laboratory work in sequencing the BoLA-DQA1 gene.

REFERENCES

Adaszek, L., & Winiarczyk, S. (2008). Molecular characterization of *Babesia canis canis* isolates from naturally infected dogs in Poland. *Veterinary Parasitology*, 152, 235–241.

Almeria, S., Castellà, J., Ferrer, D., Ortuño, A., Estrada-Peña, A. et al. (2001). Bovine piroplasms in Minorca (Balearic Islands, Spain), a comparison of PCR-based and light microscopy detection. *Veterinary Parasitology*, 99, 249–259.

Al-Waith, H.K., Mohamed, T.R., & AL-Anbari, N.N. (2018). Association between DQA1 gene polymorphism and reproductive, immunity performance and heat tolerance in Holstein cattle. *Plant Archives*, 18(2), 2681-2686.

Awadi, A., Ben Slimen, H., Smith, S., Knauer, F., Makni, M. et al. (2018). Positive selection and climatic effects on MHC class II gene diversity in hares (*Lepus capensis*) from a steep ecological gradient. *Scientific Reports*, 8, 11514.

Balasubramaniam, S., Bray, R.D., Mulder. R.A., Sunnucks, P., Pavlova, A. et al. (2017). New data from basal Australian songbird lineages show that complex structure of MHC class II β genes has early evolutionary origins within passerines. *BMC Evolutionary Biology*, 16, 1–11.

Bandelt, H.J., Forster, P., & Roèhl, A. (1999). Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution*, 16, 37-48.

Belguesmi, K. (2023). *Détection par PCR de certaines maladies parasitaires et polymorphisme du gène BoLA-DQA1 chez quelques populations bovines en Tunisie*. Msc (in French), Higher Institute of Biotechnology of Béja, Tunisia.

Bohórquez, M.D., Ordoñez, D., Suárez, C.F., Vicente, B., Vieira, C. et al. (2020). Major Histocompatibility Complex Class II (DRB3) Genetic Diversity in Spanish Morucha and Colombian Normande Cattle Compared to Taurine and Zebu Populations. *Frontiers in Genetics*, 10, 1293.

Bono, M.F., Mangold, A.J., Baravalle, M.E., Valentini, B.S., Thompson, C.S. et al. (2008). Efficiency of a recombinant MSA-2c-based ELISA to establish the persistence of antibodies in cattle vaccinated with *Babesia bovis*. *Veterinary Parisotology*, 157, 203–210.

Brown, C.G. (1990). Control of tropical theileriosis (*Theileria annulata* infection) of cattle. *Parassitologia*, 32, 23–31.

Buckler, E.S., Thornsberry, J.M., & Kresovich, S. (2001). Molecular Diversity, Structure and Domestication of Grasses. *Genetics Research*, 77, 213-218.

de Castro, J.J. (1997). Sustainable tick and tick-borne disease control in live-stock improvement in developing countries. *Veterinary Parisotology*, 71, 77–97.

Fu, Y.X., & Li, W.H. (1993). Statistical tests of neutrality of mutations. *Genetics*, 133, 693-709.

Goszczynski, D.E., Ripoli, M.V., Takeshima, S.N., Baltian, L., Aida, Y. et al. (2014). Haplotype determination of the upstream regulatory region and the second exon of the BoLA-DRB3 gene in Holstein cattle. *Tissue Antigens*, 83, 180–183.

Hall, T.A. (1999). BioEdit, A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, 41, 95-98.

Klein, J. (1986). *Natural History of the Major Histocompatability Complex*. John Wiley and Sons, New York.

Klein, J., Satta, Y., O'hUigin, C., & Takahata N. (1993). The molecular descent of the major histocompatibility complex. *Annual Review of Immunology*, 11, 269–295.

Konnai, S., Imamura, S., Nakajima, C., Witola, W.H., Yamada, S. et al. (2006). Acquisition and transmission of *Theileria parva* by vector tick, *Rhipicephalus appendiculatus*. *Acta Tropica*, 99, 34–41.

Kulaj, D., Pokorska, J., Ormian, M., & Dusza, M. (2015). New alleles at the BoLA-DQA1 locus in Holstein-Fresian cattle. *Canadian Journal of Animal Science*, 95, 161-164.

Lew, A.E., Bock, R.E., Minchin, C.M., & Masaka, S. (2002). A msp1alpha polymerase chain reaction assay for specific detection and differentiation of *Anaplasma marginale* isolates. *Veterinary Microbiology*, 86 (4), 325-35.

Librado, P., & Rozas, J. (2009). DNAsp v5. A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451-1452.

Martinez, M.L., Machado, M.A., Nascimento, C.S., Silva, M.V., Teodoro, R.L. et al. (2006). Association of BoLA-DRB3.2 alleles with tick (*Boophilus microplus*) resistance in cattle. *Genetics and Molecular Research*, 5, 513-524.

Mejdell, C.M., Lie, O., Solbu, H., Arnet, E.F., & Spooner, R.L. (1994). Association of major histocompatibility complex antigens (BoLA-A) with AI bull progeny test results for mastitis, ketosis and fertility in Norwegian cattle. *Animal Genetics*, 25, 99-104.

