Genotypes diversity of env gene of Bovine leukemia virus in Western Siberia

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From 11th International Young Scientists School "Systems Biology and Bioinformatics" – SBB-2019
Novosibirsk, Russia, 24-28 June 2019

Abstract

Background: This study describes the biodiversity and properties of Bovine leukemia virus in Western Siberia. This paper explores the effect of different genotypes of the env gene of the cattle leukemia virus on hematological parameters of infected animals. The researchers focused on exploring the polymorphism of the env gene and, in doing so, discovered the new genotypes Ia and Ib, which differ from genotype I. Several hypotheses on the origin of the different genotypes in Siberia are discussed.

Results: We obtained varying length of the restriction fragments for genotypes I. Additionally using restrictase HaeIII were received fragments was named genotype Ia, and genotype Ib. There are 2.57 ± 0.55% (20 out of 779) samples of genotype Ib which does not differ significantly from 1% (χ2 = 2.46). Other genotypes were observed in the cattle of Siberia as wild type genotypes (their frequency varied from 17.84 to 32.73%). The maximum viral load was observed in animals with the II and IV viral genotypes (1000–1400 viral particles per 1000 healthy cells), and the minimum viral load was observed animals with genotype Ib (from 700 to 900 viral particles per 1000 healthy cells).

Conclusions: The probability of the direct introduction of genotype II from South America to Siberia is extremely small and it is more likely that the strain originated independently in an autonomous population with its distribution also occurring independently. A new variety of genotype I (Ib) was found, which can be both a neoplasm and a relict strain.

Keywords: Bovine leukemia virus, Cattle, Env gene, Genotypes, Agriculture

Background

Viruses are the most variable form of life, and BLV is not an exception. The origin of new strains is a continuous process [1–4]. Monitoring the origin of new virus mutations is of great importance for veterinary and animal husbandry, as every new strain may have unique features of interaction with the host organism. Accordingly, every new strain of the virus can be cause of completely another symptoms of the disease [5]. That’s why, the monitoring of the spread and emergence of virus strains is relevant in every separated geographical region.

One of the most common and dangerous diseases of cattle is leukemia caused by Bovine leukemia virus (BLV), which belongs to the family Retroviridae [6, 7]. One of its features is a high proportion of latent virus carriers (70–90%) and low detestability of clinical symptoms by hematological methods, which complicates measures to identify sick animals and prevent the further spread of the disease [8–10]. BLV it is a virus transmitted by direct contact between animals. The transmitted
BLV from cattle to humans is possible but clinical symptoms in Homo sapiens have been registered in singular cases [11]. The probable reason for this is the tendency of Retroviridae representatives to embed into the carrier genome [4, 12]. Therefore, it is important for viruses to have such a protein composition of the capsid, so as not to be interpreted by the immune system of the carrier as a danger. In BLV, capsid proteins are encoded by env gene. The env gene is located between the pol gene and the 3’LTR-area; it is 1547 bp in length (from nucleotides 4826 to 6373, numbering from the BLV env sequence of the isolate FLK-BLV, GenBank accession number EF600696). 4615–6160 bp). This gene encodes the glycoprotein viral membrane of gp51 and the transmembrane protein gp30. The proteins -gp51(SU) and gp30 (TM) are glycosylized [13–16]. Protein gp30 contributes to the contact between viral particles and B- and T- cells and its properties affect the ability of the sheep immune system to recognize the viral particles [17, 18]. This protein can define the ability of BLV to affect the immune system cells of a carrier [19]. The gp51 protein reacts with monoclonal cattle antibodies and determines the degree of BLV virulence [20]. Therefore, qualitative changes in the gp30 and gp51 proteins that are caused by gene mutations may become the factors that affect the intensity of the immune response. So for specialists involved in coevolution of BLV and cattle, it is of great interest to study the relationship between individual mutations and genotypes of the virus with the phenotypic manifestation of leukemia.

The BLV genome includes four structural genes (env, pro, gag, and pol), of which env is the most suitable for detecting the pathogen by PCR analysis [21–23]. There are 10 genotypes of the env gene in the world [24, 25] and each creates slight differences in the biological properties of the virus. This research aims to define the virulent properties of the BLV genotypes that have been discovered in Western Siberia as well as the newly discovered forms of the genotypes on the env gene.

**Results**

PCR-RFLP analysis of the env gene detected 5 genotypes that differed on the lengths of fragment restriction. This restriction is caused by restrictases HaeIII and BstYI (Table 1). The effect of the restriction endonuclease HaeIII differed from the supposed effect. The fragment restriction lengths of 316, 27, 95, and 5 bp were expected to show up on the electrophorogram as they are considered to be the standard products of genotype I restriction [26]. However, additional fragments of restriction were found as genotypes Ia and Ib (Table 1, Fig. 1).

