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Association of haplotypes for SNPs in the LTR regions of bovine leukemia virus with hematological indices of cattle

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Molecular typing of *BLV* samples isolated from Holsteinized Russian Black Pied cattle was carried out, and various cytofluorometric and morphological blood indices were examined. We performed the total count of white blood cells (WBC), lymphocyte (lymf), granulocyte (gran), monocyte (mon), red blood cell (RBC), hemoglobin (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW), and platelet crit count (PCT). The LTR-region of *BLV* was haplotyped. Only viruses of haplotypes I (0.33 ± 0.03) and III (0.67 ± 0.03) of the eight possible were detected. The ratio of hematologically sick, healthy, and suspected carriers of *BLV* of haplotypes I and II was comparable with the results of other researchers. The numbers of leukocytes, erythrocytes and platelets in the blood of carriers of haplotype III exceeded the corresponding parameters of cattle affected by the virus of haplotype I. It is interesting to note that the difference in the hemolytic status of animals was manifested not only by the concentration of leukocytes as direct immune agents but also by the count of erythrocytes and platelets, which are not directly involved in the immune response. The number of particles of haplotype III of the *BLV* circulating in the blood of infected individuals exceeded that of the carriers of haplotype I. In this connection, an assumption was made about the evolutionary advantage of the more virulent haplotype III. However, the results of our own research in conjunction with the data of other scientists indicate that the high virulence of individual virus strains is a consequence of the tendency to implement the maximum possible intensity of the synthesis of virus particles but not of the high damaging effect alone. It is shown that high lethality is evolutionarily disadvantageous for viruses, since the extinction of the carrier as a biological species is fraught with the disappearance of the virus itself.
Key words: *BLV*; LTR-region; haplotypes; hematological indices; leukocytes; cattle.

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Связь гаплотипов SNP LTR-области *BLV* с гематологическими показателями крови крупного рогатого скота

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Проведено молекулярно-генетическое типирование образцов вируса лейкоза крупного рогатого скота (*BLV*), выделенного из образцов крови черно-пестрых голштинизированных коров, у которых были исследованы различные цитофлюорометрические и морфологические показатели крови. Оценивали общее содержание лейкоцитов (WBC), содержание лимфоцитов (lymf), гранулоцитов (gran), моноцитов (mon), эритроцитов (RBC), гемоглобина (HGB), гематокрит (HTC), средний объем эритроцитов (MCV), среднее содержание гемоглобина в одном эритроците (MCH), концентрацию гемоглобина в эритроцитарной массе (MCHC), индекс распределения эритроцитов (RDW), количество тромбоцитов (PLT), средний объем тромбоцитов (MPV), индекс распределения тромбоцитов (PDW) и тромбокрит (PCT). Определены гаплотипы SNP LTR-области *BLV*. Из восьми возможных были обнаружены только вирусы гаплотипов I (0.33 ± 0.03) и III (0.67 ± 0.03). Соотношение гематологически больных, здоровых и подозрительных носителей вируса лейкоза крупного рогатого скота I и III гаплотипов было сопоставимо с результатами других исследователей. Количество лейкоцитов, эритроцитов и тромбоцитов в крови носителей III гаплотипа превышало аналогичные параметры крупного рогатого скота, пораженного вирусом I гаплотипа. Интересно отметить, что разница

гематологического статуса животных проявилась не только в концентрации лейкоцитов, непосредственных иммунных агентов, но и в содержании эритроцитов и тромбоцитов, не имеющих к иммунному ответу непосредственного отношения. Количество частиц III гаплотипа *BLV*, циркулирующих в крови зараженных особей, превышала таковое значение носителей I гаплотипа. В связи с этим выдвинуто предположение об эволюционном преимуществе III гаплотипа как более вирулентного. Впрочем, результаты настоящего исследования в совокупности с данными других ученых показывают, что высокая вирулентность отдельных штаммов вируса есть следствие стремления к реализации максимально возможной интенсивности синтеза вирусных частиц, а не достижения высокого поражающего эффекта как такового. Показано, что высокая смертоносность эволюционно невыгодна вирусам, так как вымирание носителя как биологического вида чревато исчезновением и самого вируса.
Ключевые слова: *BLV*; LTR-область; гаплотипы; гематологические показатели крови; лейкоциты; крупный рогатый скот.

