New mutations of *env* gene and its impact on virulence properties for *Bovine leukemia virus*

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This study is the biodiversity and properties of *bovine leukemia virus* in Western Siberia. The researchers focused on exploring the polymorphism of the *env* gene and, in doing so, discovered the new genotypes I_a and I_b, which differ from genotype *I*. Restrictase *Hae III* sections the nucleotide sequence of the *env* gene intofragments with lengths of 316-27-95-5 bp (genotype *I*), 31-285-27-95-5 bp (genotype I_a), and 31-85-200-27-100 bp (genotype I_b). There are 2.57±0.55% (20 out of 779) samples of genotype I_b which does not differ significantly from 1% (χ^2=2.46). Other genotypes were observed in the cattle of Siberia as wild type genotypes (their frequency varied from 17.84 to 32.73 %). This paper explores the effect of the *env* gene of the cattle leukemia virus on hematological parameters of infected animals. 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 I_b (from 700 to 900 viral particles per 1000 healthy cells). Several hypotheses on the origin of the different genotypes in Siberia are discussed. 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* (I_b) was found, which can be both a neoplasm and a relict strain.

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Abstract

This study is the biodiversity and properties of *bovine leukemia virus* in Western Siberia. The researchers focused on exploring the polymorphism of the *env* gene and, in doing so, discovered the new genotypes I_a and I_b, which differ from genotype *I*. Restrictase *Hae III* sections the nucleotide sequence of the *env* gene intofragments with lengths of 316-27-95-5 bp (genotype *I*), 31-285-27-95-5 bp (genotype I_a), and 31-85-200-27-100 bp (genotype I_b). There are 2.57±0.55% (20 out of 779) samples of genotype I_b which does not differ significantly from 1% (χ^2=2.46). Other genotypes were observed in the cattle of Siberia as wild type genotypes (their frequency varied from 17.84 to 32.73 %). This paper explores the effect of the *env* gene of the cattle leukemia virus on hematological parameters of infected animals. 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 I_b (from

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Introduction

Evolution based in mutations, altering the structures and functional properties of proteins (Lewin, 2008; Markov, 2018; Spirin, 1986). The impact of mutations in the structural genes of viruses is important to understand in the fields of livestock farming and veterinary medicine in order to appreciate the virulence of these hazardous viruses (Agol, 2015; Moelling, 2016). The high proportion of latent Bovine leukemia virus carriers (70–90 %) that do not possess clinical symptoms indicates a need to improve upon these dated practices and develop new laboratory methods for detecting *BLV* (Gutiérrez et al., 2014; Gyles, 2016; Juliarena et al., 2017). Old methods – the isolation of sick animals, and the slaughter of infected individuals – are not effective enough(Veterinary Encyclopedia, 2013; Acaite et al., 2007; Knapen et al., 1997; Nuotio et al., 2003; Polatet al., 2017). The most promising new method is PCR analysis (Florins et al., 2012). The *BLV* genome includes four structural genes, of which *env* is the most suitable for detecting the pathogen by PCR analysis (Bateneva, 2015; Dequedt et al., 1995).

The *env* gene is one of four structural *BLV* genes in addition to the *pro*, *gag,* and *pol* genes. The *env* gene is located between the *pol* gene and the 3`LRT-area; it is 1545 bp in length (4615–6160 bp). This gene encodes the glycoprotein viral membrane of *gp*51 and the

transmembrane protein *gp*30. The proteins -*gp*51(SU) and *gp*30 (TM) are glycolized (Sagata et al., 1985; Florins et al., 2007;Barez et al., 2015;UniProtKB - P51519). Protein *gp*30 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 (Alber et al., 1993; Rice et al., 1984). This protein can define the ability of *BLV* to affect the immune system cells of a carrier (Reichert et al., 2001). The *Gp*51 protein reacts with monoclonal cattle antibodies and determines the degree of *BLV* virulence (Johnston et al., 2002). Therefore, qualitative changes in the *gp*30 and *gp*51 proteins that are caused by gene mutations may become the factors that affect the intensity of the immune response. There are 10 genotypes of the *env* gene in the world (Marawan et al., 2017; Rodriguez et al., 2009) and each creates slight differences in the biological properties of the virus.

