The genotype determination and molecular characterization of bovine leukemia virus in Turkey

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ABSTRACT

Bovine leukemia virus (BLV) infection is widespread worldwide and causes significant economic losses in the livestock industry. In this study, to identify the genotypes and to investigate the molecular characterization of BLV strains, we analyzed 25 BLV strains from cattle housed in six herds located in several Turkish geographical regions. Phylogenetic analysis, based on a partial or full-length sequence of the *env* gp51 gene of BLVs, showed that all Turkish BLVs belonged to genotype 1. The alignment of the deduced amino acid sequences demonstrated fourteen amino acid substitutions in different regions of the *env* gp51. This study updates our knowledge of BLV in Turkey. Further studies are needed to understand the molecular epidemiology and implement a comprehensive BLV control program in Turkey.

Key words: bovine leukemia virus; genotype 1; envelope glycoprotein (gp51); molecular characterization; Turkey

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Introduction

Bovine leukemia virus (BLV) is known as the etiologic agent of enzootic bovine leucosis, which is the most common neoplastic disease of cattle (GAUTAM et al., 2018). Generally, BLV-infected animals remain persistently infected with no outward signs of infection (POLAT et al., 2015). BLV causes significant economic losses in the livestock industry by decreasing the milk yield, reducing reproduction efficiency, increasing diagnostic and veterinary care, reducing carcass value, shortening lifespan, and also affecting international trade (POLAT et al., 2017b; PHIRI et al., 2019).

BLV belongs to the genus *Deltaretrovirus* of the family *Retroviridae,* and its genomic organization possesses structural and enzymatic *gag, pro, pol*, and *env* genes, the regulatory genes *tax* and *rex*, accessory genes *R3* and *G4*, and two identical long terminal repeats (LTR) (POLAT et al., 2017a; GAUTAM et al., 2018). The *env* gene encodes the envelope (Env) glycoprotein which is composed of mature extracellular protein, gp51, and a transmembrane protein, gp30. The *env* gp51 has an essential role in viral infectivity, and is also the primary target of neutralizing antibodies (MAMOUN et al., 1990;

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JOHNSTON and RADKE, 2000). The N-terminal half of BLVgp51 plays an important role in viral infectivity and syncytium formation, and comprises three conformational epitopes, F, G, and H, whereas the C-terminal half contains the linear epitopes A, B, D, and E (BRUCK et al., 1982). Because of the important biological functions of gp51, studies of BLV genotypes for phylogenetic and epidemiological analyses have primarily focused on the *env* gp51 (POLAT et al., 2016; BAZZUCCHI et al., 2019; PHIRI et al., 2019). Globally, at least 11 different BLV genotypes (genotype 1 to genotype 11) have been identified (YU et al., 2019). The literature on the distribution of the genotypes shows an interaction between some geographical regions and genotypes of BLV. Thus, genotypes 2, 5, 6, and 9 were found mostly in South American countries, genotypes 7 and 8 in Russia and Eastern European countries, genotype 10 in Thailand, China, and Myanmar, and genotypes 1, 3, and 4 on almost all continents (FECHNER et al., 1997; MORATORIO et al., 2010; OCHIRKHUU et al., 2016; POLAT et al., 2016; YANG et al., 2016; PANDEY et al., 2017; POLAT et al., 2017a; 2017b). Genotype 11, most recently identified, was detected in China (YU et al., 2019). To date, little is known about the genotypes of BLV in Turkey (ALKAN et al., 2011; DEGIRMENCI, 2011). This study presents the genotypes and the molecular characterization of BLV strains from herds located in several Turkish geographical regions, updates our knowledge of BLV in Turkey, and also contributes to future epidemiological studies.

Materials and methods

History of the study and samples. In this study, 95 blood samples were used from six organized farms, which are closed and have been restocked solely from internal animal sources, located in different regions (Fig. 1, Table 1). Animals over one year of age are kept under the same roof, although newborns are grouped under different roofs for every 3 months of age. However, they do mingle freely during grazing. First, blood samples which had been detected as positive for antibodies against gp51 by a commercial indirect ELISA kit (IDEXX, Europe B.V., Hoofddorp, the Netherlands) were used to determine the presence of BLV by PCR. Then the partial or full-length sequences of the *env* gene of BLVs from the selected samples (n = 25) were analyzed (Table 1). These were selected as samples representing different farms and sampling years.

