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Microsatellites polymorphism of major histocompatibility complex and serological profile in local Brazilian cattle Curraleiro Pé-Duro

[*Polimorfismos de microssatélites no complexo principal de histocompatibilidade e perfil sorológico na raça bovina brasileira Curraleiro Pé-Duro*]

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ABSTRACT

Microsatellites designed for regions of bovine lymphocyte antigen (BoLA) genes are used to understand the variability of immune responsiveness between individuals and populations against pathogens. The objective of this study is to characterize polymorphisms in 13 microsatellites present in genes of immune response and associate to *Brucella abortus, Leptospira* sp., *Neospora caninum*, leukosis, rhinotracheitis and bovine viral diarrhoea infections in the Brazilian local cattle breed Curraleiro Pé-Duro. Genotypes of 812 animals of both sex and various ages, using microsatellite markers were analyzed by Fisher test and linear regression model to associate infections with alleles of BoLA gene and natural resistance-associated macrophage protein 1 (SLC11A1) gene. All microsatellites exhibited moderate to high levels of polymorphism. The expected heterozygosity (He) ranged from 0.2803 to 0.8742 and the observed heterozygosity (Ho) ranged from 0.4103 to 0.8317. Allele RM185*88 were present in all leukosis positive, RM185*90 in all neosporosis positive and leukosis negative, LA54*155 in all IBR positive and 312*295 in neosporosis negative animals. The result showed that 415*457, 1778*243, 312*292, 1687*223 and other alleles are likely to play an important role in the host immune response. No significance to environmental factors was observed on regression model.

Keywords: bovine lymphocyte antigen, Curraleiro Pé-Duro, genetic resistance

RESUMO

Marcadores microssatélites relacionados a genes do antígeno linfocitário bovino (BoLA) auxiliam a compreensão da resposta imune entre indivíduos e populações. O objetivo deste estudo foi caracterizar polimorfismos em 13 microssatélites presentes em genes relacionados à ativação da resposta imune e testar a associação com Brucella abortus, Leptospira *sp.,* Neospora caninum *e com os vírus causadores da leucose, rinotraqueíte infecciosa e diarreia viral bovina na raça bovina brasileira Curraleiro Pé-Duro. Os genótipos de 812 animais de ambos os sexos e de várias idades foram obtidos pela análise de microssatélites e foram relacionados por teste de Fisher e regressão linear às infecções que culminam na ativação de genes do complexo BoLA e da proteína natural associada a macrófago (SLC11A1). Todos os microssatélites apresentaram níveis moderados de polimorfismos, com heterozigosidade esperada (He) variando entre 0,2803 e 0,8742 e heterozigosidade observada variando entre 0,4103 e 0,8317. O alelo RM185*88 estava presente em todos os animais soropositivos contra o vírus da leucose; RM185*90 em todos os soropositivos contra* Neospora caninum *e negativos contra leucose; LA54*155 em todos os positivos para IBR; e 312*295 em animais não reagentes a* N. caninum*. Foram encontrados 415*457, 1778*243, 312*292, 1687*223 e outros alelos associados a uma função importante na resposta imune do hospedeiro. Não se observou significância em relação a fatores ambientais no modelo de regressão.*

Palavras-chave: antígeno leucocitário bovino, Curraleiro Pé-Duro, resistência genética

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INTRODUCTION

The host can develop mechanisms of resistance or tolerance to infectious diseases and tick infestation to survive the infection. The mechanisms of resistance to diseases have different pathological and epidemiological effects than the mechanisms of the ability to tolerate infections. Host defense capacity is determined by resistance or tolerance. Resistance is the self defensive capacity to limit the pathogen burden and tolerance is the ability to regulate the immune response to limit the health impact of the infection (Schneider and Ayres, 2008).

Low infectious doses cause low mortality and low expression of resistance. Besides natural infection can provide great data of resistance genetic variation, cases of incomplete exposure to pathogen, problems on diagnosis and environmental interference can reduce heritability and the capacity to associate the disease with DNA polymorphisms, causing a decrease in the capacity to estimate the genetic effects (Bishop and Woolliams, 2014).

A resistance phenotype is costly and logistically difficult to measure. Thus, genomic studies are used to simplify the identification of resistant animals according to DNA-base selection, looking for quantitative trait loci (QTLs) or single nucleotide polymorphisms SNPs involved in the most efficient immune response (Bishop and Woolliams, 2014).

Curraleiro Pé Duro (CPD) is local cattle breed living in Brazilian savana and semi-arid regions, under high temperatures and lengthy drought (Mariante and Egito, 2002). The breed is popularly known by its traits of rusticity and resistance to plant intoxication (Serodio, 2013) and diseases. Some studies have shown that CPD cattle has better immune responsiveness to *Mycobacterium bovis* challenge of BCG vaccine and lymphocyte profile, when comparing with Nelore breed, demonstrating their resistance to combat intracellular pathogens (Moraes et al., 2009, Maggioli et al., 2013). CPD also has shown good neutrophils phagocytic index, producing more oxygen radicals than other crossbred cattle (*Bos taurus* x *Bos indicus*) demonstrating capability to defend itself against infections (Juliano et al., 2016).

One of the components of bovine immune system is the major histocompatibility complex (MHC), wich in cattle is called bovine leukocyte antigen (BoLA), that is considered a complex with many genes that stimulates the response to intracellular and extracellular infections. Alleles on BoLA gene influence epitope specificity of the T-lymphocyte receptors to antigens and immune response to vaccines (Sommer, 2005; Untalan *et al*., 2007; Giovambattista *et al*., 2013).

Bovine solute carrier family 11, member 1 (SLC11A1) gene contribute to bactericide activity of macrophages (Feng *et al*., 1996, Paixão *et al*., 2012). The gene is a candidate locus for natural resistance to *Brucella abortus* (Martinez *et al*., 2008), tuberculosis (Kadarmideen *et al*., 2011) and paratuberculosis infections (Pinedo *et al*. 2009).