The theory of exploring viruses and their carriers in the medium organism virus system is gaining popularity. This system implies a mutation in the genome of one of the components that provokes changes in the genome of another component. Cases have been observed when a single mutation in a species facilitates chain of mutations, which can affect entire ecosystems at times (Markow, Naymark, 2015). Coevolution of the parasite carrier is seen as a progress stimulator for both organisms and the variety of antigens of cattle and *BLV* strains makes the research relevant within individual geographical locations. This research aims to definethe 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.

Materials and 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 2016 from the subclavian vein using sterile catheters with EDTA added as an anticoagulant. Cytofluorometric 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 (*gp*51 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 1).

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:

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

Where, m is theamount 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. FLK *BLV* was applied as the positive control and a DNA buffer was used as the negative control. Table 2 illustratesthe sufficient number of components for each reaction.

Primers from Table 3 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. The restriction was realized by the enzymes *HaeIII* and *BstYI*, which were produced by the "SibEnzyme". Statistical processing was conducted using the standard methods (Lakin, 1973; Zhivotovskiy, 1991) with STATISTICA 10 software and Microsoft Excel 2007.

Research 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 4). The effect of the restriction endonuclease *HaeIII* differed from the supposed effect. The fragment restriction lengths of 315, 27, 95, and 5 bp were expected to show up on the electrophorogram as they are considered to be thestandard products of genotype *I* restriction (Smirnov, Bateneva, 2012). However, additional fragments of restriction were found as genotypes I_a and I_b (Table 4, Fig. 1).

Restriction fragments (length 27 bp) are considered to be a common trait for all three genotypes of the family *I*. The common factors for genotypes *I* and I_a are fragments of 95 and 5 bp (Fig.1). Genotype I_b differs from related genetic elements as it combines these fragments into a single one (100 bp length).The common restriction fragments of genotypes I_a and *I* reveal a short sequence (31 bp), whereas the long fragment thatis typical for genotype I_a (285 bp), is represented by two separate factions (85 and 200 bp) (Fig. 1).

The prevalence of thepreviously discovered and explored genotypes varied from 17.84 to 32.73 % with the exception of the I_b genotype (Fig.2). The number of samples with genotype I_a was 20.95±1.46 % (171 out of 779), which differed significantly (χ^2=398; P<0,001) from the statistical and methodological error and is equal to 1% (Lewin, 2008). The number of samples with genotype I_b was 2.57±0.55 % (20 of 779) and didn't differ significantly from 1% (χ^2=2.46). Therefore, of the two new genetic formations that were observed in the cattle of Siberia, only the I_a 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 *I* genotype. 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 I_b 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 5). The differences in the hematological status of the carriers of genotypes I_a, *II,* and *IV* were not significant or they were of a low reliability (P<0.05).

The carriers of the cattle leukemia virus *I*, I_a, *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 5).

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 5). 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 besufficiently high, but not identical.

The investigation into the bovin viral status had unexpected result. The typical sequence of viral load distribution for genotypes on the LRT-area (Blazhko et al., 2019) (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 (Blazhko et al., 2019). 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 5); the highest viral load was observed in the animals infected with *BLV* of *II*- and *IV* genotypes (Fig. 3.). In this case, the degree of immune response is determined not only by the number of viral particles but also by the qualitative changes in *gp*51 (SU) and *gp*30 proteins encoded by the *env* gene (Reichert et al., 2001; Johnston et al., 2002).

