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Co-infection with *Mycobacterium bovis* does not alter the response to bovine leukemia virus in BoLA DRB3*0902, genetically resistant cattle

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ABSTRACT

High proviral load (HPL) profile in bovine leukemia virus infected animals poses increased risk of transmission, and development of HPL or low proviral load (LPL) profile may be attributed to host genetics. Genetic resistance and susceptibility has been mapped to the Major Histocompatibility Complex class II DRB3 gene (BoLA DRB3). The aim of this work was to determine the effect of *Mycobacterium bovis* infection on certain virological and host immunological parameters of BLV experimental infection. Twenty-six Argentinian Holstein calves carrying the resistance-associated marker allele BoLA DRB3*0902, susceptibility-associated marker allele BoLA DRB3*1501, or neutral BoLA DRB3 alleles, exposed to *M. bovis* were used. Twenty calves were inoculated with BLV, three were naturally infected and other three were BLV-negative. Seven from twenty six (27%) of the animals resulted positive to the PPD test. The proviral load, absolute leukocyte and lymphocyte counts, time to seroconversion, antibody titer against BLV, and viral antigen expression *in vitro* at various times post inoculation were determined and compared between PPD + and PPD − animals. From a total of 23 BLV positive animals (naturally and experimentally infected), 13 (56.5%) developed HPL, and 10 (43.5%) developed LPL. None of the investigated parameters were affected by infection with *M. bovis*. We concluded that the ability of cattle carrying resistance-associated marker to control BLV and to progress towards a LPL phenotype was not altered by *M. bovis* co-infection.

1. Introduction

Enzootic Bovine Leukemia/Lymphoma (EBL) is a disease caused by the bovine leukemia virus (BLV), an exogenous retrovirus that mainly infects B lymphocytes. The disease is characterized notably by lympho-proliferation in the absence of chronic viraemia. Lymphosarcoma or lymphoma (LS) is induced in approximately <10% of the infected population (Bartlett et al., 2014; Thurmond et al., 1985; Tsutsui et al., 2016); and persistent lymphocytosis (PL), a benign but often pre-tumoural condition, appears in approximately 30% of the infected animals (Burny et al., 1985). PL is a polyclonal expansion of B lymphocytes carrying the virus integrated as a provirus into the host cell DNA (Ferrer, 1980). The presence of antibodies in the serum of infected animals does not correlate with protection against disease progression (Florins et al., 2007). However, several studies indicate that the quality of the cell-mediated immune response against BLV may influence the progression of the disease (Konnai et al., 2003; Orlik and Splitter, 1996; Pyeon et al., 1996; Pyeon and Splitter, 1998; Yakobson et al., 2000). Moreover, there is evidence that the immune response is altered in BLV-infected animals with PL rendering them potentially more susceptible to other diseases (Brenner et al., 1989; Emanuelson et al., 1992; Trainin et al., 1996).

Polymorphism of the Major Histocompatibility Complex (MHC) is an important factor involved in the immunity against pathogens (Ellis and Codner, 2012; Trowsdale and Knight, 2013), causing distinctive responses to certain antigens in different individuals (Glass, 2004), and thus determining the resistance or susceptibility to certain infections (Takeshima and Aida, 2006). Bovine Leukocyte Antigen (BoLA, MHC complex in cattle) DRB3*0902 allele (according to the International

Abbreviations: EBL, Enzootic Bovine Leukosis; BLV, bovine leukemia virus; TB, bovine tuberculosis; PPD, Purified Protein Derivative; DPI, days post inoculation; HPL, high proviral load; LPL, low proviral load; BoLA, Bovine Leukocyte Antigen; MHC, Major Histocompatibility Complex; FET, Fisher Exact Test.

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Society for Animal Genetics (ISAG) nomenclature of BoLA alelles) is the best-identified marker of resistance to the development of high proviral load (HPL) in BLV-infected cattle and subsequent development of advanced stages of the disease (Juliarena et al., 2008). Animals carrying the bovine MHC allele DRB3*16 (ISAG*1501 or 1503) showed a significant association with the development of HPL in peripheral blood. The majority of the animals carrying the BoLA DRB3.2*11 (ISAG*0902) develop a low proviral load profile (LPL), while other BoLA DRB3 alleles were found not to affect the infection profile, and hence designated as neutral (N) (Juliarena et al., 2008). Animals carrying the ISAG*0902 allele can become infected, but they are able to control the infection in a short period of time, greatly limiting the transmission of the virus.