Restriction fragments (length 27 bp) are considered to be a common trait for all three genotypes of the family I.

| Genotype | Restrictase |
|----------|-------------|
| I        | HaeIII      |
| Ia       | 316–27–95–5 |
| Ib       | 31–285–27–95–5 |
| II       | 31–85–200–27–100 |
| IV       | –          |
| I        | BstYI       |
| Ia       | –          |
| Ib       | 529–322–143 |
| II       | –          |
| IV       | 672–322    |

The table highlights the fragment restrictions with length (b.p.)

The common factors for genotypes I and Ia are fragments of 95 and 5 bp (Fig. 1). Genotype Ib differs from related genetic elements as it forms these fragments into a single one (100 bp length). The common restriction fragments of genotypes Ia and I reveal a short sequence (31 bp), whereas the long fragment that is typical for genotype Ia (285 bp), is represented by two separate factions (85 and 200 bp) (Fig. 1).

The prevalence of the previously discovered and explored genotypes varied from 17.84 to 32.73% with the exception of the Ib genotype (Fig. 2). The number of samples with genotype Ia was 20.95 ± 1.46% (171 out of 779), which differed significantly (χ² = 398; P < 0.001) from the statistical and methodological error and is equal to 1% [2]. The number of samples with genotype Ib was 2.57 ± 0.55% (20 of 779) and didn’t differ significantly from 1% (χ² = 2.46). Therefore, of the two new genetic formations that were observed in the cattle of Siberia, only the Ia genotype is thought to occur normally (not mutant) in population frequency.

The highest number of leukocytes was observed in the blood of animals infected with the BLV of the genotype I. The hematological status was different in sick animals (P<0.01: P<0.001) and the total sample (P <0.001). The carriers of the Ib genotype were seen as the only exceptions; the difference in the number of leukocytes in the animals of the I genotype was not reliable (Table 1). The differences in the hematological status of the carriers of genotypes Ia, II, and IV were not significant or they were of a low reliability (P<0.05).

The carriers of the cattle leukemia virus I, Ia, II, and IV genotypes were observed among animals that were sick, healthy, and those that were suspected of having leukemia. There was a heterogeneous relationship among the animals of different hematological statuses and animals infected by different virus genotypes (Table 2).

The highest number of lymphocytes was observed in the blood of genotype I carriers, while the lowest number of lymphocytes was found in the animals with genotype II (Table 2). The similarity of the qualitative blood parameters of the cattle infected with BLV in different
genotypes of the env gene, expressed through Wilks’ lambda statistic (0.82285), can be considered with some to be sufficiently high, but not identical.

The investigation into the bovine viral status had unexpected result. The typical sequence of viral load distribution for genotypes on the LRT-area [27] (sick > suspected > healthy) is not clearly shown in the present experiment (Fig. 3). Observed a non-typical relationship between the physiological status and viral status in the animals infected with genotype I. The maximum number of viral particles was observed in the blood of healthy animals, slightly lower in suspected animals, and minimally in animals with hematolytic leukemia (Fig. 3).

Discussion
The current research does not prove that the higher the viral load, the higher the immune response of the
leucocytes, despite the earlier results that showed that the genotypes of the LRT area affect the type of cattle leukosis [27]. The research shows that the highest number of leukocytes was observed in the blood of the cattle infected with the virus of I genotype (Table 1); the highest viral load was observed in the animals infected with BLV of II-and IV genotypes (Fig. 3).

The research results are unique due to the high diversity of the discovered virus genotypes (4 genotypes of the standard frequency and 1 genotype of the frequency indistinguishable from 1%) (Table 2, Fig. 2). Early research detected one or two BLV genotypes of the env gene in one population of cattle [24, 28]. The observed diversity of the virus may be caused by the massive

