

Immuno-biological evaluation of individual genetic variants of bovine leukemia virus in the conditions of the Ural region

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Abstract— Leukemia in animals is a serious disease of the cancer nature – the hemoblastosis, the etiologic factor of which is the bovine leukemia virus belonging to the family Retroviridae, the genus Deltaretrovirus. The causative agent is widely spread all over the world, including the Russian Federation. The main symptom of the disease is the malignant proliferation of cells of the hematopoietic organs with a violation of their maturation. Developing quantitative and qualitative disorders of the hematopoietic system are noted in the diseased organism. This paper presents data on the effects of leukemia virus on the process of hematopoiesis in cattle in the conditions of the Ural region. Infected animals of the Tyumen region were identified by serological studies (RID, ELISA) and molecular genetic studies (PCR) of isolates of bovine leukemia virus. The RFLP method established the predominant presence of the “Belgian genotype” in the samples. The env (glycoprotein envelope) gene of leukemia pathogen was sequenced. The immuno-hematological studies of cattle, infected with leukemia virus in the conditions of the Ural region, were conducted. The obtained data showed a significant increase in the number of leukocytes, lymphocytes in the blood of leukemic animals, a sharp decrease in the number of eosinophils, monocytes, CIC (circulating immune complexes), phagocytosis, T and B lymphocytes.

Keywords— *leukemia virus, cattle, immunological evaluation, genetic classification, Ural region, leukocytes, lymphocytes, phagocytosis, CIC.*

I. INTRODUCTION

BLV is a cancerous lymphoproliferative disease of cattle, and widely spread all over the world, including the Russian Federation. The pathogen genome consists of regulatory genes (gag, ltr, pol, env, tax, rex, R3 and G4), encoding structural proteins (gag-gene), envelope glycoprotein (env-gene), reverse transcriptase (pol-gene) and others. The env gene encodes the surface glycoprotein gp51 (SU) and the transmembrane protein gp30 (TM) of the virus capsid [11].

These proteins (mainly the gp 51 (SU)) induce the expression of specific immunoglobulins in infected animals that exhibit a pronounced neutralizing activity to the structural epitopes (F, G, H) of the antigen [6].

According to numerous authors, genetic pathogen is characterized by genetic variability and heterogeneous geographical distribution of individual genetic variants; genetic polymorphism is described in some detail and the phylogenetic classification of the env gene segment is made possible, forming up to 10 different genetic groups from classified BLV isolates [2, 4, 9].

It was established that mutations were characterized by some differences in the amino acid sequences of the gene region. Analysis of the amino acid sequences of isolated strains revealed that the main changes were localized in the C-part of the CD4 + epitope, the zinc binding peptide region, CD8 + T cell epitope and overlapping linear epitope E., and the greatest number of changes were noted in G4 (“Belgian type”) [12].

It is noted that during the infection, the majority of BLV infected animals remain clinically healthy, however, one third of the infected animals develop resistant lymphocytosis as a result of the polyclonal proliferation of B lymphocytes, mainly CD5 + cells, and only 0.1–10% of animals develop lymphoid tumors [10]. It has also been shown that some genotypes may influence serological diagnosis. Thus, many authors reported a link between some variants of BLV and the impossibility of detecting antibodies in infected cattle. [6] It is assumed that the identified point mutations in the pathogen genome indicate adaptive features that cause an increase in the antigenic load on a susceptible organism, by increasing the immunogenic properties of the virus. This thesis was to be established by studying the molecular-genetic properties of the pathogen and the immunological characteristics of animals in the Ural region.

The study was carried out in the laboratory of leukemia of the Department for Monitoring and Prediction of Infectious Diseases, the Laboratory of Immunology and Patho-biochemistry of the Department of Ecology and Non-contagious Pathology, Ural Federal Agricultural Scientific Research Center of Ural Department of Russian Academy of Sciences.

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II. METHODS

Serological (ELISA, RID) and molecular genetic research methods were used for the diagnostic screening.

Studies by ELISA were performed using an IDEXX Leukosis Serum Screening test, produced by IDEXX Montpellier SAS, France, for the detection of antibodies to bovine leukemia virus. We used a diagnostic kit, produced by the Kursk biofactory "BIOK", for the RID diagnostics.