Bovine tuberculosis (TB) caused by infection with *Mycobacterium bovis*, is a well-known zoonotic, endemic and chronic disease affecting cattle populations worldwide.

Both field and experimental studies have shown that *M. bovis* induces a cell-mediated immune response in the first eight weeks upon infection (Pollock et al., 2005), producing granulomatous lesions mainly in the respiratory tract and associated lymph nodes (Domingo et al., 2014), emphasizing that aerogenic transmission is the major infection pathway in cattle (Menzies and Neill, 2000). Initially, the protective immune response is characterized by the presence of macrophages and a predominant Th1-type response as described for humans (O'Garra et al., 2013). The progression towards disease involves a transition from the Th1-type response to a non-protective response with predominance of Th2 T cells (Welsh et al., 2005). It has been shown that *M. tuberculosis* can suppress the production of protective Th1-type cytokines, by inducing secretion of IL-1β in dendritic cells, thus causing dysregulation of host protective adaptive immune response (Dwivedi et al., 2012).

In the present study, calves from a farm with history of *M. bovis* infection, carrying the ISAG*0902 or the ISAG*1501 markers, as well as neutral BoLA DRB3 alleles (Juliarena et al., 2008) were experimentally inoculated with BLV. Three animals were already naturally infected, and matching calves were kept as negative controls without BLV infection. Kinetics of proviral load was followed during 178 days post-inoculation (DPI). The effect of infection by *M. bovis* on different virological and immunological parameters was evaluated during the course of the experimental BLV infection in cattle carrying defined BoLA DRB3 alleles.

2. Materials and Methods

2.1. Animals and Experimental Infection

Twenty-six castrated males, Argentinian Holstein calves approximately 6–9 months old (130–225 kg), progeny of six different Holstein sires, were obtained from a local dairy farm. The selected animals tested negative for anti-BLV antibodies by ELISA 108 (Gutierrez et al., 2001). Absence of BLV infection was confirmed by PCR (Juliarena et al., 2007). Eight of the calves carried the BoLA DRB3*0902 allele (resistance marker) in heterozygosity, eight carried the BoLA DRB3*1501 allele (susceptibility marker, six heterozygotes and two homozygotes), three carried both *0902 and *1501 alleles, and the rest of the animals ($n = 7$) carried neutral alleles (N/N) with regard to the development of HPL or LPL (Juliarena et al., 2008). A list of experimental animals, their genotype and BLV infection status is presented in Table 1. Twenty of these animals were inoculated subcutaneously with 60 μl of infected blood from a HPL, PL cow (24,012 lymphocytes/mm^3 of peripheral blood and 275,291 BLV copies/μg of DNA from peripheral blood leukocytes), diluted in 1 ml of PBS pH 7.2. The copy number of the inoculum was determined by qPCR (Gutiérrez and Forletti, 2016). The remaining calves were not inoculated with BLV and were used as controls. Blood samples were taken at 0, 3, 7, 14, 30, 38, 45, 61, 88 and 178 DPI to determine the kinetics of viral and immunologic parameters. All procedures performed in studies involving animals are in accordance with the ethical standards and approved by the Animal Welfare Committee of the Faculty of Veterinary Sciences, Universidad Nacional del Centro de la Provincia de Buenos Aires (http://www.vet.unicen.edu.ar/index.php/facultad/comite-bienestar-animal).

Table 1
List of experimental animals, their genotype and BLV infection status.

BLV Infection status	Animal	BoLA DRB3 (ISAG)
BLV natural infection	F131	*1201/1501
	F076	*0601/1201
	E979	*0902/1101
BLV negative	F067	*0902/1501
	F079	*902/1501
	F032	*2703/1501
BLV experimentally inoculated	E989	*1501/1501
	E998	*0101/0902
	F001	*0902/1501
	F039	*0902/1101
	F040	*0902/1101
	F052	*0902/0201
	F057	*0301/1101
	F058	*0101/0101
	F065	*1501/1501
	F080	*0101/0902
	F086	*0101/0101
	F088	*0101/0101
	F089	*1201/1501
	F091	*0902/1001
	F092	*0101/0902
	F095	*0101/2703
	F105	*1201/1501
	F124	*1101/1201
	F135	*1501/2703
	F140	*0101/1501