Introduction

The bovine leukosis epizootic situation inspires intensive development of strategies aimed at preventing the spread of the disease. It comes down to the isolation of sick animals from healthy ones or to the slaughter of infected individuals (Knapen et al., 1993; Nuotio et al., 2003; Acaite et al., 2007). The latter method proved to be very effective in the countries of Western Europe, New Zealand and Australia, where the purification of herds from the pathogen (*BLV*) is complete or almost complete (Polat et al., 2017).

Despite these measures, bovine leukosis is by far the most common epizootic disease in Russia and some other countries (Juliarena et al., 2017). It was identified in 28 regions of the Russian Federation (175 adverse sites) in 2017. The largest number of adverse sites for the disease, 45, was found in Kaluga region; in the Republic of Crimea, 32; Novosibirsk oblast, 27; and Moscow oblast, 20 (Novikova et al., 2018). According to some data (Kozyreva, Gulyukin, 2017), leukosis constituted about 65–66 % of the cases of infectious diseases in 2015.

One of the likely reasons for the low efficiency of bovine leukosis control is the high percentage (70 to 90 %) of animals with the asymptomatic stage (Ernst et al., 1997; Smirnov Yu.P. et al., 2015; Gyles, 2016; Juliarena et al., 2017), as characterized, among other things, by the normal nonpathological number of leukocytes, in particular, lymphocytes. The clinical stage is typically observed in 4 to 5-year-old animals, where, in the overwhelming majority of cases, the economic use of dairy cows is nearing its completion (Smirnov P.N. et al., 2015). Sometimes the latent period can be delayed to 8 years of age (Kettmann et al., 1994). In some cases, slaughter of animals infected with *BLV* but culled for other reasons not related to the clinical manifestations of leukosis was recorded (Mishchenko et al., 2018).

PCR diagnostics (Smirnov P.N. et al., 2015) and enzyme-linked immunosorbent assay (ELISA) (Syurin et al., 2001) are effective methods for identifying *BLV* carriers, but their high cost significantly hinders widespread use.

Another complicating factor is the high mutational variability associated with viruses (Lewin, 2008). In particular, there is a hypothesis about the accumulation of *BLV* mutations that allow the virus to avoid the host's immune response (Blood et al., 1979; Syurin et al., 2001; Buehring et al., 2003; Smirnov, 2007; Smirnov et al., 2011; Batenyova, 2015). For the virus itself, such mutations are undoubtedly beneficial and therefore must be supported by natural selection.

The LTR region contains the so-called housekeeping genes, among which there are regulators of mRNA transcription and translation. The collinear nucleotide sequence of the virus studied shows that at least some of the evolutionary phenotypic "acquisitions" stem from mutations not in protein-coding genes, but in the household genes, due to which mutagenesis is accelerated (Barrick et al., 2009). Indeed, mutations in the nucleotide sequence of the LTR region can activate mutagenesis in *BLV* (Merezak et al., 2001). A study of the LTR regions of other viruses gave similar results (Moelling, 2016). It is logical to assume that it was mutations in the LTR-region of *BLV* that could contribute to the evolutionary flexibility of the virus and provoke its ability to avoid the host's immune response. It is likely that more virulent strains that efficiently translate mRNA have the maximum advantage, thereby causing the greatest damage to the carrier. The aim of our research is to evaluate the hypothesis of the evolutionary advantage of mutant *BLV* strains due to higher virulence.

Materials and methods

Experiments were done with total DNA samples isolated from 780 cows of the Holsteinized Russian Black Pied breed. Blood samples were taken from the tail vein with sterile catheters using EDTA as an anticoagulant in 2015–2016. DNA was isolated with the DNA-Sorb-B kit (Central Research Institute of Epidemiology, Russia).

Cytofluorimetric and morphological blood indices were determined with an automatic veterinary haematology analyzer PCE-90 Vet.

Oligonucleotide primers were designed with regard to the mutational status of the isolate sequences. The annealing temperature of the primers was calculated from the percentages of nucleotides in the oligonucleotide sequences (Table 1).

Primers were synthesized according to the sequence in an automatic oligonucleotide synthesizer. Primer purity was monitored by High Sensitivity Gas Chromatography and found to be no less than 95 %. Primers were stored at –20 °C for no more than six months.

