Genetic Analysis of the pX Region in Bovine Leukemia Virus genotype 1 in Holstein Friesian Cattle with Different Stages of Infection

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Abstract

The pX genetic region of the Bovine Leukemia Virus (BLV) includes four genes with overlapping reading frames that code for the Tax, Rex, R3 and G4 proteins. These proteins are involved in the regulation of transcriptional and post-transcriptional viral expression, as well as having oncogenic potential. Our goal was to determine the pathogenic role associated with BLV genotype 1 pX region genetics in terms of lymphocytosis, lymphomas and proviral load.

We screened 724 serological samples from mixed-age Holstein Friesian cattle from six states in Mexico. Once peripheral blood leukocytes were isolated from whole blood with anticoagulant, we extracted genomic DNA using a commercial kit. Then, we designed *in silico* primers that hybridize in conserved regions of the BLV pX region, which allowed for PCR standardization to detect proviral DNA in infected cells. Positive amplicons were sequenced using the Sanger method, obtaining 1156 nucleotide-long final sequences that included the four pX region genes. The 30 heads of cattle that formed the genetic study population included 12 with lymphocytosis, 12 without lymphocytosis and six with lymphoma. Lymphoma presence was determined in six bovine tumor tissues using histopathology, and we identified BLV presence with in situ hybridization.

Phylogenetic analysis determined that the 30 sequences were associated with genotype 1, and genetic distance between the sequences ranged from 0.2% - 2.09%. We identified two sequences in the G4 gene, one with a three-nucleotide deletion (AGU_7488L, in a cow with lymphocytosis), and one with a nine nucleotide deletion (AGU_18A, in a cow without lymphocytosis). PX region analysis identified positive selection in the G4, *rex* and R3 genes, and we found no difference in proviral load between the studied groups. It was not possible to establish an association between pX region variability and the development of lymphocytosis, lymphoma, asymptomatic status and proviral load in BLV-infected cattle.

Introduction

Enzootic Bovine Leukosis (EBL) is the disease most associated with neoplasm development in cattle, and mainly affects dairy cattle [1,2]. EBL reduces milk production, causes carcasses to be seized at the slaughterhouse due to the presence of lymphosarcomas [3], and decreases the productive longevity of animals, as well as hindering their export [4, 5]. Most infected cattle are asymptomatic, about a third may develop persistent lymphocytosis (LP) characterized by polyclonal B cell expansion, and an additional 5% to 10% of cattle may develop lymphoma or fatal B cell lymphosarcoma after a long latency period in adult animals [6–8]

Bovine leukemia virus (BLV) is the causal agent of EBL. It belongs to the order *Ortervirales*, family *Retroviridae* and genus *Deltaretrovirus* [9, 10]. BLV is B-lymphotropic and oncogenic, and related both structurally and biologically to human T-lymphotropic viruses HTLV-I and HTLV-II [11]. All retroviruses comprise essential, *gag*, *pro*, *pol* and *env* genes, which are necessary for infectious virus production. These are flanked by two identical long terminal repeat sequences (LTRs; [12]). Unlike simple retroviruses,

the *Deltaretrovirus* genome is made up of accessory genes with overlapping reading frames that code for various regulatory and accessory proteins (Tax, Rex, R3, and G4) in the pX region [13].

Tax and Rex proteins are important in nuclear export of viral RNA to the cytoplasm, for viral transcription regulation, and in the transformation of BLV-induced leukemogenesis [14]. Tax proteins also interact with Ha-ras oncogenes inducing *in vitro* immortalization and transformation of rat embryo fibroblasts. These transformed cells cause tumors in nude mice [15, 16]. Accessory proteins R3 and G4 contribute to high viral load maintenance and, additionally, G4 proteins exhibit transforming potential in primary rat embryo fibroblasts when co-expressed with the Ha-ras oncogene [17].

During the chronic infection stage, deltaretroviruses promote infected cell proliferation through transient accessory protein (Tax) action, and propagate during the mitotic division of infected cells. In primary tumor cells, only highly sensitive methods, such as reverse transcription PCR (RT-PCR), and *in situ* hybridization can detect the limited quantities of deltaretroviral RNA. EBL development requires Tax expression, at least during early infection stages. Viral expression activation in latently infected cells and escape from cytotoxic lymphocyte (CTLs) response are essential steps in pathogenesis [18].

BLV genotypes 1 and 3 have been identified in dairy cattle in Mexico using phylogenetic analyses based on the *env* gene [19, 20]. However, there are no studies on the pX region and its role in pathogenesis. The aim of our study was to genetically analyze the pX region of BLV genotype 1, and its possible association with proviral load, lymphocytosis or development of lymphomas in Holstein Friesian cattle.

Materials And Methods

Animals and samples

The study population was made up of 724 Holstein Friesian cows that were between two and three years old. We used non-probabilistic sampling for convenience with cooperating ranchers, from dairy herds in six states across Mexico (Aguascalientes, Coahuila, Guanajuato, Hidalgo, Puebla and Tlaxcala). Whole-blood samples were obtained by coccygeal venipuncture from each animal in tubes with sodium heparin (BD Vacutainer®, USA). Over a six-month period, we collected tumors from different organs in animals destined for slaughter in the state of Hidalgo. These tumors were preserved in 10% buffered formalin while other tissues were frozen at -75ºC. Formalin-fixed samples were embedded in paraffin and 4 µm histological sections were made for subsequent staining with hematoxylin-eosin. Other samples were intended for *in situ* hybridization. The study was endorsed by the Internal Committee on Animal Use, and Experimentation of the Graduate Program in Animal Production and Health Sciences at the National Autonomous University of Mexico, under code MC-2017 / 1-7-UNAM.