DNA extraction and PCR amplification of env gene. All blood samples were analyzed for amplification of the partial sequence of the BLV env gene using nested PCR. Total DNA was extracted by using the QIAamp® DNA Mini Kit (QIAGEN, Germany) following the manufacturer's recommended protocol. All extracts were stored at -80 ºC until tested. PCR was performed for detection of BLV using the primers targeting the *env* gene region, as suggested by the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE (World Organization for Animal Health), 2018). For *env* gene amplifications, *env*-1 (5'-TCTGTGCCAAGTCTCCCAGATA-3') and *env*-2 (5'-AACAACAACCTCTGGGAAGGG-3') were used as outer primers; *env*-3 (5'-CCCACAAGGGCGGCGCCGGTTT-3') and *env*-4 (5'-GCGAGGCCGGGTCCAGAGCTGG-3') were used as inner primers, as previously described (FECHNER et al., 1996). Also, in order to detect the full-length BLV *env* gp51 gene (903 bp), PCR was performed using the following primers: forward, 5'-ATGCCYAAAGAACGACGG-3'; and reverse, 5'-CGACGGGACTAGGTCTGACCC-3' (MORATORIO et al., 2010). PCRs were performed using DreamTaq DNA Polymerase (Thermo Scientific, USA) according to the manufacturer's recommendations. The amplification products were analyzed by 1% agarose gel electrophoresis and visualized under UV light. The purified products of the partial and full-length *env* gp51 gene with expected sizes were sequenced in both directions, with the same primers used for amplification.

Cognate sequences of reference BLVs representing different genotypes for gp51 protein-encoding genes were retrieved from GenBank through the BLAST engine. Multiple sequence alignments were prepared by the MUSCLE algorithm, as implemented in Aliview Software (LARSSON, 2014). The phylogenetic tree of a 400 bp fragment of BLV *env* g51 sequence was

Table 1. Distribution of the samples according to dairy farms, years and their accession numbers based on partial/full-length env gp51 gene

| Farm No / City | Sampling Year | The number of samples | | Accession No. (partial/full-length* env gp51) |
		BLV positive	Sequenced	
I / Kirklareli	2012	22	3	MH500043 MH500044* MH500045
II / Balikesir	2012	4	2	MH500040 MH500046
III / Bursa	2012	3	2	MH500041 MH500047
	2013	10	2	MN928514 MN928528*
	2014	5	2	MN928526 MN928525
	2015	5	2	MN928515 MN928516
IV / Ankara	2014	9	1	MN928517
	2015	5	2	MN928518 MN928519
V / Adana	2016	9	1	MN928527
VI / Sanliurfa	2016	23	8	MH500038 MH500039 MH500042* MN928520 MN928521 MN928522 MN928523 MN928524

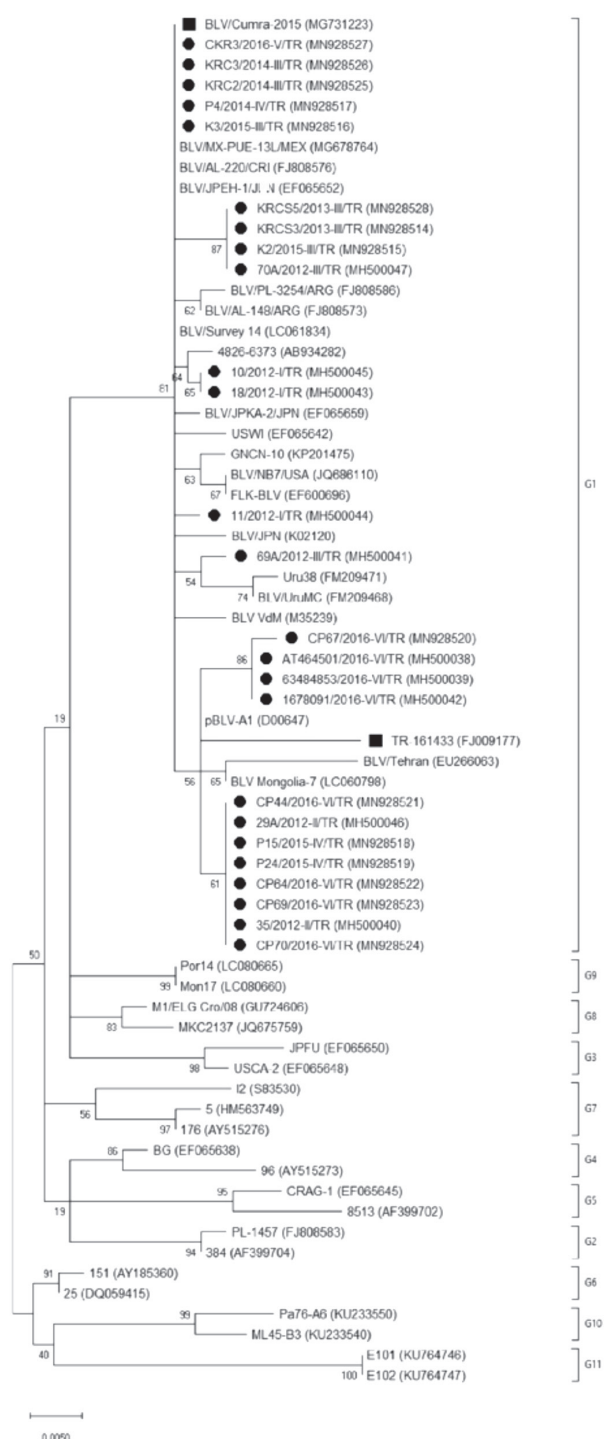Fig. 1. The map showing the cities (with gray colors) where the farms (I to VI) sampled in this study