Table 1. Characteristics of oligonucleotide primers flanking the LTR region of *BLV*, 443 bp

Criterion	Forward primer	Reverse primer
Sequence (5′->3′)	CCCCATRCGACCGGTTACAC	AGAGRRCTCRGGACCGAGAG
Flanking start site	8021	8444
Flanking end site	8040	8463
Annealing t°	60.18	60.11
GC, %	60.00	60.00

Table 2. The amplification mode for LTR 443 bp

Number of cycles	Temperature, °C	Time, min
1	95	3.0
35	95	0.5
	61	0.5
	72	0.5
1	72	3.0

Table 3. Composition of the PCR mixture (per one reaction)

Components	Required volume, µL
PCR buffer	2.5
$MgCl_2$	1.0
dNTP	1.0
Pr 1, 50 ng	1.0
Pr 2, 50 ng	1.0
Taq pol	1.5
Water	15.0
DNA, 50 ng	2

Before starting the polymerase chain reaction, the amplification mode was programmed. Based on the calculated annealing temperature of the primers, a special program was used to program the amplifier (Table 2).

The required number of components of the reaction was calculated for the required number of samples in order to prepare the PCR mixture. We determined the total number of reactions as $n + 3 + 1$, where n is the number of DNA samples that need to be diagnosed; 3 is the number of controls used in the reaction (IC is the internal control of the PCR setting; NC is the negative control reactions; PC is the positive control of the reaction); 1 is for the PCR mixture for an additional calculated sample. The calculation of the volume of each component of the mixture was made in accordance with Table 3.

The following reagents were added to the control tubes: positive control (PC), internal control (IC), and negative control (NC). Standard BLV FLK was used as a PC; a pair of specific primers for bovine DNA was used as an IC; DNA buffer was used as a NC.

Twenty microliters of mineral oil were layered on top of the mixture (in case of using amplifiers with a nonheated lid). The amplification products were resolved by electrophoresis in an agarose gel slab and visualized on a transilluminator.

To purify the amplification products from nonspecific fragments, the luminous strips were cut out of the gel on a transilluminator and the amplificates were isolated by the spin column method.

Maps of hypothetical LTR-region restriction sites were compiled. The restriction sites were: *BstMA* I (237 bp), *Bse*1 (378, 370 bp), and *BspAC* I (262 bp). To analyze possible combinations of substitutions on the selected sequence of the *BLV* genome region, a typing method was developed. Substitutions at the genome sites of 8034 and

Table 4. The formation of haplotype layout

Haplotype	The products of restriction fragments		
	BstMA I – GTCTCN↑CAGAG$(N)_5$↓	*Bse*1 – ACTGGN↑TGAC↓CN	*BspAC* I – C↑CGC GGC↓G
I	237 (A)	378 (GC)	262 (CG)
II	237 (A)	378 (GC)	262 (AG)
III	237 (A)	370 (CN)	262 (CG)
IV	237 (A)	370 (CN)	262 (AG)
V	– (N)	378 (GC)	262 (CG)
VI	– (N)	378 (GC)	262 (AG)
VII	– (N)	370 (CN)	262 (CG)
VIII	– (N)	370 (CN)	262 (AG)

8139 base pairs were analyzed by restriction fragment length polymorphism. According to the results of PCR, two viral haplotypes were identified: I and III (Table 4).

Statistical processing of the data obtained was carried out by conventional methods (Lakin, 1973; Zhivotovsky, 1991) using the STATISTICA 10 software package.

Results

Infected animals of the studied population ($n = 780$) were represented by carriers of haplotypes I (0.33 ± 0.03) and III (0.67 ± 0.03) out of the eight possible haplotypes of *BLV*. When analyzing the blood indices of animals infected with leukemia of different haplotypes, it was found that the animals carrying haplotype III had a higher ($p < 0.001$) absolute content of all types of leukocytes. However, the percentage of monocytes did not show significant differences (Table 5). The following hematological indices were estimated: total leukocyte count (WBC), lymphocyte count (lymf), granulocyte (gran), monocyte (mon), erythrocyte (RBC), hemoglobin (HGB), hematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), erythrocyte distribution width (RDW), platelet count (PLT), mean platelet volume (MPV), platelet distribution width (PDW) and platelet crit count (PCT) (See Table 5).

