Virus Research 171 (2013) 97–102

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Analysis of the pX region of bovine leukemia virus in different clinical stages of Enzootic Bovine Leukemia in Argentine Holstein cattle

Carlos Javier Panei[a,b,2], María Soledad Serena[a,1], Germán Ernesto Metz[a,1], María Emilia Bravi[a], Ester Teresa González[a], María Gabriela Echeverría[a,*,2]

[a] Department of Virology, Faculty of Veterinary Sciences, National University of La Plata, 60 and 118, CC 296, 1900 La Plata, Argentina
[b] Department of Immunology, Faculty of Veterinary Sciences, National University of La Plata, Argentina

ARTICLE INFO

Article history:
Received 30 April 2012
Received in revised form 31 July 2012
Accepted 6 August 2012
Available online 16 November 2012

Keywords:
Bovine leukemia virus
pX region
Clinical stages

ABSTRACT

Bovine leukemia virus (BLV) infection in cattle causes Enzootic Bovine Leukemia (EBL). About 30% of infected cattle develop persistent lymphocytosis (PL), a 0.1–5% develops tumors, and a 70% remains asymptomatic in an aleukemic stage (AL). Regulatory genes of BLV (Tax, Rex, R3 and G4) are located in a region known as pX_{BLV}. The variability of those genes had been postulated with the progression of the disease. The aim of this work was to compare the wild-type proviral pX_{BLV} region at different stages of BLV natural infected cattle from Argentine Holstein. Pairs of primers were designed to amplify the proviral pX region of 12 cattle by PCR, and products were then sequenced, aligned and compared both with each other and with the reference sequence. Results show a divergence percentage from 0 to 6.1 for the Tax gene, from 0 to 9.4% for the Rex gene, from 0 to 12.1% for the R3 gene and finally from 0 to 6.5% for the G4 gene. Results obtained with hierarchical clustering showed two clusters well differentiated, where the members of each cluster are cattle that had tumor, PL and AL, not allowing differentiate those two cluster by clinical stage.

1. Introduction

Enzootic Bovine Leukemia (EBL) is a chronic disease caused by bovine leukemia virus (BLV). BLV belongs to the *Retroviridae* family (genus *Deltaretrovirus*) and is widely distributed in cattle populations in many countries, including Argentina. The genome of BLV consists of two identical RNA molecules that are transcribed to a double-stranded DNA by the reverse transcriptase enzyme, and then integrate into the "B" cell genome as provirus. Approximately 30% of infected cattle develop persistent lymphocytosis (PL), 0.1–5% may develop tumors that invariably lead to death, and about 65–70% remain as asymptomatic seropositive carriers in an aleukemic state (AL). The virus has transcriptional and post-transcriptional regulatory genes and structural genes (Gag, Pol and Env). The BLV regulatory genes are located in a region situated between the Env gene and the LTR-3′: a region known as pX_{BLV}, which includes the Tax, Rex, R3 and G4 genes. The pX_{BLV} genes are involved in tumorigenesis by affecting transcriptional and post-transcriptional regulation. The expression of BLV is regulated by the Tax gene at transcriptional level. The Tax gene encodes a 34 kDa protein (p34) localized in the nucleus of the cell (Miller et al., 1969; Lagarias and Radke, 1989). p34 plays a role in the activation of viral transcription by binding to sequences present in the LTR-5′. These sequences have three repetitions of 21 base pairs in the LTR U3-5′ region, called TxREs (Tax response elements) (Derse, 1987; Katoh et al., 1989). p34 does not bind directly to these TxREs (Yoshida, 2001) but indirectly by binding to a protein called b-ZIP (basic-zipper) -P34t/b-ZIP-. This protein complex acts through cellular factors such as cAMP response element-binding (CREB) and activating transcription factor ATF (Adam et al., 1994). CREB is a protein that acts as a transcription factor, therefore, binds to certain sequences (TGACGTCT) called cAMP response element (CRE) found in TxREs (Wagner and Green, 1993; Anderson and Cresswell, 1994; Perini et al., 1995; Yin and Gaynor, 1996) which increases or decreases the downstream transcription regulated by these genes. This protein complex may also recruit co-activators such as CBP/p300 (a co-activator binding protein), which facilitate transcription initiation of the cell to have chromatin remodeling activity. In addition to its role as a transcriptional activator, Tax can induce immortalization of rat embryo fibroblasts and is responsible for malignant transformation by activating cellular proto-oncogenes (Willems et al., 1990). The Rex gene is a post-transcriptional regulator of viral replication by stabilizing the viral RNA to facilitate translation (Stott et al., 1991). It is a nuclear

* Corresponding author. Tel.: +54 221 425 7980; fax: +54 221 425 7980.
E-mail address: gecheverria@fcv.unlp.edu.ar (M.G. Echeverría).
[1] Fellows of CONICET-CCT La Plata (Scientific Research Council).
[2] Members of CONICET-CCT La Plata (Scientific Research Council).

