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# Polymorphism of Haplotypes of Bovine Leukemia Virus

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***Abstract*— The study of molecular structure in LTR regions of the BLV genome (bovine leukemia virus), which is present in the holsteinized Black Pied breed in the Novosibirsk Region, with the subsequent differentiation into haplotypes. Screening for the presence / absence of BLV was performed by PCR analysis. Hematological studies were performed using a PCE-90 Vet hematology analyzer. The affiliation of the virus to haplotypes was determined by several specific sites using a set of three endonucleases. Hydrolysis with BstMA I endonuclease showed the presence of fragments 237 bp long and 206 bp long in all the studied samples. Restriction of BspI in 100% of the samples revealed the presence of fragments 262 and 161 bp, which indicates the monomorphism of the site for nucleotide A (adenine). Fragments of 370 and 73 bp, which corresponded to haplotype I, as well as 378 and 65 bp, which were interpreted as haplotype III, were obtained from Bse1 restriction results. The fact of presence / absence of the dependence of the number of leukocytes on the haplotype of the virus is analyzed. The results showed the absence of significant hematological differences in blood samples of carriers of I and III haplotype. Various hypotheses of evolutionary models of the relationship between BLV and cattle are considered, where the LTR polymorphism of the virus region could be a determining factor. The totality of our own research and literature data indicates the failure of the hypothesis about the effect of mutations in the LRT region on the degree of recognition of BLV by the immune system of cattle.**

***Keywords—SNP, bovine leukemia virus (BLV), LTR region, restriction fragment length polymorphism, leukemia, haplotype.***

## I. INTRODUCTION

At present, leukemia is the most common epizootic disease of cattle in Russia and an in number of foreign countries [1-3]. It is the cause of 40% to 66% of infectious pathologies of cattle [4, 5]. Economic losses from the period from 2010 to 2014 amounted to 11225954.3 thousand rubles [6]. Therefore, it is by no means surprising that some authors [7] call bovine leukemia for “disease of the century”. The causative agent of bovine leukemia is BLV – an RNA-containing virus of the Retroviridae family. BLV is integrated into the genomic DNA of B-lymphocytes, as an intermediate DNA form (provirus) [8, 9]. Vital processes of viruses, which are reduced to replication, are in principle impossible outside the host organism [10], which has become the reason for the concept of “virus – organism – environment” gaining popularity [11, 12]. It is believed that the symbiotic and parasitic relationships of microorganisms, in particular viruses, with carriers are quite powerful co-evolutionary factors [13].

Of all living organisms, viruses are most susceptible to mutagenesis [14]. Although BLV is a virus with a rather conservative nucleotide sequence [15, 16], the RNA molecule is still a rather unstable and mutable structure [17]. Over the past ten years, many new BLV strains have been reported in the world [18], while there is still no guarantee that the researchers have described all existing varieties [19]. In turn, it is the nucleotide sequence of the virus itself which is the target for the most modern and accurate screening method – PCR analysis [20-22]. At the same time, a single mutation is quite enough for the existing test systems to give a false negative result in the analysis. Thus, it is desirable to have several test systems on the “armament” of the diagnostic laboratory that can identify the presence of BLV in samples of cattle biological material.

BLV genes such as pol, env, and gag are quite well studied and are used in screening test systems [23–25]. At the same time, the BLV LTR region is considered to be a rather poorly studied structure [19], which makes it important not only to study its molecular structure, but also creates the prospect of developing test systems based on primers specific to the LTR region.

It is known that the so-called mutator genes that catalyze mutagenesis are localized in the LTR region of BLV [2]. Some studies have shown the relationship of mutations in the LTR region of BLV with the characteristics of the course of infection [2, 26]. Long circular repeats of the proviral DNA of the BLV virus contain sequences predisposed to the formation of such noncanonical DNA structures as G4 quadruplexes [11, 25, 27] and triplexes [28]. It was found that a relatively long purine-pyrimidine track 13 bp long, predisposed to the formation of intermolecular triplexes of DNA-RNA, and characterized by relatively increased conservatism both in sequenced GenBank sequences and BLV-induced lymphomas, is localized in areas of the second section of TxRE (Tax-responsiveelements) [11, 29]. It can be expected that insertions of proviral DNA with such flanks predisposed to the formation of non-canonical DNA structures have a significant effect on the expression of adjacent genes. The TxRE region that binds the proviral DNA transcription activator (Tax) is of particular interest because it is located in the enhancer region of the LTR region. Tax is directly involved in the regulation of transcription of proviral BLV DNA and has a significant effect on the expression of host