Many genomic regions can be studied to demonstrate the potential association between allelic expression and diseases resistance in cattle. The alleles and haplotypes may vary across cattle populations, living under various selection pressures (Fritz, 2009). In general, local cattle managed under poor conditions of food and sanity, well-adapted to environment, exhibit a high degree of resistance to tropical disease because of many years of natural selection (Giovambattista *et al*., 2013).

The aim of this study was to investigate the alleles of microsatellites markers within the BoLA complex associated to host resistance to viral, bacterial and parasitic infection in bovine. Associating analysis with the serological phenotype were carried out to estimate the genetic of disease resistance of the local brazilian cattle breed Curraleiro Pé-Duro.

ETHICAL ASPECTS

The research was submitted to the *Ethics Committee in the Use of Animals* of FEDERAL UNIVERSITY OF GOIÁS, protocol code N. 041/16, approved on 12 September 2016.

MATERIAL AND METHODS

A total of 1,028 blood and serum samples were obtained from animals of both sexes with different ages, collected in 2011 from 20 herds located on several districts of Goiás, Piauí and Tocantins, Brazil (Fig. 1 and Table 1). The samples belong to the Pró Centro-Oeste Network

for characterization and conservation of bovine Brazilian genetic resources.

Total genomic DNA was obtained from frozen whole blood using the salting out extraction method described by Miller (Miller *et al*., 1988). DNA was isolated at the Federal University of Goiás, Genetics and Biodiversity Laboratory. DNA samples were quantified in NanoDrop spectrophotometer (NanoDrop Technologies, DE, USA) and all samples were diluted to 20ng/µL to be used in PCR reactions. The samples and controls were genotyped in GeneMapper software. Samples with poor quality DNA and samples with low call rates were discarded resulting in a final data set containing 812 individuals.

Figure 1-A. Curaleiro Pé-Duro specimen showing phenotypic traits of the breed; B. Map of sampling sites for Curraleiro Pé-Duro breed herds in Brazil (Freitas, 2014). Yellow: farms in Piauí; green: farms in Tocantins; red: farms in Goiás

Table 1. Genotyping of Curraleiro Pé-Duro breed samples with microsatellite markers for BoLA gene, distributed by farm code, location and samples number analyzed

Farm	Location	State	SN	TS
G1	Cavalcante	GO	95	367
G2	Monte Alegre		19	
G3	Planaltina		47	
G4	Campestre		58	
G5	Mimoso de Goiás		42	
G6	Pilar de Goiás		19	
G7	Água Fria		55	
G8	Pirenópolis		32	
T1	Guaraí	TO	73	129
T2	Porto Nacional		30	
T3	Sucupira		17	
T4	Chapada da Natividade		9	
P1	São João do Piauí	PI	88	316
P2	Campo Maior		29	
P3	Campo MaiorI		29	
P4	Palmeiras		40	
P5	Oeiras		40	
P7	Elesbão Veloso		44	
P8	Campo Maior		15	
P9	Campo Maior		31	
TOTAL			812	812

SN: sample number after data filtering; TS: total sample number by state

Serological results were obtained from epidemiological studies developed by researchers of Pró Centro-Oeste Network. Serum samples were tested against brucellosis and leptospirosis by the microagglutination test (MAT). Leptospirosis infection was tested against 19 serovars (Autumnalis, Hardjo, Andamana, Australis, Bratislava, Butembo, Canicola, Castellonis, Copenhageni, Gryppothyphosa, Hebdomadis, Icterohaemorrhagiae, Patoc, Pomona, Pyrogenes, Shermani, Sentot, Tarassovi, Wolfi) and the positive cutoff was considered 1:100. Neosporosis infection was determined by indirect immunofluorescence using nc-1 of *Neospora caninum* and the cutoff was considered 1:100. Vírus detection of IBR, BVD and leucosis was determined by indirect ELISA using comercial kit (Idexx Laboratories), considering the cutoff determined by the manufacturer.

Epidemiological questionnaires of Pró Centro-Oeste Network database were used to obtain age, sex, herd size, quarantine, occurrence of abortion, leptospirosis and brucellosis vaccine, presence of rodents, herd management practices, flooded areas, slaughter on farm, and veterinary service. Risk factor analysis for all infection sero-positivities were conducted using Fisher's exact test and generalised linear models (Asakura *et al*., 2018).

Environmental data was obtained from Lapig Maps, Google Earth, and Instituto de Meteorologia (INMET). Longitude, latitude, state, rain, evapotranspiration, precipitation, temperature, humidity, vegetation index, cellulose index, atmospheric pressure, global radiation, and wind direction average data from 2000 to 2011 were used in Fisher's exact test and generalised linear models for association with alleles and infections (Lobo, 2018).

A previously described panel of 13 microsatellites was amplified by primers labeled with different fluorochromes (FAM, NED, PET & VIC) at the 5'end (Table 2) as presented in the ISAG-FAO. The microsatellites used in this study have been characterized in previous studies of BoLA gene (Ellegren *et al*., 1993, Untalan *et al*. 2007, Fritz, 2009) and included locus from BTA23 and SLC11A1 gene in BTA2. The alleles identified on GeneMapper were statistically analyzed for possible association with resistance to *Brucella abortus*, *Leptospira* spp., *Neospora caninum*, bovine leukosis virus, bovine herpesvirus type 1 (of infectious bovine rinotrhacheitis), and bovine diarrhoea virus.

Polymerase chain reaction (PCR) was performed in the in the Núcleo de Pesquisas Replicon (NPR) of the Pontifical Catholic University of Goiás. The Multiplex PCR reactions were performed with 2µL DNA (20ng/µL) and 8µL of PCR mix. The PCR mix was prepared with 5µL buffer (Taq PCR Master Mix Kit - QIAGEN®), 3µL primer mix (containing 0,2-0,4µM of each primer). Cycling conditions were 95°C for 30s, 34 cycles of 95°C for 10s, 55°C for 20s, 72°C for 30 s, and a final extension period of 72°C for 10min. Following amplification, PCR products were diluted in 80µL of ultrapure water and 1µL of this was added to 10µL of formamide with an internal size standard (for each sample was used 10µL formamide and 0,18uL of GenescanLIZ 600® - Life Technology and Applied Biosystems, Foster City, CA, USA).