0168-1702/$ – see front matter © 2012 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.virusres.2012.08.001

phosphoprotein that acts post-transcriptionally through *cis*-acting elements in the LTR, to regulate levels of expression of genes encoding virion components, thus determining whether infectious virions are produced (McGirr and Buehring, 2005). Sequence analysis of several viral isolates has shown that the Rex gene is conserved, with a variation of less than 5% (Choi and Hope, 2005). Moreover, the pX_{BLV} region has two known regulatory gene accessories: G4 and R3. The G4 gene is expressed mainly in the leukemic phase in vivo (Alexandersen et al., 1993), and when expressed in primary cells it induces their immortalization (Kerkhofs et al., 1998). Although the functions of these two genes are not clearly established, their biological significance has been demonstrated. In fact, mutant proviruses without the R3 and G4 genes retain their infectivity (Willems et al., 1994; Kerkhofs et al., 1998). Therefore, R3 and G4 are dispensable for infectivity in vivo, but the integrity of these genes is essential to allow efficient virus spread in the host (Mammerickx et al., 1981; Radke et al., 1992; Adam et al., 1994).

Mutated viruses in the pX_{BLV} region are infectious and capable of inducing leukemia and lymphoid tumors in sheep (Twizere et al., 2000). The genetic variability present in the pX_{BLV} region could determine the clinical course of the disease. Therefore, it is important to monitor the appearance of genetic variants at the molecular level of pX_{BLV} capable of determining the different clinical states of the disease. For that reason, our main objective was to compare the proviral pX_{BLV} region at different stages (BLV+ animals with: tumors, PL or AL) of EBL in Argentine Holstein cattle.

2. Materials and methods

2.1. Bovine samples

Blood samples with EDTA were collected from 217 cattle from three dairy farms located in Córdoba, Santa Fe and Buenos Aires Provinces, Argentina. This area is the most productive dairy area and contains the highest concentration of Holstein cows in Argentina. It is located in the Northwest of the Province of Buenos Aires, South the Province of Santa Fe and South of the Province of Cordoba. From these 217 cattle, 203 animals were detected as BVL positive by AGID and PCR tests (68 out of 70 from Cordoba herd, 41 out of 45 from Santa Fe herd and 94 out of 102 from Buenos Aires farm). The selection of 12 bovines was according to the population size (203 positive to BLV), with an expected variability of pX_{BLV} region of 2%, an accepted error of 8%, and a level of confidence of 95%, with the WinEpiscope 2.0 software. These 12 bovines were selected according to three their clinical stage: BLV^+ tumor: animals T1–T4; BLV^+ PL: animals PL5–PL8 and BLV^+ AL: animals AL9–AL12 (category I, II and III, respectively). The cutoff value used for the classification between PL and AL positive to BLV was 10,000 cells/μl (Tolle, 1965). In the category I were studied 2 animals from Cordoba herd (between 6 and 8 years old) one from Santa Fe and one from Buenos Aires herds, both between 2.5 and 3.5 years old; in category II, 2 Holstein from Santa Fe herd, one from Cordoba and one from Buenos Aires herds, between 2.5 and 3.5 years old. In category III, 2 bovines from Buenos Aires, one from Santa Fe and one from Cordoba herds, all between 2.5 and 3.5 years old were studied.

2.2. DNA extraction

Bovine DNA was obtained from peripheral blood mononuclear cells isolated from 10 ml of blood by centrifugation over Histopaque (Sigma–Aldrich). Genomic DNA was extracted from peripheral blood leukocytes using a commercial kit (DNA Purification Kit, Promega, WI, USA). The DNA concentrations were determined using a Nano Spectrophotometer Vue (GE Healthcare) (Institute of Veterinary Genetics Ing. Fernando Noel Dulout-IGEVET, Faculty of Veterinary Sciences, National University of La Plata, Argentina). In all cases, DNA concentrations were above 5 mg/ml.

2.3. Proviral detection

In order to confirm the seropositive cattle and analyze the presence of provirus, a nested PCR of a segment of the BLV gp51 was performed by using the following primers: env 5032 5′-TCTGTGCCAAGTCTCCCAGATA-3′-forward-, env 5608r: 5′-AACAACAACCTCTGGGAA-3′-reverse-, resulting in the amplification of a 598 bp fragment and env 5099 5′-CCCACAAGGGCGGCGCCGGTTT-3′-forward-, and env 5521r: 5′-GCGAGGCCGGGTCCAGAGCTGG-3′-reverse- resulting in a 444 bp fragment, for the first and second rounds, respectively (Licursi et al., 2003). PCR was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA). PCR reactions were carried out according to the manufacturer's protocols, using PCR Master Mix (Promega, WI, USA). First, an initial incubation at 94 °C for 9 min was performed followed by 40 cycles, each consisting of denaturation at 95 °C for 30 s, primer annealing at 62 °C (external primers) or at 70 °C (internal primers) for 30 s, and polymerase extension at 72 °C for 60 s. This was followed by a final extension at 72 °C for 4 min. Amplified products were visualized on 1.5% agarose gels in TBE buffer (40 mM Tris–acetate pH 7.8, 40 mM boric acid, and 1 mM EDTA) by staining with ethidium bromide. The bands were identified according to their size using a 100 bp DNA ladder as a marker (Gibco BRL).

