VETERINARSKI ARHIV 83 (6), 581-591, 2013

Eradication of bovine leukosis virus on a diary farm through improved virus detection

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LOJKIĆ, I., D. BALIĆ, N. RUDAN, M. KOVAČIĆ, Ž. ČAČ, M. PERIŠKIĆ, T. BEDEKOVIĆ, B. ROIĆ, I. CIGLAR GROZDANIĆ: Eradication of bovine leukosis virus on a diary farm through improved virus detection. Vet. arhiv 83, 581-591, 2013.

ABSTRACT

The aim of this study was to perform a diagnosis and molecular characterisation of bovine leukosis virus (BLV) on a Croatian dairy farm. For that purpose we compared the diagnostic tools used in our study, made sequence and phylogenetic analysis of our BLV and tried to describe the difficulties in the process of EBL eradication on the examined farm after reintegration into Croatian territory after the war. From 1998 to 2008 blood samples from a dairy farm in the North-eastern part of Croatia were tested serologically using AGID and ELISA. In 2002, 2003 and 2004 37%, 22% and 10% of animals were serologically positive, respectively. After the initial eradication steps, the disease reappeared in 2008, when all examined blood samples reacted positively in BLV-specific nested PCR. Finally, at the end of 2010, after an extended eradication program, which included the implementation of PCR together with regular ELISA testing for detection of positive animals, the farm obtained the status of "free of BLV". Sequence and phylogenetic analysis of 524-nt fragment of BLV *env* gp51 encoding gene showed the greatest identity with the Croatian BLV genotype 8 isolates, so it was phylogenetically clustered with those isolates.

Key words: bovine leukosis virus, gene *env*, gp51, ELISA, nested PCR, phylogenetic analysis

Introduction

Bovine leukemia virus (BLV) is a member of the *Deltaretrovirus* genus (family *Retroviridae*) and can cause persistent lymphocytosis and lymphosarcoma in cattle,

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ISSN 0372-5480
Printed in Croatia

and is described as enzootic bovine leukosis (EBL) (MILLER et al., 1969). BLV has been described worldwide and has been recognized in Croatia since 1956 (LOJKIĆ et al., 2001).

Most BLV infected cattle do not show any clinical symptoms. Some infected cattle may show nonspecific clinical symptoms such as weakness and emaciation, and later, if the tumour appeared on an internal organ, its function or the function of the surrounding organs or tissues could be affected. After a latency period of 1-8 years, only 1-5% of the infected cattle develop malignant B-cell lymphosarcomas (SCHWARTZ and LEVY, 1994) However, 30% of cattle naturally infected with BLV develop persistent lymphocytosis with non-malignant polyclonal expansion of CD5+ B-cells, the majority of which harbour BLV provirus (MIRSKY et al., 1996).

Once infected, cattle remain infected, showing a serological response a few weeks after infection. Maternally derived antibodies may take up to 6 or 7 months to disappear (ANONYMOUS, 2012). Since there is no vaccination, the presence of antibodies is an accurate indicator of natural infection. So, eradication and control of the disease is exclusively based on screening for antibodies and segregation of serologically positive animals from negative animals. The agar-gel immunodiffusion (AGID) and enzyme linked immunosorbent assay (ELISA) are used widely for routine detection of antibodies against BLV (JOHNSON and KANEENE, 1992). Polymerase chain reaction (PCR) has been described as an alternative method, which directly detects the presence of proviral DNA in BLV infected cattle, with low, transient or absent antibody titres (KITTELBERGER et al., 1996; KLINTEVALL et al., 1994).

The envelope glycoproteins of BLV are crucial for the virus ability to enter the cells, and are the target for neutralizing antibodies (MAMOUN et al., 1990). They also contain a variable region with the sequence coding for epitopes involved in antigen-antibody reactions and virus-host relations (BAN et al., 1992). The BLV envelope gene (*env*) codes a polyprotein precursor (gpr72), which is cleaved to the gp51 envelope and gp35 transmembrane glycoprotein (CALLEBAUT et al., 1993). Genetic variation analyses and comparison of gp51 sequences of different isolates have been described by several authors (PORTETELLE et al., 1989; MAMOUN et al., 1990; FECHNER et al., 1997; CAMARGOS et al., 2002; ZHAO and BUEHRING, 2007). Also the classification of BLV strains into groups, clusters or genotypes has been proposed over the last decade (MAMOUN et al., 1990; FELMER et al., 2005; CAMARGOS et al., 2007; RODRIGUEZ et al., 2009). Recently, a new Genotype 8 of BLV has been discovered (BALIĆ et al., 2012).

