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Bull Vet Inst Pulawy 59, 447-450, 2015
DOI:10.1515/bvip-2015-0065

SHORT COMMUNICATION

Genetic diversity of the long terminal repeat of bovine leukaemia virus field isolates

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Received: November 25, 2015   Accepted: December 04, 2015

Abstract

In this study the sequences of the long terminal repeat (LTR) of field isolates of the bovine leukaemia virus (BLV) were analysed. These isolates came from emerging cases of BLV infection in cattle from herds having BLV-free status. We found several sequence variations within regulatory motifs in the LTRs like GRE, DAS and interferon binding site. These mutations can possibly affect transcriptional activity of the virus, leading to its silencing.

Keywords: bovine leukaemia virus, long terminal repeat, genetic variability.

Introduction

Bovine leukaemia virus (BLV) belongs to *Deltaretrovirus* in the *Retroviridae* family and is an etiological agent of enzootic bovine leukosis (EBL). Most of BLV-infected cattle remain asymptomatic, but one-third of them suffer from persistent lymphocytosis and around 5%-10% develop lymphoid tumours (12). BLV genome, as all retroviruses, consist of *gag*, *pro*, *pol*, and *env* structural genes. In addition, a region X contains the open reading frames (*orfs*) for Tax, Rex, R3, and G4 as the regulatory proteins (9, 18). Whole BLV proviral DNA is flanked by two identical long terminal repeat (LTR) fragments divided into the U3, R, and U5 regions. Transcription of the virus initiates at the promoter located in 5'LTR. The U3 region possesses the following motifs associated with transcriptional upregulation: three a 21 bp sequence of Tax response elements (TREs) containing an E box and cyclic-AMP responsive element (CRE) (1); the promoter CAAT and TATA boxes and a poly (A) signal (PAS) (5); a glucocorticoid responsive element (GRE) (14), and a PU.1/Spi-B site (4). Additionally, BLV expression is regulated by a downstream activator sequence (DAS) (R region) (11) and an interferon regulatory factor (IRF)-binding site (U5 region) (10).

BLV infection is distributed worldwide. Nevertheless most EU countries are now free of the disease (8). An official BLV control programme has been applied intensively in Poland since 2006 and consequently more than 90% of districts have already obtained BLV-free status. However, in some herds officially free from BLV emerging infections were noted. This can be linked to the late seroconversion caused by a low level of virus replication. Like in other retroviruses genetic variability of LTR might influence interactions with cellular transcription factors and affect virus replication (2, 15, 16). Therefore, the aim of our study was a genetic characterisation of several BLV LTR sequences, representing virus isolates from emerging cases of BLV infection in herds recognised as BLV-free.

Material and Methods

DNA extraction. Blood samples were collected from five cows, coming from herds located in geographically distinct regions of Poland, in which a new infection with BLV was identified. This infection was confirmed by serological testing (IDEXX Leukosis Blocking Ab ELISA Test, Montpellier, France). Peripheral blood leukocytes (PBLs) were isolated from 10 mL of blood by centrifugation at 1500 g for 25 min. Erythrocytes were haemolysed by osmotic shock with H_2O and 4.5% NaCl. Afterwards,

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the PBLs were washed twice with PBS, aliquoted (5×10^6 cells), and stored as pellet at -80ºC until DNA extraction. Genomic DNA was extracted using DNeasy Blood & Tissue Kit (Qiagen), following manufacturer's instructions. For each sample, genomic DNA quantities were measured using IMPLEN Nanophotometer and the samples were stored at – 20ºC until examination.