### Table 2 Cytometric and morphological parameters of blood of animals-carriers of different genotypes of BLV

| env | Hematological status | WBC, 10^9/l | lymf, 10^9/l |
|-----|----------------------|-------------|-------------|
|     |                      | X          | S_x         | X          | S_x         |
| I   | suspected            | 12.30      | 0.132       | 12.04      | 0.124       |
|     | sick                 | 24.18      | 0.452       | 23.29      | 0.295       |
|     | healthy              | 4.95       | 0.369       | 4.22       | 0.287       |
|     | On average           | 12.36      | 0.180       | 12.01      | 0.127       |
| Ia  | suspected            | 11.26      | 0.256       | 10.76      | 0.116       |
|     | sick                 | 19.82      | 0.429       | 18.98      | 0.264       |
|     | healthy              | 7.06       | 0.197       | 6.67       | 0.231       |
|     | On average           | 9.93       | 0.240       | 9.44       | 0.210       |
| II  | suspected            | 9.73       | 0.195       | 9.35       | 0.150       |
|     | sick                 | 17.40      | 1.109       | 15.22      | 1.540       |
|     | healthy              | 6.81       | 0.307       | 6.21       | 0.265       |
|     | On average           | 9.08       | 0.246       | 8.60       | 0.212       |
| IV  | suspected            | 11.70      | 0.233       | 11.24      | 0.176       |
|     | sick                 | 17.25      | 0.960       | 15.36      | 0.920       |
|     | healthy              | 8.36       | 0.173       | 8.02       | 0.157       |
|     | On average           | 9.71       | 0.210       | 9.30       | 0.190       |

Wilks’ lambda statistic = 0.82,285, F(12, 1530) = 13,056, p<0.001

WBC – number of white blood cells; lymf – number of lymphocytes X – the arithmetic average; S_x – error of the arithmetic average; -5 and + 5% are denoted the corresponding deviation of the attribute in the greater and lesser direction relative to the arithmetic mean; n – number of animals

![Fig. 3 Distribution of viral load at different stages of infection progress in relation to heterogeneity of env gene BLV (Wilks’ =0.66,912, F = 28.406, p < 0.001; confidence intervals-95%)](image)
import of cattle to Siberia from other regions, particularly from abroad.

Interestingly, genotypes I and IV and their subtypes were found in BLV isolated from the biological material of cattle that were bred in different countries. Genotype I was found in the cattle from Japan, the United States, Australia, Germany, Korea, Iran, Brazil, Colombia, and the Dominican Republic [24, 29–34]. Genotype IV was observed in the cattle from Brazil, Belgium, Poland, Ukraine, and France. In Russia, and particularly in Siberia, only genotype IV BLV by the env gene was observed early [29, 33]. The cattle and sperm from Europe, the US, and Canada were delivered to Siberia in order to improve local livestock productivity [35, 36], the presence of the BLV genotypes I and IV in the explored sample is logically explained.

Interestingly the presence of the genotype II, peculiar only to South American cattle populations, had an frequency of 17.84% in the blood samples (Fig. 2). Cattle delivery from South American countries, where genotype II is widespread, to Siberia is not observed in the scientific literature. The cattle from South America have similar dairy productions to Russian cattle [37, 38]. The period of delivery and the different climate conditions between Siberia and South America assume lack of breeding and economic efficiency of black and white cattle when considering their delivery from South America to Siberia. Therefore, the introduction of genotype II BLV seems highly doubtful.

Phylogenetically, genotype II BLV is a separate complex that is distant from genotypes I and IV [39]. The research on isolated laboratory strains of E. coli [40] and red salmon inhabiting the rivers and lakes of Alaska [41] showed the independent directions of mutagenesis in the isolated populations. The model of allopatric speciation is based on geographical isolations [42] that helps divergence of morphogenetic characteristics of the strains, breeds, or populations [3, 43, 44]. Therefore, the possibility of an independent formation of the Siberian and South American BLV genotype II by convergent processes accepted as unlikely. It may be that genotype II BLV originated in a certain, presumably European or North American, cattle population and was then further introduced in the another regions. It is in Europe and North America that are the main suppliers of bull semen with a maternal productivity of 15,000 kg of milk per 305 days of lactation to other regions of the world [37].

Therefore, the assumption of the origin of the II genotype in the European or North American cattle population looks quite reasonable. Subsequently, probably, there was its independent introduction to Siberia and to South America together with importation of animals or semen of bulls. However, this hypothesis, despite its logic, remains only a working version that requires additional experimental verification.

The map of restriction fragments (Fig. 1) defines the line of mutations caused by genotype I to genotype Ia (Fig. 4a). This statement is arguable based on the controversial idea that if something had not been discovered before, then it did not exist. Our previous article and other papers [27, 45, 46] have highlighted the evolutionary advantage of more viral strains over less viral ones. Changes, as a rule, mostly aim at improving the functional properties of an object and its complex structural organization [3, 47]. Therefore, the general evolutionary vector should aim at increasing viral capacity it is supported by the rapid synthesis of viral particles and contributes to the evolutionary advantage of the strain as an intra specific generation. Analyzing the maximum number of leukocytes and the viral load, the observed genotype Ib as the least virulent and prevalent, followed by genotype Ia. The virus with genotype I (Table 2, Fig. 3) caused the highest immune response.