Fig. 2. Maximum likelihood phylogenetic tree based on the partial nucleotide sequences of *the env* gp51 gene (400 bp). The strains investigated in this study are indicated by black dots (name/year-farm no/ Turkey). The genotype 1 strains previously detected in Turkey are indicated by black squares.

constructed using the Find Best Model in MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms (KUMAR et al., 2018). Thus, Maximum Likelihood methods with the Kimura 2-parameter model, plus gamma distribution and 1000 bootstrap replicates were performed. The nucleotide (nt) and amino acid (aa) identities were calculated using online tools (SIAS, http://imed. med.ucm.es/Tools/sias.html).

Results

The results showed that all samples (n = 95) yielded amplicons of *env* gp51 fragments of the expected size. Of these, 25 selected amplicons were sequenced. Also, it was possible to obtain sequencing data for the full-length gp51 gene region of three samples from different farms. The genome sequences of the BLVs identified in this study were deposited in GenBank under the following accession numbers: MH500038-MH500047 and MN928514-MN928528 (Table 1).

The phylogenetic tree, based on partial *env* gp51 sequences of the Turkish strains and the reference strains representing genotypes 1-11 from different countries that were available from GenBank, showed that all of our strains belonged to genotype 1 (Fig. 2). Our partial *env* gp51 sequence (400 bp) identities calculated by SIAS showed 98.5-100% nt and 98.5-100% aa identity to each other, and 97.7-100% nt and 96.2-100% aa identity to *env* gp51 sequences in genotype 1 from several other countries. In addition, the nearly full-length sequence consensus (874 bp) of *env* gp51 for 3 BLVs (MH500042, MH500044, and MN928528) shared 98.8-99.1% and 98.6-99.6% nt identity to each other and *env* gp51 sequences in genotype 1 from several other countries, respectively. They also shared 98.6-99.3% and 97.9-99.6% aa identity to each other and *env* gp51 sequences in genotype 1 from several other countries, respectively.