The challenge of this study was to eradicate the disease on a dairy farm located in Eastern Slavonia, territory that was under occupation for eight years. To accomplish this goal we needed to find the best diagnostic tools for detecting all BLV positive animals. Together with that, we made sequence and phylogenetic analysis of the BLV that was circulating on the observed farm.

Materials and methods

Samples. From 1998 to 2008 blood samples were taken from a dairy farm in the North-eastern part of Croatia. The cows were divided into two barns. At the beginning of the test the farm had 500 dairy cows, mostly Holstein-Friesian breed, and a variable number of heifers were brought from other farms as calves. Blood samples for serological examination were collected during the whole observation period of 10 years, but only in 2004, 2008 and 2009 were samples submitted for PCR testing. Samples were also collected from gravid cows. Peripheral blood was obtained from the jugular vein with and without anticoagulant (EDTA 0.2 M v/v). From sera collection, the blood was allowed to clot and was then centrifuged at 1500g for 15 min. In cases when sera and whole blood were not submitted to analysis immediately, they were frozen at -20 °C until used.

AGID test. The AGID test kit (Mevak A. S. Nitra, Nitra, Slovak Republic) was used to detect antibodies to BLV gp51 according to the manufacturer's recommendations, using 75 µL of serum and reagent per well. Results were read after 24, 48 and 72 hrs in a humid chamber at room temperature.

Commercially available ELISAs. Individual sera samples were tested using Svanovir® BLV-gp51-Ab ELISA test kit (Svanova Biotech AB, Uppsala, Sweden). Positive results of the tests of both individual and pooled samples were confirmed by the Bovine Leucosis Serodiagnosis - verification kit (Institute Pourquier, Montpellier, France), according to the manufacturer's instructions.

Bovine genomic DNA extraction and BLV nested PCR. In cases when fresh blood was received, bovine DNA was obtained from peripheral blood mononuclear cells (PBMC). PBMCs from cattle were harvested according to the manufacturer's protocol on Histopaque 1.077 (Sigma-Aldrich, Steinheim, Germany), washed twice with phosphate buffer saline (PBS) pH 7,4 and stored at -20 °C until DNA extraction. When frozen whole blood was used for the DNA extraction, the iPrep PureLink gDNA Blood Kit (Invitrogen, Carlsbad, CA, USA) with iPrep instrument platform (Invitrogen) were used following the manufacturer's instructions.

A 444 bp fragment of the *env* gene was amplified by nested PCR (BEIER et al., 2001) in a total volume of 50 µL, using the JumpStart REDTaq Ready Mix (Sigma-Aldrich, Steinheim, Germany). The sequences of the outer and inner primers were forward 5'-TCT GTG CCA AGT CTC CCA GAT A-3', reverse 5'-AAC AAC AAC CTC TGG GAA GGG-3' and forward 5'- CCC ACA AGG GCG GCG CCG GTT T-3', reverse 5'-GCG AGG CCG GGT CCA GAG CTG G-3', respectively. The first round conditions were: denaturation at 94 °C/2 min followed by 40 amplification cycles at 94 °C/30 s; 57 °C/30 s; and 72 °C/45 s and a final extension step at 72 °C for 5 min. For the second round of PCR, 3 µL of the product were taken from the first amplification and re-amplified with the same conditions as above except that annealing temperature was increased to 68 °C. Ten

μL of PCR products were analyzed by 1 or 2 % agarose gel electrophoresis and stained with ethidium bromide. Those products that were positive in the first round (598 bp) have been submitted for sequencing. Products were purified by QIAquick purification kit (Qiagen, Hilden, Germany). Sequencing was carried out in both directions using the Big Dye Terminator 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) as recommended by the manufacturer. Sequencing analysis was performed on an automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequence obtained from our BLV strain designated M1/ELG-Cro/08 was deposited in GenBank.

Multiple alignment and phylogenetic analysis. The sequence data were initially aligned to known sequences using the basic BLAST search program. Sequence analysis was performed with the MEGA version 4 (TAMURA et al., 2007). Phylogenetic analyses of the 524-bp fragment of BLV *env* gene were conducted using the same program with 1000 replicates for the Neighbour-joining analysis. Names and accession numbers of the BLV strains used in study are shown in Fig. 1.