It is noteworthy that the difference was noticed not only in the concentration of leukocytes but also in the number of erythrocytes and platelets, which do not have a direct relationship to the immunity of animals (See Table 5).

The facts indicate that the recognition of a particular strain of leukemia virus by the immune system of cattle begins even before the transition of the disease to the clinical stage. The level of leukocytes in the blood of animals affected by the virus of haplotype III was higher ($p < 0.001$) regardless of whether the animals had clinical symptoms of leukemia, had a latent stage, or belonged to the group with suspected leukemia (Table 6). In animals with a high content of leukocytes in the blood, which are classified as leukemia suspects, the levels of leukocytes in carriers of different haplotypes of *BLV* differed significantly. However, due to the clear limitation of the level of the indicator in this group the difference between the groups was more than 2×10^9/L.

On the average, differences in the leukocyte count in the diseased and the suspected animals exceed those between the suspected and the healthy animals (See Table 6). This is especially noticeable in visual comparison, when the cluster of infected individuals shows a varying degree of dependence on which *BLV* haplotype infected the studied cattle (Fig. 1). Differences in leukocyte count between animals of different hemolytic status were significant

Table 5. Cytometric and morphological blood indices of animals carrying different *BLV* haplotypes

Index	Haplotype I				Haplotype III				t_ϕ
	$\overline{X}$	$S_{\overline{X}}$	$D[\overline{X}]$	95 %	$\overline{X}$	$S_{\overline{X}}$	$D[\overline{X}]$	95 %	
WBC, 10^9/L	8.87	0.10	5.47	0.26	14.12	0.27	18.65	0.54	$p < 0.001$
lymf, 10^9/L	5.98	0.07	2.25	0.17	9.85	0.21	11.50	0.42	$p < 0.001$
mon, 10^9/L	0.73	0.01	0.06	0.03	1.15	0.03	0.17	0.05	$p < 0.001$
gran, 10^9/L	4.16	0.06	1.71	0.15	5.89	0.10	2.66	0.20	$p < 0.001$
lymf, %	54.33	0.42	93.28	1.09	67.72	0.76	147.13	1.50	$p < 0.001$
mon, %	8.43	0.08	3.55	0.21	8.55	0.19	9.11	0.37	nsd
gran, %	43.24	0.42	92.25	1.09	33.56	0.73	132.16	1.43	$p < 0.001$
RBC, 10^{12}/L	5.10	0.04	0.78	0.10	5.39	0.07	1.15	0.13	$p < 0.001$
HGB, g/L	90.23	0.75	293.64	1.93	93.20	1.21	365.85	2.38	$p < 0.05$
HTC, %	26.50	0.20	20.67	0.51	27.55	0.32	25.45	0.63	$p < 0.01$
MCV, fl	52.26	0.18	17.49	0.47	51.65	0.35	30.85	0.69	nsd
MCH, pg	17.78	0.13	9.50	0.35	17.57	0.23	13.75	0.46	nsd
MCHC, g/L	341.58	2.30	2772.97	5.94	337.98	3.92	3875.45	7.72	nsd
RDW, %	15.43	0.05	1.29	0.13	15.93	0.07	1.24	0.14	$p < 0.001$
PLT, 10^9/L	236.78	4.52	10725.82	11.69	214.60	5.22	6854.52	10.27	$p < 0.001$
MPV, fl	6.93	0.03	0.47	0.08	6.78	0.04	0.44	0.08	nsd
PDW	16.99	0.02	0.30	0.06	16.85	0.03	0.28	0.07	nsd
PCT, %	1.26	0.64	211.71	1.64	0.13	0.00	0.00	0.01	nsd

Notes: t_ϕ – the significance of differences determined by Student's test; nsd – no significant difference.