2.2. Genotyping

Heparinized blood samples were obtained by jugular venipuncture. DNA was extracted from peripheral blood leukocytes after lysis of erythrocytes with ammonium chloride solution (150 mM NH_4Cl, 8 mM Na_2CO_3 and 6 mM EDTA, pH = 7) using the Illustra Blood Genomic Prep Mini Spin kit (GE Healthcare, USA). The purified genomic DNA was quantified at 260 nm (Nanodrop, Thermo Fisher Scientific Inc., USA). Detection of BoLA DRB3*0902 and *1501 alelles was carried out by real-time PCR (Forletti et al., 2013), and confirmed by amplification and sequencing from both ends of a 284 bp fragment of the BoLA DRB3 gene as described (Baxter et al., 2008). Allele assignment from the sequence data was done using the script Haplofinder (Miltiadou et al., 2003).

2.3. Skin Test Reaction

All animals were inoculated by the intradermal route, in the middle third of the left caudal fold with 0.1 ml of bovine PPD (*Mycobacterium bovis* strain AN5, 1 mg/ml 32.500 IU, CEVA Salud Animal S.R.L., Argentina). The sensitivity of the skin test has been estimated between 75 and 82%, and the specificity between 96 and 99% (Bernardelli, 2007). The response to bovine PPD was evaluated by clinical observation of signs of inflammation (swelling, pain, necrosis, induration) and by the increase in skin thickness at the site of innoculation. Prior to inoculation, skin thickness was measured with a 0.05 mm precision caliper (Essex, Essex Junction, V, USA). At 72 h post-inoculation, skin thickness was again measured, and the increase in size was recorded. All records were carried out by the same person. The interpretative criterion for this test in Argentina is as follows: if skin thickness is increased ≥5 mm is considered positive as PPD reaction; from 3.0 to 4.9 mm, doubtful; and <3 mm, negative. If skin thickness is increased ≥5 mm in any animal of a herd, animals with intradermic reactions of ≥3 mm in size in the same herd are classified as positive.

2.4. Absolute Leukocyte and Lymphocyte Count

Absolute leukocyte count was determined in heparinized blood samples using an automatic hematological analyzer (Cell dyn 1400, Abbott Laboratories, Illinois, USA). Differential leukocyte count was performed by visual observation of May-Gründwald-Giemsa stained blood smears under the microscope with 100× magnification).

2.5. Serological Diagnosis of BLV Infection and Determination of Anti-BLV Antibody Titer

Plasma was obtained by centrifugation of heparinized blood samples. The presence of anti BLVgp51 antibodies was determined by the competitive ELISA 108 (Gutierrez et al., 2001) in the sequential samples obtained at all the time points, and time to seroconversion was determined. Two-fold dilutions of plasma samples starting at 1:25 were tested in ELISA 108 to determine the anti BLV-gp51 titer of antibodies.

2.6. Proviral Load

Absolute quantification of proviral load was carried out by real time PCR (qPCR). The detailed procedure of the qPCR is described elsewhere (Gutiérrez and Forletti, 2016). Briefly, a 59 bp fragment of the *pol* gene of BLV was amplified in an ABI 7500 Real Time PCR System (Applied Biosystems), with the following cycling conditions: initial incubation at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Amplification was monitored by incorporation of SYBR Green® dye and subsequent melting curve analysis was used to check for specificity. A standard curve was prepared with DNA obtained from fetal lamb kidney (FLK) cell line, which is persistently infected with 4 proviral copies of BLV per cell. The limit of detection of the method is 10 proviral copies per reaction (30 ng of DNA). Calculation of the proviral copy number of samples was carried out by interpolation of the obtained Cq value within the calibration curve.

Cattle that reached a proviral copy number below 200 proviral copies/30 ng DNA at 88 DPI or undetectable proviral copy number at 178 DPI were classified as LPL profile. If the copy number was above this limit, cattle were classified as HPL profile.

2.7. In vitro BLV Expression

Peripheral blood mononuclear cells (PBMC) were obtained by centrifugation on Ficoll Paque gradient (Ficoll-Paque™ Plus, GE Healthcare) as described (Gutierrez et al., 2009). These cells were suspended in RPMI supplemented with 10% fetal calf serum and 10 μg/ml of Concanavalin A (Sigma Chemical Co. St Louis, MO, USA) at 5×10^6 cells/ml and cultured in 6-well plates. Cultures were maintained for 20–24 h at 37 °C with 5% CO_2.

