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*ₐ, which differ from genotype *I*. Restrictase *Hae III* sections the nucleotide sequence of the *env* gene into fragments 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*ₐ). There are 2.57±0.55% (20 out of 779) samples of genotype *I*ₐ which do not differ significantly from 1% ($\chi^2$=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*ₐ (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*ₐ) 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 into fragments 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% ($\chi^2=2.46$). 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.

**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 gp51 and the
transmembrane protein gp30. The proteins -gp51(SU) and gp30 (TM) are glycolized (Sagata et al., 1985; Florins et al., 2007; Barez et al., 2015; UniProtKB - P51519). 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 (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 Gp51 protein reacts with monoclonal cattle antibodies and determines the degree of BLV virulence (Johnston et al., 2002). 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. 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 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.

**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 (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 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 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. FLK BLV was applied as the positive control and a DNA buffer was used as the negative control. Table 2 illustrates the 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 the standard 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 that is typical for genotype I_a (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 $I_b$ genotype (Fig.2). The number of samples with genotype $I_a$ was 20.95±1.46 % (171 out of 779), which differed significantly ($\chi^2=398; P<0.001$) 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% ($\chi^2=2.46$).

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 be sufficiently 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 gp51 (SU) and gp30 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 in one 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 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 (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 Canada were delivered to Siberia in 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 from South 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 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 (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 directions of mutagenesis in 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 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 (Fitzsimmons et al., 2018; Korboukh et al., 2014; Blazhko et al., 2019) 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 (Markov, Naymark, 2015; Markov, 2018). Therefore, the general evolutionary vector should aim at increasing virulence as 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’s 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

1. The research discovered 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%.

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, varying from 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
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.

**Conflict of interest**

The authors declare no conflict of interests

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Figure 1

Scheme of *Haelll* restriction with formation of genotypes
Figure 1. Scheme of *HaeIII* restriction with formation of genotypes
Figure 2

Frequency of env gene genotypes (%) in Western Siberia
Figure 2. Frequency of env gene genotypes (%) in Western Siberia
Figure 3

Distribution of viral load at different stages of infection progress in relation to heterogeneity of env gene BLV

\[ \lambda_{\text{Wilks'}} = 0.66912, F = 28.406, p = 0.0000, \text{ confidence intervals } -95\% \]
Figure 3. Distribution of viral load at different stages of infection progress in relation to heterogeneity of env gene BLV ($\lambda$ Wilks' $\chi^2$ =0.66912, F = 28,406, p=0.0000, confidence intervals - 95 %)
Figure 4

Hypotheses of *BLV* genotype I₄ on env gene origination

Are presented several variants for origin of *BLV* genotype I₄ on env gene in Western Siberia.
Figure 4. Hypotheses of BLV genotype I on env gene origination
Table 1 (on next page)

Temperature profile of PCR
Table 1. Temperature profile of PCR

<table>
<thead>
<tr>
<th>Number of cycles</th>
<th>Temperature, °C</th>
<th>Period of time</th>
<th>Number of cycles</th>
<th>Temperature, °C</th>
<th>Period of time</th>
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<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>3 min</td>
<td>1</td>
<td>95</td>
<td>3 min</td>
</tr>
<tr>
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<td>95</td>
<td>30 sec</td>
<td>34</td>
<td>95</td>
<td>30 sec</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>30 sec</td>
<td></td>
<td>66</td>
<td>30 sec</td>
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<td>2 min</td>
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<td>1</td>
<td>72</td>
<td>10 min</td>
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<tr>
<td>Storage</td>
<td>4</td>
<td>&lt; 12 hours</td>
<td>Storage</td>
<td>4</td>
<td>&lt; 12 hours</td>
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Table 2 (on next page)