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 5, Fig. 2). Early research detected one or two *BLV* genotypes of the *env* gene inone population of cattle (Marawan et al., 2017; Gendzhiyeva, 2012). The observed diversity of the virus may be caused by the massive 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 ofcattle 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 (Marawan et al., 2017; Behavides et al., 2017; Heenemann et al., 2014; Lee et al., 2015;Rola-Łuszczak et al., 2013; Yang et al., 2016; Bateneva et al., 2011). Genotype *IV* was observed in the cattle from Brazil, Belgium, Poland, Ukraine, and France (Lee et al., 2015; Rola-Łuszczak et al., 2013). In Russia, and particularly in Siberia, only genotype *IV BLV* by the *env* gene was observed early. (Rola-Łuszczak et al., 2013; Bateneva et al., 2011). The cattle and sperm from Europe, the US, and Canadawere delivered to Siberiain order to improve local livestock fertility (Zheltikov et al., 2010; Durov et al., 2014), 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 fromSouth America have similar dairy productions to Russian cattle (Klimenok et al., 2014; Picoli et al., 2015). The period of delivery and the different climate conditions betweenSiberia 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* (Lojkić et al., 2013). The research on isolated laboratory strains of *E. coli* (Barrick et al., 2009) and red salmon inhabiting the rivers and lakes of Alaska (Gomez-Uchida et al., 2012) showed the independent directionsof mutagenesisin the isolated populations. The model of

allopatric speciation is based on geographical isolations (Guerrero, Hahn MW, 2017) that helps divergence of morphogenetic characteristics of the strains, breeds, or populations (Markov, 2018; Nikitin et al., 2014; Nikitin, Knyazev, 2015). 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.

The map of restriction fragments (Fig. 1)defines the line of mutations caused by genotype *I* to genotype I_b (Fig. 4A).This statement is arguablebased on the controversial idea that if something had not been discovered before, then it did not exist. Our previous article and other papers (Fitzsimmons et al., 2018; Korboukh et al., 2014;Blazhko et al., 2019) have highlighted the evolutionary advantage of more viral strains overless viral ones. Changes, as a rule, mostly aim at improving the functional properties of an object and its complex structural organization (Markov, Naymark, 2015; Markov, 2018). Therefore, the general evolutionary vector should aim at increasing virulenceas it is supported by the rapid synthesis of viral particles and contributes to the evolutionary advantage of the strain as an intraspecific generation. Analyzing the maximum number of leukocytes and the viral load, the observed genotype I_b as the least virulent and prevalent, followed by genotype I_a. The virus with genotype *I* (Table 5, 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 I_b can be considered as the initial form, and then, due to the accumulation of mutations, the strain I_a 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 I_a is the ancestral one and the mutations that formed genotypes I and I_b were accumulated independently (Fig. 4C). This model seems to be the least attractive as it admits the appearance and consolidation of genotype I_b with a frequency indistinguishable from the mutant one, with the lower number of viral particles than that of genotype I_a, and lower rate of their synthesis. This means a lower reproduction of a virus'sown copies and an evolutionary unattractiveness to this new formation.

Two of thethree 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

1. The research discovered5 *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%.
2. The highest number of leukocytes was observed in the blood of animals infected with the BLVI genotype. Hematological status differed in respect to sick animals (P<0,001) and the sample as a whole (P<0,001).
3. 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 was slightly lower, varyingfrom 700 to 900.
4. 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

231 hypotheses, genotypes I_a and I_b are new growths that originated in Western Siberia due to
232 mutagenesis. Two other theories suggest that the explored genotypes are relict forms of BLV that
233 have been displaced in the cattle by more progressive strains.

234 **Conflict of interest**
235 The authors declare no conflict of interests
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Figure 1