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genes associated with cell proliferation and differentiation, immortalization of primary cell populations in the in vitro system, and an increase in the mutation rate by suppressing excision DNA re-evaporation [11, 25]. Despite the wide range of targets for the regulatory effects of the Tax protein, it is believed that genes encoding the proteins of the immune system are one of the leading targets for the regulatory effects of this protein [11, 25]. There is an assumption about the effect of single nucleotide substitutions in long end-repeats (LTR regions) on the recognition of the virus by the body, that is, to hide the virus from the body's immune system, thereby increasing the chance of the virus being inserted into the host cell [29]. Thus, the study of mutations in the LTR region of the BLV is a very interesting aspect. Contribution to its resolution is the objective of this study.

## II. METHODS

We used samples of total DNA isolated from the whole blood of 288 cows of holsteinized Black Pied breed that were bred in the territory of the Novosibirsk Region. Blood samples were taken in May 2016 from the tail vein with sterile catheters using EDTA as an anticoagulant. DNA was isolated using the DNA-Sorb-B kit (FGUN Central Research Institute of Epidemiology, Russia). Screening studies for the presence of BLV in the samples were carried out using the AmpliSens® kit (Central Research Institute of Epidemiology, Russia). Cytofluoremetric and morphological blood indices were determined using the automatic veterinary hematological analyzer PCE-90 Vet.

The BLV infected were detected by PCR analysis, since this method is considered the most reliable [20-22]. The methodology for PCR analysis of the LTR region of BLV and the primers used in the study are proprietary. The direct testing technique is described in detail in the previous work [31]. For typing the polymorphism of the LTR region, restriction enzymes BstMA I, Bse1, BspI were used. A restriction map was compiled using bioinformation programs for working with sequences of decrypted genomes using sequences from the International NCBI (Gene-Bank) database. The lengths of the restriction fragments are indicated in accordance with the "SibEnzim" campaign product catalog [32]. The statistical significance of the differences in the characteristics was evaluated by Student's test criterion.

## III. RESULTS

The association of the results of screening for BLV virus and biochemical testing of blood samples shows that BLV carriers were detected in 63 out of 288 examined individuals (Table 1). Of the total number of infected animals, only 15% of blood counts remained within normal limits. The hematological stage of the course of leukemia infection with severe lymphocytosis was diagnosed in 14 animals.

TABLE I. DISTRIBUTION OF BLV-INFECTED ANIMALS BY THE MANIFESTATION INTENSITY OF LEUKEMOID REACTIONS

| Indicator | n | % |
|---|---|---|
| Infected | 63 | 100 |
| Unchanged hematological parameters | 16 | 25 |
| Severe leukocytosis > 10 x 10 9/l | 33 | 53 |
| Hematological stage of leukemia | 14 | 22 |

Taking into account the substitutions at the genome sites of 8034 and 8139 nucleotides detected by the method of polymorphism of the lengths of the restriction fragments, a scheme for the formation of haplotypes was compiled (Table 2). The haplotype was indicated by the corresponding abbreviation for the nucleotide, in the case of cutting, and the symbol N, that is, the unknown nucleotide in the absence of a cut. Given the combination of substitutions (Table 2), a haplotype formation table was compiled for each sample, according to which all samples were divided into 2 haplotypes – I and III. The occurrence of haplotype I (n = 42; p = 0.667; mp = 0.059) was two times higher (P <0.001) than haplotype III (n = 21; q = 0.333; mq = 0.059).

TABLE II. HAPLOTYPE PATTERN

| Haplotype | Restriction fragments | | |
|---|---|---|---|
| | ***BstMA I – GTCTCN↑ CAGAG(N)5↓*** | ***Bse1 - ACTGGN↑ TGAC↓CN*** | ***Bsp I – C↑CGC GGC↓G*** |
| I | 237 (A) | 378 (GC) | 262 (CG) |
| II | 237 (A) | 378 (GC) | - (AG) |
| III | 237 (A) | 370 (CN) | 262 (CG) |
| IV | 237 (A) | 370 (N) | -( AG) |
| V | – (N) | 378 (GC) | 262 (CG) |
| VI | – (N) | 378 (GC) | - (AG) |
| VII | – (N) | 370 (CN) | 262 (CG) |
| VIII | – (N) | 370 (CN) | - (AG) |

The research results showed the presence of: homogeneous wild type, homogeneous with the presence of SNP and two heterogeneous types, that is, having wild type haplotypes and polymorphisms. The restriction enzyme BstMA I forms the same sections for all the studied samples, visualized as fragments 237 bp and 206 in length, respectively. Thus, nucleotide No. 237 in the studied sample was monomorphic, represented by adenine (A), which corresponds to the so-called wild-type virus (Fig. 1, Table 2).