Composition of reaction mixture
Table 2. Composition of reaction mixture

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<tr>
<th>Mixture components</th>
<th>Necessary amount, mcl</th>
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<td></td>
<td>Reaction1</td>
</tr>
<tr>
<td>10x of optimized DNA-buffer</td>
<td>5.0</td>
</tr>
<tr>
<td>10 mM MgCl₂</td>
<td>1.0</td>
</tr>
<tr>
<td>10 mM dNTP</td>
<td>1.0</td>
</tr>
<tr>
<td>10 mM primerAP (direct)</td>
<td>1.0</td>
</tr>
<tr>
<td>10 mM primerZM2 (reverse)</td>
<td>1.0</td>
</tr>
<tr>
<td>2 U/µLTAGpol</td>
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</tr>
<tr>
<td>Water</td>
<td>13.0</td>
</tr>
<tr>
<td>DNA, 50 ng</td>
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</tr>
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</table>
Table 3 (on next page)

Primers used for PCR analysis
Table 3. Primers used for PCR analysis

<table>
<thead>
<tr>
<th>Title of the primer</th>
<th>Sequence of oligonucleotides (5’-&gt;3’)</th>
<th>Site of flanking beginning</th>
<th>Site of flanking end</th>
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<tr>
<td>Forvard primer AP</td>
<td>GCTCTCCTGGCTACTGACC</td>
<td>4772</td>
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<tr>
<td>Reverse primer ZM2</td>
<td>CTCTGATGGCTAAGGGCAGACACGC</td>
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<td>Reverse primer ZM5</td>
<td>GCTAGGCCCTAAGGTCAGGGCCGC</td>
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<td>5766</td>
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</table>
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.).

<table>
<thead>
<tr>
<th>Haplotype</th>
<th><em>Hae III</em></th>
<th><em>BstYI</em></th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>316-27-95-5</td>
<td>–</td>
</tr>
<tr>
<td>I&lt;sub&gt;a&lt;/sub&gt;</td>
<td>31-285-27-95-5</td>
<td>–</td>
</tr>
<tr>
<td>I&lt;sub&gt;b&lt;/sub&gt;</td>
<td>31-85-200-27-100</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>–</td>
<td>529-322-143</td>
</tr>
<tr>
<td>IV</td>
<td>–</td>
<td>672-322</td>
</tr>
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</table>
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
Table 5. Cytometric and morphological parameters of blood of animals-carriers of different genotypes of \textit{BLV}

<table>
<thead>
<tr>
<th>env</th>
<th>Hematological status</th>
<th>WBC, $10^{9}$/l</th>
<th>lymf, $10^{9}$/l</th>
<th>n</th>
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<tr>
<td></td>
<td></td>
<td>$\overline{x}$</td>
<td>$S_{x}$</td>
<td>-95%</td>
</tr>
<tr>
<td>I</td>
<td>suspected</td>
<td>12,30</td>
<td>0,072</td>
<td>11,31</td>
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<tr>
<td></td>
<td>sick</td>
<td>24,18</td>
<td>1,197</td>
<td>19,49</td>
</tr>
<tr>
<td></td>
<td>healthy</td>
<td>4,95</td>
<td>0,294</td>
<td>3,53</td>
</tr>
<tr>
<td></td>
<td>On average</td>
<td>12,36</td>
<td>0,175</td>
<td>11,06</td>
</tr>
<tr>
<td>Ia</td>
<td>suspected</td>
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<td>0,251</td>
<td>9,48</td>
</tr>
<tr>
<td></td>
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<td>19,82</td>
<td>0,633</td>
<td>16,75</td>
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<tr>
<td></td>
<td>healthy</td>
<td>7,06</td>
<td>0,242</td>
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<td>9,93</td>
<td>0,291</td>
<td>7,80</td>
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<tr>
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<td>suspected</td>
<td>9,73</td>
<td>0,201</td>
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<td>On average</td>
<td>9,08</td>
<td>0,230</td>
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<td>11,70</td>
<td>0,352</td>
<td>8,94</td>
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<td>healthy</td>
<td>8,36</td>
<td>0,259</td>
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<td>9,71</td>
<td>0,313</td>
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<tr>
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<td>11,99</td>
<td>0,378</td>
<td>9,78</td>
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Wilks' lambda statistic=0.82285, F(12, 1530)=13.056, p=0.0000