For the isolation of DNA from the blood of cattle, we used a set of reagents "Diatom DNA Prep 200", produced by "IsoGen" (Moscow). Laboratory studies were performed by the method of the Nested PCR. The env gene fragment was amplified using the following primers: env 5032 tct-gtg-cca-agt-ctc-cca-gat-a, env 5608 aac-aac-aac-ctc-tgg-gaa-ggg-t, env 5099 ccc-aca -agg-gcg-gcg-ccg-gtt-t, env 5521 gcg-agg-ccg-ggt-cca-gag-ctg-g [1]. For carrying out the reaction, a set of BioMaster HS-Tag PCR, produced by "Biolabmix" (Novosibirsk), was used. PCR was performed in a 50 µl volume of the reaction mixture per sample (25 µl BioMaster set HS-Tag PCP (2^x), 1.5 µl of each primer (10 µM), 1 µl MgCl₂ (50 µM), 500 ng of genomic DNA, diluted bidistilled water.

Amplification was performed using an Applied Biosystems 2720 thermal cycler (Singapore) with the following cycle parameters: 2 minutes at 94°C (1 cycle), 30 seconds at 95°C, 30 seconds at 62°C (external primers) or 30 seconds at 70°C (internal primers), 60 seconds at 72°C (40 cycles), 4 minutes at 72°C. Accounting for the reaction was carried out by horizontal electrophoresis using a 1.5% agarose gel with the addition of ethidium bromide as an intercalating dye for the DNA. The equipment used in the work was a Mini-Sub Cell GT mini camera manufactured by Bio-Rad (USA), a Bio-Rad CHEMIDOC XRS + camera (USA), which visualized under the ultraviolet radiation. To determine the size of the amplicons, after carrying out PCR with primers, we used the "Step100" marker of Biolabmix (Novosibirsk). As a control, DNA isolated from cell culture FLK-BLV was used.

At the first step of staging Nested PCR, samples were counted as positive, containing amplicons, whose electrophoretic mobility corresponded to a fragment length of 600 bp. In the second step of Nested PCR, positive amplicons corresponded to a length of 444 bp. The DNA concentration was measured on the MaxLife H100 Mod.2 kit of "MVM Diagnostics" (Barnaul).

For the genotyping of the env region (444bp-gp51 region), a polymorphism reaction (RFLP) was used; BamHI, BclI, PvuII (Thermo Fisher Scientific, USA) were used as restriction enzymes.

Incubation was carried out using an Applied Biosystems 2720 thermal cycler (Singapore) with the following cycle parameters: BamHI, PvuII - 37°C for 2 hours; BclI - 55°C for 2 hours. PCR was performed in a reaction volume of 20 µl per sample (5 µl of PCR product, 1 µl of enzyme, 2 µl of buffer, 12 µl of bidistilled water). The results of the carried out restriction were taken into account by electrophoresis in accordance with the tabular data (Tab. 1).

TABLE I. DISTRIBUTION OF RESTRICTION SITES BY THE GENOTYPE

BLV Type	BamHI	PvuII	BclI
japanese	316, 128	444	219, 121, 104
australian	316, 128	444	225, 219
belgian	444	280, 164	225, 219

For a comparative analysis, the "GenPak DNA PCR test for DNA amplification of Bovine leukemia virus", produced by "IzoGen" (Moscow), was used. The work was carried out in accordance with the manufacturer's instructions for the use of test systems.

Hematological parameters (determination of the absolute number of leukocytes and lymphocytes in the blood) were determined on an Abacus Junior Vet automatic hematology analyzer (Austria), using standard reagent kits from Diatron (Hungary). The leukocyte formula was counted in blood smears stained according to Romanovsky – Giemsa method. Definition of indicators of cellular and humoral immunity: the content of T-lymphocytes (E-ROL) - in the reaction of spontaneous rosetting with sheep erythrocytes (MA Bazhin, 1989; R.V. Petrov, 1992), B-lymphocytes (M-ROL) - by the method of spontaneous rosetting with mouse erythrocytes (D.K. Novikov, V.I. Novikova, 1996), the ratio of T- and B-lymphocytes was calculated (T / B index). Phagocytic activity of neutrophils was studied by the method of opson-phagocytic reaction (P.N. Smirnov, 1989), with use of latex. Accounting for the reaction was performed on a Micros MCX 100 binocular microscope (Austria).