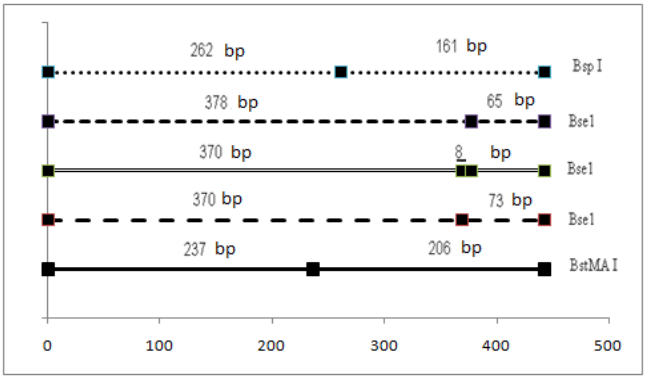


Fig. 1. Scheme of fragment formation during hydrolysis of a 443 bp BLV genome with the indicated restriction enzymes

BspI endonuclease restriction products were identical in the all studied samples with the formation of fragments 262 and 161 bp, which indicates the presence of cytosine (C). The absence of hydrolysis characteristic of the presence on this site of adenine (A), characteristic of adenine (A) at the restriction site (Fig. 1, Table 2). Thus, 100% of the formed amplificons were of the wild type.

Hydrolysis with Bse1 endonuclease was the most informative. Two groups of patterns are distinguished on the electrophoregram (Fig. 2): the first segment of the amplificon at the level of 378 bp is clearly visible; the second at 65 bp is poorly distinguishable at the photo, although it is very clear visually. The difference in the quality of visualization is explained by electrophoresis on an agarose gel, in which, as a

rule, short fragments are poorly visualized [33]. In the second group of patterns, the amplificon part of 370 bp is visible, and the second part – 73 bp, is also poorly visualized (Fig. 2).

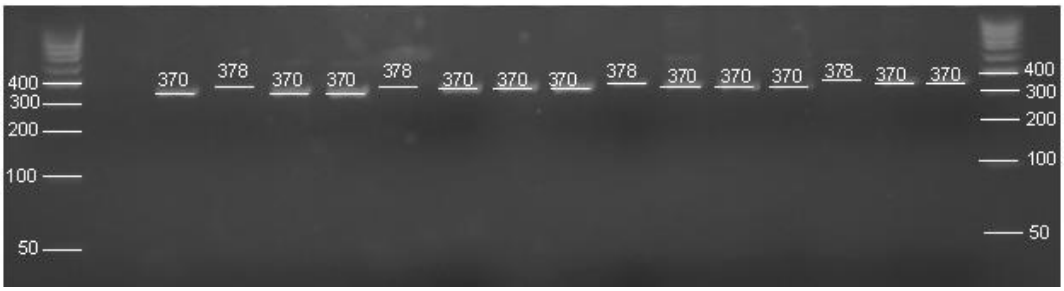


Fig. 2. Visualization of hydrolysis of amplification products Bse1 - CN/GC

This distribution image of restriction fragments was typical for the all samples. Only two haplotypes were determined. Moreover, there should be paid attention to the fact that at the level of 370 bp, the fragment is cut with an endonuclease if there is a substitution in the sequence from G to C, while a cut at the 378 bp site is possible with a wild-type sequence, that is, without replacement at haplotype C (Fig. 2, 3). Possible substitutions (SNPs) are indicated in parentheses for the nucleotide that they replace. Thus, the factor determining the BLV haplotype in the studied samples was nucleotide mutations determined by hydrolysis upon exposure to Bse1 endonuclease.