White blood cell count, peripheral blood leukocyte and plasma collection

We used 1ml of blood with heparin (Vacutainer® BD U.S.A) to carry out leukocyte counts in a Neubauer chamber, as well as differential counts by means of smears stained with diff quick (Hycel de México®,

Mexico). The rest of the blood sample (7 ml) was centrifuged at 350 □ g for 15 minutes to separate plasma, which was collected in 1.5 mL microtubes. Peripheral blood leukocytes (PBLs) were then processed with lysis solutions [21]. Plasma samples and PBLs were stored at -70° C until use.

In situ hybridization (ISH)

We placed 4 µm tissue sections on electrocharged slides (Probe On Plus, Fisher Scientific, Pittsburg, USA), heated them on a thermal stage at 60^{o} C for 30 minutes, and then de-paraffinized them in three 5-minute xylene passes. The tissue samples were then rehydrated in ethanol gradients (two 5-minute passes in 100% ethanol, one 2-minute pass in 95% ethanol, and one 2-minute pass in 70% ethanol), followed by one 1-minute pass in 1X automation buffer (Automation buffer, Biomeda Corp., Foster City CA, USA).

ISH reactions were performed in a Microprobe Manual Staining System workstation (Fisher Scientific, Pittsburg, USA) following a standard protocol that consisted of the following steps: predigestion and digestion with 0.25% pepsin (Fisher, Scientific, Pittsburg, USA) in a 2.0 pH automation buffer. Prehybridization with 100% formamide (Promega Corp. Madison, WI, USA), hybridization with 2.5 ng / µl of the specific BLV DNA probe in hybridization solution (chondroitin sulfate 7.5%, 5X Citrates / SSC saline solution, 50 nM Phosphate Buffer, 0.25% Blocking Agent, 22.5% Formamide). Followed by high stringency washes with 0.5% and 0.2% SSC (Promega Corp. Madison, WI, USA), blocking with Buffer 1 (NaCl 0.15M, 0.1M Tris HCl, pH 7.5) supplemented with 0.3% Triton X-100 (Fisher Scientific, Pittsburg, USA) and 1% sheep serum.

Detection was performed with a 1:500 dilution of anti-digoxigenin conjugated with alkaline phosphatase (Roche Applied Sciences, Mannheim, Germany) and developed with 0.45% blue-nitro-tetrazolium (NBT) and 5-bromocresyl-3-indolylphosphate (BCIP) at 0.35% (Roche Applied Sciences, Mannheim, Germany) in Buffer 2 ($MgCl_2$ 0.06M, NaCl 0.12M, Tris 0.1M pH 9.5) supplemented with Tween 20 at 0.4% (Hycel de México SA de CV) and Brij 35 0.25% (Fisher Scientific, Pittsburg, USA). The reaction was stopped with distilled water and fast green (Hycel de México S.A. de C.V.) was applied as a contrast dye and permanent mounting medium (Entellan, Merck, Hamburg, Germany). Each ISH protocol had a positive and a negative control. Likewise, each problem lamella was processed in the absence of the labeled probe as an internal hybridization control.

The probe was obtained by direct labeling with digoxigenin, using a set of commercial reagents (PCR DIG Probe synthesis Kit, Roche Biochemical, Diagnostic, Mannheim, Germany). We used the 202 bp product of the BLV *pol* gene as a template for labeling.

Serological analysis

To determine the presence of antibodies against BLV, we evaluated plasma with an indirect enzyme-linked immunosorbent assay (ELISA) that identifies antibodies against the gp51 protein of the virus, following the manufacturer's instructions (VMRD Inc., Pullman, WA. USA).

DNA extraction

We performed DNA extraction from PBLs using a commercial kit (FavorPrepTM TissueGenomic DNA Extraction Mini Kit Favorgen, Taiwan), following the manufacturer's instructions. DNA was quantified at 260-280 nm absorbance in a nanodrop (Thermo fisher Scientific, USA) and stored at -70º C until use.

Primers

Nucleotide sequences used for primer design were some of the available GenBank sequences of BLV genotypes 1-4, 6, 9 and 10. We used BioEdit and Primer3 input V4.0 programs to design three sets of overlapping primers to amplify the entire pX region (Table 1).

BLV pX region PCRs

To amplify the pX region from BLV proviral DNA, we standardized three PCRs based on the amplification of positive controls, which were obtained from a PCR-positive field sample and confirmed by sequencing and inserted into a plasmid following manufacturer's instructions for the commercial Thermo Scientific InsTAclone PCR Cloning kit (Thermo Fisher Scientific ™ USA).

The PCR reaction mixture was: 1X buffer (Amplificasa® Biotecmol, Mexico), 1.5 mM $MgCl_2$ (Amplificasa® Biotecmol, Mexico), 230 µM dNTPs (Kappa Biosystems, USA), 400 nM of each primer, 5U of Taq DNA Polymerase (Amplificasa® Biotecmol, Mexico), and 500 ng of DNA for a final 30 µL volume.

