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Genotyping the Isolates of Bovine Leukemia Virus, Circulating In the Stavropol Territory.

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ABSTRACT

The bovine leukemia virus (BLV) circulating on the territory of the Stavropol Territory of Russia is characterized according to the modern classification of subspecies membership. Proviral deoxyribonucleic acid (DNA) of BLV has been separated out of the peripheral blood of animals that had been naturally infected with this virus. Subsequent amplification, sequencing and phylogenetic analysis of a fragment of the *env*-gene with the length of 444 pairs of nucleotides (p.n.) made it possible to classify the studied isolates as genotypes 4 and 6. Besides, we have separated out the BLV isolate located apart from clusters of isolates of all eight known genotypes, which made it possible to classify it as atypical. In course of sequencing a section of the *env* gene of BLV provirus isolates separatedin this area, the presence of 31 point mutation in the studied locus has been detected, with 11 of them being significant: 2 transversions and 9 transitions.

Keywords: Bovine leukemia virus, the Stavropol Territory, polymerase chain reaction, sequencing, phylogenetic analysis, genotype.

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INTRODUCTION

Bovine leukemia virus (BLV) is a causative agent of enzootic leukemia, which is a malignant disease of the lymphatic system. The virus belongs to the *Deltaretrovirus* genus of the *Retroviridae* family, which also includes T-lymphotropic viruses in humans and monkeys – human T-cell leukemia/lymphotropic viruses I and II (HTLV-I and II), simian T-cell leukemia/lymphotropic viruses I and II (STVL-I and II). The main biochemical feature for all members of the *Retroviridae* family is the presence of the reverse transcriptase (RNA-dependent DNA polymerase) [1, 2, 3] in the virions.

Numerous studies have shown that as a result of infecting immunocompetent BLV cells, persistent lymphocytosis develops in 30% of infected animals; in 60-65% - oncologic cells transformation occurs against the background of lymphoid leukocytosis, and the tumor form of leukemia develops. In some cases (up to 10%), the tumor phase is not preceded by leukocytosis (aleukemic tumoral leukemia).

The analysis of the data about viral oncogenesis has shown that retroviruses can induce transformation of a cell with integration of its proviral DNA near inactive cellular oncogenes. Since the proviral DNA contains long terminal repeats of successors (LTR) that have promoters similar to the cellular ones for transcription of the informational riziform, integration of proviral DNA immediately after inactive oncogene leads to expression of this oncogene. Informational riziform translation results in synthesis of the oncogenic product, which is involved in the process of cell transformation.

Particularly interesting is the fact that the lymphoid cells infected with the bovine leukemia virus transmit the viral genome to the offspring during their own reproduction period. The virus may be transmitted from cell to cell without producing viral particles by the mechanism of «cellular kissing».

Bovine leukemia is a disease that is widely spread in the world. As for Russia, over the past 15 years, the level of BLV infection has not virtually changed, and remains within 10.3-14.7%, while the share of leukemia in the structure of infectious diseases in cattle exceeds 50% [4, 5, 6].

The BLV genome, like other retroviruses, is presented by the 70S RNA, which consists of two 38S subunits. The DNA provirus of BLV consists of 8714 pairs of nucleotides; its complete nucleotide sequence has been decoded. The genetic structure of BLV is as follows: *s-LTR-gag -pol-env-pXbl-LTR-3*. LTR consists of 530 base pairs. The donor splice site is located in the R-locus. LTR is followed by a leader sequence of 97 pairs. The first open reading frame is the *gag* gene (nucleotides 628-1806). This gene encodes translation of the group-specific protein, which is the precursor of structural proteins, R24 in particular. 500 pairs after the end *gag* triplet, the second reading frame starts for *pol,* which encodes 852 amino acids (positions 2317-4875). The *pol* gene encodes polypeptides with revertase (RNA-dependent DNA-polymerase) activity. It is possible that the same gene encodes endonuclease. The third frame is the *env* gene (positions 4821-6368), which encodes 515 amino acids that constitute the gp51 surface glycoprotein and the gp30 transmembrane protein that are directly involved in the infecting the cell and cause strong immune response in infected animals [7,8].