Diluted PCR products were added with formamide-GeneScan Liz600® and the mix was denatured for 5 minutes in 95°C and separated by capillary electrophoresis in an automatic sequencer ABI 3500 (Applied Biosystems, Foster City, CA, USA). For every run, two reference samples were used to correct variations in allele size assignation.

The alleles identified were analyzed for possible association with serological profile against *Brucella abortus*, *Leptospira* spp., *Neospora caninum*, bovine leukosis virus, bovine herpesvirus type 1, and bovine diarrhoea virus resistance.

GeneMapper software was used to allele size assignation. The results were tabulated on Excel and the Microsatellite Toolkit for Excel complement was used to transform data to textfile format and to obtain number of alleles by direct counting with 95% confidence interval (CI), allele frequencies, expected and observed heterozygosity, and polymorphic information content (PIC).

Potential deviations from Hardy-Weinberg exact test were estimated with Genepop software. Reynolds' and Nei's genetic distance matrix and the phylogenetic trees were estimated using the Populations (Langella, 1999) software. Mega software version 7.0.26 was used to build phylogenetic trees.

Table 2. Microsatellites for BoLa genes investigated in Curraleiro Pé-Duro breed for infectious diseases association

BTA	MHC	Microsatellite	Primer forward	Primer reverse	Interv	Ref
23	IIa	LA54 (FAM)	GAGAGTTCACTGTGCAG GAAGTATCT	CGCGAATTCCCAGAGTGAGT	149-227	1
23	IIa	DRBP1 (FAM)	ATGGTGCAGCAGCAAGGTGAGCA TTTG	GGGACTCAGTCTCTCTATCTC	110-140	2
23	IIa	171(FAM)	TTTCCCAGTCACGACGTTGACTTCA GCCAACAGGTAATGCT	TGGAGAGGGITGTITTCAGA	343-363	2
23	III	1268 (FAM)	TTTCCCAGICACGACGTTGCTGGAA AAGCCCCACTAAAGTA	GGCACAACTAACACACTCCA AA	242-280	2
23	I	312 (FAM)	TTTCCCAGTCACGACGTTGTGTTTC CTCCTCGACACTGA	ACCCCTGAACTTCTCCCACT	450-484	2
23	I	415 (PET)	TTTCCCAGTCACGACGTTGTTGAGA GGAAGGCAACCAAC	TCCAGCCATTTCTCTCCATC	437-487	2
23	I	1870 (PET)	TTTCCCAGTCACGACGTTGAAAAC AGAGAACACCAGCATCC	ACAATGGCCTTTCTAGCCCTT C	158-186	2
2	-	SLC11A1 (PET)	GGAGGACCTCGGGATGAG	TTGGCTGCCTTCCAGAAC	279-288	4
23	II	RM185 (FAM)	TGGCCTGTCTATGCTTGCATC	GAGTTTCCTTTGCATGCCAGT C	80-108	5
23	III	AGER (VIC)	TTTCCCAGTCACGACGTTGAAAAG GGGAGACGTGGAAAG	CACCTGGGGAAAAATCAACT	404-414	2
23	IIa	DRB3 (NED)	CGCTCCTGTGACAGATCTAT CC	CACCCCCGCGCTCACC	163-200	2
23	I	1687 (PET)	TTTCCCAGTCACGACGTTGTCCTTT TCTGCCTCACCCTA	AGGAGGGAAGATTTGGTGCT	388-436	2
23	I	1778 (VIC)	TTTCCCAGTCACGACGTTGCTTTGA GATGAGGCCAGCAG	CCAACATCACTGGAAGACAG AA	246-282	2

BTA: Bos taurus autossome; Interval: allele range in base pairs; Ref.: reference of the microsatellites 1. Outteridge *et al*. (1996); 2. Fritz (2009); 3. Pinedo *et al*. (2009); 4. Vázquez-Flores *et al*. (2006), 5. Ihara *et al*. (2004).

Genetix software (Belkhir *et al*., 2003) was used to calculate allele number, heterozygosity, FIS, FIT and Fst. The Weir and Cockerman's method of Wrigth F-statistic (Wright, 1951, Weir and Cockerham, 1984) was used to evaluate within breed variability. Populations structures were determined on Structure software (Pritchard *et al*., 2000) using a Bayesian clustering method. All samples were tested with a length burn-in period of 100,000 generations and 500,000 iterations after burn-in. A set from K=1 to K=10 was analyzed assuming admixture with five iterations for each K.

Effects of the alleles on serological status (positive/negative/suspected) were also evaluated by multivariate regression analysis and generalized linear models (GLM) procedures at 5% level of significance using the software R (R Development Core Team, 2011). The diseases were the dependent variable and molecular, epidemiological, and environmental data were the explanatory variables.

The alleles were classified on absent/absent (*/*), present/absent or absent/present (present/*) and present/present. Significant differences between genotypes */*, present/* and present/present frequencies were tested for all microsatellite alleles by analysis of Fisher test, considering $p<0.05$.

The alleles located on BTA23 were tested on Phase software to obtain haplotypes. Two microsatellites data were excluded from sampling, namely SLC11A1 because it is located on BTA2, and LA54, which had so many alleles and interfer on software analysis.

All alleles were included on multivariable logistic regression as well as epidemiological data (age, sex, herd size, presence of veterinary assistance, other breeds, quarantine, flooded area, abortion, vaccination, rodents) and environmental data (average of 10 years of longitude, latitude rain, evapotranspiration, precipitation, temperature, humidity, atmospheric pressure data). The final model included genotypes class, alleles and haplotypes as explanatory variable of interest controlling for age, sex, breed, epidemiological and environmental variables.