Scheme of *HaeIII* restriction with formation of genotypes

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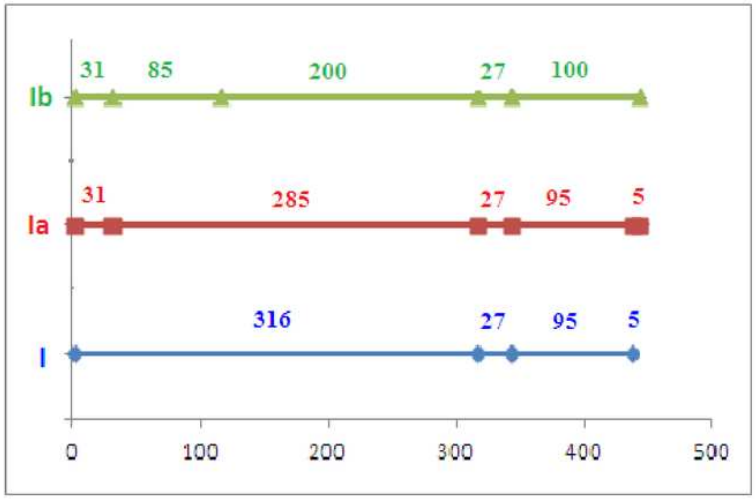

Figure 1.Schemeof*HaeIII* restriction with formation of genotypes

Figure 2

Frequency of *env* gene genotypes (%) in Western Siberia

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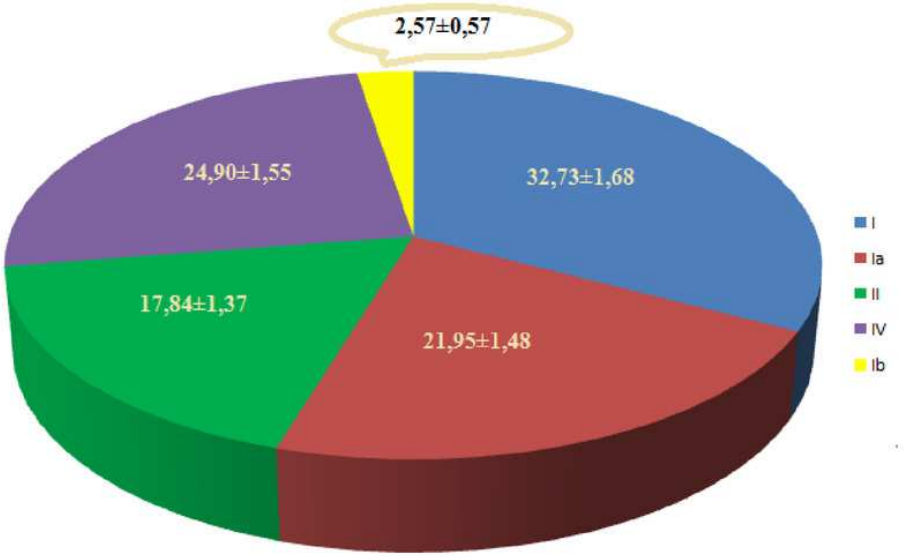

Figure 2. Frequencyof *env* gene genotypes (%)in Western Siberia

Figure 3

Distribution of viral load at different stages of infection progress in relation toheterogeneity of *env* gene *BLV*