PCR amplification. Six different primers (Table 1) were used for the amplification of full-length LTR region, according to a model described by Zhao *et al.* (17). The primers amplifying LTR were designed based on conserved regions shared by major BLV genotypes (Fig. 1). LTR region was amplified by two separate semi-nested PCRs conducted to obtain two fragments of LTR region: 5'LTR fragment (P3) and 3'LTR fragment (P1) respectively. The amplification was carried out in a 50 µL volume. The mixture contained: 5 µL of buffer (10 x), 1 µL of dNTP solutions (10 µM), 1 µL (2 U) of Taq polymerase (DyNAzyme II DNA), 1 µL of $MgCl_2$ (25 mM), 1 µL of each of the primers (10 µM) (Genomed, Poland), and 500 ng of DNA template. The reaction was performed by initial denaturation for 3 min at 94ºC followed by 30 cycles (I round) or 42 cycles (II round) consisting of denaturation for 30 s at 94ºC, annealing for 30 s at 68ºC, and extension for 2 min at 72ºC Final extension was performed for 7 min at 72ºC. A volume of 4 µL of the first round PCR mixture was used as a template in the second PCR which was performed under the same condition for the P1 and P3 fragments. The semi-nested PCR products, 5 µL mixed with 1 µL of loading buffer, were visualised by electrophoresis (data not shown).

DNA sequencing and sequence characterisation. PCR products were purified using the NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). The samples were sequenced using 3730xl DNA Analyzer (Applied Biosystems) with the Big Dye Terminator v3.1 Cycle Sequencing Kit (Genomed, Poland). Sequence chromatograms of P1 and P3 fragments of LTR were analysed and subsequently assembled to reconstruct full-length LTR region using the Geneious Alignment module. The whole LTR nucleotide sequences were compared using the ClustalW alignment algorithm in Geneious. BLV LTR sequences obtained in this study were compared to the reference sequence, FLK-BLV subclone pBLV913 (NCBI, EF600696), for the evaluation of genetic variability.

Table 1. Sequences of the primers used in two semi-nested PCRs and sequencing

Primer name	Primer sequence (5' → 3')	Positions (nt)
P1out5	CATTATCGGGCACG	7628–7642
P1outin3	AGGGGAAGTTGGGGAGGTA	8310–8292
P3out5	TGTATGAAAGATCATGCCGA	1–20
P3outin3	GTTAGGGTTCCGGGGTGATC	571–552
P1in5	TCGATACCCTCCTTGTGGACC	7736–7756
P3in5	TAGGAGCCGCCACCGC	23–38

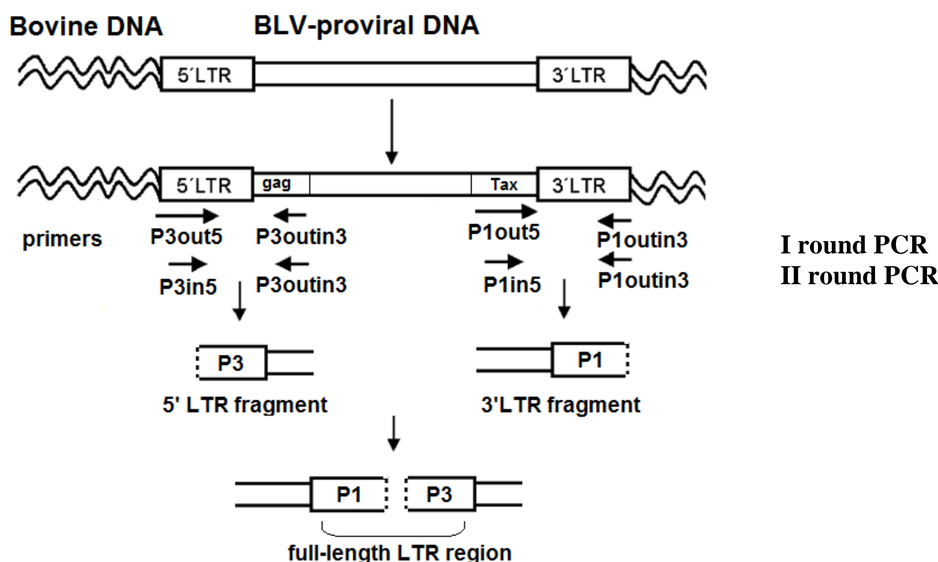

Fig. 1. Scheme of the two-step PCR procedure. The two-step PCR method was adapted and modified from Zhao *et al.* (17)