RESULTS

Serological investigations considered antibody frequency by breed, farms and state are shown in Table 3. The antibody frequencies had differences between populations. The lower frequencies for *Brucella* infection were expected due to a national program of vaccination, diagnostic, and sacrifice of positive animals. *Leptospira* sp., *Neospora caninum*, leukosis, IBR, and BVD frequencies showed heterogeneity regarding the immunological profile of the animals. In some farms the frequencies were above the average frequency for the region.

Table 3. Antibody frequencies against infectious diseases in Curraleiro Pé-Duro (CPD) breed from Brazil

State	Farm	*Brucella*	*Leptospira*	*Neospora*	Leukosis	IBR	BVD
TO	T1	0%	85%	28%	36%	62%	46%
TO	T2	0%	10%	5%	40%	93%	70%
TO	T3	0%	16%	5%	5%	58%	21%
TO	T4	0%	28%	6%	89%	78%	50%
PI	P1	0%	39%	56%	1%	55%	46%
PI	P2	0%	33%	93%	7%	83%	53%
PI	P3	0%	86%	97%	32%	66%	38%
PI	P4	0%	50%	8%	18%	95%	28%
PI	P5	0%	27%	6%	59%	67%	41%
PI	P7	0%	4%	22%	8%	61%	33%
PI	P8	0%	80%	20%	40%	53%	7%
PI	P9	0%	94%	75%	36%	61%	78%
GO	G1	0%	50%	11%	20%	56%	36%
GO	G2	0%	60%	18%	0%	52%	23%
GO	G3	0%	33%	52%	42%	56%	13%
GO	G4	0%	28%	6%	89%	78%	50%
GO	G5	1%	12%	65%	10%	49%	17%
GO	G6	6%	40%	47%	21%	79%	30%
GO	G7	0%	28%	6%	89%	78%	50%
GO	G8	2%	61%	31%	19%	71%	23%

Pathogens investigated: *Brucella abortus*, *Leptospira sp.*, *Neospora spp.*, Leucosis virus, Bovine Herpesvirus type 1 (causing infectious bovine rinotracheitis – IBR) and BVDV (bovine viral diarrhoea virus).

A Bayesian cluster analysis on Structure software indicated that the genotypes are better clustered in two clusters, considering the delta K value of Evano's method, but the results showed differences when phylogenetic tree were made using different methods (Nei and Reynolds). It is possible to see a particular cluster when k=10 (marked with yellow color on Fig. 2), suggesting that more clusters can be tested. Fig. 2 shows the animals grouped in clusters according to the proportion of animal genome (represented by color and size). Animals of the same color are grouped together.

The antibody frequencies had no significant difference between the clusters. The results showed that cluster 1 and 2 have similar frequencies for all infections (Table 4).

The cluster analysis did not show resistance or susceptibility profiles. It was observed that alleles were grouped in 949 possible combinations and the samples allocated in 932 haplotypes blocks, using 11 microsatellites. No significant association was observed between the haplotypes and pathogens.

The analysis of population diversity revealed significant deviations of the Hardy-Weinberg equilibrium ($P<0.01$) in all the loci analyzed, excluding *DBRP1* (Table 5). Piauí populations are mostly in equilibrium and Goiás populations has more markers in disequilibrium. The 1778 marker is in disequilibrium in more herds.

The allele number expected and observed heterozygosity were tested for each farm (Table 6). The number of alleles ranged from four to nine alleles per locus. He and Ho ranged from 0.5642 to 0.7088 and Ho 0.4870 to 0.7521, respectively.

All herds showed high number of alleles. Although He and Ho indicates that microsatellites were highly informative, the observed heterozygosity (Ho) was lower than the expected heterozigosity (He) in 15 herds, suggesting excess of homozygotes.

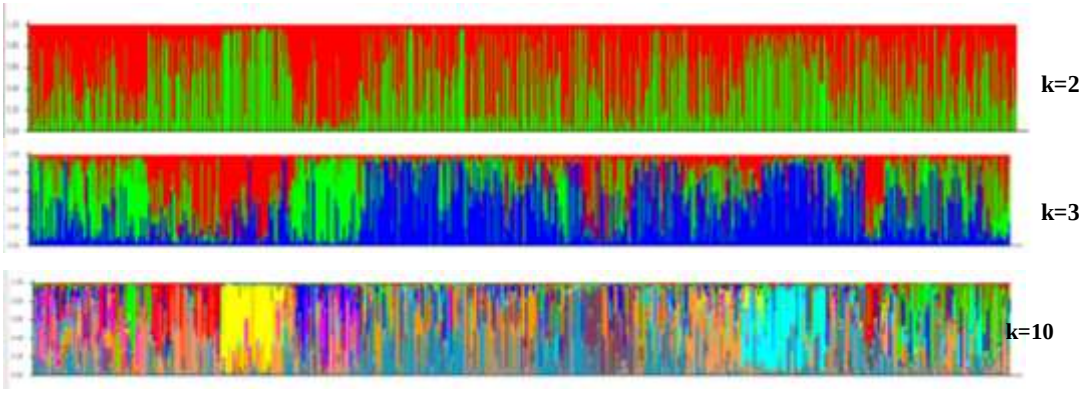

Figure 2. Bayesian clustering assignment of Curraleiro Pé-Duro breed representing the clusters according to the genome proportion of alleles on markers linked to BoLA gene, and the better k=2

Table 4. Prevalence of infections in Curraleiro Pé-Duro breed clustered by microsatellites of immune response

Pathogen	Prevalence cluster 1	Prevalence cluster 1
Brucella abortus	0,5% (2/397)	0,96% (4/415)
Leptospira spp.	39,80% (158/397)	48,16% (197/409)
Neospora caninum	31,06% (123/396)	37,22% (153/411)
Leukosis virus	45,29% (178/393)	21,48% (87/405)
Herpesvirus type 1	67,34% (266/395)	64,79% (265/409)
Diarrhoea virus infection	43,95% (171/389)	40,63% (167/411)