The level of circulating immune complexes in the blood serum was determined by PEG-precipitation with polyethylene glycol using the "CIC-HEMA" kit on a UV-1800 spectrophotometer, manufactured by SHIMADZU (Japan).

The obtained quantitative indicators are processed mathematically on a Pentium PC using the Microsoft Excel statistical package.

III. RESEARCH RESULTS

We have selected a group of cows that was imported from the Netherlands to the Tyumen region in 2014, 3-5 years of age, 100% of the Holstein breed, in the amount of 24 units, responding positively to leukemia in the RID. Bearing in mind the well-being of the countries of the European Union, the cattle were infected from a local herd, as was shown by the results of DNA sequencing. Animals were further studied by ELISA and PCR methods.

In all 24 samples, a specific DNA region of leukemia virus was obtained, according to the Nested PCR, which was 100% consistent with serological identification (ELISA) methods.

It was established by the RFLP method, that the amplicons of 23 isolates were assigned to the "Belgian genotype" with the following lengths of restriction sites: BamHI- 444 bp; PvuII - 280, 164 bp; BclI - 225, 219 bp. Conformity with the "Australian genotype" was determined in the same DNA fragment of the leukemia virus (№19). During the polymorphism reaction, the length of the restriction sites corresponded to the following parameters: BamHI - 316,128 bp; PvuII - 444 bp; BclI - 225.219 bp. (Fig. 1).

TABLE II. SUMMARY TABLE OF THE RESULTS OF MOLECULAR-GENETIC (PCR, NESTED-PCR, RFLP) AND SEROLOGICAL (AGID, ELISA) STUDIES OF CATTLE IN THE TYUMEN REGION

№	AGID	ELISA	PCR	Nested PCR	RFLP
1	+	+	+	+	belgian type
2	+	+	+	+	belgian type
3	+	+	+	+	belgian type
4	+	+	+	+	belgian type
5	+	+	+	+	belgian type
6	+	+	+	+	belgian type
7	+	+	+	+	belgian type
8	+	+	+	+	belgian type
9	+	+	+	+	belgian type
10	+	+	+	+	belgian type
11	+	+	+	+	belgian type
12	+	+	+	+	belgian type
13	+	+	+	+	belgian type
14	+	+	+	+	belgian type
15	+	+	+	+	belgian type
16	+	+	+	+	belgian type
17	+	+	+	+	belgian type
18	+	+	+	+	belgian type
19	+	+	+	+	australian type
20	+	+	+	+	belgian type
21	+	+	+	+	belgian type
22	+	+	+	+	belgian type
23	+	+	+	+	belgian type
24	+	+	+	+	belgian type

We isolated the causative agent of leukemia, given its genetic characterization by the method of polymorphism. It was established that on the territory of the Tyumen region the antigenic landscape in the dominant value is represented by the “Belgian genotype” (23 isolates) and only in one case of the “Australian genotype”. Detailed phylogenetic characterization is to be established by DNA sequencing of the env gene fragment of the leukemia virus.

In parallel with molecular genetic studies of leukemia virus isolates, we also carried out an immunological examination of the infected cattle.

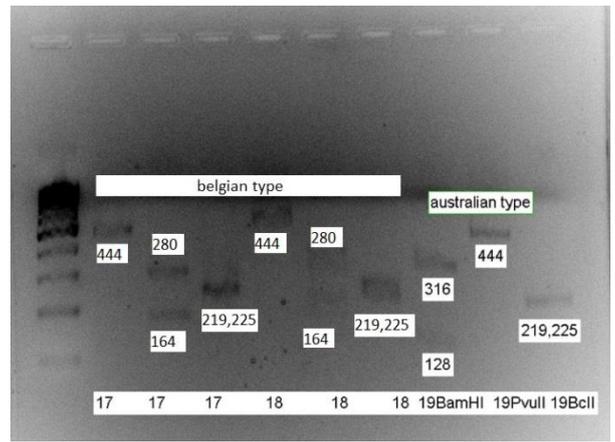


Fig. 1. Electrophoregram of the distribution of restriction sites in the RFLP reaction: M - marker M100 step

Hematological examination of cows showed no changes in the obtained indicators of red blood cells. The hemoglobin content and the hematocrit index, which shows what part of a unit of blood volume is occupied by the volume of red blood cells, were not found in all the studied cows within the physiological norm; blood clots or thinning were not established. In 16.6% of cows, a slight (by 7.6%) increase in the number of erythrocytes was observed.