Table 6. Decomposition of hypotheses about the influence of the LTR haplotype on the course type of infection

LTR	HS	Leukocytes, 10^9/L				Lymphocytes,10^9/L				*n*
		$\overline{X}$	$S_{\overline{x}}$	–99.90 %	+99.90 %	$\overline{X}$	$S_{\overline{x}}$	–99.90 %	+99.90 %	
III	Suspected	12.762	0.128	12.333	13.190	5.788	0.107	5.431	6.145	240
III	Diseased	21.889	0.938	18.536	25.243	12.982	0.697	10.490	15.473	7
III	Healthy	10.900	0.125	10.711	11.240	3.700	0.105	3.500	4.000	281
I	Suspected	10.423	0.104	10.077	10.769	4.682	0.086	4.396	4.969	211
I	Diseased	17.314	0.040	17.074	17.555	10.014	0.571	6.612	13.416	38
I	Healthy	7.339	0.082	7.066	7.612	3.222	0.052	3.049	3.396	3

Notes: HS is the hematological status; $\lambda = 0.982$ at $p < 0.001$; F (4.1546) = 3.3451.

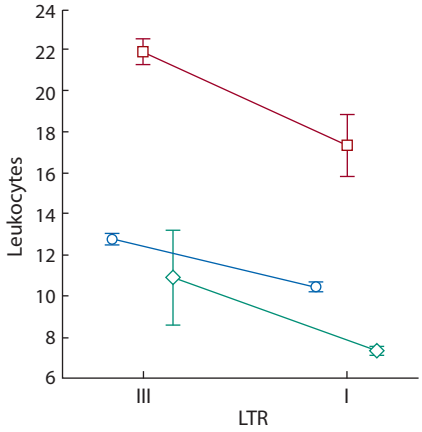

Fig. 1. The haplotype influence on the leukocyte count in healthy, suspected, and diseased animals ($\lambda = 0.982$; F (4.154) = 3.345; $p = 0.0098$).

The red line indicates the number of leukocytes in animals with clinical symptoms of leukemia, blue – in individuals with suspected leukemia, green – in healthy.

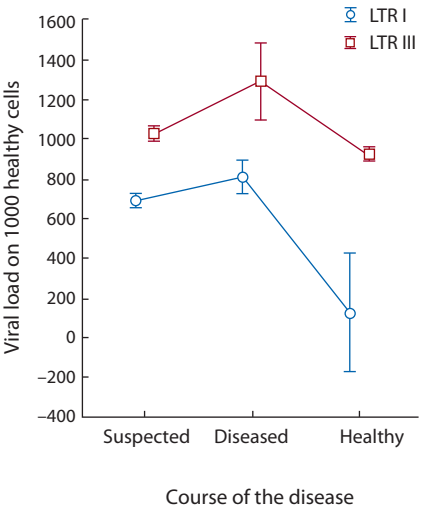

Fig. 2. Viral load in animals with different *BLV* haplotypes.

($p < 0.001$) except for the "healthy – suspected" difference for haplotype III. The proportion of infected animals is 15.08 % in cattle infected by the virus of haplotype I and 1.33 % in carriers of haplotype III.

A similar picture was observed in lymphocyte count in the blood of the animals. Haplotype certainly affects the change in the level of lymphocytes, although the groups had a low level of discrimination (See Table 6).

The level of viral load was in direct proportion to the status of the animals. The greatest number of viral particles was found in the blood of infected animals and the smallest, in animals without clinical signs of infection. It is interesting to note that in general, viral load was higher in cattle infected with haplotype III (Fig. 2).

For comprehensive evaluation of the hypothesis of the influence of categorical features on continuous variables, multidimensional criteria were used (Table 7). The values of the Wilks criteria (Wilks' Lambda, WL), Pillai's criteria (Pillai's Trace, PT), Hotelling's criteria (Hotelling's Trace, HT), and Roy's criteria (Roy's Largest Root, RLR) reveal a significant association of *BLV* LTR haplotypes with the process type of the infection, which is expressed, among other indices, in the count of leukocytes.

Discussion

A greater number of different types of leukocytes were found in animals infected with *BLV* haplotype III compared to the carriers of haplotype I (See Table 5). This observation points to a stronger immune response of the cattle organism to this particular type of the virus. It is possible that the virus of haplotype III is more virulent, as also indicated by the larger number of virus particles in comparison to haplotype I (See Fig. 2). The fact that the former haplotype is more common is indicative of an evolutionary advantage of more virulent strains over less virulent. Similar results were obtained in many experiments with RNA viruses of mice, rats, and rabbits (Furió et al., 2012; Elsworth et al., 2014; Korboukh et al., 2014; Fitzsimmons et al., 2018). Moreover, in some cases there was an increase in the evolutionary flexibility of viruses. For example, *H273R* – due to changes in the nucleotide sequences of mutant genes that accelerate mutagenesis (Korboukh et al., 2014).