Table 5. Hardy-Weinberg test for microsatellite loci in the Curraleiro Pé-Duro populations

Prop	Microssatelite markers												
	171	*312*	*415*	*1268*	*1687*	*1778*	*1870*	*AGER*	*DBR3*	*DBRP1*	*LA54*	*RM185*	*SLC11A1*
G1	**	-	-	**	-	**	*	-	*	-	**	*	-
G2	-	**	-	-	-	**	**	**	*	-	-	-	-
G3	**	-	**	-	-	**	**	-	-	-	-	-	*
G4	-	-	-	-	-	**	-	-	-	-	-	-	*
G5	-	-	*	-	-	-	-	-	-	-	**	-	-
G6	-	*	**	*	-	**	-	-	-	-	-	-	-
G7	*	-	**	-	*	**	**	**	-	-	*	**	**
G8	-	-	-	-	**	**	*	-	-	-	-	-	*
P1	-	-	-	-	-	-	**	-	-	-	**	**	-
P2	-	-	-	-	-	**	-	-	-	-	-	-	-
P3	-	-	*	-	*	**	-	-	-	-	*	-	-
P4	-	-	-	-	-	-	-	-	-	-	-	-	-
P5	-	-	-	*	-	**	-	-	-	-	-	-	-
P7	-	-	-	-	-	*	-	-	-	-	*	-	*
P8	-	*	-	-	**	**	-	-	-	-	-	-	-
P9	-	-	-	-	-	**	-	-	-	-	-	-	*
T1	**	-	**	-	-	**	**	-	-	-	-	*	**
T2	-	-	-	-	-	*	**	-	-	-	-	-	-
T3	-	*	**	-	-	-	*	**	-	-	**	*	**
T4	-	-	-	*	-	-	-	-	-	-	*	-	-

* P<0.05, ** p<0,01

Table 6. Analysis of heterozygosity and allele number of microsatellite for BoLA and SLC11A1 gene by herd of Curraleiro Pé-Duro breed

Population	Nº	He	He SD	Ho	Ho SD	Alleles	Alleles SD
G1	95	0.6815	0.0509	0.6112	0.0140	9.38	5.28
G2	19	0.6045	0.0794	0.4870	0.0370	5.38	2.50
G3	47	0.6847	0.0454	0.6771	0.0192	6.85	2.41
G4	58	0.5907	0.0434	0.5780	0.0193	5.69	2.29
G5	42	0.5642	0.0765	0.5866	0.0213	5.23	1.74
G6	19	0.6789	0.0446	0.6722	0.0308	5.46	1.90
G7	55	0.7088	0.0522	0.5685	0.0189	7.69	3.22
G8	32	0.6559	0.0641	0.6080	0.0263	7.23	3.47
P1	88	0.6452	0.0548	0.6254	0.0143	7.69	3.40
P2	29	0.6720	0.0555	0.6785	0.0242	7.08	3.35
P3	29	0.7033	0.0498	0.7045	0.0238	6.38	2.57
P4	40	0.6448	0.0548	0.6626	0.0209	6.15	2.67
P5	40	0.6719	0.0565	0.6425	0.0211	6.46	2.50
P7	44	0.6636	0.0586	0.6189	0.0203	7.92	4.01
P8	15	0.7234	0.0523	0.6150	0.0378	6.15	2.67
P9	31	0.6419	0.0645	0.6138	0.0249	7.46	3.41
T1	73	0.6162	0.0458	0.5278	0.0166	7.77	2.98
T2	30	0.6880	0.0586	0.6526	0.0243	7.23	3.52
T3	17	0.6291	0.0605	0.4948	0.0354	5.46	2.33
T4	9	0.6687	0.0482	0.7521	0.0399	4.38	1.94

He: Unbiased heterozigozity; Ho: observed heterozigozity; SD: standard deviation

The global F statistics (FIT, FST, and FIS) (Table 7) were performed to show genetic variability within and between groups of the breed, and to estimate excess of homozygosity on each locus.

FST revealed the moderate genetic diversity for all loci. Similarity of allele frequencies between populations could mean the capacity of immune response was similar in all herds. Heterozygous deficiency was observed and could lead to the decrease in the hability to resist infections.

Table 7. Microsatellites and endogamic coefficients of Curraleiro Pé-Duro breed

Microsatellite	FIS	FIT	FST
171	0.0624	0.1772	0.1224
312	0.0716	0.1868	0.1241
415	0.0542	0.1389	0.0895
1268	0.0702	0.1849	0.1233
1687	0.0672	0.1820	0.1230
1778	0.0435	0.1513	0.1127
1870	0.0566	0.1730	0.1234
AGER	0.0657	0.1799	0.1223
DBR3	0.0722	0.1866	0.1233
DBRP1	0.0727	0.1881	0.1245
LA54	0.0554	0.1710	0.1224
RM185	0.0640	0.1788	0.1227
SLC11A1	0.0576	0.1738	0.1233

All microsatellites (Table 8) exhibited moderate to high levels of polymorphism, except the 415 locus. The markers had higher He than Ho. The lowest He, Ho and PIC values were found in the 415 marker while the higher values were found on DRB3 marker. The expected heterozygosity (He) ranged from 0.2803 (415) to 0.8742 (DRB3) and the observed heterozygosity (Ho) ranged from 0.4103 to 0.8317 on the same locus. The result contrast with Haikukutu (2018), that found Ho 0.244 and He 0.428 in the DRB3 marker. PIC values varied between 0.2631 (415) and 0.8617 (DRB3).