Over the last two decades, the approaches to taxonomic classification of the bovine leukemia virus, which are built exclusively on the molecular-genetic principles of identifying the pathogen, have undergone significant changes, ranging from various classification strategies based on interpretation of generated PCR-RFLP profiles [9, 10, 11] to modern assessment of BLV genotypic diversity based on phylogenetic analysis of the *env* gene locus of certain viral pathogen [12, 13, 14]. Herewith, until the end of year 2010, the BLV strains isolated around the world was classified into seven different genotypes. Today, according to the classification proposed by M. Rola-Luszczak (2013), eight evolutionary genotypes of the leukemia virus are classified.

In this paper, we make the first report about the identification of the BLV circulating in the Stavropol Territory (Russian Federation). To determine the genotype of the virus of this disease, we performed sequencing and subsequent phylogenetic analysis of the BLV isolates that contain the corresponding fragment of the *env* gene.

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METHODS

As clinical material, we used samples of whole blood from 29 heads of the black-motley cattle of the Holsteined and the Red steppe breeds in the age over four years from 4 disturbed by leukemia farms in the Stavropol Territory. The genomic DNA of the cattle was extracted by the nucleo-sorption method with the use of commercial sets of reagents “DNA-Sorb-B” (Federal Budget Institution of Science at the Central R&D Institute of Epidemiology of Rospotrebnadzor, Russia) according to the manufacturer's instructions.

The performance of the polymerase chain reaction (PCR) of the BLV gene *env*. The fragment of the BLV proviral *env* gene was amplified by the method of PCR in two rounds (“nested” PCR) using the *ENV*1-*ENV*4 primers:

*ENV*1 (5032-5053) - 5´-TCTGTGCCAAGTCTCCCAGATA-3´;
*ENV*2 (5629-5608) - 5´-AACAACAACCTCTGGGAAGGGT-3´;
*ENV*3 (5099-5120) - 5´-CCCACAAGGGCGGCGCCGGTTT-3´;
*ENV*4 (5542-5521) - 5´-GCGAG GCCGGGTCCAGAG CTGG-3´.

The primers were synthesized by the phosphoramidite method at the set of equipment of LLC “Biosset” (Russia) according to the manufacturer's instructions. The primers were purified by the method of reversed-phase high-performance liquid chromatography (RP-HPLC).

PCR was performed 50 µl of the reaction mixture. The reaction mixture for PCR with a set of *ENV*1 – *ENV*4 primers contained: 20.0 µl of PCR-mix-2 red (2.5 x PCR-buffer, cresol red, 5.5 mM of MgCl2), 4.8 µl of 10× solution of dNTP-mix deoxynucleotides (“InterLabService”, Russia), 0.2 µl of each primer at the concentration of 100 PM/µl (*ENV*1 and *ENV*2 of the first round, *ENV*3 and *ENV*4 for the second round), 4,8 µl of RNA-eluent, and 20 µl of BLV genomic DNA.

The first round of “nested” PCR with external *ENV*1-*ENV*2 primers resulted in amplification of the product by the length of 598 p.n. After the second round with the *ENV*3-*ENV*4 internal primers, a fragment with the length of 444 p.n. was synthesized. PCR was performed with the following temperature and time parameters: initial incubation at 94°C for 2 min; denaturation at 95°C at 30 sec; annealing at 62°C for the pair of primers *ENV*1-*ENV*2, or at 68°C for the pair of primers *ENV*3-*ENV*4 for 30 sec; synthesis at 72°C for 60 sec; the number of cycles was 50. For the 1st and the last 5 cycles of the synthesis, the duration was increased up to 75 sec;and the duration of the final synthesis was 4 min.

After the 1st round of amplification with external primers, 3 µl of the reaction mixture was taken and used as the matrix for the 2nd round of PCR with the *ENV*3-*ENV*4 internal primers, with the same temperature and time parameters. In order to visualize amplicons, the amount of 20 µl of the PCR product was separated by means of electrophoresis in 2% agarose gel, followed by dyeing with ethidium bromide.