2.4. pX_{BLV} detection

To study the pX_{BLV} region of the provirus genome, three pairs of primers were designed using the DNAstar program (Tamura et al., 2007), dividing the 1817 bp pX_{BLV} region in three parts as follows: $pX_{BLV}1$ (Forward: 6910–6929, 5-TATTCCACCCTCGCAAGGC-3, Reverse: 7498–7519, 5-GGGCAGTTGATCCAGAGTCGT-3); $pX_{BLV}2$ (Forward: 7405–7424, 5-ATCAACTGGACCGCCGATG-3, Reverse: 8053–8074, 5-CGTAGGGTCATGAAGGAAGCG-3) and $pX_{BLV}3$ (Forward: 7862–7881, 5-CCTTCTCTCCTAATGTCCC-3, Reverse: 8292–8302, 5-AGGGGAAGTTGGGGAGGTA-3). Conditions of PCR amplification were 30 cycles as follows: for $pX_{BLV}1$, denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. The expected product is of about 609 bp. For $pX_{BLV}2$ and $pX_{BLV}3$, the conditions were: denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 30 s and final extension at 72 °C for 5 min. The expected amplification products were of 669 bp and 478 bp for $pX_{BLV}2$ and $pX_{BLV}3$, respectively. All products were visualized in 2% agarose gel in TBE buffer stained with ethidium bromide. The PCR products were purified according to the manufacturer's protocols, using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Both chains of each product were sequenced using the same primers as for PCR using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, USA) in an ABI3130XL Sequencer (Applied Biosystems, USA), Unidad Genómica, INTA Castelar, Argentina. The sequencing reads for each sample were analyzed and matched with MEGA version 4.0 using the ClustalW algorithm.

2.5. Data analysis

The nucleotide sequences for the pX_{BLV} region of each bovine were translated to their corresponding amino acids with DNAman (Lynnon BioSoft 1994–1999). The sequences were then aligned and compared with a consensus sequence obtained from GenBank K02120 (Sagata et al., 1985) and with the Argentine BLV sequence AF257515 (Dube et al., 2000) with MEGA version 4.0 using the

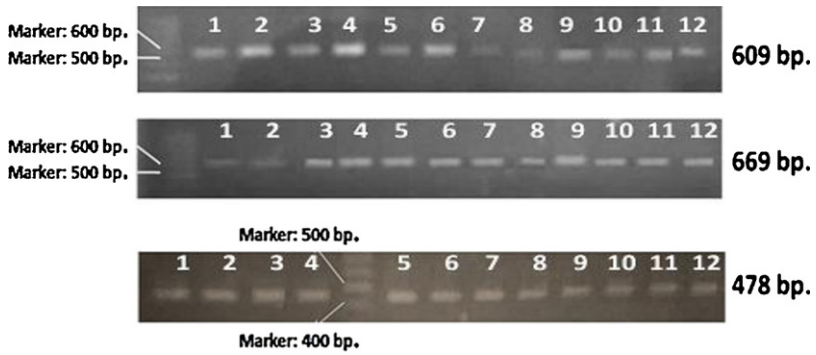

Fig. 1. Amplification of $pX_{BLV}1$ (609 bp), $pX_{BLV}2$ (669 bp) and $pX_{BLV}3$ (478 bp) region in BLV-provirus from 12 Argentine Holstein.

ClustalW algorithm. The sequence pair distances were calculated by DNAstar (Tamura et al., 2007). Hierarchical clustering explorer program 3.0 was used for hierarchical clustering and dendogram analyzes (www.cs.umd.edu/hcil/hce/).

3. Results

The Env region of BLV provirus was amplified in all the samples used, giving a product of 444 bp (data not shown). The partial pX_{BLV} region from 12 Argentine Holstein cattle was also successfully amplified. The products of amplifications were: pX_1, 609 bp; pX_2, 669 bp and pX_3, 478 bp (Fig. 1). Each sequence of the pX_{BLV} region belonging to these 12 bovines was annotated in GenBank under accession numbers JF288763 to JF288774. Subsequently, the nucleotide sequences of each bovine were translated to their corresponding amino acids for each of the genes in this pX_{BLV} region. These sequences also were aligned and compared both with each other and with the reference sequence obtained from GenBank K02120 (Sagata et al., 1985) and with the Argentine BLV sequence AF257515 (Dube et al., 2000) (Table 1). These results show a divergence percentage from 0 to 6.1 for the Tax gene, from 0 to 9.4% for the Rex gene, from 0 to 12.1% for the R3 gene and finally from 0 to 6.5% for the G4 gene. The amino acid changes observed in the three categories of Argentine BLV-positive Holsteins and the reference sequence K02120 are described in Table 2. Amino acid substitutions in the Tax protein can be observed in different positions: 56, 64, 69, 130, 148, 164, 216, 236, 240, 241 and 289 with respect to the reference sequence in the three categories of BLV-positive Holsteins studied. Amino acid substitutions in the Rex protein were observed in positions 31, 64, 72, 93, 94, 103, 118 and 138, whereas those in the R3 protein were observed in positions 5, 19 and 27, and those in the G4 protein were observed in positions 2, 10, 20, 22, 27 and 30. Tax protein had less amino acid variation rate (3.25%) than the Rex, R3 and G4 proteins (4.72%, 11.11% and 7.5% respectively). Besides, there are amino acids substitutions characteristics in the BlvArg38 strain in positions 48, 78–84, 146–147 and 166 for Tax protein and 56, 86–88 and 90 for Rex protein.