Table 8. Results of univariate analysis for association of diseases and microsatellite alleles on BoLA gene in Curraleiro Pé-Duro breed

Microsatellite	He	Ho	PIC	Allele	Disease	P-value
AGER	0.6974	0.6304	0.6446	310, 312, 314, 316, 320	Neosporosis	<0.05
				310	Leukosis	<0.05
				310, 312, 314, 316, 320	IBR	<0.05
DBRP1	0.8159	0.797	0.7941	115, 131	Leukosis	<0.05
DRB3	0.8742	0.8317	0.8617	162, 164, 168, 174, 176, 178, 182, 184, 186, 188, 196	Neosporosis	<0.01
				178, 188, 190, 192	BVD	<0.05
LA54	0.8266	0.6729	0.8133	159, 177, 193	Leptospirosis	<0.01
				193	Leukosis	<0.05
				155, 191, 199	IBR	<0.05
1687	0.8327	0.7529	0.8203	239	Brucelosis	<0.05
				213, 221, 223, 235, 237, 239, 241, 245, 249, 253	Leptospirosis	<0.05
				221, 223, 231, 241, 245	Neosporosis	<0.001
				211, 213, 215, 217, 221, 223, 231, 237, 239, 243, 245, 253	Leukosis	<0.01
				211	IBR	<0.05
				217, 231	BVD	<0.05
171	0.6049	0.5007	0.5524	324, 326	Brucelosis	<0.01
				326, 328, 340	Neosporosis	<0.001
				322, 324, 326	Leukosis	<0.001
				324	IBR	<0.01
				328, 338	BVD	<0.05
1778	0.8378	0.4697	0.82	239, 241	Brucelosis	<0.05
				237, 239, 241, 243, 245, 247, 249, 253, 261, 273	Leptospirosis	<0.05
				241, 245, 247, 263, 273	Neosporosis	<0.001
				241, 271	Leukosis	<0.05
				231, 239, 263	IBR	<0.05
				261, 273	BVD	<0.05
1870	0.6789	0.5532	0.6486	151, 153	Brucelosis	<0.05
				145, 147, 149, 153, 155, 161	Leptospirosis	<0.001
				141, 151, 153, 157, 161	Neosporosis	<0.05
				145, 153	Leukosis	<0.05
				145	BVD	<0.05
312	0.7934	0.7611	0.7646	292	Leptospirosis	<0.01
				292	Neosporosis	<0.05
				292, 295, 311	BVD	<0.05
415	0.2803	0.1272	0.2631	455	Brucelosis	<0.05
				453, 455, 457	Leptospirosis	<0.05
				457	Neosporosis	<0.001
				453, 455, 461	Leukosis	<0.05
				461	IBR	<0.05
				457	BVD	<0.001
RM185	0.8573	0.7317	0.8411	88, 90, 96, 98, 104, 106, 108,	Leptospirosis	<0.05
				90, 94, 98, 102, 104, 106	Neosporosis	<0.05
				88, 90, 100	Leukosis	<0.05
				100, 102, 104, 106	BVD	<0.01
				88, 90, 92, 94, 96, 98	BVD	<0.05
SLC11A1	0.4917	0.4103	0.4597	279, 281, 283, 285, 287	Leptospirosis	<0.01
				279	Neosporosis	<0.01
				291	IBR	<0.05
1268	0.7836	0.7426	0.7566			

He: expected heterozygous; Ho: observed heterozygous; PIC: polymorphic information content; p-value of chi square test.

Some of the alleles were significant to univariate analisis (showed on Table 7) were present in 100% of animals positive or negative for one of the infections. Although some alleles were not significant to logistic regression analysis, the allele RM185*88 were present in all leukosis positive and neosporosis negative (6 animals), RM185*90 in all neosporosis positive and leukosis negative (16 animals), LA54*155 in all IBR positive (5 animals) and 312*295 in neosporosis negative (4 animals).

All microsatellites and ordinary alleles were tested against each disease by univariate analysis and logistic regression. The alleles and risk factors evolved on positive or negative results for each infection is reported in Table 9.

Table 9. Multivariate analysis of the individual animal odds of sorology for infections and alleles among brazilian Curraleiro Pé-Duro breed