DNA sequencing. The sequence of PCR products was determined with the use of the BigDye Terminator v3.1 Cycle Sequencing Kit (“Applied Biosystems”, USA) that contained *ENV*3 and *ENV*4 primers. The reaction of cyclic sequencing was performed in an automated thermocycler with the heated lid function in accordance with the thermal cycling profile: initial incubation at 95°C for 3 min; denaturation at 95°C for 15 sec; annealing at 50°C for 5 sec; elongation at 60°C for 4 min; the number of cycles was 25.

The nucleotide sequences of the sequencing reaction products were analyzed by means of automatic capillary electrophoresis with the use of an ABI 3500 Genetic Analyzer (“Applied Biosystems”, USA).

The sequencing data was processed in Sequencing Analysis Software v 5.4 (“Applied Biosystems”, USA). The sequences were compared with the use of FinchTV 1.4.0 software (“Geospiza”, USA), Chromas 2.3.3, and the BLAST algorithm (NCBI). Each studied fragment was analyzed separately using the sequences of the reference strains of leukemia pathogen from the Genbank database.

The phylogenetic tree was built based on the obtained nucleotide sequences with the use of the CLC Sequence Viewer software package using the "Neighbor – Joining" grouping algorithm.

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MAIN PART

According to the results of monitoring the epizootic situation with bovine leukemia, in 2014, the level of livestock infection at livestock farms of the Stavropol Territory averaged 15% (based on serologic testing (immunodiffusion test, EIA) performed at 15 farms in 8 districts of the Stavropol Territory).

For the purpose of determining the subspecies membership of the BLV circulating in this territory, we performed sequencing of the sequences of highly conservative locus fragment of the *env*-gene from the Stavropol isolates with the length of 444 p.n. The reference was the nucleotide sequence of isolate HQ902258.1 from Belarus that was taken from the Genbank international database, which is the most similar to the structure of the studied locus (similarity 99%).

In the research, we detected 31 nucleotide substitutions in the analyzed locus, which in 11 cases resulted in missense mutations, of which there were 2 transversions and 9 transitions (Table 1).

Table 1: Characteristic of oligonucleotide polymorphisms identified in the locus of the *env* gene of the BLV provirus circulating in the Stavropol Territory

No.	Position	Kind of mutation	Frequency of occurrence, %	Amino-acid substitution	Type of mutation
1	2	3	4	5	6
1	30	G→ A	6.90	R→K	transition
2	84	A→G	6.90	H→R	transition
3	104	A→G	100.00	N →D	transition
4	143	A→G	6.90	N →D	transition
5	153	T →C	10.34	I →T	transition
6	167	C→G	6.90	L→V	transversion
7	170	A→C	6.90	K→Q	transversion
8	171	A→G	27.59	K→R	transition
9	278	A →G	3.45	Q→R	transition
10	338	G →A	3.45	A→T	transition
11	342	G →A	3.45	R→Q	transition

Analysis of the obtained data made it possible to establish a number of features that, in our opinion, are interesting. So, for the nucleotide sequences of the *env*-gene of all isolates from the Stavropol Territory, mutation A→G accompanied by appropriate amino acid substitution (N→D) is characteristic for position 104.

Most frequently, among studied isolates (65.52% of cases) transitions occur at position 61 with substitution G→A, and in position 401 with substitution C→T, which do not result in changing the amino acid sequence. In 27.59% of cases (farms of the Kochubeevsky district of the Stavropol Territory), transition A→G was detected at position 171, resulting in substitution of lysine for arginine. In the nucleotide sequences of three isolates from the Novoaleksandrovsky region, the presence of missense mutation T→C was detected at position 153 with substitution of isoleucine for threonine in the corresponding amino acid sequence.