Results

The first serological tests (AGID) were conducted in 1998, when EBL was first diagnosed on the observed farm. That year, from a total of 488 examined cows 61 were recorded as positive (12.5%). Next, AGID tests were undertaken in 2002, 2003 and 2004, when 37%, 22% and 10% of the animals were positive, respectively. Initial veterinary measures which were carried out, starting in 2002, and led to eradication of the disease, but the farm did not obtain BLV-free status. Those measures included separation of serologically positive animals from others and continued use of the milk of all cows in diary production. In 2004, 8 AGID-serologically positive and 12 negative samples were examined with ELISA and PCR as well. Among 12 AGID negative sera, three were positive with ELISA, and four more with PCR. Two samples that were AGID positive were negative by PCR (Table 1).

Table 1. Comparison between AGID, ELISA and nested PCR results of examination of 20 cow sera and blood samples in the 2004

Cow	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
AGID	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-	+	+	+
ELISA	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+
PCR	-	-	+	-	-	+	+	-	-	+	+	-	+	+	+	-	+	+	+	+

Blood samples from 12 cows were submitted for PCR and all reacted as positive. Three samples were positive even after the first PCR reaction. Those PCR products were sequenced and 524-nucleotide fragment of BLV *env* gp51 encoding gene was analysed.

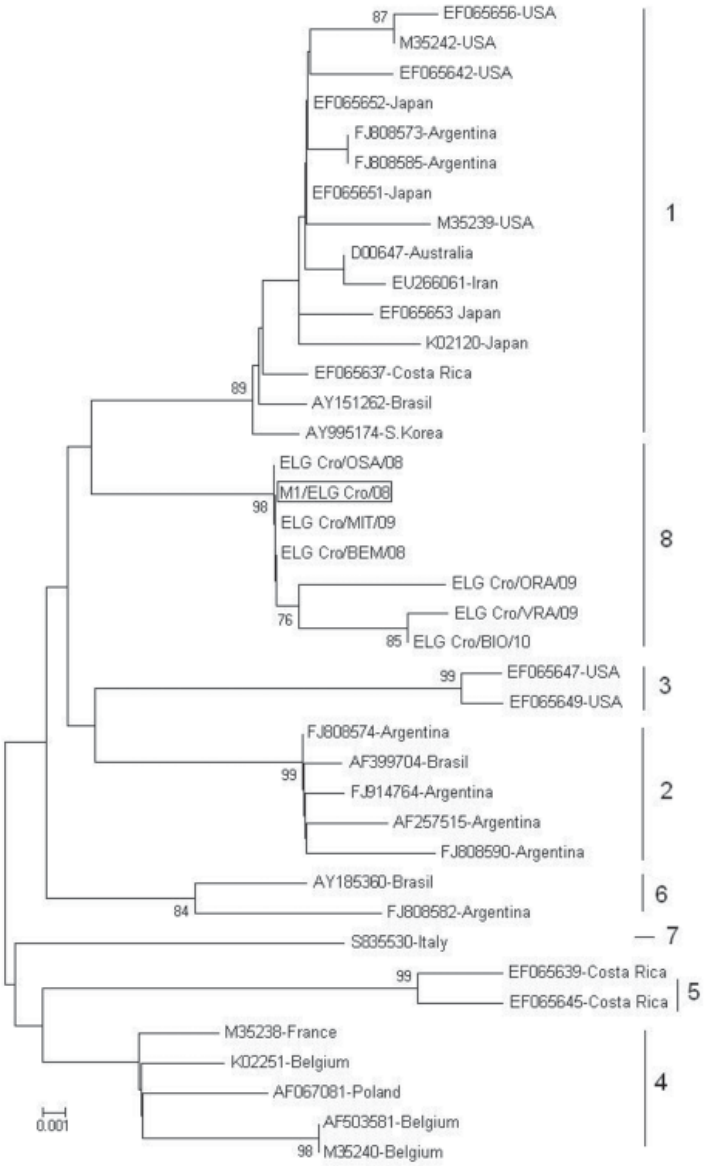

Fig. 1. Neighbour-joining phylogenetic tree of the partial *env* sequences from different geographical locations worldwide. All Croatian isolates are genotype 8 BLVs. Isolate M1/ELG-Cro/08 from the studied dairy farm is given in the box. Genotypes 1-8 are identified with vertical lines.