Applying hierarchical clustering analyze, clustering was obtained according with similarity of each amino acid sequences in each pX_{BLV} region. Two clusters well differentiated in the four genes (Tax, Rex, R3 and G4) were observed, where the members of each cluster are cattle that tumor, PL and AL, not allowing differentiate those two cluster by clinical stage. Also, similarities and divergences on the sequences between them and with the reference and BA38 sequences, is shown where red boxes denoting maximum differences and green boxes denoting maximum similarities between each pX_{BLV} regions analyzed. Dark boxes denote an intermediate level of similarities/divergences between them (Fig. 2).

4. Discussion

Several researchers have described different functional areas of the Tax gene: a putative zinc finger motif (amino acids 30–53), a transactivating domain (amino acids 157–197), and two phosphorylation sites (amino acids 106 and 293) (Willems et al., 1990, 1991, 1992). All these researchers found that three cysteine and two histidine residues between amino acids 30 and 53 were critical for the proper transactivator function of the Tax gene, and that when cysteine and histidine were mutated to glycine and alanine, the protein lost this function. All amino acids sequences including the cysteines and histidines remain conserved in the region (amino acids 30–53) in all 12 bovines compared with the reference sequence K02120 and with BA38 strain; also the phosphorilation sites remain invariable. Considering the region between amino acids 95 and 147, other authors have postulated that mutations in this region do not destroy the transactivation function of Tax because its structural integrity is less strictly required (Sakurai et al., 1991). In the sequences analyzed here, only one change was observed in position 130 (isoleucine to valine –I x V–) in animals T3, T4, PL5, AL11 and AL12. The BA38 Argentine strain had characteristic amino acids substitutions (position 146–147). The sequence conservation in this region would be less stringent. The region between amino acids 78 and 84 was not variable between the 12 sequences in comparison with the reference strain. In contrast, other authors found a great difference in this region (Dube et al., 2000). Also, this region has been postulated to be critical for Tax transactivation (Sakurai et al., 1991). According to Willems et al. (1991) the region between amino acids 157 and 197 of the Tax gene is a leucine-rich region that acts as an activation domain. Deletions in this region would destroy Tax's ability to transactivate. The analysis in this region in the 12 Argentine Holsteins studied in this work showed a leucine-rich region with only one amino acid substitution (position 164) in five animals of all three categories (T3, T4, PL5, AL11 and AL12). These results demonstrate that the function of Tax as a transactivactor gene would be intact in all bovines studied, since no leucine residues were changed. Although Willems et al. (1991) did not explore every individual amino acid change, and some changes we found do not correspond to the ones these authors investigated, it is possible that amino acid changes in this region could have an impact on transactivation function. Only BA38 Argentine strain had a characteristic amino acid substitution in position 166. As described by other authors (Sakakibara et al., 1998) the amino acids between positions 111 and 150 of the Tax gene act as a T cell epitope. All twelve Argentine Holstein sequences differed by one amino acid in position 148 (R-----G). As the change did not involve cysteine, the disulphide bond formation is nule. Changes in these epitopes could potentially result in cytotoxic T-lymphocyte (CTL) escape mutants; also this fact could be a strategy for the virus to evade the immune system. Studies

Table 1
Percentage of amino acid divergence in the proviral pX_{BLV} region between different clinical stages of EBL in 12 Argentine Holstein cattle. (A) Tax: lower triangle, Rex: upper triangle. (B) R3: lower triangle; G4: upper triangle. T1–T4: *category I* (BLV+ tumor); PL5–PL8: *category II* (BLV+ PL); AL9–AL12: *category III* (BLV+ AL); Ref.: Reference strain K02120 (Sagata et al., 1985), BA38: BlvArg38 – AF257515 (Dube et al., 2000).

(A)