In addition to screening for the presence / absence of the BLV in samples of the biological material of cattle, a biochemical analysis of blood serum was performed. Blood counts of animals infected with leukemia of different haplotypes did not have significant differences in the content of both the total leukocyte count and their individual fractions, which is confirmed by the Student's t-test below the standard for $P < 0.05$ ($t = 2.00$ at $df = 61$). It is worth noting that carriers of the haplotype I were distinguished by slightly higher leukocytosis due to lymphocytes and granulocytes (Table 3). The leukocyte formula of animals of both groups significantly exceeded the physiological standard [34].

TABLE III. HEMATOLOGICAL PARAMETERS OF CARRIERS OF DIFFERENT BLV HAPLOTYPES

| **Indicator** | **Carriers of haplotype I** ***p±mp*** | **Carriers of haplotype III** ***p±mp*** | ***tf*** |
|---|---|---|---|
| Leukocytes, 10 9/l | 17.07± 2.78 | 14.52±3.05 | 0.618 |
| Lymphocytes, 10 9/l | 9.14± 2.46 | 8.35± 2.75 | 0.214 |
| Monocytes, 10 9/l | 1.50± 0.35 | 1.27± 0.51 | 0.372 |
| Granulocytes, 10 9/l | 6.43±1.22 | 4.90±1.93 | 0.670 |
| Lymphocytes, 10 9/l | 51.95±10.03 | 57.10±11.56 | 0.337 |
| Monocytes,9/l | 8.99±1.31 | 8.78±2.96 | 0.065 |
| Granulocytes, 10 9/l | 36.69±7.51 | 34.14±12.17 | 0.178 |

[a.] tf – Student's t-test

## IV. DISCUSSION

It is noteworthy that only 25% of all selected infected animals did not show clinical signs of leukemia expressed by the leukocyte formula. Earlier studies show that the latent leukemia is characteristic for at least 70% of the carriers [2, 35, 36]. Moreover, both BLV haplotypes are more aggressive in this study, causing a large proportion of clinical cases of leukemia, even in comparison with the results of typing of animals from other farms in the Novosibirsk Region [31]. However, this can be explained not so much by the unaccounted genetic factors of the virus or cattle, as by the unequal conditions of feeding and managing. It it been well known [38] that malnutrition is a very significant factor for the progression of infectious agents, which is impossible if there are enough natural antiviral and antimicrobial factors such as vitamins and minerals in the animal body.

It is important to note that it is the haplotype I that carries the SNP determined by the endonuclease BseI, which makes it possible to verify the assumption of a number of scientists [30, 38] about the need for these substitutions so that the virus can "escape" from the reaction of the host's immune system. On the other hand, the LRT region contains genetic elements for regulating the rate of synthesis of viral particles of BLV [19], and as our recent studies [38] show, the intensity of replication of viral particles may well be a consequence of mutations in the LRT region. But it is worth remembering that an increase in the rate of reproduction, all other things being equal, will certainly provoke a more intense response of the immune system, which can lead to the death of both the virus carrier [38] and the virus if it fails to penetrate the body of the next host. Thus, the rate of virus reproduction is limited by the recognition of the immune system, which, in fact, allows us to determine the second evolutionary vector aimed at reducing the recognition of the virus by the immune system and, ultimately, on the transition of virus-host relationships from parasitic to symbiotic.

Data from the previous study indicate an increase in viral load and white blood cell count when C> G is replaced [38], which clearly refutes the assumption that the BLV masking vector is determined by the LRT region. A comparison of our own studies and published data [2, 19, 36] more likely confirms the hypothesis about the function of the LRT region – a regulator of the viral particles synthesis. Taking into account the principles of genetic control, it is difficult to predict [14] that the regulator itself could be a factor in recognition of the virion by the host's immune system on the basis of the "friend or foe" principle. The only possible effect of the regulator on camouflage function may be to reduce the synthesis of the regulated product. Much more logical seems to be that the camouflage function performs the virion envelope proteins, which are encoded by the env gene in the BLV [26, 40, 41].

## V. CONCLUSIONS

1. Typing of the LRT region of BLV revealed a single nucleotide substitution C> G, which was detected as the formation of restriction fragments with Bse1 endonuclease, 370 + 73 bp long (corresponding to haplotype I) and 378 + 65 bp (haplotype III).

2. Incidence of haplotype I was 0.667 ± 0.059; haplotype III 0.333 ± 0.059

3. White blood cell count in the blood of carriers of haplotypes I and III did not have significant differences.

4. Totality of our studies and literature data indicates the inconsistency of the hypothesis about the influence of the LRT region mutations on the degree of recognition of the BLV by the immune system of cattle.

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