PCR amplification conditions were carried out in a thermal cycler (BIOER, China), and consisted of one initial denaturation cycle at 95° C for 5 min, followed by 30 denaturation cycles at 95° C for 30 s, hybridization at 54° C (PCR-1) / 56° C (PCR-2) / 47° C (PCR-3) for 40 s and extension at 72° C for 50 s, with a final extension cycle at 72° C for 15 min. To visualize PCR products, these were separated via electrophoresis in 1.5% agarose gels, stained with EtBr ethidium bromide (5 µg / µl). A base pair marker (Cleaver Scientific, UK) was used as a reference, applying electrical current at 110 volts for one hour for later visualization under UV light in a transilluminator (UVP®, USA).

Sequencing

We purified positive samples using a commercial FavorPrep GEL/PCR Purification Kit (Favorgen, Taiwan) following the manufacturer's instructions. The purified PCR product was sequenced using the Sanger method with the ABI 3130x1 Genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequencing was carried out in both directions with the specific primers for each product.

Sequence analysis and phylogeny

We used ClustalW for sequence alignment, and the similarity matrices were determined with BioEdit v7.0.4. Amino acid deduction and genetic distance measurements were performed with Geneious v11.0.3 using a variance estimation method (p-distance). The tree was built with the GENEIOUS®, USA program,

using the MrBayes algorithm which is based on the Markov Monte Carlo (MCMC) model [22]. FigTree® v1.4.3. was used to edit the tree. We determined the synonymous and non-synonymous substitution rate using SNAP (Synonymous Non-synonymous Analysis Program) v2.1.1 [23]; https://www.hiv.lanl.gov/content/sequence/SNAP/SNAP.html.

Proviral load

We determined proviral load using qPCR in 20 µl reactions in a commercial Sensi Fast SYBR Lo-Rox Master Mix kit (Bioline, London, UK). We used 50 ng of cDNA, with the Strategene Mx3005P detection and amplification system (Agilent Technologies, USA). Each sample was evaluated in duplicate and with a non-tempered control reaction (NTC / Non Template Control) in 96-well optical plates (Agilent Technologies, USA). We used the following conditions: 95° C for 2 min, followed by denaturation 5 sec. at 95° C, alignment 10 sec. at 60° C, elongation 10 sec. at 72° C, for 45 cycles. Following amplification, we increased the temperature to 95° C to construct a dissociation curve and determined the specificity of the amplified product.

Quantification method

We used a relative quantification method, comparing the cross-point/CP. Constitutive gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) was used as the internal control, and the procedure reported by Livak and Schmittgen [24] was used as the calculation method.

Results

BLV infection detection

The presence of antibodies against BLV was demonstrated in 405 animals (55.9%), which were then classified as cattle without lymphocytosis if their leucocyte counts fell below 7,500 leukocytes/mm^3, and as cattle with lymphocytosis when their leucocyte counts were above 10,000 leukocytes/mm^3 [25]. Differential counts confirmed lymphocytosis in the second group (Table 2).

Histopathology and *in situ* hybridization

At necropsy, nine cows presented tumoral lesions in the abomasum, heart, lymph nodes and kidneys. These tissues were analyzed by means of histopathology, and all cases showed cellular alterations suggestive of BLV infection (Figure 1 A, B, D and E). Lymphomas only presented positive ISH signals in six cases (Figure 1 C and F).

Determination of proviral load

To determine the association between proviral load and the different test groups (with lymphocytosis, without lymphocytosis and with lymphoma), we tested the proviral load of the 30 sequenced samples.

Then we ran ANOVAs and Tukey tests ($p < 0.05$) on the results between the groups, and used the $\log 10^{2-\Delta\Delta Cp}$ value as a dependent variable, using the Statgraphics Centurion 18 statistical package.

Cattle groups with and without lymphocytosis showed proviral load values of 3.325 and 3.265 respectively, and both of these were higher than values from the group with lymphoma (2.655), however the values between groups were not statistically different ($p > 0.05$).

PCR amplification of the pX region of BLV

We carried out PCRs on seropositive samples to amplify the complete BLV pX region and sequenced two samples per region and six lymphomas. We obtained a total of 30 samples from 12 cattle without lymphocytosis, 12 with lymphocytosis and six with lymphoma.

Phylogeny

We constructed a phylogenetic tree (Figure 2) to determine the prevalent BLV genotype in our samples (n=30). The tree included reference sequences from the pX region of BLV genotypes 1-4, 6, 9 and 10, that are available in GenBank. The sequences we obtained in this study were deposited in GenBank and are available under accession numbers MN707591 to MN707620. All of our samples, independently of which study group they belonged to (without lymphocytosis, with lymphocytosis and lymphoma), or geographical provenance, were associated with genotype 1. Samples were distributed heterogeneously throughout the clade and there was no association by infection phase nor geographic region (Figure 2).

Sequence analysis

Genetic variability found between the BLV nucleotide sequences we obtained in the study showed genetic distances ranging from 0.2 to 2.09 (Table 3). We determined there was negative selection in the proportion of synonymous to non-synonymous mutations in most of the genes and study groups. We also found positive selection in the *G4* gene for cattle in all the three study groups, in the *rex* gene in cattle with lymphoma, and in the *R3* gene in cattle with lymphocytosis (Table 3). We found different changes among *R3*, *G4*, *rex* and *tax* gene derived residue alignment from the 30 sequences, as well as deletions in the protein expressed by the *G4* gene in a pair of sequences.