The most pronounced changes in the morphological image were obtained in terms of white blood cells. An initial (aleukemic) stage of the leukemic process was established in 25% of the studied cows, a moderate increase in the number of leukocytes was noted from $15.2 \cdot 10^9/l$ to $19.8 \cdot 10^9/l$. In 20.8% of cows, the number of leukocytes in the blood increases (subleukemic leukemia) and is already from $25.8 \cdot 10^9/l$ to $33.6 \cdot 10^9/l$; and in 8.3%, the increase in the number of leukocytes reaches the leukemic level and corresponds to $43.9 \cdot 10^9/l - 47.7 \cdot 10^9/l$ [7, 13] (Fig.2).

The transition from aleukemic to leukemic stage of disease is accompanied by an increase in the content of absolute and relative count of lymphocytes in the blood [5, 8].

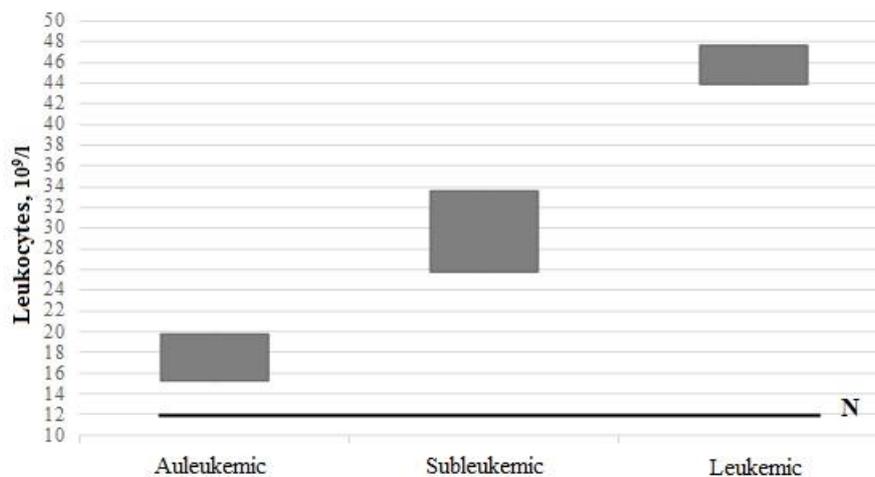


Fig. 2. The number of leukocytes at different stages of the leukemic process

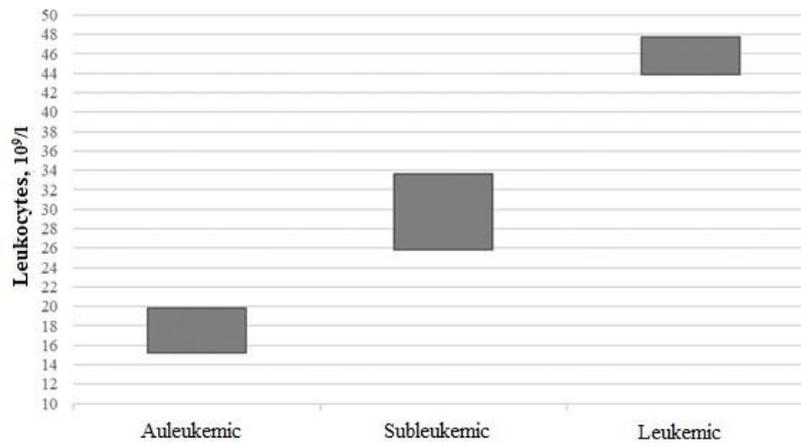


Fig. 3. Absolute lymphocyte count at various stages of the leukemic process

In cows of the first group, the absolute count of lymphocytes is 9.4-11.7 *10⁹/l; the second group – 13.14-29.3 *10⁹/l; the third group – 43.9-47.7 *10⁹/l, which is 3.21-3.54 times higher; and the content of the relative count of lymphocytes increased by 9.7-14.8%, compared with the animals of the initial stage of leukemia process (Fig.3).

Leukocyte formula is an integral indicator of the balance of all homeostatic systems of the body. The cause of leukocyte rearrangements is the general mobilization of the protective mechanisms of the body.