Miyasaka, T., Takeschima, S.N., Matsumoto, Y., Kobayashi, N., Matsuhashi, T. et al. (2011). The diversity of bovine MHC class II DRB3 and DQA1 alleles in different herd of Japanese Black and Holstein cattle in Japan. *Gene*, 472, 42-49.

M'ghirbi, Y., Hurtado, A., Brandika, J., Khlif, K., Ketata, Z. et al. (2008). A molecular survey of *Theileria* and *Babesia* parasites. in cattle, with a note on the distribution of ticks in Tunisia. *Parasitology Research*, 103, 435–442.

Murrell, B., Wertheim, J.O., Moola, S., Weighill, T., Scheffler, K. et al. (2012). Detecting individual sites subject to episodic diversifying selection. *PLoS Genetics*, 8, e1002764.

Murrell, B., Moola, S., Mabona, A., Weighill, T., Sheward, D. et al. (2013). FUBAR, A Fast, Unconstrained Bayesian AppRoximation for Inferring Selection. *Molecular Biology and Evolution*, 30, 1196-1205.

Nascimento, C.S., Machado, M.A., Martinez, M.L., da Silva, M.V.G.B., Guimaraes, M.F.M. et al. (2006). Association of the bovine major histocompatibility complex (BoLA) BoLADRB3 gene with fat and protein production and somatic cell score in Brazilian Gyr dairy cattle (*Bos indicus*). Genet. *Molecular Biology*, 29, 641-647.

Nayel, M., El-Dakhly, K.H., Aboulaila, M., Elsify, A., Hassan, H. et al. (2012). The use of different diagnostic tools for *Babesia* and *Theileria* parasites in cattle in Menofia, Egypt. *Parasitology Research*, 111, 1019–1024.

Pond, S.L.K., & Frost, S.D.W. (2005). Datamonkey, rapid detection of selective pressure on individual sites of codon alignments. *Bioinformatics*, 21, 2531–2533.

Rupp, R., Hernandez, A., & Mallard, B.A. (2007). Association of bovine leukocyte antigen (BoLA) DRB3.2 with immune response, mastitis, and production and type traits in Canadian Holsteins. *Journal of Dairy Science*, 90, 1029-1038.

Schwab, A.E., Geary, T.G., Baillargeon, P., Schwab, A.J., & Fecteau, G. (2009). Association of BoLA DRB3 and DQA1 alleles with susceptibly to *Neospora caninum* and reproductive outcome in Quebec Holstein cattle. *Veterinary Parisotology*, 165, 136-140.

Stephens, M., Smith, N.J., & Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics*, 68 (4), 978-89.

Takeshima, S.N., & Aida, Y. (2006). Structure, function and disease susceptibility of the bovine major histocompatibility complex. *Animal Science Journal*, 77, 138–150.

Takeshima, S., Miki, A., Kado, M., & Aida, Y. (2007). Establishment of a sequence-based typing system for BoLA-DQA1 exon 2. *Tissue Antigens*, 69, 189-199.

Takeshima, S., Matsumoto, Y., Chen, J., Yoshida, T., Mukoyama, H. et al. (2008). Evidence for cattle major histocompatibility complex (BoLA) class II DQA1 gene heterozygote advantage against clinical mastitis caused by *Streptococci* and *Escherichia* species. *Tissue Antigens*, 72, 525-531.

Takeshima, S.N., Miyasaka, T., Polat, M., Kikuya, M., Matsumoto, Y. et al. (2014). The great diversity of major histocompatibility complex class II genes in Philippine native cattle. *Meta Gene*, 2, 176–190.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6, Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30, 2725-29.

Zanotti, M., Poli, G., Ponti, W., Polli, M., Rocchi, M. et al. (1996). Association of BoLA class II haplotypes with subclinical progression of bovine leukaemia virus infection in Holstein-Friesian cattle. *Animal Genetics*, 27, 337-341.

Zhang, T., Zhao, N., & Liu, Q. (2013). The effects of artificial selection on genetic variation of some immune genes in *Gallus gallus*. *Archives Animal Breeding*, 56, 691–699.

Yang Z. (2007). PAML 4, phylogenetic analysis by maximum likelihood. *Molecular Biology and Evolution*, 24 (8), 1586–91.

Yang, Z., Nielsen, R., Goldman, N., & Pedersen, A.M. (2000). Codon-substitution models for heterogeneous selection pressure at amino acid sites. *Genetics*, 155, 431–449.

Yoshida, T., Furuta, H., Kondo, Y., & Mukoyama, H. (2012). Association of BoLA-DRB3 alleles with mastitis resistance and susceptibility in Japanese Holstein cows, *Animal Science Journal*, 83, 359-366.

Weber, D.S., Stewart, B.S., Schienman, J., & Lehman, N. (2004). Major histocompatibility complex variation at three class II loci in the northern elephant seal. *Molecular Ecology*, 13(3), 711-8.