Compared to each other, 100% identity was found. When aligned with the corresponding nucleotide sequences published for BLV isolates worldwide, the greatest (98,6-100 %) was found with other Croatian BLV sequences (JN990069-JN990074), so it was phylogenetically clustered with those isolates, belonging to the new BLV Genotype 8 (Fig. 1).

In 2009, two more tests were performed aimed at eradicating the disease from the farm. Samples from seven cows were received in spring, and eight in the summer. The first test showed agreement with the results obtained by ELISA and PCR (Table 2.). In the last test, of eight sera, six were positive by ELISA. All eight samples tested positive by PCR (Table 3.).

Table 2. Comparison between ELISA and nested PCR results of examination of 7 cow sera and blood samples in the spring 2009

Cow	1	2	3	4	5	6	7
ELISA	-	+	+	+	+	+	+
PCR	-	+	+	+	+	+	+

Table 3. Comparison between ELISA and nested PCR results of examination of 8 cow sera and blood samples in the summer 2009

Cow	1	2	3	4	5	6	7	8
ELISA	+	+	-	+	+	-	+	+
PCR	+	+	+	+	+	+	+	+

Discussion

The process of BLV eradication is a long term process, which demands extensive effort, especially in larger herds. The eradication process, as well as searching for the optimal diagnostic tool for BLV diagnostics, was difficult on the observed farm. The reason is largely the slow reintegration of Eastern Slavonia into Croatian territory after the war.

In the 1998, when the first serological tests were completed, EBL was confirmed on the farm. At that time, the farm had 500 cows, the majority of which were Holstein Friesian breed. Heifers had been brought from several neighbouring farms considered BLV free.

First, veterinary measures separated serologically positive animals from others in separate facility within the infected farm but use of milk of all the cows in dairy production continued. Further serological tests included all cows from the neighbouring dairy farms

and relocation of positive animals to the same facility on the infected farm from where the cows were taken to the slaughterhouse after pregnancy. Similar approaches were applied and studied by SARGEANT et al. (1997) and ACAITE et al. (2007). Extra measures such as the single use of needles and obstetrical sleeves, and replacement of whole milk feeding with high-quality milk replacer, as described by SPRECHER et al. (1991) and ACAITE et al. (2007) were also carried out. Those measures were also found to be effective, even without the segregation of positive animals. Finally, at the end of 2010 the farm obtained the status "free of BLV". During that whole period, neither farm veterinarians nor veterinary inspectors at the slaughter house recorded any clinical or pathomorphological changes related to EBL.

The BLV eradication program in Croatia has been in effect since 1985. Until 1989, only AGID was used in the diagnosis but from that year on ELISA was used as well. One of the reasons for this is that AGID cannot detect serologically positive pregnant animals, as in our trial in 2004. According to the study of CHOI et al. (2002), AGID test sensitivity was not sufficient to identify correctly all the samples from the analyzed group, failing to detect up to 30% of animals positive for BLV by other methods. Today, veterinary laboratories in Croatia use ELISA tests for monitoring and another ELISA test for verification of BLV positive animals and eradication of EBL.

During the past decade, in several experiments, we have tried to introduce PCR as a diagnostic tool for EBL. In 2004 we performed PCR on the same samples we had tested by ELISA and AGID, and in 2008 and 2009 we performed PCR on the samples which were confirmed positive with ELISA. The test that was undertaken in 2008 showed agreement between the results obtained by ELISA and PCR, but in 2004 and 2009, PCR was able to detect BLV proviral DNA in blood from animals that tested negative in the serological tests. According to JACOBS et al. (1992) the sensitivity of PCR permits detection of bovine leukaemia provirus in 6.8% of serologically negative BLV-exposed cattle. However, the sensitivity of BLV PCR is not 100%. The PCR assay performed with serologically positive animals fails in about 1.4-9.6 % of cases (JACOBS et al., 1992; EAVES et al., 1994). This is in agreement with our results from 2004, when PCR failed to detect three samples that were ELISA positive. Some investigations suggest that the negative results obtained by PCR are attributable to extremely low amounts of provirus genetic material in the lymphocytes of infected animals (MOLLOY et al., 1994), or to infection confined to lymphoid tissues rather than circulating lymphocytes (KLINTEVALL et al., 1994).