The alignment of the nucleotide sequences (partial or full-length *env* gp51) of the 25 Turkish BLV strains with that of the Japanese K02120 demonstrated that a total of 32 mutations were found in our BLV strains.These mutations were G10A, A11G, C75T, G83C, T99C, T172C, A207C, T261C, C270T, C280A, C283A, G286A G363C,
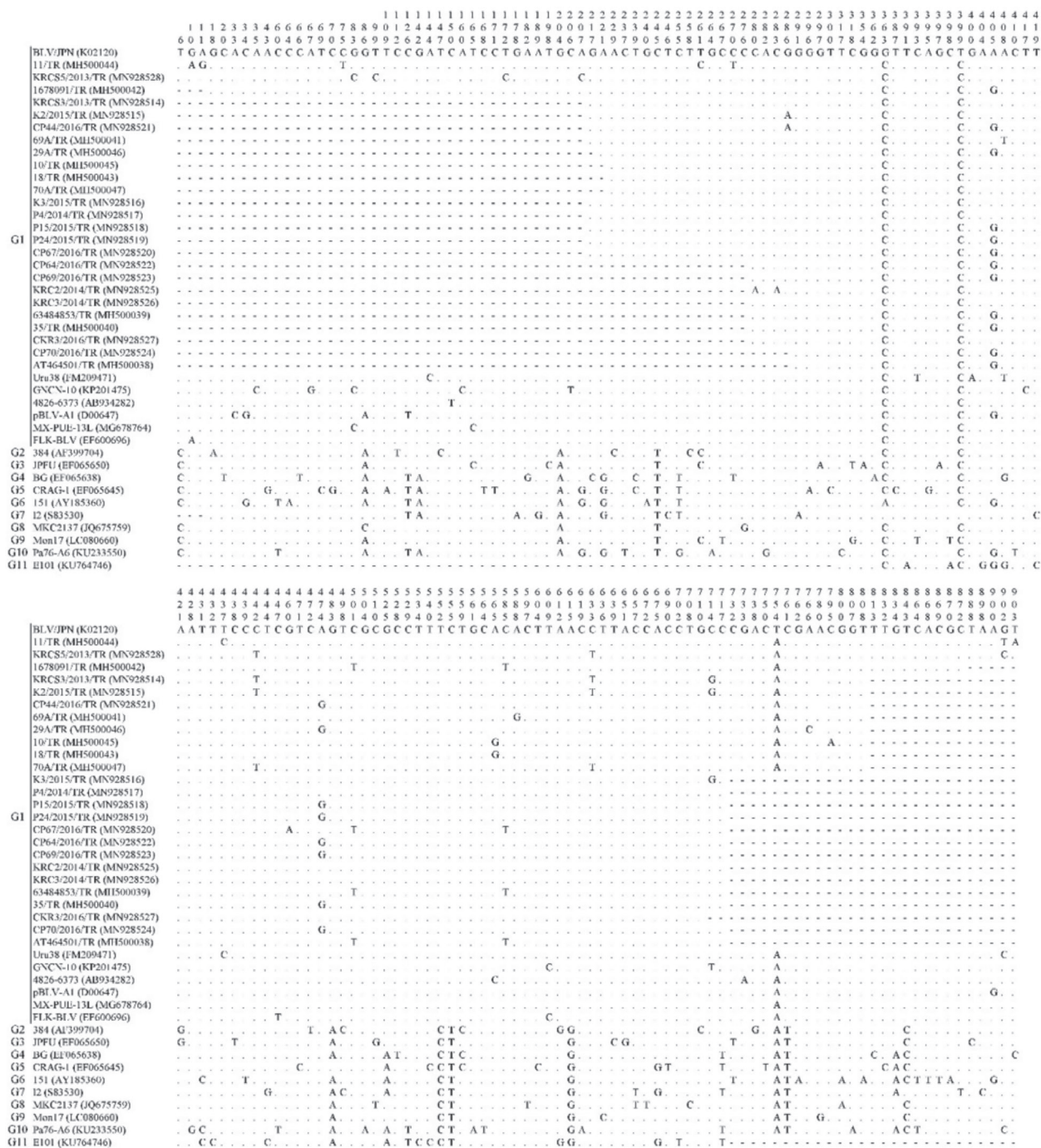
Fig. 3. Alignment of BLV partial and full-length *env* gp51 nucleotide sequences of 25 Turkish BLV strains and reference sequences, representing all 11 BLV genotypes, from GenBank. All strains are shown by name and GenBank accession numbers in the parentheses. The strains investigated in this study are indicated by (*). Dots indicate identity with the Japanese strain (K02120), which was used as a reference in this study, and numbers above the sequences denote the nucleotide numbers of the reference.

Fig. 4. Alignment of partial and full-length deduced amino acid sequences of BLV *env* gp51 of 13 Turkish strains and other reference strains. These Turkish strains have amino acid substitutions compared with the reference, Japanese strain (K02120), and the numbers above the sequences indicate the amino acid residue number of the reference. Dots represent amino acids identical to the reference sequence. BLV genotypes are indicated on the left and all strains are shown by name and GenBank accession numbers in the parentheses. The strains investigated in this study are indicated by (*). The neutralizing domains (ND) and other epitopes are shown at the top of the alignment. The genotype (G1, G4, or G7) is indicated by a black bar at the far left of the figure.

T399C, A405G, A408T, T437C, C442T, G460A, A474G, C501T, A565G, C585T, A588G, C633T, C714G, T754A, A766C, C795A, G902C, G902T and T903A (Fig. 3).

To provide greater insights into the genetic diversity of the Turkish BLV genotype 1 strains, we aligned the deduced amino acid sequences of the 25 *env* gp51 sequences (partial or full-length). The data demonstrated that fourteen different amino acid substitutions were identified among the thirteen strains, whereas there were no differences among the remaining twelve Turkish BLVs (Fig. 4).