At position 167 in the nucleotide sequences of isolates from the Kochubeevsky region of Stavropol Territory, transversion C→G was detected with amino-acid substitution of leucine for valine. In 6.90% of isolates (the Trunovsky region), transversion A→C was detected, which led to lysine substitution for glutamine. In positions 84, 143 and 278, missense mutations with transition A→G were detected, which resulted in substitution of histidine for arginine, asparagine for aspartic acid and glutamine for arginine, respectively. Transition G→A at position 30 was the reason for substitution of arginine for lysine, at position 338 - alanine for threonine and at position 342 – arginine for glutamine.

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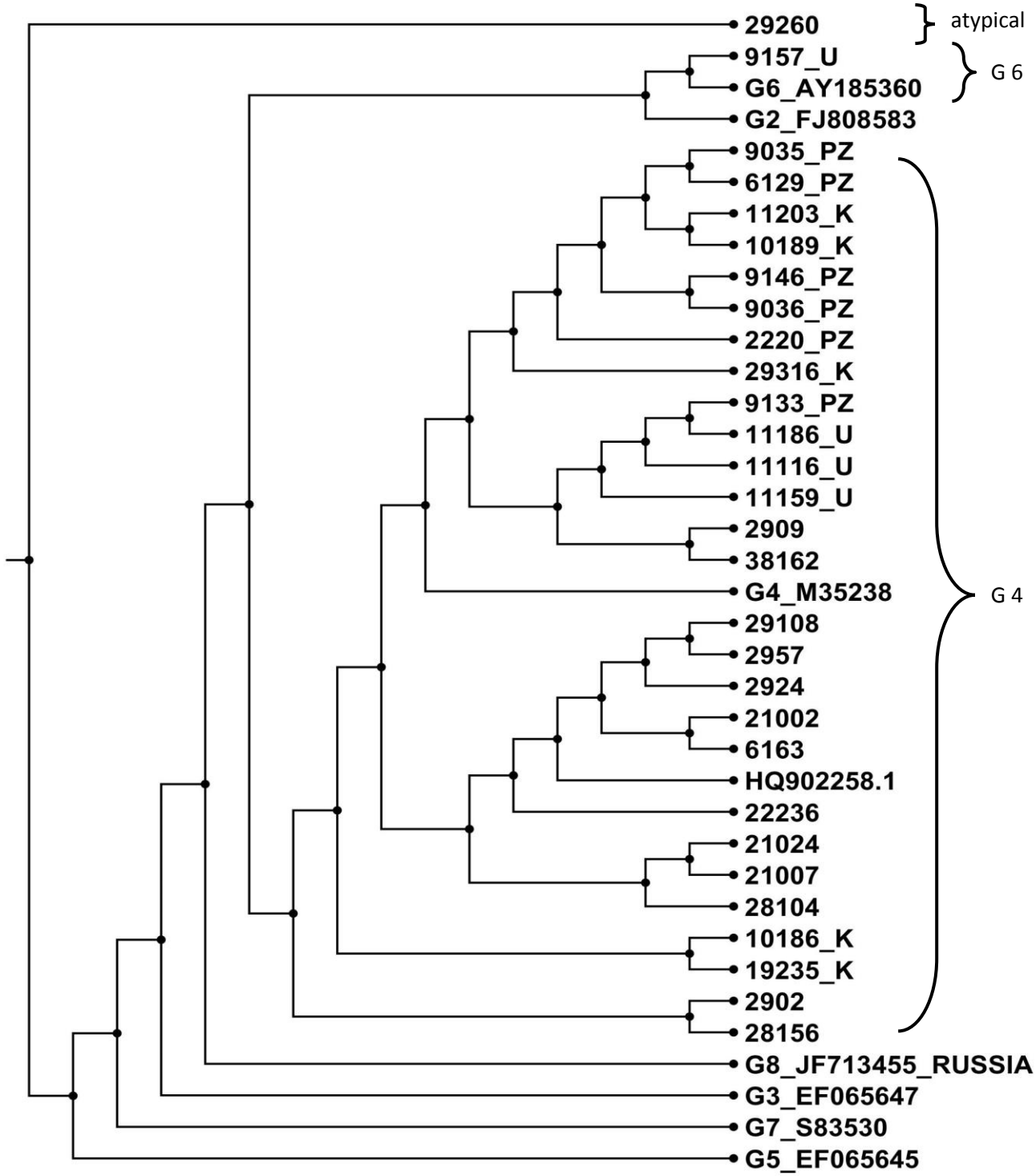

Figure 1: Cluster analysis of 37 strains that represent the reference (n=8) strains, and the studied isolates (n=29) based on the results of sequencing the *env* locus of BLV.