The changes concentrated in the residues from the four proteins are shown in Figure 3 A, B, C, D. In the *tax* gene phosphorylation sites, B-cell epitope and multifunctional domain were conserved, while we found four changes in the Zn finger domain, 13 in the T-cell epitopes, and three in the Leucine-rich activation domain. We identified four residue changes in the nuclear export signal (NES) and six changes in the nuclear localization signal (NLS) in the *rex* gene. In the case of *G4*, we observed nine changes in myb-like motif (MYB) and four in the arginine-rich nucleus targeting RNA-binding region (ARR). Lastly, in the *R3* gene, we identified nine residues with changes in five study sequences.

Discussion

It was not possible to establish an association between genetic variability in the BLV pX region and the development of lymphocytosis, tumor development, asymptomatic status and proviral load in Holstein Friesian cattle infected with BLV genotype 1.

We obtained 30 sequences measuring 1156 nt in length that corresponded to the BLV pX region. Phylogenetic analysis showed that all of these sequences were associated with BLV genotype 1, which is widely distributed throughout Mexico [19, 20]. Furthermore, the sequences were grouped heterogeneously throughout the genotype 1 reference sequence clade, not displaying any relationship to infection phase (with and without lymphocytosis or lymphoma) nor geographic origin of the samples.

The R3 protein is made up of 44 amino acids with a hydrophilic portion at the N-terminus followed by a hydrophobic region. [26] described premature stop codons in two sequences obtained from tumor lesions in the *R3* gene, but we did not find any premature stop codons in the 30 sequences analysed for this study. While we did find nine non-synonymous mutations in five sequences in the three study groups, we did not identify their possible biological implications (Figure 3 D).

Although the functions of G4 and R3 proteins have not been fully clarified, they are known to be of great importance for viral propagation *in vivo* [27]. The oncogenic potential of G4 protein stands out among other known functions. This protein includes an amino terminal stretch of hydrophobic residues, followed by possible proteolytic cleavage sites and a region rich in arginines located in the center of the protein [28]. This region is key in the interaction of G4 with farnesyl pyrophosphate synthetase (FPPS;[29]), an enzyme that participates in the pathway to lipidation of a great variety of nuclear lamin proteins, Ras, other regulatory binding proteins (GTP), as well as various kinases and phosphatases [29] (Lefebvre *et al.*, 2002).

The G4-FPPS interaction has been demonstrated in cell transformation. Mutations in the alpha helix of the arginine-rich region of the G4 protein, prevent the immortalization of primary cells and the induction of tumors in nude mice [29]. The interruption of the G4-FPPS interaction could alter the process of oncogenesis. In our study sequences, we found four non-synonymous mutations in the arginine-rich region, two of them in abomasum and heart tumor tissues (Figure 3 C). Although we identified positive selection in the *G4* gene in all infected animal groups, the high conservation of the arginine-rich region in the G4 protein may be necessary for it to exert its oncogenic function. We also found many non-synonymous mutations in the MYB motif, and this many accumulated changes have not been previously described in the literature for this gene. Additionally, we found two sequences with AGU_7488L residue deletions, and three residues (AGU_18A) that coincide with descriptions by Murakami *et al.* [30]. These authors carried out *in vitro* analyses and reported a deletion of four amino acids in the G4 gene linked to a decrease in viral production and replication. The high number of changes identified in this region may impact viral replication, but *in vitro* studies are necessary to demonstrate this.

The Rex protein facilitates viral RNA export from the nucleus to the cytoplasm via nuclear localization (NLS) and nuclear export (NES) signals [31]. NLS directs Rex proteins to the nucleus, except when it binds to viral RNA. This binding masks the NLS, allowing the NES to direct the viral RNA to the cytoplasm

through nuclear pores [32, 33]. In our study sequences we found ten non-synonymous mutations (Figure 3 B), four of them were in the NES region in cattle with lymphocytosis, and six in the NLS region. Three of the latter were in cattle without lymphocytosis, two were in cattle with lymphocytosis, and one was in an animal with lymphoma. We only identified mutations in the NES region in cattle with lymphocytosis, where a serine residue exchange for leucine dominated.

The *tax* gene is involved with transcription of viral and cellular genes, and may allow oncogenic transformation through inhibition of DNA repair pathways in infected cells [34]. The presence of a zinc finger motif, a transactivator motif and two phosphorylation sites have been identified in the Tax protein [26]. A study found that Tax mutants with substitutions in amino acids 240 and 265 had a greater transcriptional capacity directed to LTR than what is seen naturally with the Tax protein [35]. We did not find these substitutions in our study. Den Breoeke *et al.* [36] reported a mutant with a single substitution (E303K) that turned out to be replication defective, but this substitution was also absent in the sequences we obtained.

Phosphorylation sites, sites associated with transactivation, and B-cell epitopes were conserved in all sequences. Only four mutations were observed in the zinc finger motif, three mutations in the leucine-rich domain in three animals without lymphocytosis, and 13 mutations in T-cell epitopes. None of the substitutions observed in the Tax protein were associated with the previously described regions that impact virus transcription and replication (Figure 3A). While other studies have described mutations in functional domains and important epitopes of this protein, phosphorylation sites are generally conserved [18].

Previous studies have revealed that different genetic and epigenetic mechanisms can silence the *tax* gene, which is essential for non-progression of tumoral processes [37]. We found high degrees of conservation in our analysis of *tax* gene sequences, and it was not possible to identify sequence patterns that could be associated with the development of cell transformation, especially in animals with lymphocytosis and tumor tissues.