In all the studied cows, a sharp increase in the number of immature stab neutrophils was found, by a factor of 3-5 (from 9% to 25%, with a physiological rate of 2-5%). Significant changes in the size of lymphocytes were marked – 3.3 times in one direction or another. The cell contours had an abnormal structure of the nucleus, increased basophilicity in the cytoplasm, with pronounced azurophilic granularity, the amount of chromatin was increased, and the structure of the chromatin core was soft-mesh, with the presence of from 3 to 5 nuclei. An increase in leukocytes and neutrophils indicates an adequate immune response, young forms, which include myeloblasts, metamyelocytes and band-aided neutrophils, begin to rapidly release from the bone marrow, the

inflammatory process that has begun in the body, and the development of a malignant disease [5].

Basophils protect the body from the effects of infectious agents, they are involved in allergic processes, thereby ensuring the movement of other white cells to the site of inflammation. Their decrease in 62.5% of cows confirms the presence of an infectious disease in the studied animals.

Eosinophils and monocytes are involved in phagocytosis, it allows the body to naturally deal with alien bodies, the elimination of the elements of pathological microorganisms, as well as malignant cancer cells. There was a decrease in eosinophils in 41.6% of the studied animals, and the absence of eosinophils in 16.6% and monocytes in 90%, indicating a deterioration in the functional ability of macrophages in the diseased animals.

The mononuclear phagocytic system cells (macrophages, monocytes) carry out the effector reaction of antitumor, in this case anti-leukemic immunity. Macrophages, T and B lymphocytes being cell populations, are directly involved in all parts of the immune response. Macrophages carry out regulatory functions in reactions of the immune response, concentrating and focusing antigen molecules on their surface, facilitate access to it by T and B lymphocytes,

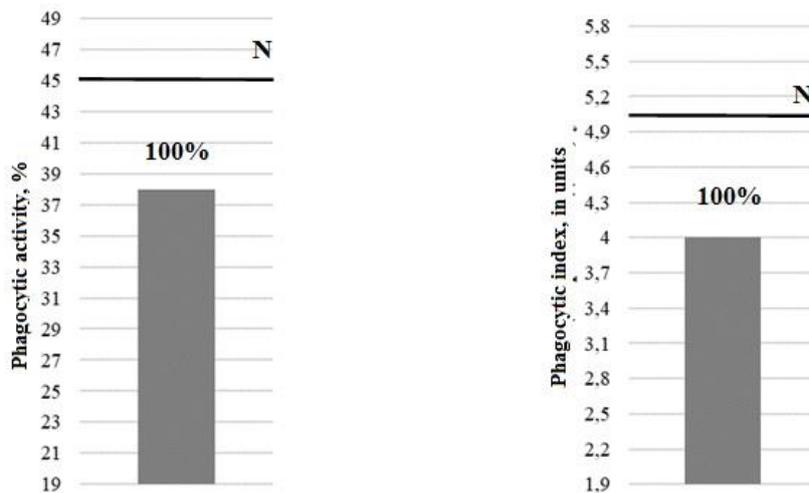


Fig. 4. Phagocytosis indices of the infected cattle

thereby stimulating lymphocytes for the immune response. Activated macrophages have a nonspecific antitumor property; by activation they mean morphofunctional changes in macrophages, including an increase in phagocytosis. [3, 8]

In the blood of the all studied cows, a decrease in phagocytic activity was established by 1.5-3 times (up to 19-38%), and a phagocytic index by 2.5-5.2 times (up to 1.9– 4.0 cu), which indicates that the inflammation process is becoming chronic and the autoimmune process is maintained, the decrease of the destruction function and removal of immune complexes from the body, the loss of antitumor properties by macrophages (Fig.4).

Cellular immunity is of great importance in the antitumor defense of the body. Cellular immune responses

are known to be carried out by T lymphocytes, producing such biologically active mediators as lymphokines and lymphotoxins. Cellular immune reactivity is considered as a good prognosis, but a decrease or lack of reactivity is associated with tumor progression. [7, 8] The detection of a given population of lymphocytes during leukemia is an important criterion for the classification of a given disease and the assessment of the body's immune reactivity. In the pathogenesis of leukemia, the characterization of leukemia according to their T and B cell significance is essential.