Cells were harvested by centrifugation and cell extracts prepared as described (Zandomeni et al., 1992). Total protein concentration in cell extracts was determined (Bradford, 1976) using the Bio—Rad dye reagent and bovine serum albumin as the standard. The quantification of the major core protein of BLV (BLVp24) in cell extracts was carried out by a capture ELISA as described (Gutierrez et al., 2009). Results were expressed in nanograms of BLVp24 per milligram of total protein. The limit of detection of the capture ELISA is 11 ng/ml.

2.8. Statistical Analysis

The Fisher's Exact Test of Independence (FET) was applied to analyze association of different alleles with PPD skin reaction and interaction of *M. bovis* co-infection with development of LPL profile in naturally or experimentally BLV-infected cattle. Kaplan-Meier curves were analyzed to compare time to seroconversion and Log Rank statistics was performed. The Student's *t*-test was used to compare anti-BLVgp51 antibody titers between groups,

The leukocyte and lymphocyte absolute count, proviral copy number and *in vitro* BLV expression variables were log transformed for statistical analysis. Repeated measurements ANOVA with time, PPD group and interaction effect of time and PPD was used to test for changes in the four mentioned variables. Statistical analyses were conducted with PROC MIXED procedures of SAS v9.3 (SAS Institute Inc., Cary, NC, USA).

3. Results

The BLV provirus was detected in blood leukocytes at very low levels between 7 and 14 DPI in twelve of the twenty BLV-infected animals and the remaining eight animals were detected positive for provirus at 30 DPI. The proviral load at 30 DPI was very high (>3700 proviral copies/30 ng DNA) in all the experimentally infected cattle. All the experimental animals developed specific antibodies to BLV at 30 ± 7 DPI, except for one animal that seroconverted at 88 DPI. The negative control animals remained serologically negative throughout the study. Intradermal positive reaction to PPD (PPD+) was developed in one BLV naturally infected and five BLV experimentally infected animals, and in one non-inoculated, BLV-negative control animal (Table 2). Only one of these PPD+ reactor calf developed symptom compatible with advanced tuberculosis disease (animal N°F086), while the rest of the animals retained a good condition, without clinical signs. The mean size and standard error for the delayed type hypersensitivity (DTH) reaction in the PPD+ group was 11 ± 1.5 mm for the animals carrying the genotype ISAG *0902/N; and 8.5 ± 7.8 mm for the animals carrying the genotype ISAG*1501/N.

The positive reaction to intradermal PPD injection was not significantly associated with any of the BoLA DRB3 genotypes in the population studied (FET, $p > 0.05$) (Table 3).

To identify a possible effect of *M. bovis* co-infection on the expected BLV infection profiles in the PPD+ animals, different immunological (time to seroconversion, specific antibody titer, leukocyte count and

Table 2
Size of the delayed type hypersensitivity (DTH) reaction to PPD, and BLV infection profile developed by the experimental animals.

	DTH induration				
	Pre-PPD	Post-PPD	Difference		
Animal	(mm)	(mm)	(mm)	PPD reaction	Infection profile[a]
E979	1	12	11	+	LPL
E989	1	1,5	0,5	−	HPL
E998	<1	1	1	−	LPL
F001	1	10	9	+	LPL
F032[b]	1	4	3	+	–
F039	1	1	0	−	HPL
F040	<1	1	0	−	LPL
F052	1,5	1	−0,5	−	LPL
F057	<1	1	0	−	HPL
F058	1	3	2	−	LPL
F065	1,5	1	−0,5	−	HPL
F067[b]	1	1	0	−	–
F076	1	1	0	−	HPL
F079[b]	<1	1,5	0,5	−	–
F080	1,5	14	12,5	+	LPL
F086[c]	1	11	10	+	HPL
F088	<1,5	1	0	−	LPL
F089	1	1	0	−	HPL
F091	1	1,5	0,5	−	LPL
F092	2	11,5	9,5	+	LPL
F095	<1,5	2,5	1	−	HPL
F105	1	15	14	+	HPL
F124	1	1	0	−	HPL
F131	1	1.5	0.5	−	HPL
F135	0.5	1	0.5	−	HPL
F140	<1.5	1	0.5	−	HPL

[a] Proviral load determined at 88 days post experimental infection.
[b] BLV negative controls.
[c] Animal with clinical symptoms of TB.

Table 3
Association between BoLA DRB3 genotype and infection with *Mycocterium bovis* determined by the tuberculin test (PPD).