λWilks' =0,66912, F = 28,406, p=0,0000, confidence intervals -95 %

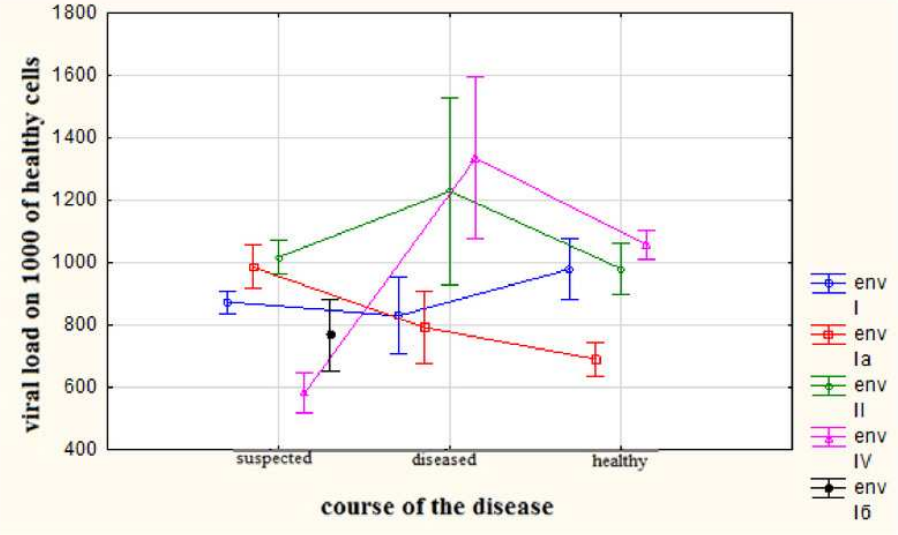

Figure 3. Distribution of viral load at different stages of infection progress in relation to heterogeneity of *env* gene *BLV* (λWilks' =0,66912, F = 28,406, *p*=0,0000, confidence intervals - 95 %)

Figure 4

Hypotheses of *BLV* genotype I_b on env gene origination

Are presented several variants for origin of *BLV* genotype I_b on env gene in Western Sibiria.

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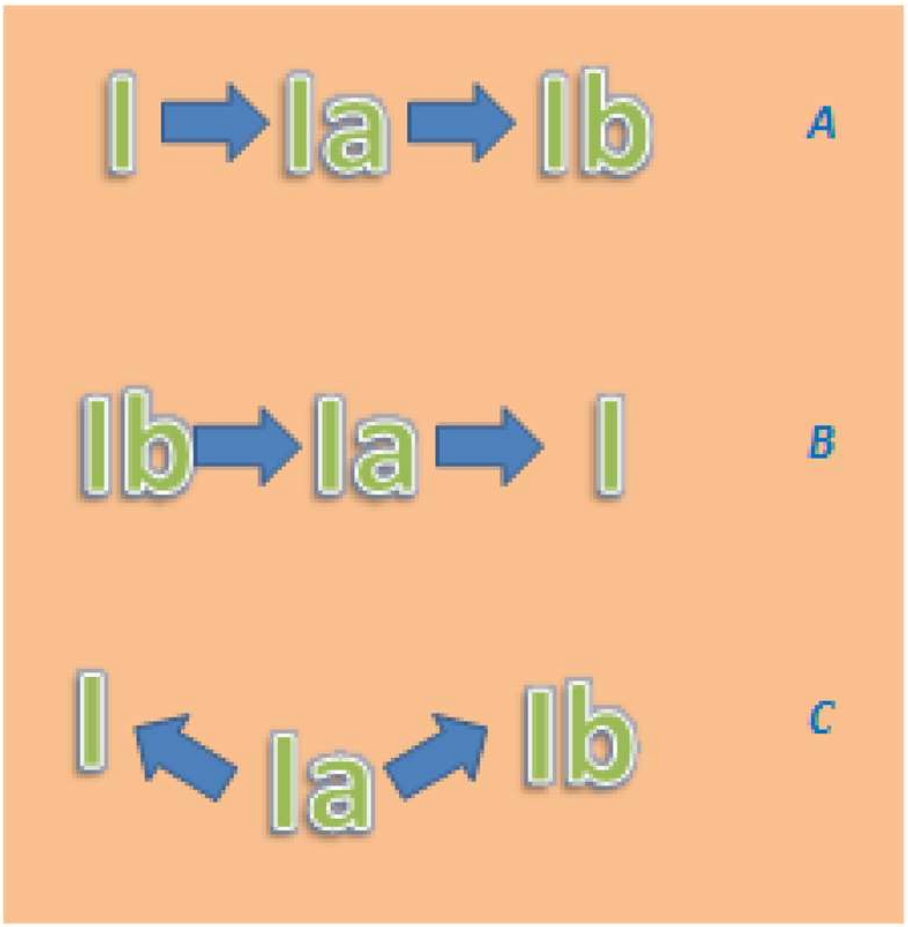

Figure 4.Hypotheses of *BLV* genotype I on *env* gene origination

Table 1(on next page)

Temperature profile of PCR

1 Table 1. Temperature profile of PCR

Reaction 1			Reaction 2		
Number of cycles	Temperature, °C	Period of time	Number of cycles	Temperature, °C	Period of time
1	95	3 min	1	95	3 min
34	95	30 sec	34	95	30 sec
	62	30 sec		66	30 sec
	72	2 min		72	2 min
1	72	10 min	1	72	10 min
Storage	4	< 12 hours	Storage	4	< 12 hours