Thus, the second hypothesis can be put forward for the chronology of BLV genotypes originating from the env gene, where genotype Ib can be considered as the initial form, and then, due to the accumulation of mutations, the strain Ia emerged, and the last and most progressive form is the carrier particles of genotype I (Fig. 4b).

Considering the analysis of restriction fragments (Fig. 1), there is third hypothesis which states that genotype Ia is the ancestral one and the mutations that formed genotypes I and Ib were accumulated independently (Fig. 4c). This model seems to be the least attractive as it admits the appearance and consolidation of genotype Ib with a frequency indistinguishable from the mutant one, with the lower

![Fig. 4 Hypothetical schemes originiation of BLV genotype I on env gene](image-url)
number of viral particles than that of genotype $I_a$ and lower rate of their synthesis. This means a lower reproduction of a virus' own copies and an evolutionary unattractiveness to this new formation.

Two of the three mentioned hypotheses make the case that genotypes $I_a$ and $I_b$ are not new growths that emerged in Western Siberia as a result of mutagenesis. They are relict forms of $BLV$, preserved in the cattle and displaced in other places by progressive strains.

**Conclusions**

The research discovered of 5 $BLV$ genotypes in the $env$ gene, where genotype $I_b$ was observed with a frequency (2.57 ± 0.57%) indistinguishable from that of the mutant genotype (1%). Genotypes $I, I_a, II, IV$ were found to have a frequency of 17.84 to 32.73%. The highest number of leukocytes was observed in the blood of animals infected with the $BLV I$ genotype. Hematological status differed in respect to sick animals ($P<0.001$) and the sample as a whole ($P<0.001$). The maximum viral load was observed in the carriers of genotypes $II$ and $IV$ (1000–1400 viral particles per 1000 healthy cells). The number of viral particles in the family $I$ genotypes vary from 700 to 900. For the first time, the paper describes genotypes $I_a$ and $I_b$. Three hypotheses were suggested that try to explain the stages in the origination of genotype $I$. According to one of the hypotheses, genotypes $I_a$ and $I_b$ are new growths that originated in Western Siberia due to mutagenesis. Two other theories suggest that the explored genotypes are relict forms of $BLV$ that have been displaced in the cattle by more progressive strains.

**Methods**

Samples of whole blood were taken from black and white Holstein cows ($n = 779$) located in the Novosibirsk region in Russia. The blood samples were obtained in May 2019 from the sub clavian vein using sterile catheters and EDTA added as an anticoagulant. Cytological and morphological parameters of the blood were determined by means of the RSE-90 Vet automatic veterinary hematological analyzer.

The total DNA was isolated by DNA-Sorb-B (Central Research Institute of Epidemiology, Russia) in order to conduct PCR analysis. The screening tests for observation of $BLV$ in the blood samples were conducted by AmpliSens® (Central Research Institute of Epidemiology, Russia). The authors explored the $env$ gene sequence by means of PCR-RPF analysis ($gp51$ fragment of $env$ gene) according to the practice of the National Veterinary Institute (Poland, Pulava). Amplification was accomplished using the nest method in two stages with the use of 3 primers. The number of cycles, their temperature, and time parameters were set in agreement with the methodology of the National Veterinary Institute (Table 3). As the control strain was used FLK isolate The FLK strain (GenBank inventory number EF600696).

The calculation of the number of components necessary for a reaction in line with the recommended number of samples was obtained according to the formula (1):

$$M = m \times n + 3 + 1$$

Where, $m$ is the amount of reaction mixture per a sample, $n$ is the number of samples for virus tests, 3 is the number of controls in the reaction ($IC =$ internal control for PCR, $NC =$ negative control, $PC =$ positive control), 1 is the safety amount of reaction mixture equal to the reaction mixture per a sample. $BLV$ was applied as the positive control and a DNA buffer was used as the negative control. Table 4 illustrates the sufficient number of components for each reaction.

Primers from Table 5 were produced by an automated synthesizer of oligonucleotides that was purchased from the “SibEnzyme” (Novosibirsk, Russia). The accuracy of the primers was defined by the HPLC method and its accuracy was at least 95%.