	BoLA DRB3				
	*0902/N	*1501/N	*902/*1501	N/N	Total
PPD −	5	6	2	6	19
PPD +	3	2	1	1	7
Total	8	8	3	7	26

Number of animals in each category. (p = 0.9249; FET). BoLA DRB3 alleles designated according to ISAG nomenclature. N: neutral allele with regard to the development of HPL or LPV profile.

Table 4
Anti-BLVgp51 antibody titers in animals experimentally infected with BLV that tested positive and negative for *Mycobacterium bovis* PPD (mean ± standard error).

	M. bovis infection		
	PPD −	PPD +	
Anti-BLVgp51 antibody titer at 45 DPI	383 ± 106	200 ± 71	p = 0.3988
Anti-BLVgp51 antibody titer at 88 DPI	787 ± 143	520 ± 279	p = 0.3774

DPI = days post-inoculation.

lymphocyte absolute count) and virological parameters (*in vitro* expression of viral antigen and proviral load) were measured during the course of the BLV experimental infection.

Mean time to seroconversion was higher in the PPD + group. Median value of sero-conversion was 30 days in PPD + group (excluding animal F086, that seroconverted at 88 DPI). Time to sero-conversion in both groups is described by the Kaplan-Meier curves (Fig. 1). No significant difference between the survival curves from PPD + and PPD − groups was found (p = 0.393).

The antibody titer against viral structural protein BLVgp51 in the twenty experimentally infected calves grouped according to their reaction in the PPD test is summarized in Table 4.

The mean antibody titer against BLVgp51 at 45 DPI and 88 DPI was lower in the PPD + group compared to the PPD − group, although these differences were statistically non-significant (Table 4).

The leukocyte count per mm^3 of blood obtained from experimental animals at different time points during the experimental infection is shown in Fig. 2. This parameter showed a similar behavior until 88 DPI in both PPD + ($n = 5$) and PPD − ($n = 15$) reactor animals. The increase in the leukocyte counts observed at approximately 30 DPI, subsided at about 40 DPI. A second expansion of total leukocytes was also detected approximately at 90 DPI. Lymphocyte counts showed a similar pattern as of leukocyte counts (Fig. 3), exhibiting an increased mean number at the same time points during the experimental infection. Although the PPD + group had increased values for both parameters compared to the PPD − group, the differences were not statistically significant (p > 0,05).

As BLV is maintained transcriptionally silent *in vivo*, PBMCs were isolated and cultured for 24 h before the expression of internal BLVp24 was determined. The BLVp24 structural protein was detectable at very low levels (<150 ng/mg total protein) in only 5 (4 PPD − and 1 PPD +) from 18 tested calves at 7 DPI. Fig. 4 shows the kinetics of the *in vitro* expression of the BLV main core protein in the 18 animals tested (13 PPD − and 5 PPD +). At 30 DPI all the BLV–inoculated calves, both the PPD − and PPD +, exhibited a high level of *in vitro* expression of BLV, which reached a maximum value at this time point. Then, the level of expression declined in both groups, and was maintained detectable in 11 (7 PPD − and 4 PPD +) from 18 inoculated calves at 61 DPI. There was not statistically significant difference in BLVp24 expression between the *M. bovis* infected and non-infected animals.

The BLV proviral load was determined at all time points in each inoculated animal. Fig. 5 shows the kinetics of proviral load in the experimental cattle grouped according to their PPD test results. The statistical analysis showed that infection with *M. bovis* did not significantly affect the variation of proviral load over time (p > 0.05). Cattle were classified as HPL or LPL according to the criteria defined in material and methods section. Table 5 shows the outcome of the BLV infection profile in animals grouped according to their PPD test result. From a total of 23 animals with BLV infection, 13 (56.5%) developed HPL profile, and 10 (43.5%) developed LPL profile. The *M. bovis* infection was not significantly associated with any of the two BLV infection profiles defined (p > 0.05).