The genetic distance values obtained from our study *R3*, *G4*, *rex* and *tax* gene nucleotide sequences were 0.2 - 2.09%, 0.94 - 1.18%, 0.5 - 0.8% and 0.73 - 0.8% respectively. Other studies on genotype 1 BLV have identified genetic distances ranging from 0 - 12.1%, 0 - 6.5%, 0 - 9.4% and 0 - 6.1% for the *R3*, *G4*, *rex* and *tax* genes respectively [38]. The maximum genetic distance values described by Panei et al. [38] exceeded the values obtained in our study by up to six times. This may be due to some analyzed sequences being phylogenetically related to genotype 2 (JF288766 and JF288767; Figure 2).

Zhao *et al.* [34] identified that the *tax* gene has the highest mean nucleotide variation rate (1.86%) with respect to the *R3*, *G4* and *rex* genes (1.24, 1.29 and 1.40%, respectively), and in this case *tax* had the second lowest average variation rate (0.77%) compared to *rex*, *G4* and *R3* (0.66, 1.1 and 1.12, respectively). Genetic distances ranging from 0 - 2.8% and 0 - 4.7% have been identified for the *rex* and *tax* genes respectively. These values are greater than the ones found in our study, and these differences may be explained by the inclusion of genotype 1 and genotype 4 sequences in the analysis [39]. Genetic

distances in the pX region of genotype 1 genes found in our study showed low variability, regardless of infection phase or geographical origin.

One method for determining genetic variability entails measuring the substitution-rate, primarily in overlapping reading frames, because a synonymous change in one gene may not be neutral in the other. Purifying selection, also known as negative selection, is a type of natural selection in which genetic diversity decreases as a particular trait value (phenotype frequency) stabilizes in the population. In comparison, positive selection increases the frequency of certain variations and occurs when equilibrium in the population has not yet been reached [34]. The proportion of synonymous to non-synonymous substitutions (dN / dS) in our result sequences for the three analyzed cattle groups with respect to the *tax*, *R3* (cattle without lymphocytosis and with tumor development) and *rex* (cattle with and without lymphocytosis) genes established a negative selection. Similar results for *tax* and *rex* genes were described by McGirr and Buehuring [40]. Zhao *et al.* [34] reported negative selection for the BLV *tax* gene, which could be due to a higher percentage of its sequence not being superimposed, in comparison to other pX region genes.

The *tax* gene has been found to be more conserved than *rex* in primate lymphotropic T viruses 1 and 2, which are classified in the same genus as BLV. This is consistent with Tax being the most important regulatory protein for Deltavirus behavior. We determined positive selection for experimental sequences obtained for the *R3* (cattles with lymphocytosis) and *rex* (with lymphoma) genes, in addition to all BLV *G4* gene sequences obtained from cattle. This would indicate that the virus tries to modify its genome and thus avoid the host's immunity mechanisms, including APOBEC [41].

A first approach to EBL diagnosis can be carried out through clinical signs in cattle with tumors and the subsequent histopathological study of biopsies from these tissues. Tumor tissue histopathological analyses allowed the identification of lymphomas characterized by the proliferation of neoplastic lymphocytes with marked anisocytosis and anisokaryosis, as well as a large number of mitoses. Using the ISH technique, we identified the proviral genome and observed a positive signal in tumor tissues. Similar studies have detected BLV in organ samples from an ISH assay directed at non-coding RNA [42]. Marking in lymphomas was low intensity (Figure 1 C and F), indicating few infected cells. However, these data suggest that the ISH technique may be useful in the study of EBL.

We categorized cattle as having lymphocytosis based on an absolute count of 10,000 lymphocytes/mm^3, and out of 405 BLV seropositive cattle, 54% (n = 221) had lymphocytosis. These results differ from those described in other studies, including Khudhair *et al.* [43], who identified 29% of animals with lymphocytosis. This could be due to low infection rates, as only 7% of animals in that study were seropositive. It is important to mention that the high numbers of animals with lymphocytosis identified in our study cannot all be associated with BVL infection, as at least three continuous samplings are required to determine persistent lymphocytosis [44] and this was not done in our study.

The use of real-time PCR allowed us to identify proviral loads across study groups (cattle with and without lymphocytosis, tissues with lymphoma) however we did not find statistically significant differences between them. This may be related to the number of infected cells in animals with lymphocytosis, as observed in tumor cells, which did not allow us to identify differences between the study groups. The lack of difference in proviral load could also be related to the number of samples analyzed in each group [19]. Previous studies have also been unable to correlate proviral load with different infection phases [45, 46]. On the other hand, Chieh-Wen [47] showed that BLV-induced lymphoma and proviral load are associated with different alleles of BoLA-DRB3 in Holstein cows in Japan, and Kobayashi [48] found that high proviral loads in blood are significant for identifying cattle at high risk for developing lymphomas. While available information on proviral loads suggests that these are an important factor in disease progression, our study did not identify any relation between proviral load, pathogenesis and disease.

Numerous diagnostic methods have been used in BLV infection detection studies (including seroneutralization, radioimmunoassay, IDGA, ELISA, western blot and PCR; [49]), and ELISA and PCR have proven to be highly efficient diagnostic techniques at the herd level [20, 50]. We identified BLV infection in six regions across Mexico via antibody detection using a commercial ELISA test. We found 55.9% seropositivity in the study group of 724 Holstein Friesian dairy cattle. Across states, BLV seropositivity ranged between 41% and 80%, which reveals high infection levels in Mexican dairy herds, as well as a notable increase in BLV infection from previous serological studies [51]. Increasing infection levels coincide with reports from other countries such as the United States with 83.9% [45, 52] and Taiwan with 81.8% [53].