	Tax													Rex	
	Ref.	T1	T2	T3	T4	PL5	PL6	PL7	PL8	AL9	AL10	AL11	AL12	BA38	
Ref.	*	4.9	4.9	3.5	3.5	3.5	2.8	4.2	4.9	4.9	4.9	3.5	3.5	9.4	Ref.
T1	1.3	*	0	2.8	2.8	2.8	2.1	0.7	0	0	0	2.8	2.8	4.2	T1
T2	1.3	0	*	2.8	2.8	2.8	2.1	0.7	0	0	0	2.8	2.8	4.2	T2
T3	1	1.6	1.6	*	0	0	0.7	2.1	2.8	2.8	2.8	0	0	5.6	T3
T4	2	2.7	2.7	1	*	0	0.7	2.1	2.8	2.8	2.8	0	0	5.6	T4
PL5	1.3	2	2	0.3	0.7	*	0.7	2.1	2.8	2.8	2.8	0	0	5.6	PL5
PL6	0.3	1	1	0.7	1.6	1	*	1.4	2.1	2.1	2.1	0.7	0.7	6.4	PL6
PL7	1.3	0	0	1.6	2.7	2	1	*	0.7	0.7	0.7	2.1	2.1	4.9	PL7
PL8	1.3	0	0	1.6	2.7	2	1	0	*	0	0	2.8	2.8	4.2	PL8
AL9	1.3	0	0	1.6	2.7	2	1	0	0	*	0	2.8	2.8	4.2	AL9
AL10	1.3	0	0	1.6	2.7	2	1	0	0	0	*	2.8	2.8	4.2	AL10
AL11	1.6	2.3	2.3	0.7	1.6	1	1.3	2.3	2.3	2.3	2.3	*	0	5.6	AL11
AL12	1.6	2.3	2.3	0.7	1.6	1	1.3	2.3	2.3	2.3	2.3	0	*	5.6	AL12
BA38	5.8	5.1	5.1	5.4	5.8	5.1	6.1	5.1	5.1	5.1	5.1	6.1	6.1	*	BA38
	Ref.	T1	T2	T3	T4	PL5	PL6	PL7	PL8	AL9	AL10	AL11	AL12	BA38	

(B)

	R3													G4	
	Ref.	T1	T2	T3	T4	PL5	PL6	PL7	PL8	AL9	AL10	AL11	AL12	BA38	
Ref.	*	6.5	6.5	3.9	3.9	3.9	3.9	6.5	6.5	6.5	6.5	3.9	3.9	3.9	Ref.
T1	12.1	*	0	6.5	6.5	6.5	6.5	0	0	0	0	6.5	6.5	3.9	T1
T2	12.1	0	*	6.5	6.5	6.5	6.5	0	0	0	0	6.5	6.5	3.9	T2
T3	0	12.1	12.1	*	0	0	0	6.5	6.5	6.5	6.5	0	0	2.5	T3
T4	0	12.1	12.1	0	*	0	0	6.5	6.5	6.5	6.5	0	0	2.5	T4
PL5	0	12.1	12.1	0	0	*	0	6.5	6.5	6.5	6.5	0	0	2.5	PL5
PL6	0	12.1	12.1	0	0	0	*	6.5	6.5	6.5	6.5	0	0	2.5	PL6
PL7	12.1	0	0	12.1	12.1	12.1	12.1	*	0	0	0	6.5	6.5	3.9	PL7
PL8	7.8	3.8	3.8	7.8	7.8	7.8	7.8	3.8	*	0	0	6.5	6.5	3.9	PL8
AL9	7.8	3.8	3.8	7.8	7.8	7.8	7.8	3.8	0	*	0	6.5	6.5	3.9	AL9
AL10	7.8	3.8	3.8	7.8	7.8	7.8	7.8	3.8	0	0	*	6.5	6.5	3.9	AL10
AL11	0	12.1	12.1	0	0	0	0	12.1	7.8	7.8	7.8	*	0	2.5	AL11
AL12	0	12.1	12.1	0	0	0	0	12.1	7.8	7.8	7.8	0	*	2.5	AL12
BA38	7.8	3.8	3.8	7.8	7.8	7.8	7.8	3.8	7.8	7.8	7.8	7.8	7.8	*	BA38
	Ref.	T1	T2	T3	T4	PL5	PL6	PL7	PL8	AL9	AL10	AL11	AL12	BA38	