4. Discussion

Enzootic Bovine Leukosis has been successfully eradicated from countries like Australia, New Zealand, Western and some Eastern European states. (Acaite et al., 2007; Maresca et al., 2015; Nuotio et al., 2003; Voges, 2011) (World Animal Health Information Database, WAHIS Interface). However, in the Americas (Erskine et al., 2012; Nekouei et al., 2015a) and some Eastern European countries (Rola-Łuszczak et al., 2013), Africa (Morris et al., 1996; Oyejide et al., 1987) and Asia (Ma et al., 2016; Mousavi et al., 2014; Murakami et al., 2010; Sevik et al.,

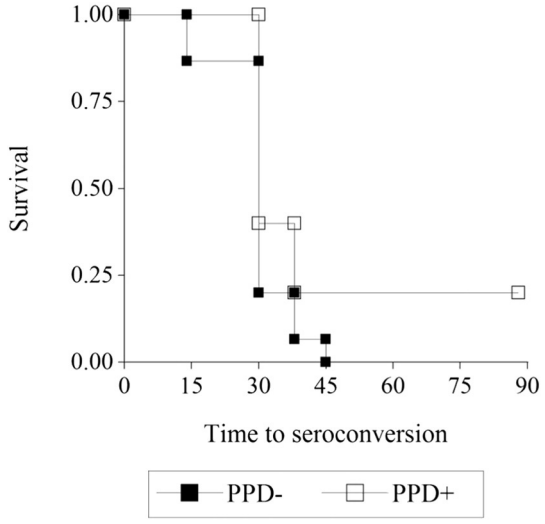

Fig. 1. Kaplan-Meier curves showing the time to seroconversion for animals with negative (close squares) and positive (open squares) PPD tests (Log Rank test = 0.730 p = 0.392790).

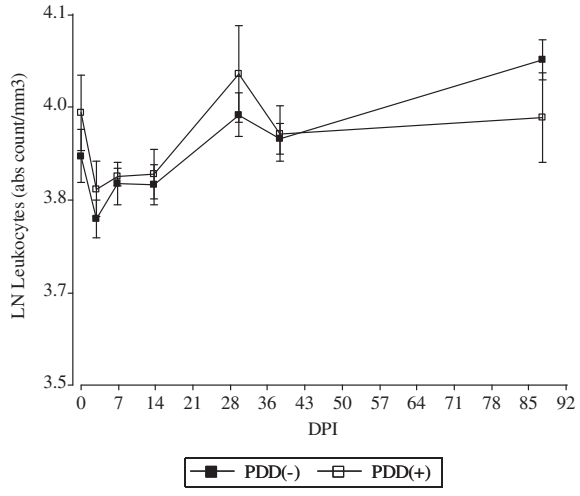

Fig. 2. Leukocyte counts/mm3 at various intervals post experimental inoculation with BLV (DPI) in animals with negative (close squares) and positive (open squares) PPD tests (comparison between groups, repeated measures ANOVA p = 0.4644). LN: natural logarithm.

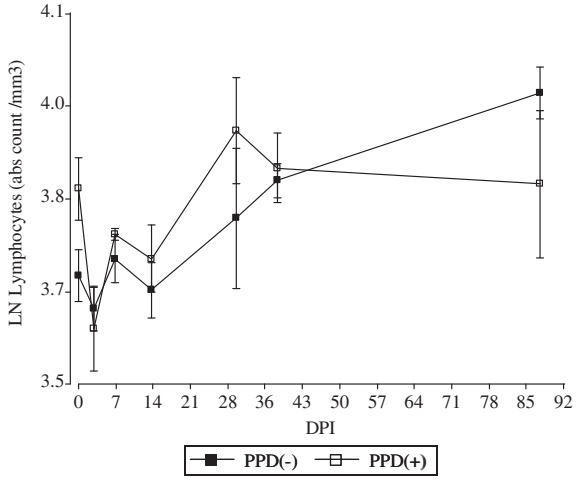

Fig. 3. Absolute lymphocyte count/mm^3 at various intervals post experimental inoculation with BLV (DPI) in animals with negative (close squares) and positive (open squares) PPD tests (comparison between groups, repeated measures ANOVA p = 0.4526). LN: natural logarithm.

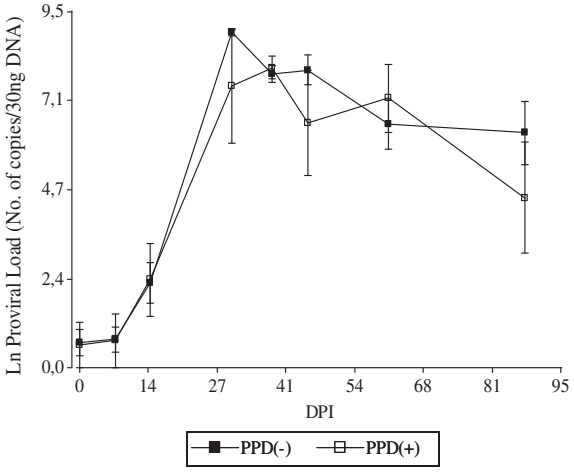

Fig. 5. Proviral load determined by qPCR in animals with negative (closed squares) and positive (open squares) PPD tests at various intervals post-experimental inoculation with BLV (DPI) (comparison between groups, repeated measures ANOVA p = 0.4819). Ln: natural logarithm.