Most BLV phylogenetic work has focused on the *env* gene, and particularly on the gp51 region because of its importance as a ligand and antigen, among other functions [54, 55]. A detailed study in 2007 identified seven BLV genotypes through *env* gene analysis [56]. Genotype 8 was first described in Croatia and later distributed across other geographical regions [57]. Genotypes 9 and 10 were described in Bolivia in 2016 [58], Thailand [59] and Myanmar [12]. Our results show that the pX region may be useful in BLV phylogenetic analyses, however it is necessary to generate sequences that include all the genotypes previously identified via *env* gene analysis to consolidate this proposal.

In conclusion, we did not find an association between BLV pX region (*R3*, *G4*, *rex* and *tax*) genetic variability and infected cattle with lymphomas, and with or without lymphocytosis. Although we identified positive selection in three of the four genes that make up the pX genetic region, we could not find implications of this in BLV pathogenesis. Proviral load quantification did not show significant differences between the three study groups. We identified high BLV seropositivity in the study regions, and an overall a high frequency of BLV genotype 1 infecting dairy cattle in Mexico.

Declarations

Conflicts of interest

The authors have no financial or personal interests that could influence or bias the content of this article. The authors declare that they have no competing interests. All authors have seen and approved the manuscript.

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Tables

Table 1. Primers used for amplification of the BLV pX region.

Designation	Sequence (5´- 3´)	Primer position*
Fw1	TCAGTGTACCATCACAAGCC	6426-6445
Rv1	ACCAACAACACTTGCTTAAA	7241-7260
Fw2	AACAGTGCCCATAAAGTCC	7100-7118
Rv2	CGGTTAGGAATAGGTCGAT	7978-7996
Fw3	ATGGCCAATTTTTCCTTACT	7708-7727
Rv3	ACGTCTCTGTCTGGTTTACG	8225-8244

*Primer position was based on the OIE reference sequence with accession number K02120 [60]

Table 2. Serology and absolute lymphocyte count for all analyzed samples, with cattle classed according to provenance from different states.

STATE	ELISA seropositive	With lymphocytosis	Without lymphocytosis
Aguascalientes	96	50	46
Coahuila	92	47	45
Guanajuato	57	33	24
Hidalgo	34	16	18
Puebla	41	35	6
Tlaxcala	85	40	45
Total	405	221(54%)	184 (46%)

Table 3. Genetic distance and ratio analyses of BLV synonymous (dS), nonsynonymous (dN) and nucleotide sequences of the Tax, Rex, G4 and R3 genes in infected Friesian Holstein cattle.

	Substitutions			Genetic distance
Gene and group	Average dN	Average dS	Average dN/dS	Percentage (%)
tax With lymphocytosis	0.0050	0.0218	0.229	0.8
tax Without lymphocytosis	0.0075	0.0211	0.355	0.8
tax lymphoma	0.0067	0.0190	0.352	0.73
rex With lymphocytosis	0.0116	0.0241	0.481	0.5
rex Without lymphocytosis	0.0136	0.0169	0.804	0.8
rex lymphoma	0.0253	0.0045	5.6	0.69
G4 With lymphocytosis	0.0153	0.0152	1	0.94
G4 Without lymphocytosis	0.0197	0.0175	1.1	1.18
G4 lymphoma	0.0308	0.0228	1.3	1.18
R3 With lymphocytosis	0.0632	0.0609	1.03	2.09
R3 Without lymphocytosis	0.0150	0	0	0.2
R3 lymphoma	0.0149	0.0639	0.23	1.07