The products of amplification were analyzed by means of fragment restriction acceleration that was caused by the horizontal electrophoresis method in agarose gel. Samples suspected of short fragments were additionally detected by vertical electrophoresis in polyamide acrylic gel [48]. The restriction was realized by the enzymes.

| Table 3 | Temperature profile of PCR |
|---------|---------------------------|
| Number of cycles | Temperature, °C | Period of time |
| 1 | 95 | 3 min |
| 34 | 95 | 30 s |
| 62 | 95 | 30 s |
| 72 | 72 | 2 min |
| 1 | 72 | 10 min |
| Storage | 4 | < 12 h |

| Table 4 | Composition of reaction mixture |
|---------|-------------------------------|
| Mixture components | Necessary amount, mcl |
| Reaction1 | Reaction2 |
| 10×of optimized DNA-buffer | 5.0 | 5.0 |
| 10 mM MgCl₂ | 1.0 | 1.0 |
| 10 mM dNTP | 1.0 | 1.0 |
| 10 mM primerAP (direct) | 1.0 | 1.0 |
| 10 mM primerZM2 (reverse) | 1.0 | 1.0 |
| 2 U/μL TAGpol | 1.0 | 1.0 |
| Water, ml | 13.0 | 10.0 |
| DNA, 50 ng | 2.0 | 2.0 |
Table 5 Primers used for PCR analysis

| Title of the primer | Sequence of oligonucleotides (5’ → 3’) | Site of flanking beginning | Site of flanking end |
|---------------------|-----------------------------|---------------------------|---------------------|
| Forward primer AP   | GCTCCTCGTGGCTACTGACC        | 4772                      | 791                 |
| Reverse primer ZM2  | CTTGATGCGCTAAGGGCAGACGCGC  | 5822                      | 848                 |
| Reverse primer ZM5  | GCTAGGGCCTAAGGTCAGGGGC CG  | 5743                      | 766                 |

**Haelli** and BstYI, which were produced by the “SibEnzyme”. Restriction fragments, which were used to determine the genotype of the virus (Table 1), were compared with fixed-length nucleotide sequences, which were synthesized to order in the company “SibEnzyme”.

The significance of the differences was determined by the Student’s criterion [49]. The error of genotype frequency was calculated by Zhivotovsky’s formula [50]. Comparison of genotype frequencies with the maximum permissible mutant allele frequency of 1% [2] was carried out using the Chi-square criterion [49]. Discriminant analysis was conducted using STATISTICA 10 software.

**Abbreviations**

BLV: Bovine Leukemia Virus; PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; IC: Internal control; NC: Negative control; PC: Positive control; LRT-area: Long terminal repeats – the area of the BLV genome; gp: glycoprotein; bp: Bases pair

**Acknowledgements**

The authors thank to the directorship of Novosibirsk State Agricultural University for assistance in funding this publication. We also express our gratitude to the management and team of specialists of the “Doroninskoe farm” (Novosibirsk region, Russia) for their help in obtaining experimental data.

**Declarations**

The manuscript is deposited as a preprint (https://doi.org/10.7287/peerj.preprints.27902v1 | CC BY 4.0 Open Access | rec: 15 Aug 2019, publ: 15 Aug 2019).

**About this supplement**

This article has been published as part of BMC Genetics Volume 21 Supplement 1, 2020: Selected Topics in Systems Biology and Bioinformatics - 2019: genetics. The full contents of the supplement are available online at https://bmcgenet.biomedcentral.com/articles/supplements/volume-21-supplement-1.

**Authors’ contributions**

All authors read and approved the final manuscript. NB development of methods of PCR analysis of LRT-region of BLV, development of the general scientific concept of the article, processing of primary data, statistical processing of experimental data and writing the primary text. SV solution of the manuscript and all chapters of the final text of the article, statistical analysis of the research results, hypothesizing the emergence of new strains of haplotype I, as well as the origin and distribution of haplotype II, preparing the manuscript for publication, working with the editor. VR, KP, AKh, SP – direct sampling of biological material, DNA isolation, PCR reactions and interpretation of its results.

**Funding**

This study was funded by the Ministry of agriculture of Russian Federation (NP AAAA-A20–120012230079-3). The funding body played no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript. Publication costs have been funded by budgetary funds of Novosibirsk State Agrarian University.

**Availability of data and materials**

All data generated or analyzed during this study are included in this published article.

**Ethics approval and consent to participate**

The research was carried out in compliance with existing Russian and international regulations including Basel Declaration (https://www.basel-declaration.org/basel-declaration/). The results of the research were reported on compliance with ethical standards for the treatment of animals at a meeting of the Ethics Committee of the Novosibirsk State Agrarian University, where a unanimous decision was made to allow the article to be published in an international journal. The manuscript does not contain any data about individuals. The data of this article concerning the physiological state of animals (cattle) are submitted for publication with the permission of the head of the Doroninskoye farm (Toguchin district, Novosibirsk region, Russia).

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare no conflict of interests.

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**Published: 22 October 2020**

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