2015) the BLV infection is a continuing problem, showing a gradual increase in its prevalence over the years (Bartlett et al., 2014; Mousavi et al., 2014; Murakami et al., 2010; Nekouei et al., 2015b; Sevik et al., 2015).

Bovine tuberculosis has a complex epidemiology, involving several domestic and wild animal species, that poses great difficulties for eradication (Fitzgerald and Kaneene, 2013; Miller et al., 2013). *M. bovis* infection in cattle exists in most countries of Latin America and the Caribbean Region, especially concentrated in dairy farms. In Argentina, in spite of intensive eradication attempts by the animal health authorities, new cases of TB are occasionally diagnosed (Garro and Garbaccio, 2015; Kantor et al., 2012). EBL is widely distributed in Argentina, mainly in dairy herds (Ghezzi et al., 1997; Trono et al., 2001). Moreover, the detrimental effects of the BLV infection on the immune response of PL cattle could predispose to tuberculosis disease, as it is the case in Human Immunodeficiency Virus infected humans (Aaron et al., 2004). Consequently, co-infection of cattle and a potential syndemic interaction between BLV and *M. bovis* can be expected in farms where both microorganisms are present (Sledge et al., 2009).

BLV is a retrovirus that can be transmitted among cattle through contact with body fluids containing infected cells. Blood transfusion in cattle has been shown to be a major route of transmission of the disease. It has been shown that minute amounts of blood from a LP or HPL cow is sufficient for infection (Burny et al., 1987; Juliarena et al., 2007), and that once infected, the animal becomes a lifetime carrier of the provirus. The contact with neighboring infected cattle poses a risk for the uninfected animals (Kobayashi et al., 2015). This risk is elevated when the infected animals have a HPL profile, resulting in the perpetuation of the infection within the herd. Taking into account that, to date, there are no vaccines or treatments for prevention or elimination of BLV infection, the only way to control the spread of the virus is based mainly on elimination or segregation of infected cattle and improvement in hygienic care. The costs of implementation of a control program depends on the initial prevalence levels. In the Americas (where prevalence levels are high in most cases there is no official economic compensation for the worth of the animals that should be eliminated; therefore the costs of implementation of such a strategy exceeds the potential benefits. Countries such as USA, Canada, Argentina and Japan lacking financial compensatory policies usually failed to obtain adherence to enroll in these programs.

A control program for BLV infection, based on genetic selection of naturally resistant cattle has been proposed (Esteban et al., 2009). The best marker identified to date for this program is the BoLA DRB3*0902 allele, which has been strongly associated with a phenotype of LPL (Juliarena et al., 2008; Xu et al., 1993).

The introduction into the herd of animals that, upon infection, are able to restrict the virus replication (i.e.: animals with LPL infection profile) offer a more sustainable choice to control the disease. As these animals carry negligible numbers of infected leukocytes in peripheral blood, they cannot serve as an efficient source of infection for other animals. This fact was demonstrated by the subcutaneous administration of blood from BLV positive animals to sheep, a species highly susceptible to BLV infection. The inoculation of a high volume of blood (100 ml)

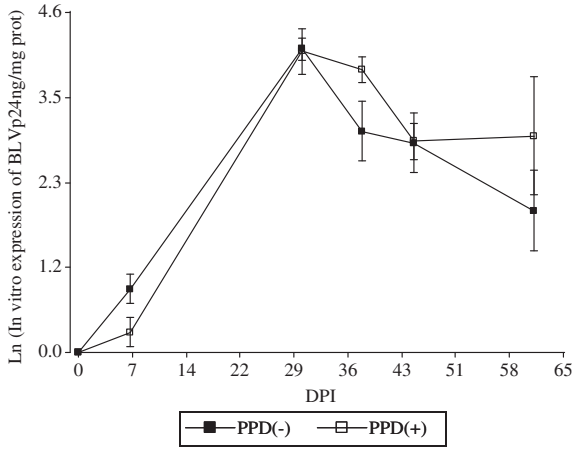

Fig. 4. *In vitro* expression of BLVp24 in animals with negative (close squares) and positive (open squares) PPD tests at various intervals post-experimental inoculation with BLV (DPI) (comparison between groups, repeated measures ANOVA p = 0.7137). Ln: natural logarithm.