Figures

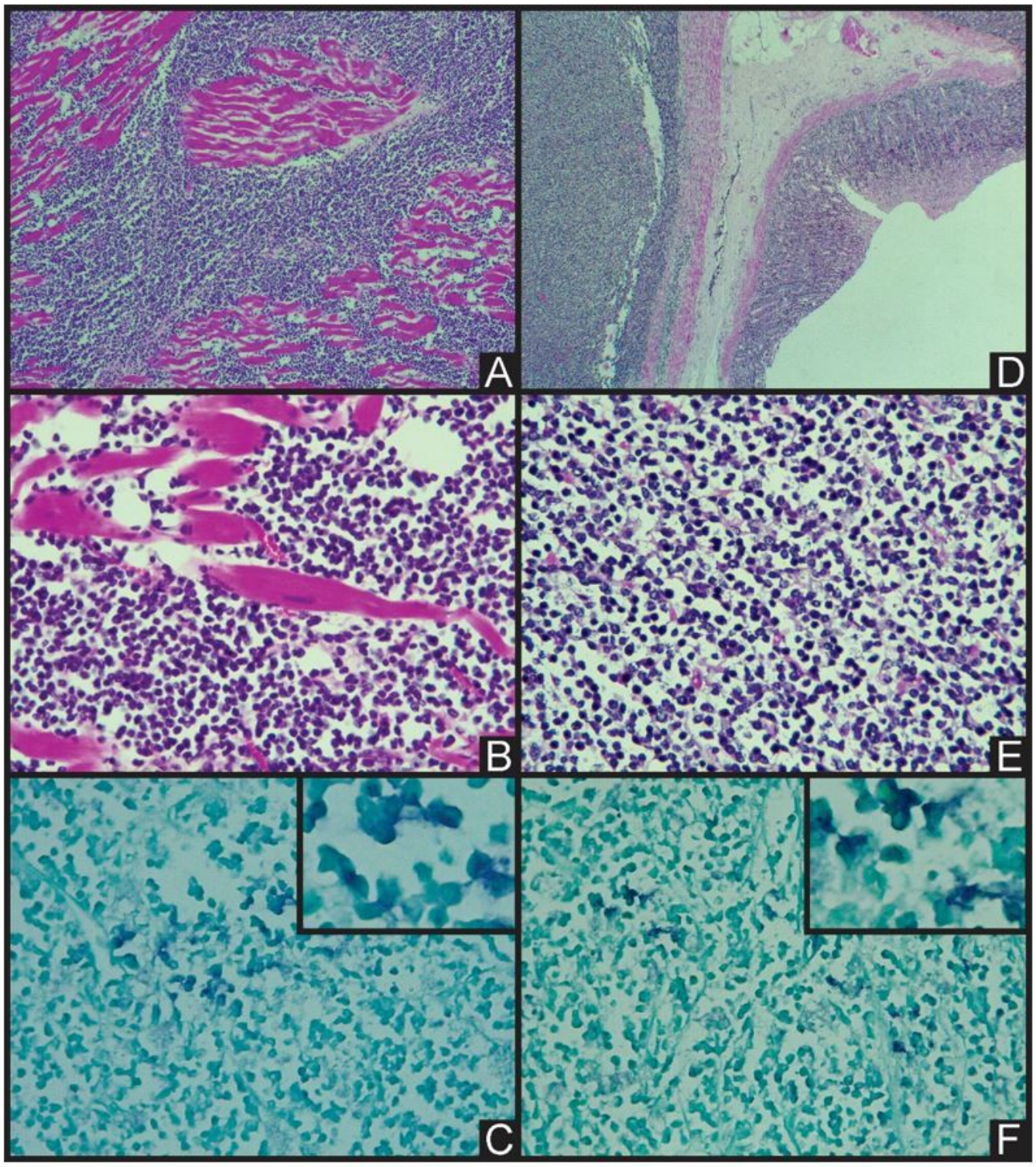

Figure 1

H-E staining, histopathological changes. Abundant neoplastic lymphocyte aggregates are visible with marked anisocytosis, located between myocardial fibers and in the mucosa and muscle of the abomasum 40x (A, D) and 400x (B and E). In situ hybridization with fine signals shown in blue, in neoplastic cells located in the heart and abomasum of an animal with lymphomas, with 400x fast green staining. In the upper right frame, images C and F show an enlargement of the positive signals.