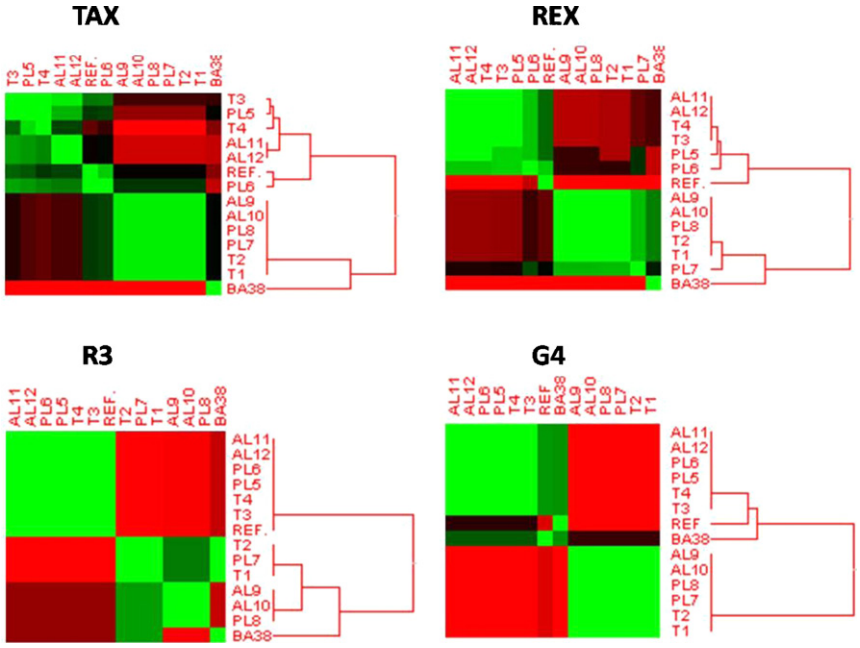

Fig. 2. Hierarchical clustering analyze between pX_{BLV} region of cattles studied. The analyze was categorized by an average linkage hierarchical clustering program. Red boxes denoting differences and green boxes denoting similarities between each pX_{BLV} regions of 12 cattle. Dark boxes denote an intermediate level of similarities or divergences. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Amino acid substitutions in the four regulatory genes from 12 BLV-positive Argentine Holstein cattle, in respect to reference strain K02120 (Sagata et al., 1985). Substitutions for BA38: BlvArg38 – AF257515 (Dube et al., 2000) are indicated in a separated column.

Gene	Position	Amino acid	Bovine number	BA38
TAX				
	48	L------H	None	*
	56	N------ T	T1,T2; PL7, PL8; AL9, AL10	*
	64	C-------F	T1,T2; PL7, PL8; AL9, AL10	*
	69	T------A	T1,T2; PL7, PL8; AL9, AL10	*
	78 to 84	ETRPQGP-----RDPAPRA	None	*
	130	I------V	T3, T 4; PL5; AL11, AL12	*
	146-147	VL-----CP	None	*
	148	R-----G	1 to 12	R
	164	S------P	T3, T4; PL5; AL11, AL12	*
	166	D-----N	None	*
	216	R------G	AL11, AL12	R
	236	T------P	AL11, AL12	T
	240	S------L	T4	S
	241	W-----Q	T4	W
	289	I------L	T4; PL5	L
REX				
	31	M-----T	T1, T2; PL8; AL9, AL10	*
	56	S-----T	None	*
	64	T------P	T1,T2; PL7, PL8; AL9, AL10	*
	72	A-----S	T1,T2; PL7, PL8; AL9, AL10	*
	86-88	RPA-----ETR	None	*
	90	K-----Q	None	*
	93 and 94	E------D	1 to 12	*
	103	S------P	1 to 12	*
	118	S------G	1 to 12	*
	138	L-----S	T3, T4; PL5; AL11, AL12	*
R3				
	5	V------I	T1, T2; PL7	*
	19	I-------V	T1, T2; PL7, PL8; AL9, AL10	I
	27	F------L	T1, T2; PL7, PL8; AL9, AL10	*
G4				
	2	H------R	T1, T2; PL7, PL8; AL9, AL10	H
	10	F------S	T1, T2; PL7, PL8; AL9, AL10	*
	20	F------S	T1, T2; PL7, PL8; AL9, AL10	F
	22	R-----Q	1 to 12	*
	27	H-----L	T3, T4; PL5, PL6; AL11, AL12	H
	30	V------L	T1,T 2; PL7, PL8; AL9, AL10	
		V-----F	T3,T 4; PL5, PL6; AL11, AL12	*

T1–T4: *category I* (BLV+ tumor); PL5–PL8: *category II* (BLV+ PL); AL9–AL12: *category III* (BLV+ AL).
* Indicates similar amino acid substitution; proper amino acid substitutions of BA38 are shaded.

related by Tajima and Aida (2000) in Tax proteins from mutants and wild-type BLV-provirus demonstrated that the mutants proteins appeared to enhance the production of viral proteins and particles, as compared to the wild-type Tax proteins, via the LTR of a contransfectated defective recombinant provirus clone of BLV. Thus, Tax protein demonstrated that a single substitution between the residues 240 and 265 was critical for the elevated transactivation activities of the Tax mutant proteins, not so in wild-type provirus. In this study related in 12 infected cattle with wild-type BLV-provirus, we found only one BLV+ (T4) which had change in amino acids 240 and 241 (serine to leucine and tryptophan to glutamine, respectively). A cysteine in position 257 was present in all 12 bovines as well as in the reference strain K02120 and Argentine BA38 strain, which means that the disulphide bonds remain intact. Other researchers reported a change in amino acid number 48, where a leucine in the K02120 consensus was replaced by histidine in the viral Argentine strain BA38 (AF257515). This cow is a healthy BLV-positive Argentine Holstein with PL (Dube et al., 2000). In the present study we found no such replacement within the 12 Holstein cattle tested for Tax amino acids. The amino acid sequence of the Tax protein in BLV Argentine Holsteins had less variation than the Rex, R3 and G4 proteins. The results found in this work are in concordance with others authors where were reported a variability for Tax proteins in wild-type BLV-provirus less than 5% (McGirr and Buehring, 2005) being the most conserved protein of the four in the pX region (Zhao et al., 2007).

The Rex gene, which is less than half the size of Tax but contains more variation, resulted in amino acid changes located in the second third of the sequence (McGirr and Buehring, 2005), with the exception of BA38 Argentine strain, which had particular changes (amino acids 56, 86–88 and 90). As was observed by others (Alexandersen et al., 1993), the expression of G4 was specifically detected in naturally infected cows with persistent lymphocytosis. Only a variation in amino acid 22 was found in the provirus from all 12 BLV infected cattle used in this study.