Serological tests have been used more extensively to identify BLV infected cattle worldwide due to their rapidity, cost-effectiveness and easy interpretation. However, antibodies may not be produced until 14 weeks after infection. During that period, the animal could be viremic and transmitting the virus to other animals. Nested PCR for detection of BLV gp51 *env* gene is a specific and reliable method except in the cases

described above. It may be used for BLV detection in young calves fed with colostrum from seropositive cows, in tumour cases, to differentiate sporadic from infectious lymphomas, in tumour tissues from suspected cases collected in slaughterhouses, in recent infections, before the development of antibodies, in doubtful reactions or weak positive reactions in ELISA tests, for monitoring bovines in progeny tests before their use in artificial insemination centres, and in bovines used for vaccine production (ANONYMOUS, 2008). The advantage of PCR is that it can be followed by sequence and phylogenetical analysis to see the distribution of various BLV genotypes worldwide, as in our study. Compared to each other, a 100% sequence identity was found among our analysed sequences. For that reason only one sequence was chosen for GenBank submission and further analysis. After multiple alignment with the corresponding nucleotide sequences published for BLV isolates worldwide, the greatest identity (98.6-100%) was found with other Croatian BLV sequences (JN990069-JN990074) also obtained from cows in Eastern Slavonia, belonging to the new BLV genotype 8 (BALIĆ et al., 2012). Four point mutations were found specific for Croatian analysed viruses, but all four were outside the 1st, 2nd, G and T cell epitope, according to the study by ZHAO and BUEHRING (2007). Our phylogenetic analysis clustered the Croatian isolate together with other Croatian BLV sequences belonging to the recently described Genotype 8.

Although Croatia has a long history of BLV infection, this is the first investigation that showed how a long-term and laborious eradication process can be successfully completed. Some countries such as Belgium and Lithuania (KNAPEN et al., 1993; ACAITE et al., 2007) have achieved nearly complete eradication; their approaches together with successful results obtained here should encourage us to achieve eradication on a national level.

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Received: 23 November 2012
Accepted: 17 April 2013

LOJKIĆ, I., D. BALIĆ, N. RUDAN, M. KOVAČIĆ, Ž. ČAČ, M. PERIŠKIĆ, T. BEDEKOVIĆ, B. ROIĆ, I. CIGLAR GROZDANIĆ: Iskorjenjivanje enzootske leukoze goveda na farmi mliječnih krava primjenom različitih dijagnostičkih postupaka. Vet. arhiv 83, 581-591, 2013.

SAŽETAK

Cilj istraživanja bio je dijagnosticirati i molekulski karakterizirati virus enzootske leukoze goveda (VELG) na jednoj hrvatskoj farmi mliječnih krava. U tu svrhu uspoređeni su dijagnostički postupci rabljeni u istraživanju te je napravljena analiza nukleotidnog slijeda VELG i filogenetska analiza. Opisane su i poteškoće u provođenju mjera iskorjenjivanja na pretraživanoj farmi tijekom mirne integracije ovog područja Hrvatske poslije domovinskog rata. Od 1998. do 2008. uzimani su uzorci krvi krava na farmi mliječnih krava smještenoj u sjeveroistočnom dijelu Hrvatske te serološki pretraživani gel difuzijskim precipitacijskim (GDP) i imunoenzimnim testom. U 2002. na VELG bilo je pozitivno 37%, 2003. 22%, a 2004. 10% životinja. Godine 2008. svi pretraženi uzorci krvi bili su pozitivni pretragom lančanom reakcijom polimerazom. Naposljetku, krajem 2010. nakon produženih mjera iskorjenjivanja što su uključivale primjenu lančane reakcije polimerazom istodobno s redovitim pretraživanjem imunoenzimnim testom kako bi se identificirale sve životinje pozitivne na ELG, farma je postala slobodna od ELG. Na osnovi filogenetske analiza odsječka VELG što kodira za gen *env* gp51 izolati su bili svrstani u istu skupinu (VELG genotip 8) s već istraženim hrvatskim izolatima VELG.

Ključne riječi: enzootska leukoza goveda, gen *env*, gp51, imunoenzimni test, lančana reakcija polimerazom, filogenetska analiza