The assumption of the evolutionary advantage of more virulent strains of the bovine leukemia virus looks convincing. The main objective of viruses, including *BLV*, is not the destruction of the cells of the host organism but the production of the maximum possible number of their own particles, which is perfectly reasonable (Agol, 2015). It has been proven that the evolutionary advantage of any mutations is determined primarily not by qualitative changes but by the survival rate of the greatest possible number of descendants of mutation carriers. This rule applies not only to viruses but to all representatives of all taxonomic kingdoms (Markov, Naimark, 2015). And one of the truly harmful qualities of viruses is not their virulence as such but the immune responses provoked by a

Table 7. Testing the null hypothesis about the absence of haplotype influence on the type of the infection

Index	Criterion	Important	F	dfEffect	dfError	*p*
Free term	WL	0.197743	1568.054	2	773	0.000000
	PT	0.802257	1568.054	2	773	0.000000
	HT	4.057061	1568.054	2	773	0.000000
	RLR	4.057061	1568.054	2	773	0.000000
LTR	WL	0.935624	26.593	2	773	0.000000
	PT	0.064376	26.593	2	773	0.000000
	HT	0.068805	26.593	2	773	0.000000
	RLR	0.068805	26.593	2	773	0.000000
Hematological status	WL	0.619193	104.675	4	1546	0.000000
	PT	0.380882	91.038	4	1548	0.000000
	HT	0.614883	118.672	4	1544	0.000000
	RLR	0.614686	237.884	2	774	0.000000
LTR+hematological status	WL	0.982912	3.345	4	1546	0.009764
	PT	0.017150	3.347	4	1548	0.009730
	HT	0.017322	3.343	4	1544	0.009799
	RLR	0.012124	4.692	2	774	0.009430

Notes: dfEffect – degree of freedom of the test; dfError – degree of freedom of the residual error.

large number of viral particles: degradation of RNA (both viral and cellular), suppression of protein synthesis (both viral and cellular), self-destruction (apoptosis and other types of programmed cell death), and, finally, inflammation (Debacq et al., 2004; Lezin et al., 2009; Agol, 2015). When we assume that *BLV* does not alter the host complex of the synthesized proteins without introducing anything of "its own" (Kettmann et al., 1980), such a theory looks quite plausible. Since *BLV* is not a carrier of a program encoding foreign proteins, its harmfulness can increase only through accelerated synthesis of its own copies. The result is the self-destructive response of the immune system of cattle.

The fact that the higher virulence of *BLV* haplotype III is a consequence of the acceleration of its replication and not vice versa is confirmed by the following data. The virus does not program carrier cells for the synthesis of extraneous particularly harmful proteins; in contrast, it carries genetic elements that activate the immunity of the cattle (Lagarias, Radke, 1989; Juliarena et al., 2017). At the first glance, it is a "suicidal" evolutionary acquisition that should have led to the extinction of *BLV* as such. But in reality, this provoked the creation of a certain complex of coexistence of cattle and *BLV*, where the virus is the carrier of the genetic system aimed at neutralizing the inevitable harmful presence of the virus in the carrier. As a result, 70–90 % of the cattle do not show any clinical signs of leukemia, which in general is shown in the present study (See Table 6).

Thus, the *BLV* genetic program provides not only the intensification of the synthesis of its own particles, but also mitigation of the negative consequences for the cattle. And the evolutionary strategy can be traced quite clearly: the virus does not benefit from the extinction of cattle as a biological species since this would lead to its own extinction.

Conclusions

The research results are consistent with the presented concept. Mutations in the genes of the LTR region of *BLV* initially provoked an acceleration of viral particle synthesis, which in turn caused a more intense immune response in cattle (See Table 5, Fig. 1). Thus, the evolutionary advantage of haplotype III of *BLV* over haplotype I is expressed in the accelerated reproduction of its copies, which is ultimately reflected in the higher prevalence of haplotype III (0.67 ± 0.03) compared to haplotype I (0.33 ± 0.03).

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