Because of the similarities found in the provirus pX_{BLV} region of the 12 animals tested, we were not able to relate with the clinical stages of the disease. This conclusion was supported by hierarchical clustering analyze were did not show a differentiation between the clinical stage and the sequence per each cattle, where both cluster found comprised all stages of the EBL disease. Thus, the stages of EBL (tumor, PL and AL) were not differentiated in this

work in this regulatory region of the provirus. Many studies have reported factors which are involved in the disease progression of BLV infection, including aleles of DRB3.2 gen (Lewin et al., 1988; Panei et al., 2009), the changes of cytokines profiles (Pyeon et al., 1996; Pyeon and Splitter, 1998;) and the mutation of p53 gene (Zhuang et al., 1997). In this study we found no amino acid strict differences between the different categories of BLV-positive Argentine Holsteins, i.e., between those which developed tumor or PL and those which remained without clinical symptoms. Although other authors have studied the pX region by sequencing, only one strain from Argentina was analyzed, from a healthy cow with PL (Dube et al., 2000; Zhao et al., 2007), to our knowledge, no author has studied this region in reference to the clinical stages in natural infected cattle.

Acknowledgements

The technical assistance of Ms. M.C. Mondragón, Ms. A. N. Conde and Mr. C. Leguizamón are highly acknowledged.

References

Adam, E., Kerkhofs, P., Mammerickx, M., Kettmann, R., Burny, A., Droogmans, L., Willems, L., 1994. Involvement of the cyclic AMP-responsive element binding protein in bovine leukemia virus expression in vivo. Journal of Virology 68, 5845–5853.

Alexandersen, S., Carpenter, S., Christensen, J., Storgaard, T., Viuff, B., Wannemuehler, Y., Belousov, J., Roth, J., 1993. Identification of alternatively spliced mRNAs encoding potential new regulatory proteins in cattle infected with bovine leukemia virus. Journal of Virology 67, 39–52.

Anderson, K.S., Cresswell, P., 1994. A role for calnexin (IP90) in the assembly of class II MHC molecules. EMBO Journal 13, 675–682.

Choi, E.A., Hope, T.J., 2005. Mutational analysis of bovine leukemia virus Rex: identification of a dominant-negative inhibitor. Journal of Virology 79, 7172–7181.

Derse, D., 1987. Bovine leukemia virus transcription is controlled by a virus-encoded trans-acting factor and by cis-acting response elements. Journal of Virology 61, 2462–2471.

Dube, S., Dolcini, G., Abbott, L., Mehta, S., Dube, D., Gutierrez, S., Ceriani, C., Esteban, E., Ferrer, J., Poiesz, B., 2000. The complete genomic sequence of a BLV strain from a Holstein cow from Argentina. Virology 277, 379–386.

Katoh, I., Yoshinaka, Y., Ikawa, Y., 1989. Bovine leukemia virus trans-activator p38tax activates heterologous promoters with a common sequence known as a cAMP-responsive element or the binding site of a cellular transcription factor ATF. EMBO Journal 8, 497–503.

Kerkhofs, P., Heremans, H., Burny, A., Kettmann, R., Willems, L., 1998. In vitro and in vivo oncogenic potential of bovine leukemia virus G4 protein. Journal of Virology 72, 2554–2559.

Lagarias, D.M., Radke, K., 1989. Transcriptional activation of bovine leukemia virus in blood cells from experimentally infected, asymptomatic sheep with latent infections. Journal of Virology 63, 2099–2107.

Lewin, H.A., Wu, M.C., Stewart, J.A., Nolan, T.J., 1988. Association between BoLA and subclinical bovine leukemia virus infection in a herd of Holstein–Friesian cows. Immunogenetics 27, 338–344.

Licursi, M., Inoshima, Y., Wu, D., Yokoyama, T., González, E.T., Sentsui, H., 2003. Provirus variants of bovine leukemia virus in naturally infected cattle from Argentina and Japan. Veterinary Microbiology 96, 17–23.

Mammerickx, M., Portetelle, D., Burny, A., 1981. Experimental cross-transmissions of bovine leukemia virus (BLV) between several animal species. Zentralblatt fur Veterinarmedizin B28, 69–81.

McGirr, K.M., Buehring, G.C., 2005. Tax and rex sequences of bovine leukaemia virus from globally diverse isolates: rex amino acid sequence more variable than tax. Journal of Veterinary Medicine B: Infectious Diseases and Veterinary Public Health 52, 8–16.

Miller, J.M., Miller, L.D., Olson, C., Gillette, K.G., 1969. Virus-like particles in phytohemagglutinin-stimulated lymphocyte cultures with reference to bovine lymphosarcoma. Journal of the National Cancer Institute 43, 1297–1305.

Panei, C.J., Suzuki, K., Echeverría, M.G., Serena, M.S., Metz, G.E., González, E.T., 2009. Association of BoLA-DRB3.2 alleles with resistance and susceptibility to persistant lymphocytosis in BLV infected cattle in Argentina. International Journal of Dairy Sciences 4, 123–128.