Conducted immunological studies have shown that the content of T lymphocytes (E-ROL) in the blood of all studied cows is significantly reduced.

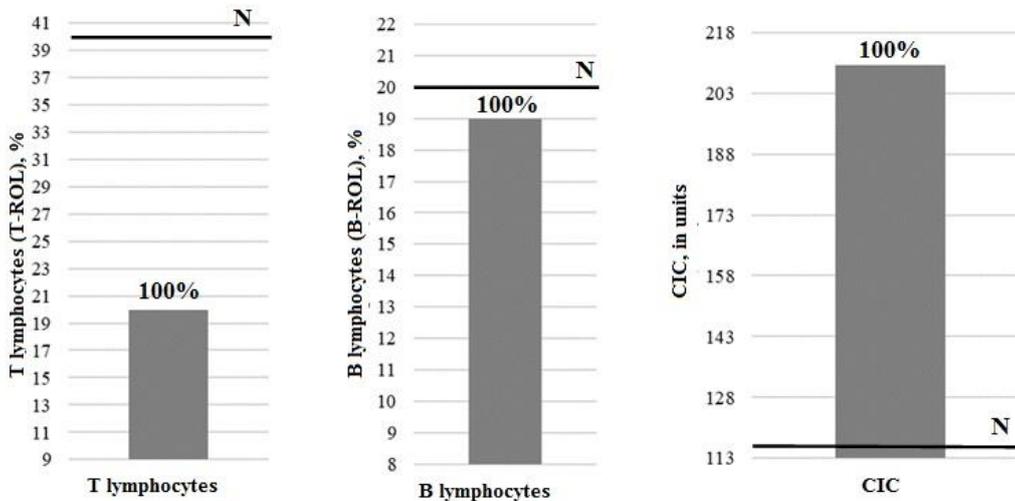


Fig. 5. The content of T lymphocytes (T-ROL), B lymphocytes (M-ROL) and circulating immune complexes in the blood of leukemic cows

IV. CONCLUSION

We performed a diagnostic screening of the BVL on a selected group of infected animals (n = 24). The diagnosis was confirmed by PCR. Using the nested PCR method, short fragments of a section of the env gene of the leukemia pathogen were obtained, the polymorphism of which was established by the RFLP method. It was established that in the Tyumen region, the antigenic landscape is dominantly represented by the “Belgian genotype” (23 isolates) and only in one case by the “Australian genotype” of the BVL. Detailed phylogenetic characterization is to be established by DNA sequencing of the env gene fragment of the leukemia virus.

A hematological study of cattle infected with the “Belgian genotype” revealed an increased content of leukocytes, mainly of the lymphoid series, as well as the presence of atypical hematopoietic cells of different degrees of maturity, indicating the oppression of the hematopoietic organs. In animals at the initial stage of the disease, neutrophilic leukocytosis was established, with no immature forms yet, and anemia was absent. With the development of the disease, the number of immature forms of neutrophils and eosinophilic leukocytes increases, younger forms with basophilic granules appear; basophilic leukocytes are found in an increased amount, their

presence in large numbers confirms the diagnosis of leukemia. As for platelets, their number in the initial stage of the leukemic process is normal or slightly elevated, thrombocytopenia and even their complete absence appears at the eruptive phase of the disease. The emerging and developing leukemic process significantly changes the body's immunological reactivity. Immunological deficiency is a central, not a secondary manifestation. In animals with the BVL, the number of T lymphocytes and circulating immune complexes was mainly changed; the pathology of the B lymphocyte system was observed, which indicates suppression of T cells, a decrease in the phagocytic activity of macrophages, which, as we know, produced by T lymphocytes. A cow with an “Australian genotype” of bovine leukemia virus in the blood has an elevated red blood cell count, slight leukocytosis, and has the immature and atypical poorly differentiated blastoma-type reticular cells. Immune insufficiency was manifested in the form of a noticeable decrease in the functional activity of T lymphocytes (E-ROL) up to $1.0 \cdot 10^9/l$ and of B lymphocytes (M-ROL) – up to $0.5 \cdot 10^9/l$; neutrophil phagocytic activity – up to 25%, neutrophil digesting capacity – up to 3.2 units.

The identified hematological and immunological changes indicate a violation of the hematopoiesis and the failure of the immune system in BLV infected cattle, regardless of the genotype of the pathogen.

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