Pathogen	Marker*allele	Positive (%)	Negative (%)	odds	CI	p
Brucella	171*326	0.23% (2/866)	99.76% (864/866)	0.15	0.03-0.75	<0.05
Leptospira	>24 months	47.53% (674/1418)	51.48% (730/1418)	3.43	2.24-5.23	<0.01
	LA54*177	19.35% (6/31)	80.64% (25/31)	3.42	0.13-0.86	<0.05
	1687*237	43.96% (240/546)	54.58% (298/546)	7.63	0.6-0.97	<0.05
	1687*241	32.98% (62/188)	66.49% (125/188)	4.82	0.34-0.68	<0.01
	1687*249	31.37% (16/51)	68.63% (35/51)	4.97	0.25-0.93	<0.05
	1778*243	88.89% (16/18)	11.11% (2/18)	8.12	1.81-3.63	<0.01
	1778*245	34.86% (38/109)	65.14% (71/109)	6.07	0.39-0.93	<0.05
	1778*253	27.59% (8/29)	72.41% (21/29)	3.86	0.16-0.91	<0.05
	1870*145	19.23% (5/26)	80.77% (21/26)	2.61	0.09-0.71	<0.01
	1870*149	22.22% (10/45)	77.78% (35/45)	3.95	0.18-0.83	<0.05
	1870*155	36.36% (40/110)	63.64% (70/110)	5.81	0.37-0.9	<0.05
	312*292	83.33% (10/12)	16.67% (2/12)	5.87	1.23-2.79	<0.05
	415*457	22.47% (20/89)	77.53% (69/89)	4.57	0.26-0.78	<0.01
Neospora	>24 months	35.47% (498/1406)	64.58% (908/1406)	2.88	1.77-4.69	<0.01
	12-24 months	52.17% (24/46)	47.83% (22/46)	3.8	1.65-8.72	<0.01
	Brucella vaccine	35.34% (164/464)	64.66% (300/464)	0.51	0.38-0.68	<0.01
	DRB3*162	46.88% (90/192)	53.13% (102/192)	2.08	1.39-3.11	<0.01
	DRB3*168	28.74% (73/254)	71.26% (181/254)	1.65	1.13-2.4	<0.01
	DRB3*176	38.87% (145/373)	61.13% (228/373)	1.61	1.17-2.22	<0.01
	DRB3*178	39.20% (49/125)	60.80% (76/125)	1.96	1.22-3.13	<0.01
	1687*221	67.74% (21/31)	32.26% (10/31)	9	3.52-2.29	<0.01
	1687*223	5.71% (2/35)	94.29% (33/35)	0.07	0.01-3.18	<0.01
	1687*231	50% (18/36)	50% (18/36)	3.05	1.43-6.48	<0.01
	171*328	66.25% (53/80)	33.75% (27/80)	4.5	2.57-7.88	<0.01
	1778*255	47.62% (20/42)	52.38% (22/42)	2.82	1.32-6.01	<0.01
	1870*141	50% (7/14)	50% (7/14)	1.03	3.12-3.37	<0.01
	1870*151	35.61% (271/761)	64.39% (490/761)	2.07	1.48-2.88	<0.01
	1870*153	35.86% (90/251)	64.14% (161/251)	2.53	1.66-3.82	<0.01
	1870*161	54.35% (100/184)	45.65% (84/184)	2.69	1.72-4.2	<0.01
	RM185*104	45.23% (109/241)	54.77% (132/241)	1.89	1.35-2.63	<0.01
	RM185*106	48.12% (64/133)	51.88% (69/133)	2.02	1.29-3.15	<0.01
	RM185*94	46.51% (60/129)	53.49% (69/129)	2.03	1.31-3.12	<0.01
Leukosis	>24 months	22.43% (318/1418)	75.04% (1064/1418)	1.93	1.18-3.14	<0.05
	AGER*310	36.51% (46/126)	62.69% (79/126)	1.66	1.07-2.56	<0.05
	DBRP1*115	15.83% (22/139)	82.01% (114/139)	0.49	0.28-0.84	<0.01
	LA54*193	57.58% (19/33)	36.36% (12/33)	4.88	2.26-10.52	<0.01
	1687*223	54.29% (19/35)	42.86% (15/35)	2.61	1.19-5.7	<0.05
	1687*231	44.44% (16/36)	55.56% (20/36)	3.79	1.78-8.04	<0.01
	1687*253	55.56% (5/9)	44.44% (4/9)	5.51	1.4-21.57	<0.01
	171*322	26.34% (54/205)	72.20% (148/205)	1.48	1.01-2.17	<0.05
	1870*153	15.14% (38/251)	82.07% (206/251)	0.53	0.36-0.78	<0.01
	415*453	25% (30/120)	72.50% (87/120)	2.23	1.29-3.84	<0.01
	415*455	23.28% (268/1151)	74.72% (860/1151)	1.85	1.3-2.63	<0.01
	415*461	75% (6/8)	25% (2/8)	1.89	3.45-103.1	<0.01
IBR	>24 months	27.64% (392/1418)	68.97% (978/1418)	3.76	2.67-5.29	<0.01
	12-24 months	56.52% (26/46)	43.48% (20/46)	1.98	1.01-3.86	<0.05
	1687*211	82.22% (37/45)	15.56% (7/45)	2.5	1.09-5.77	<0.05
	171*324	72.70% (269/370)	22.43% (83/370)	1.67	1.26-2.21	<0.01
	1778*239	76.87% (113/147)	21.09% (31/147)	1.72	1.13-2.62	<0.05
BVD	>24 months	45.70% (648/1418)	44.43% (630/1418)	4.61	3.07-6.92	<0.01
	DRB3*178	58.59% (75/128)	29.69% (38/128)	2.25	1.5-3.4	<0.01
	DRB3*188	66.67% (20/30)	30% (9/30)	2.26	1.05-5.1	<0.05
	1687*231	11.11% (4/36)	80.56% (29/36)	0.25	0.1-0.58	<0.01
	1870*145	23.08% (6/26)	73.08% (19/26)	0.37	0.15-0.91	<0.05
	415*457	20.22% (18/89)	71.91% (64/89)	0.45	0.27-0.74	<0.01

CI: confidence interval of 95%, p: p-value. Risk factors were included in the column of Marker*allele.

The genotypes heterozygous (*/allele) and homozygous (*/* and allele/allele) for each allele were tested against infections. The most frequent alleles in infected animals and in susceptible animals were different between diseases.

The most associations between markers and infections were positive on odds ratio, indicating alleles more present in infected animals. The only environmental data important on multivariate analysis was cellulose. Cellulose was a factor with ranging of 0,162965 and 0,291898 and no biological context explain the association with *Neospora caninum.*

DISCUSSION

The results showed genetic and nongenetic variables with significant association of immune responses to infectious disease agents. These results can be used on cattle management for genetic and productive improvement. The development of cattle resistant to diseases can increase the productivity, decrease the costs of the production system, decrease antibiotic and chemical usage, improve animal welfare, industry and public concerns, and reduce potential zoonosis (Bishop and Woolliams, 2014).

Here we reported 161 alleles on MHC genes. From those, 42 alleles of 1687, 171, 1778, 1870, 415, AGER, DRBP1, DRB3, LA54, and RM185 markers are likely to play an important role in the host immune response, when compared by regression analysis. Alleles of 1687 markers were significant to all infections, except *Brucella abortus*. one. Alleles of SLC11A1, 312, and 1268 markers have no significance with diseases although a difference was observed in the univariate analysis. The microsatellite alleles of DRBP1, DRB3, RM185 loci are related to immune response and are reported in animals naturally infested with ticks (Acosta-Rodríguez *et al*., 2005).

Allelic richness was found for all microsatellites tested. Although the lower observed heterozygosity than expected, the allelic richness was high and it means that although these breeds had low number of individuals, there are statistically significant differences between individuals, and it was a good indicator of genetic diversity.