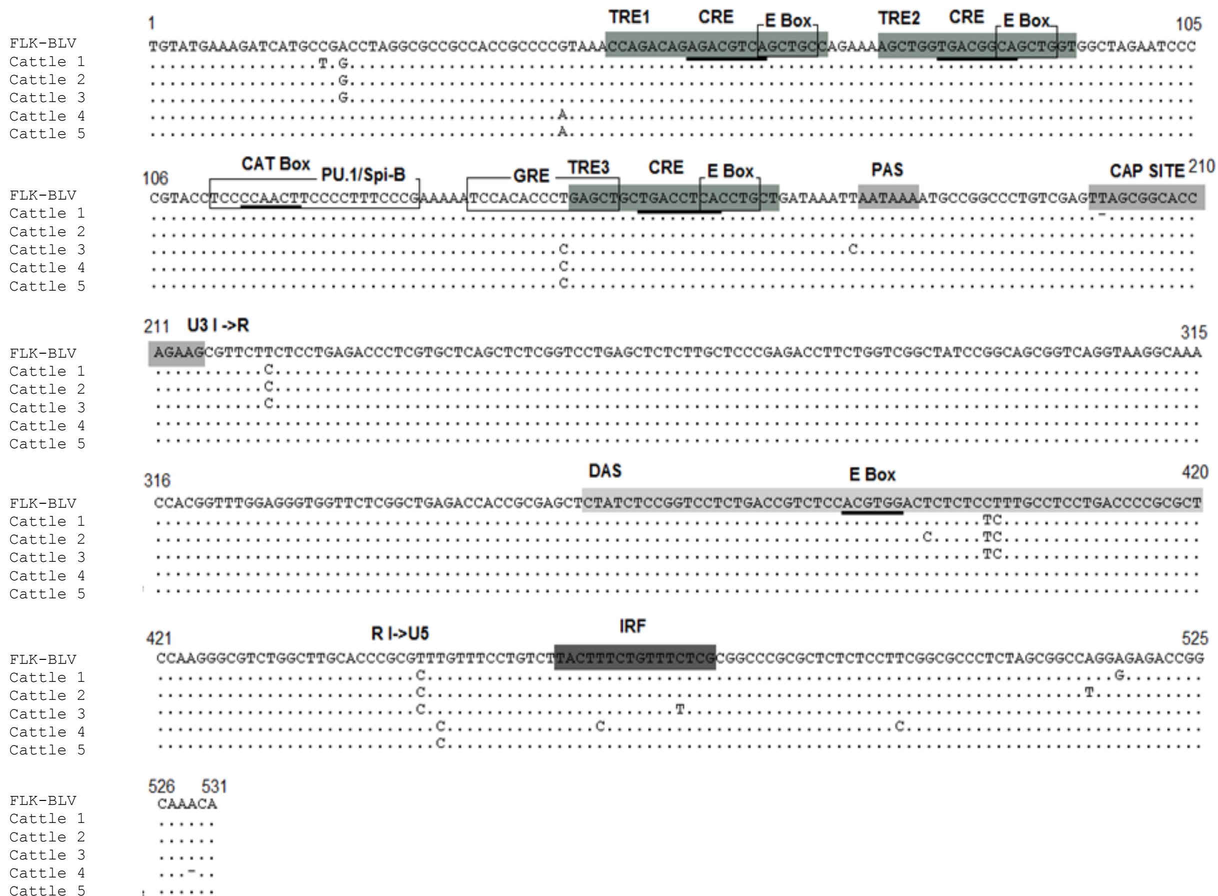

Fig. 2. Alignment of full length LTR region representing five field isolates of BLV and reference strain. FLK-BLV sequence is fully shown for comparison. Only the nucleotides different from BLV-FLK are indicated, while those identical to reference sequence are reported as (.). Dashes (-) denote deletions. The U3, R and U5 "domains" are indicated on top of the alignment. The TxRE enhancer regions, IRF, PAS, CAP SITE, and DAS are shown in dark and light grey respectively. CRE and CAT Box are underlined. E-Box, GRE, and PU.1/Spi-B sequences are indicated in black rectangle