Pyeon, D., O'Reilly, K.L., Splitter, G.A., 1996. Increase interleukin-10 mRNA expression in tumor-bearing or persistently lymphocytotic animals infected with bovine leukemia virus. Journal of Virology 70, 5706–5710.

Pyeon, D., Splitter, G.A., 1998. Interleukin-12 p40 mRNA expression in bovine leukemia virus-infected animals: increase in alymphocytosis but decrease in persistent. Journal of Virology 72, 6917–6921.

Perini, G., Wagner, S., Green, M.R., 1995. Recognition of bZIP proteins by the human T-cell leukaemia virus transactivator Tax. Nature 376, 602–605.

Radke, K., Sigala, T.J., Grossman, D., 1992. Transcription of bovine leukemia virus in peripheral blood cells obtained during early infection in vivo. Microbial Pathogenesis 12, 319–331.

Sagata, N., Yasunaga, T., Tsuzuku-Kawamura, J., Ohishi, K., Ogawa, Y., Ikawa, Y., 1985. Complete nucleotide sequence of the genome of bovine leukemia virus: its evolutionary relationship to other retroviruses. Proceedings of the National Academy of Sciences of the United States of America 82, 677–681.

Sakakibara, N., Kabeya, H., Ohashi, K., Sugimoto, C., Onuma, M., 1998. Epitope mapping of bovine leukemia virus transactivator protein tax. Journal of Veterinary Medical Science 60, 599–605.

Sakurai, M., Taneda, A., Nagoya, H., Sekikawa, K., 1991. Construction and functional characterization of mutants of the bovine leukaemia virus trans-activator protein p34tax. Journal of General Virology 72, 2527–2531.

Stott, M.L., Thurmond, M.C., Dunn, S.J., Osburn, B.I., Stott, J.L., 1991. Integrated bovine leukosis proviral DNA in T helper and T cytotoxic/suppressor lymphocytes. Journal of General Virology 72, 307–315.

Tajima, S., Aida, Y., 2000. The region between amino acids 245 and 265 of the bovine leukemia virus (BLV) tax protein restricts transactivation not only via the BLV enhancer but also via other retrovirus enhancers. Journal of Virology 74, 10939–10949.

Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: molecular evolutionary genetic analysis (MEGA) software version 4.0. Molecular Biology and Evolution 24, 1596–1599.

Tolle, A., 1965. On the determination of quantitative hematologic findings in the diagnosis of leukemia in cattle. Zentralblatt fur Veterinarmedizin B12, 281–290.

Twizere, J.C., Kerkhofs, P., Burny, A., Portetelle, D., Kettmann, R., Willems, L., 2000. Discordance between bovine leukemia virus tax immortalization in vitro and oncogenicity in vivo. Journal of Virology 74, 9895–9902.

Wagner, S., Green, M.R., 1993. HTLV-I Tax protein stimulation of DNA binding of bZIP proteins by enhancing dimerization. Science 262, 395–399.

Willems, L., Heremans, H., Chen, G., Portetelle, D., Billiau, A., Burny, A., Kettmann, R., 1990. Cooperation between bovine leukaemia virus transactivator protein and Ha-ras oncogene product in cellular transformation. EMBO Journal 9, 1577–1581.

Willems, L., Kettmann, R., Burny, A., 1991. The amino acid (157–197) peptide segment of bovine leukemia virus p34tax encompass a leucine-rich globally neutral activation domain. Oncogene 6, 159–163.

Willems, L., Grimonpont, C., Heremans, H., Rebeyrotte, N., Chen, G., Portetelle, D., Burny, A., Kettmann, R., 1992. Mutations in the bovine leukemia virus Tax protein can abrogate the long terminal repeat-directed transactivating activity without concomitant loss of transforming potential. Proceedings of the National Academy of Sciences of the United States of America 89, 3957–3961.

Willems, L., Kerkhofs, P., Dequiedt, F., Portetelle, D., Mammerickx, M., Burny, A., Kettmann, R., 1994. Attenuation of bovine leukemia virus by deletion of R3 and G4 open reading frames. Proceedings of the National Academy of Sciences of the United States of America 91, 11532–11536.

Yin, M.J., Gaynor, R.B., 1996. HTLV-1 21 bp repeat sequences facilitate stable association between Tax and CREB to increase CREB binding affinity. Journal of Molecular Biology 264, 20–31.

Yoshida, M., 2001. Multiple viral strategies of HTLV-1 for dysregulation of cell growth control. Annual Review of Immunology 19, 475–496.

Zhao, X., McGirr, K.M., Buehring, G.C., 2007. Potential evolutionary influences on overlapping reading frames in the bovine leukemia virus pXBL region. Genomics 89, 502–511.

Zhuang, W., Tajima, S., Okada, K., Ikawada, Y., Aida, Y., 1997. Point mutation of p53 tumor suppressor gene in bovine leukemia virus-induced lymphosarcoma. Leukemia 3, 344–346.