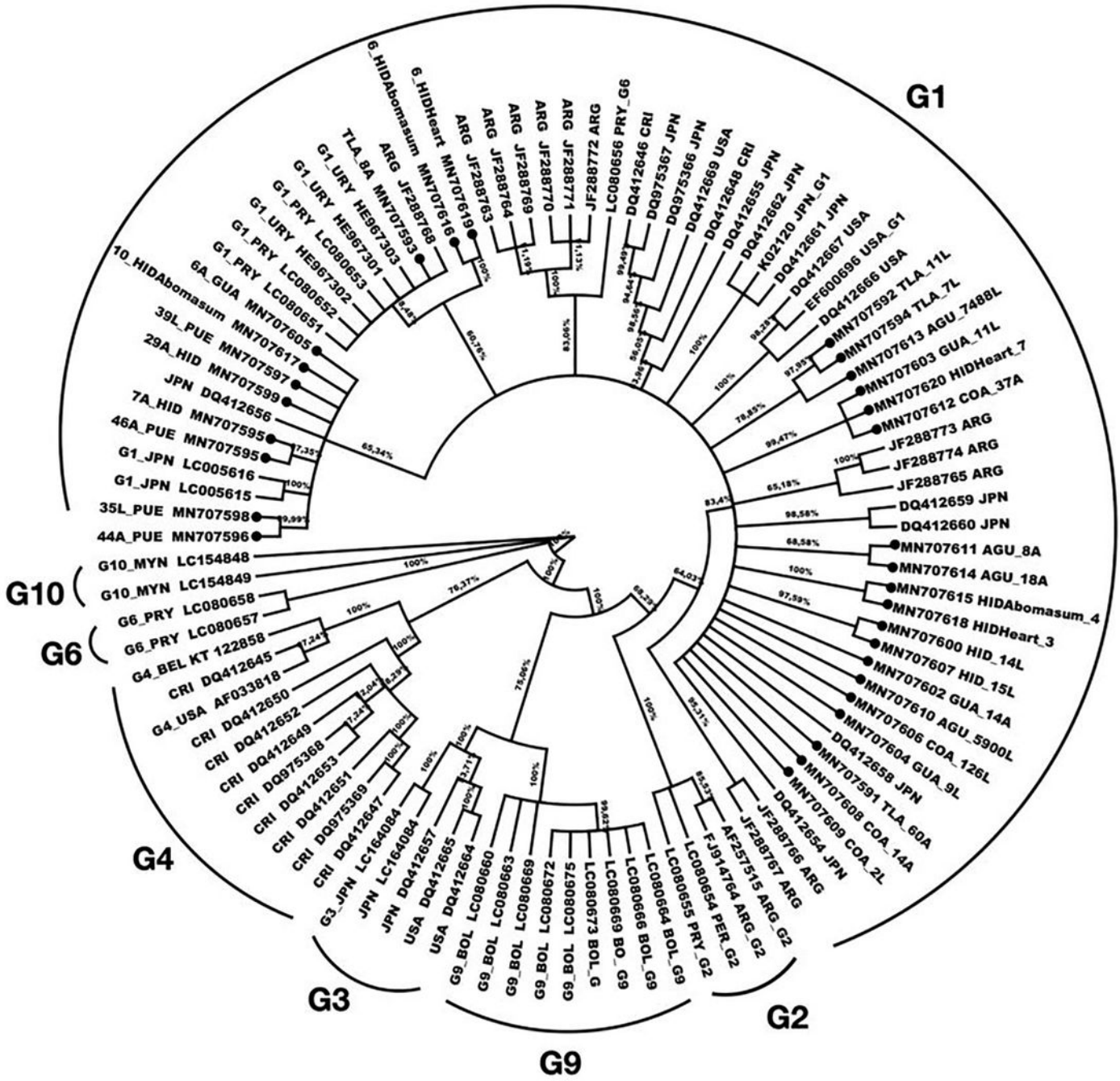

Figure 2

Phylogenetic tree constructed using the Bayesian method and 1000 bootstraps. G1, G2, G3, G4, G6, G9 and G10 show reference sequences grouping by genotype and (▯) sequences obtained in this study. Identification of sequences in the phylogenetic tree, from the center out: accession number, state abbreviation code (AGU: Aguascalientes, COA: Coahuila, GUA: Guanajuato, HID: Hidalgo, PUE: Puebla and

TLA: Tlaxcala), and animal identification. L: with lymphocytosis, A: without lymphocytosis. Samples of tumor tissue of the abomasum and heart.

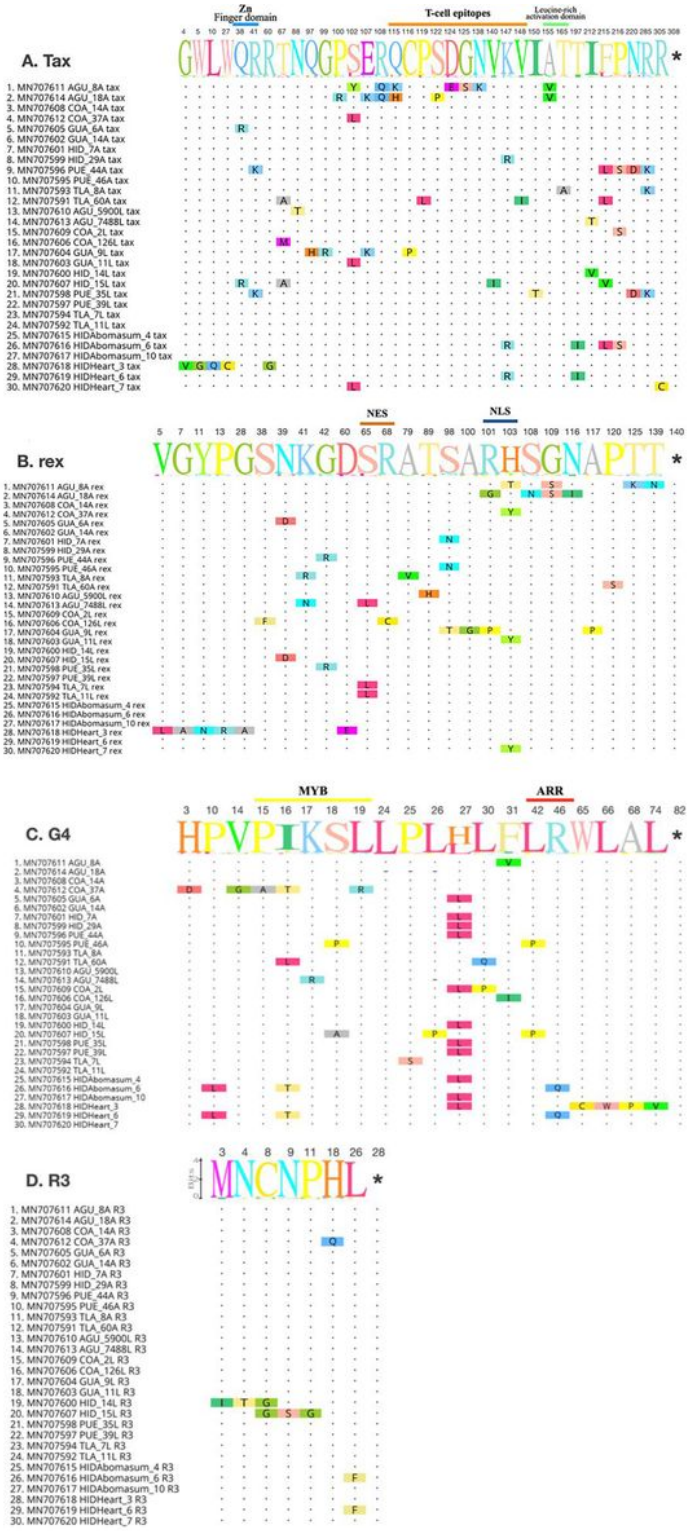

Figure 3

A, B, C, D: Alignments that show Tax, Rex, G4 and R3 protein amino acid sequences with position changes. The position and residue where at least one residue change was identified in the sequences

analysed is shown. Numbers indicate the residue number. In the upper part of the image we show a consensus weblog of the amino acids, graphically representing found changes.