In addition, there are synonymous transitions T→C in 11 cases (37.93%), C→T – in 6 cases (20.69%), A→G – in 5 cases (17.24%) and G→A – in 4 cases (13.79%).

Comparative analysis of the nucleotide sequence of the *env* DNA locus of one isolate taken at a farm in the Trunovsky region made it possible to identify five major specific deletions uncharacteristic for other samples and reference strains, which was the basis for classifying the specified isolate as atypical.

Building a phylogenetic tree based on sequences of a fragment of the *env*-gene isolated in the Stavropol Territory has made it possible to classify these BLV isolates in accordance with the current international classification [15].

The structure of the dendrogram makes it possible to clearly distinguish two branches of BLV isolates circulating in the Stavropol Territory (Fig. 1).One branch is formed by 96.55% of the studied samples, 27 of which belong to a large group that includes a strain from France (M) and the reference strain from Belarus (HQ902258.1), which represent genotype 4 (the degree of relationship is 98 to 99%). The structure of the branch may be divided into several groups and their constituent separate cladesrepresented mainly by isolates from specific cattle-breeding farms. So, one group includes isolates from farms of the Kochubeevsky region (9035_PZ, 6129_PZ, 11203_K, 10189_K, 9146_PZ, 9036_PZ and 29316_K), and the other group includes samples from the Trunovsky region (29108, 2957, 2924, 21002, 6163, 22236, 21024, 21007, 28104).

In addition, one isolate (9157_U) from the farm in the Novoaleksandrovsky region is a separate clade with isolate AY185360 from Brazil, which may signify its genetic similarity to a representative of genotype 6.

All the described 28 isolates from various regions of the Stavropol Territoryconsiderably differ from the isolates classified as genotype 8 from Russia (JF713455), genotype 3 from the USA (EF065647), genotype 7 from Italy (S83530) and genotype 5 from Costa Rica (EF065645), which form individual branches in the dendrogram.

Atypical isolate (29260) from the farm in the Trunovsky region formed a separate cluster on the dendrogram that is isolated from the previously considered isolates and reference samples, which is a consequence of the unique primary structure of the studied DNA locus, where at least 65% of the nucleotide residues had been deleted.

CONCLUSION

Thus, the results of the study showed that the BLV circulating in the Stavropol Territory of Russia is evolutionary close to the cluster of isolates classified as genotypes 4 and 6, which corresponds to the data obtained by several scientists.

However, high mutational variability of the analyzed isolates of the BLV provirus results in the necessity of further research, identifying possible impact of private mutations on the virulent properties of the virus, animals' susceptibility to a particular genotype of the leukemia virus, and developing resistance to this disease.

Currently work is underway for populating the nucleotide sequences of a fragment of the proviral *env* gene in the BLV isolates circulating in the Stavropol Territory into the Genbank international database (NCBI). At the moment, ten nucleotide sequences have been populated with registration numbers KP308390, KR007590 to KR007598.

Conclusions

Based on the obtained data, we can make the following conclusions:

- In course of sequencing a section of the *env* gene of BLV provirus isolates separated in this area, the presence of 31 point mutation in the studied locus has been detected, with 11 of them being significant: 2 transversions and 9 transitions.
- In the nucleotide sequences of this gene in all isolates from the Stavropol Territory, mutation A→G accompanied by appropriate amino acid substitution (N→D) was detected at position 104.
- Based on the phylogenetic analysis, the studied BLV isolates have been classified as genotypes 4 and 6 in accordance with the international classification.
- The isolate from the farm in the Trunovsky area cannot be typed due to altered genetic structure, the sample is classified as atypical.

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