Discussion

BLV has been circulating in Turkey for a long time, and many studies have been carried out to determine the prevalence of BLV infection since 1991 (BURGU et al.,1991; AKÇA et al., 1996; UYSAL et al., 1998; BURGU et al., 2005). However, information on its genetic characterization is limited. In this study, we aimed to gain an insight into the genotype(s) and genetic variability of the BLV strains in Turkey. The phylogenetic analysis and also amino acid data clearly showed that our BLVs belonged to genotype 1 (Fig. 2 and Fig. 4). To date, there have been only three studies reporting the molecular characterization of BLVs in Turkey that represent genotypes 1 and 4 (ALKAN et al., 2011; DEGIRMENCI, 2011; DOGAN et al., 2020). Studies from different countries/continents have revealed genotype 1 as the most dominant genotype, whereas genotype 4 is the second most common (OCHIRKHUU et al., 2016; POLAT et al., 2017b). Genotype 1 is distributed across almost all continents, and it is particularly highly prevalent in South and North America, which still have a high prevalence of BLV infection (POLAT et al., 2017b). In addition, genotype 1 is also common in Asian countries such as Japan, Korea, The Philippines, Thailand, and Mongolia (MATSUMURA et al., 2011; LEE et al., 2015; POLAT et al., 2015; LEE et al., 2016; OCHIRKHUU et al., 2016). However, in Europe genotype 1 was only detected in Germany (FECHNER et al., 1997). As stated in previous studies, the widespread distribution of BLV, and also its genotypes, within and between distant geographical locations may be based on livestock

trading (POLAT et al., 2017b). It is not known when BLV entered Turkey. However, BLV infection was first detected in imported, highly productive, pedigree Holstein and Swedish dairy cows on a farm in Turkey (HAKIOGLU, 1962). Subsequent studies also detected high seropositivity rates on this farm and other farms that had a relationship with this farm in terms of animal production (BURGU et al., 1991; BURGU et al., 2005). The higher frequency of BLV genotype 1 in Turkey might correspond to its initial entrance and, then, its spread all over the country through animal movements. On the other hand, the fact that the importation of live animals from countries with BLV infection, with high rates in herds and individual levels, should be hypothesized for subsequent studies. Live animals had been imported from Europe, the US, and also South America (Brazil, Uruguay, etc.) to Turkey for a long time, as reported in the Global Agricultural Information Network Report (Report Number: TR8027 with date 8/31/2018) (https://apps.fas.usda.gov/, https://www.trademap.org/). Different genotypes (genotypes 1, 2, 4, 5, 6, 7, and 9) were circulating in these countries (FECHNER et al., 1997; CAMARGOS et al., 2007; ZHAO and BUEHRING, 2007; MORATORIO et al., 2010). Considering the history of animal importation into Turkey, the detection of BLV genotype 1, which is the most dominant, was not surprising. Also, we believe that the detection of genotype 4 in the Marmara region (DEGIRMENCI, 2011), which is a very important region for dairy cattle production, could be related to live animal imports. The presence of more than one genotype in a country or even in a herd has previously been reported in many studies (OCHIRKHUU et al., 2016; YU et al., 2019). Briefly, additional genotypes may be circulating in Turkey and therefore further studies are needed to substantiate this opinion.

In addition to the detection of genotype(s) circulating in a herd or country, genetic characterization is very important to understand the evolution of the virus. The alignment of our BLVs with that of the Japanese K02120 strain demonstrated some nt substitutions in the *env* gp51 region (Fig. 3). Among the mutations, sixteen were synonymous mutations (C75T, T99C, A207C, T261C, C270T,