The analyses performed by AMOVA revealed significant deviations from the Hardy-Weinberg equilibrium (P<0,01) for most of the loci. Piauí populations was the only population with no loci in disequilibrium. Similar results reported significant deviations of the HWE (P<0.01) due to the lack of heterozygous in most of the analyzed loci on the CPD and other Brazilian Creole breeds (Caracu, Crioulo Lageano, Curraleiro, Mocho Nacional, and Pantaneiro) (Serrano *et al*., 2004, Oliveira *et al*., 2012).

The equilibrium deviation may suggest excess of homozygous genotypes or the presence of null alleles, due to inbreeding, subdivision, and geographic isolation. The three effects are reported on CPD populations that are adapted to its local region with some degree of isolation causing loss of genetic variation and inbreeding (Oliveira *et al*., 2012).

The variability of alleles on BoLA loci in CPD breed is similar to the parental breeds. Parental Portuguese breeds Alentejana, Arouquesa, Barrosa and Mirandesa (Mariante and Cavalcante, 2000) also exhibited higher variability, similar values of expected and observed heterozygosity, with a trend of lower observed homozygosity in BoLA microsatellites loci (Bastos-Silveira *et al*., 2009).

PIC, He and Ho ranged from 0.2631 to 0.8617, from 0.2803 to 0.8742, and from 0.4103 to 0.8317, respectively. Observed heterozygosity in CPD populations are lower and PIC values are similar compared to Bali cattle, an important breed in Indonesia. The higher diversity on Bali cattle can be due to breeding programs for conservation and improvement genetic potential (Puja *et al*., 2018). CPD cattle do not have a conservation program oriented to the breed.

The high diversity of alleles was reported on Latin American Creole cattle that had 36 alleles previously reported and three novel alleles (Giovambattista *et al*., 2013). Similarly, Curraleiro Pé-Duro has small population sizes with high degree of gene diversity, although with lower observed heterozygosity than expected. Among all observed alleles we report no exclusive alleles because CPD from our study

was not compared to other Brazilian cattle breeds.

The haplotypes had no significance with infections (p>0,05). The univariate logistic regression results agreed with the reported by Estonba *et al*. (2005), where one microsatellite allele of the SLC11A1 gene had significantly higher frequency in the seronegative group.

When the association between the alleles and the phenotypes were tested by univariate analysis, 81 alleles differ significantly (P≤0.05) in case-control animals. When classified on homozygous or heterozygous, we found statistical significance with infection, with more animals heterozygous positive than homozygous positive.

Alleles RM185*88, RM185*90, and LA54*155 were associated to all leukosis positive animals and 312*295, RM185*90 to neosporosis negative. RM185 marker is BoLA class I microsatellite that encode surface molecules important to induce and regulate immune response (Acosta-Rodríguez *et al*., 2005). LA54 primer corresponded to a sequence on intron 2 of DRB3 gene, which is functionally the most important gene on cattle expressed at a high level in peripheral blood lymphocytes. This marker has been explored as a possible marker of the expressed polymorphism in the exon region (Ellegren *et al*., 1993).

Other alleles were signiticant to infections according to a linear regression model. The 415*457 (allele 457 of 415 marker) was present in 77,53% of negative leptospirosis and 71,91% of negative to BVD, indicating a possible locus for resistance. The 1778*243 and 312*292 were present in 88,89% and 83,33% *Leptospira* positive animals, respectively, while 1687*223 was present in 94,29% of *Neospora* negative. Other loci were present in most positive animals, such as 1870*145 present in 80,77% of *Leptospira* and 73,08% of BVD positive.

The infectious status os an animal can be modified by several factors. Some data not observed in this study can modify the susceptibility to infections, like nutritional status, environmental factors, pregnancy and productivity level (Acosta-Rodríguez *et al*., 2005).

Extended research proved the significant role of the SLC11A1 gene in disease susceptibility (Martinez *et al*., 2008). Studies found potential connection between polymorphisms in SLC11A1 in seronegative group for paratuberculosis (Pinedo *et al*., 2009), lower incidence of tuberculosis (Kadarmideen *et al*., 2011) and resistance against *Brucella abortus* infection in cattle (Barthel *et al*., 2001).

The statistical analysis resulted in significant differences in allelic frequencies between seropositive and seronegative samples and microsatellite alleles. The major frequency in */* genotype (allele absent) is explained by the larger number of alleles for each loci. Animals will have low probability for a particular allele, so more animals are expected with absence than with presence of that allele. More frequent alleles were 171*326, 1870*151, 312*289, SLC*281, AGER*312, LA54*183, 415*455.

Epidemiological and environmental factors were not significant at logistic regression. The Curraleiro Pé-Duro populations presented a probable heterozygote deficit by moderate genetic differentiation (Fst between 0,0895 and 0,1245). This study showed that Curraleiro Pé-Duro presented many haplotypes conferring many phenotypes, but the endogamy can decrease the variability of this breed.

CONCLUSIONS

In the current study, we identified microsatellite alleles associated with infection and susceptibility phenotypes. Microsatellite genotypes and loci in bovine MHC region were described in high number of positive or negative animals, suggesting their usefulness as markers for susceptibility/resistance to leptospirosis, neosporosis, leukosis, rhinotracheitis and bovine diarrhoea virus infections in CPD breed. Alleles in 1687 markers are related to all infections, except *Brucella* sp. infection.

The CPD cattle has higher allelic richness and alleles associated to diseases resistance. However, the deficit of heterozygosity should be avoided with programs of genetic conservation to select animals with superior disease resistance and maintain the genetic variability of populations.

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CONTRIBUTORS

TMS Freitas: manuscript writing; TMS Freitas, JM Dias, MI Moura: data collection and laboratory analysis; AS Carmo, V Landi, MCS Fioravanti: study conception, objective, data analysis; MCS Fioravanti: acquired the funding, data analysis, discussion and approval of the version to be published.

DATA AVAILABILITY STATEMENT

Data-available – the research data are available in the repository Figshare.
https://doi.org/10.6084/m9.figshare.28836725.v1

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