Table 5
Frequency of animals with LPL and HPL infection profiles grouped according to their result in the Tuberculin intradermal test.

	BLV infection profile		
	LPL	HPL	Total
PPD +	4	2	6
PPD −	6	11	17
Total	10	13	23

LPL: Low proviral load: HPL High proviral load.

from ISAG*0902 homozygous cows with LPL profile only infected 1 out of 6 lambs (Juliarena, 2008). Moreover, it has been recently demonstrated that LPL-BoLA.

DRB3*902 heterozygous cattle did not transmit BLV to uninfected cattle in a 20-month experiment, carried out in a commercial dairy herd in Argentina (Juliarena et al., 2016). It was demonstrated that polymorphism of the BoLA DRB3 gene was not associated with neutralizing antibody titers against foot and mouth disease virus, bovine viral diarrhea virus, or bovine herpesvirus type 1, suggesting that selection of BLV-resistant cattle or, segregating BoLA alleles associated to BLV susceptibility would not affect the resistance or the predisposition to those diseases (Juliarena et al., 2009).

The BoLA region has shown association with several diseases in cattle; in particular BoLA DRB alleles have been associated with variation in T-cell responses to *M. bovis* in Holstein calves (Casati et al., 1995). In our study, no association was observed between the BoLA genotype and reactor, suggesting that genetic selection for BLV resistant animals would not increase the herd susceptibility to TB. Although our study was limited to find the association between PPD responsiveness and the BoLA genotype, and the number of animals included was limited, we consider this preliminary information in favour towards the selection of BoLA DRB3*0902 animals. However, a more extensive study is needed to confirm this observation.

Specific antibodies to BLVgp51 structural protein appear in blood after 2 to 8 weeks post infection (Klintevall et al., 1997; Miller et al., 1981; Nagy et al., 2007). Time of sero-conversion depends on several factors such as transmission route (natural *versus* experimental infection), the effective infective dose administered, the sampling period and the sensitivity of the method used for detection of specific antibodies (Evermann et al., 1986; Monti and Frankena, 2005). The sero-conversion time observed (about 30 DPI, except for animal F086) for both groups is similar to previous observations for experimentally infected animals (Klintevall et al., 1997; Lassauzet et al., 1989; Mammerickx et al., 1987; Nagy et al., 2007), but is shorter than the median seroconversion time estimated by Monti et al. (48 DPI) (Monti and Frankena, 2005). This discrepancy is probably due to the characteristics of their analysis that involved a greater number of animals, several experimental designs and the use of different serological tests. The antibody titers as well as the white blood cell counts showed the same tendency in both experimental groups during the 88 DPI period, when the TB infected animals were slaughtered. Both groups displayed a transient lymphocytosis at the time of seroconversion (around 4 weeks post-inoculation) in accordance with results from other experimental infection of calves (Klintevall et al., 1997; Ungar-Waron et al., 1999).

Compared to the PPD− calves, neither the *in vitro* viral expression, nor the development of the BLV infection profile was affected by the TB infection. At 88 DPI, a high percentage of the animals bearing the BoLA DRB3*0902 allele developed a LPL profile, while 100% of the animals carrying the genotype associated with susceptibility (BoLA DRB3*1501/N) developed a HPL profile, irrespectively of the TB infection status (data not shown). The proviral loads and lymphocyte counts of the PPD− animals were followed until the end of the experiment (178 DPI). In the resistant animals the proviral load continued to decrease and became undetectable at 178 DPI, while the susceptible calves maintained a high proviral copy number (data not shown). In the present experiment, the LPL infection profile was already defined at 12 weeks and became well established at about 24 weeks post experimental infection. Although it was not possible to follow the proviral load in the PPD+ calves, it is reasonable to expect the same behavior for the PPD+ animals, indicating that the *M. bovis* co-infection would not interfere with the capacity of the resistant animals to control the dissemination of the BLV.

5. Conclusion

M. bovis infection was not associated with any of the BoLA genotypes under study. None of the evaluated parameters concerning the host response to BLV infection were affected by the co-infection with *M. bovis*. The ability to submit or control the experimental BLV infection, and to develop a HPL or LPL profile, was not altered by the *M. bovis* co-infection in animals bearing the susceptibility or resistance BoLA genotype.

Conflict of Interest

None.

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