G363C, T399C, A405G, A408T, A474G, C501T, C585T, A588G, C633T, C714G, and C795A), the remaining sixteen were non-synonymous mutations (G10A, A11G, G83C, T172C, C280A, C283A, G286A, T437C, C442T, G460A, A565G, T754A, A766C, G902C, G902T, and T903A). Furthermore, twelve of the mutations (A11G, C75T, T172C, C280A, C283A, G286A, G460A, C501T, C585T, A588G, A766C and C795A) were identified as unique mutations. These mutations were traced to the fourteen different amino acid substitutions in thirteen BLV strains. Four of them were semi-conservative (C28S, P94T, H148Y and R301P) and five were conservative substitutions (S58P, Q95K, S189G, S252T and N256H), while the remaining five (E4R, G96R, F146S, G154R, and R301L) were non-conservative substitutions. On the basis of the alignment of the deduced amino acid sequences (Fig. 4), two different amino acid substitutions were identified in the Env leader peptide; E4R and C28S. Comparisons of conformational (F, G, and H) and linear (A, B, D, and E) epitopes revealed that A, B and G were conserved. The H-epitope at residue 58, the F epitope at residue 95, the E epitope at residue 189 and the D epitope at residue 256 differed, and the substitutions S58P, Q95K and N256H were unique. The amino acid substitution was within the conformational epitope H, which was previously found to influence epitope H-specific monoclonal antibody recognition (JOHNSTON et al., 2002). N256H amino acid substitution in the D-epitope was found in only one strain, and its biological importance is unclear. However, amino acid changes in the D epitope were previously expected to affect the fusion and infectivity of BLV (GATOT et al., 2002; LEE et al., 2016). Furthermore, S189G amino acid substitution within the E epitope was observed, similar to that previously reported (ROLA-ŁUSZCZAK et al., 2013; POLAT et al., 2015; OCHIRKHUU et al., 2016; YANG et al., 2019). Of the neutralizing domains, the 1st and 3rd were conserved, while two amino acid substitutions, F146S and H148Y, were located in the 2nd ND, and they were observed in one and three Turkish BLVs, respectively. The substitution F146S has been reported earlier (MORATORIO et al., 2010; ROLA-ŁUSZCZAK et al., 2013), whereas

the substitution H148Y is unique. Although the role of these mutations in affecting infectivity is unknown, on the basis of the predicted tertiary structure of gp51, they are located on the surface of the env gp51 and could affect viral fusion and the infectivity of BLV in vivo (GATOT et al., 2002). The substitutions at residue P94T and G96R were located in the CD4+ T-cell epitope, and they were also unique. Another mutation, G154R, which was found in only one BLV, occurred in the CD8+ T-cell epitope. However, this mutation did not seem to affect the detection capability of the serological test because all our samples were positive by both ELISA and PCR assays. Since the CD8+ T-cell epitope also overlaps with the zinc-binding peptide, which is critical for viral fusion and infectivity in vivo, any amino acid changes within this region may alter BLV infectivity (PLUTA et al., 2017). Lastly, one of the strains had proline at residue 301 (P301), similar to the strains reported from Uruguay genotype 1 strains (MORATORIO et al., 2010) and Thailand genotype 6 strains (LEE et al., 2016), whereas another had leucine at residue 301 (L301), which has not been described previously.

In conclusion, in this study, only one BLV genotype (genotype 1) was identified according to the genetic analysis of partial and/or full-length BLV env gp51. On the basis of our study and the other previous studies (ALKAN et al., 2011; DEGIRMENCI, 2011), it is possible to say that at least two BLV genotypes (genotypes 1 and 4) have been circulating over the last decade in Turkey. Molecular characterization of the env gp51 of these BLV strains showed relatively high nucleotide sequence conservation, however, some mutations in specific regions of the env gene were detected. This study, which presents the genotypes of relatively old BLV strains, will help us to better understand the genetic diversity of BLV strains and the molecular epidemiology of the infection in Turkey and the world from the past to the present. Further studies are needed to determine the genotype(s) circulating, to define immunogenicity and pathogenicity between different genotypes, and to investigate the interaction between the BLV genotypes and animal importation between the countries. They will make it possible to provide supplementary information

for the development of more effective prevention and control measures against this infection in Turkey and also other countries.

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SAŽETAK

Virus goveđe leukemije (BLV) raširen je po cijelom svijetu i uzrokuje znatne ekonomske gubitke u proizvodnji. U istraživanju, s ciljem identifikacije genotipova i molekularne karakterizacije sojeva BLV-a, analizirano je 25 sojeva BLV-a goveda iz 6 stada, uzgajanih u nekoliko turskih zemljopisnih regija. Filogenetska analiza osnovana na djelomičnoj ili punoj sekvenciji gena *env* gp51 BLV-a pokazala je da svi turski virusi goveđe leukemije pripadaju genotipu 1. Poravnanje izvedenih aminokiselinskih sekvencija pokazalo je 14 supstitucija aminokiselina u različitim regijama gena *env* gp51. Ovo istraživanje dopunjuje znanje o BLV-u u Turskoj. Potrebna su daljnja istraživanja kako bi se razumjela molekularna epidemiologija i proveo sveobuhvatan program kontrole BLV u Turskoj.

Ključne riječi: virus goveđe leukemije; genotip 1; glikoprotein ovojnice (gp51); molekularna karakterizacija; Turska