Results

Semi-nested PCRs were used for the amplification of 531bp LTR region of BLV. DNA from PBLs of all five cattle used in this study gave positive PCR amplification of both fragments of LTR region. Next, these fragments were subsequently subjected to sequencing and the resulting LTR sequences were aligned to each other, and to a well-known FLK-BLV subclone sequence. The respective results were shown in Fig. 2. In total, 18 different mutations were detected, dispersed mainly along U3 and U5 "domains" of the LTR. The observed nucleotide changes were predominantly point mutations, such as transitions between A and G, C and T, and two deletions. Some of the mutations were detected in one isolate only while some of them were found in other isolates. Out of 18 mutations, found in this study, only four namely A20G, C399T, T400C and T449C, have been already described (6, 7, 17). Six mutations were located in regulatory elements: at glucocorticoid responsive element (T147C), interferon regulatory factor-binding site (T465C, C473T), and three substitutions T- > C, C -> T (T393C, C399T, T400C) were found in downstream activator sequence. In comparison to the reference FLK-BLV strain, two deletions in position 201(CAP SITE) and 529 were characteristic for isolates from cattle 1 and 4, respectively.

Discussion

Emerging cases of BLV infection were identified in cattle, from herds having BLV-free status, by serological testing and PCR examination. The problem of the recurrence of BLV infection in disease-free herds was pointed out by Lorenz and Straub (13) and most likely it was a consequence of a latent infection reactivation. We hypothesized that BLV isolates analysed in this study could represent genetic variants which were silenced and they had diminished transcriptional activity. Since the sequences that regulate viral transcription are located in the LTR, we investigated the genetic diversity of this region. We found several variations which were localised within BLV LTR regulatory sites. Although three Tax response elements, fundamental for regulation of BLV transcription, remained unchanged, the mutation

T147C (cattle 3, 4 and 5) placed just upstream TRE3 - in glucocorticoid responsive element (GRE) (14) seemed to be interesting. This mutation could potentially affect responsiveness to dexamethasone, which upregulated BLV transcription in the presence of Tax. Other variations noted in DAS region C399T and T400C were also observed for three isolates (cattle 1, 2 and 3), but probably did not influence BLV replication, as was already described for BLV strains from Argentina (6, 7). However, another unique mutation T393C (cattle 2), also localised in DAS region, though near E box, could modify binding transcription factors USF1 and USF2, and the regulation of the LTR promoter as a result (3). Two successive mutations: T465C (cattle 3) and C473C (cattle 4) were found in U5 region, where IRF-1 and IRF-2 interact with interferon binding site and then stimulate Tax independent expression of BLV.

In conclusion, future study will focus on clarification whether natural deletions or insertions observed in multiple isolates would result in changing viral transcription using reporter-based assay *in vitro*. Furthermore, it is important to possibly extend these observations to a higher number of field isolates.

Conflict of Interests Statement: The authors declare that they have no conflict of interests regarding the publication of this article.

Financial Disclosure Statement: The study was funded by KNOW (Leading National Research Centre) Scientific Consortium "Healthy Animal - Safe Food", decision of the Polish Ministry of Science and Higher Education No. 05-1/KNOW2/2015