2

Table 2(on next page)

Composition of reaction mixture

Table 2. Composition of reaction mixture

Mixture components	Necessary amount, mcl	
	Reaction1	Reaction2
10xof optimized DNA-buffer	5.0	5.0
10 mM $MgCl_2$	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/μLTAGpol	1.0	1.0
Water	13.0	10.0
DNA, 50 ng	2.0	2.0

Table 3(on next page)

Primers used for PCR analysis

1 Table 3.Primers used for PCR analysis

Title of the primer	Sequence of oligonucleotides (5'->3')	Site of flanking beginning	Site of flanking end
Forvard primer AP	GCTCTCCTGGCTACTGACC	4772	4791
Reverse primer ZM2	CTCTGATGGCTAAGGGCAGACACGGC	5822	5848
Reverse primer ZM5	GCTAGGCCTAAGGTCAGGGCCGC	5743	5766

2

3

Table 4(on next page)

Scheme of genotypes formed by restrictases *HaeIII* and *BstYI*. The table highlights the fragment restrictions with length (b.p.)

Table 4.Scheme of genotypes formed by restrictases *HaeIII* and *BstYI*. The table highlights the fragment restrictions with length (b.p.)

Haplotype	Restrictase	
	Hae III	*BstYI*
I	316-27-95-5	–
I_a	31-285-27-95-5	–
I_b	31-85-200-27-100	–
II	–	529-322-143
IV	–	672-322

Table 5(on next page)

Cytometric and morphological parameters of blood of animals-carriers of different genotypes of *BLV*

Wilks' lambda statistic=0,82285, F(12, 1530)=13,056, p=0,0000

Table5. Cytometric and morphological parameters of blood of animals-carriers of different genotypes of *BLV*

env	Hematological status	WBC, 10 $^{9/l}$				lymf, 10 $^{9/l}$ -				*n*
		$\bar{X}$	$S_{\bar{x}}$	-95%	+95%	$\bar{X}$	$S_{\bar{x}}$	-95%	+95%	
I	suspected	12,30	0,072	11,31	13,30	5,60	0,104	4,17	7,03	210
	sick	24,18	1,197	19,49	28,87	13,65	0,447	11,90	15,41	18
	healthy	4,95	0,294	3,53	6,38	2,45	0,214	1,42	3,49	27
	On average	12,36	0,175	11,06	13,42	5,83	0,140	4,42	7,25	255
I_a	suspected	11,26	0,251	9,48	13,04	5,11	0,200	3,70	6,53	56
	sick	19,82	0,633	16,75	22,89	12,38	0,343	10,96	13,80	20
	healthy	7,06	0,242	4,92	9,30	3,08	0,155	1,98	4,10	95
	On average	9,93	0,291	7,80	12,11	4,83	0,192	3,59	6,03	171
II	suspected	9,73	0,201	7,84	11,61	4,09	0,149	2,70	5,48	97
	sick	17,40	0,201	14,58	20,22	9,36	0,566	8,60	10,12	3
	healthy	6,81	0,305	5,00	8,62	3,04	0,186	1,94	4,14	39
	On average	9,08	0,230	7,19	10,96	3,91	0,168	2,61	5,20	139
IV	suspected	11,70	0,352	8,94	14,45	5,44	0,211	3,76	7,07	68
	sick	17,25	1,300	15,16	19,39	10,50	0,514	9,69	11,38	4
	healthy	8,36	0,259	5,64	11,08	3,57	0,120	2,32	4,83	122
	On average	9,71	0,313	6,99	12,43	4,37	0,160	2,98	5,75	194
I_b	suspected	11,99	0,378	9,78	12,93	5,68	0,349	4,23	7,13	20

Wilks' lambda statistic=0,82285, F(12, 1530)=13,056, p=0,0000