References

1. Adam E., Kerkhofs P., Mammerickx M., Kettmann R., Burny A, Droogmans L., Willems L.: Involvement of the cyclic AMP-responsive element binding protein in bovine leukemia virus expression *in vivo*. J Virol 1994, 68, 5845–5853.
2. Angelopoulou K., Brellou G.D., Greenland T., Vlemmas I.: A novel deletion in the LTR region of a Greek small ruminant lentivirus may be associated with low pathogenicity. Virus Res 2006,118, 178-184.
3. Calomme C., Nguyen T.L., de Launoit Y., Kiermer V., Droogmans L., Burny A., Van Lint C.: Upstream stimulatory factors binding to an E box motif in the R region of the bovine leukemia virus long terminal repeat stimulates viral gene expression. J Biol Chem 2002, 277, 8775–8789.
4. Dekoninck A., Calomme C., Nizet S., de Launoit Y., Burny A., Ghysdael J., Van Lint C.: Identification and characterization of a PU.1/Spi-B binding site in the bovine leukemia virus long terminal repeat. Oncogene 2003, 22, 2882–2896.
5. Derse D., Casey J.W.: Two elements in the bovine leukemia virus long terminal repeat that regulate gene expression. Science 1986, 231, 1437–1440.
6. Dube S., Abbott L., Dube D.K., Dolcini G., Gutierrez S., Ceriani C., Juliarena M., Ferrer J., Perzova R., Poiesz B.J.: The complete genomic sequence of an *in vivo* low replicating BLV strain. Virol J 2009, 6, 120, doi: 10.1186/1743-422X-6-120.
7. Dube S., Dolcini G., Abbott L., Mehta S., Dube D., Gutierrez S., Ceriani C., Esteban E., Ferrer J., Poiesz B.: The complete genomic sequence of a BLV strain from a Holstein cow from Argentina J Virol 2000, 277, 379–386.
8. EFSA AHAW Panel (EFSA Panel on Animal Health and Welfare). Scientific opinion on enzootic bovine leukosis. EFSA J 2015, 13, 1-63. doi:10.2903/j.efsa.2015.4188.
9. Gillet N., Florins A., Boxus M., Burteau C., Nigro A., Vandermeers F., Balon H., Bouzar A.B., Defoiche J., Burny A., Reichert M., Kettmann R., Willems L.: Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. Retrovirology 2007, 4, 18.
10. Kiermer V., Van Lint C., Briclet D., Vanhulle C., Kettmann R., Verdin E., Burny A., Droogmans L.: An interferon regulatory factor binding site in the U5 region of the bovine leukemia virus long terminal repeat stimulates Tax-independent gene expression. J Virol 1998, 72, 5526–5534.
11. Kiss-Toth E., Unk I.: A downstream regulatory element activates the bovine leukemia virus promoter. Biochem Biophys Res Commun 1994, 202, 1553–1561.
12. Kettmann R., Burny A., Callebaut I., Droogmans L., Mammerickx M., Willems L., Portetelle D.: Bovine leukemia virus. In: *The Retroviridae*, Plenum Press, New York, 1994, pp. 39–81.
13. Lorenz R., Straub O.C.: The problem of recurrence of enzootic bovine leucosis in previously cured herds. Dtsch Tierarztl Wochenschr 1994, 101, 158–162.
14. Niermann G.L., Buehring G.C.: Hormone regulation of bovine leukemia virus *via* the long terminal repeat. Virology 1997, 239, 249–258.
15. Neto W.K., Da-Costa A.C., de Oliveira A.C., Martinez V.P., Nukui Y., Sabino E.C., Sanabani S.S.: Correlation between LTR point mutations and proviral load levels among human T cell lymphotropic virus type 1 (HTLV-1) asymptomatic carriers. Virol J 2011, 8, 535. doi: 10.1186/1743-422X-8-535.
16. Murphy B., Hillman C., Castillo D., Vapniarsky N., Rowe J.: The presence or absence of the gamma-activated site determines IFN gamma-mediated transcriptional activation in CAEV promoters cloned from the mammary gland and joint synovium of a single CAEV-infected goat. Virus Res 2012, 163, 537–545. doi: 10.1016/j.viruses.2011.12.001.
17. Zhao X, Jimenez C., Sentsui H., Buehring G.C.: Sequence polymorphisms in the long terminal repeat of bovine leukemia virus: evidence for selection pressures in regulatory sequences. Virus Res 2007, 124, 113–124.
18. Zhao X., McGirr K.M., Buehring G.C.: Evolutionary influences on overlapping reading frames in the bovine leukemia virus pXBL region. Genomics 2007, 89, 502–511. doi: 10.1016/j.ygeno.2006.12.00.