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CHANGES IN LEUKOCYTE AND ERYTHROCYTE BLOOD PROFILE AND PARAMETERS UNDER A COMBINED *Anaplasma marginale* AND BOVINE LEUKEMIA VIRUS INFECTION IN CATTLE

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Abstract

The global spread of infectious diseases and large-scale import of cattle genetic resources lead to necessity of developing screening methods that estimate the danger of co-infection with different pathogens and its impact on animal adaptiveness. In this regard here we analyzed the variability of erythrocyte and leukocyte characteristics in dairy Black-and-White holsteinized cattle naturally infected by *Anaplasma marginale*, the causative agent of bovine anaplasmosis, and bovine leukemia virus (BLV). The results showed that BLV infection of cattle did not facilitate the cross-infection with *A. marginale* in cattle since more than half of *A. marginale*-free animals were BLV-infected, and about one-third cows were characterized by leukocytosis. Except an increased number of leukocytes and lymphocytes due to retroviral infection, *A. marginale*-free animals were characterized only by the absence of statistically significant correlation between the counts of erythrocytes and neutrophils as compared to *A. marginale*-infected cows, which may point at the activation of nonspecific defense mechanisms in *A. marginale*-infected animals. Testing animals for BLV infection by agar gel immunodiffusion (AGID) and polymerase chain reaction (PCR) assays revealed the proviral DNA integration in one AGID-negative cow, whereas in seven out of thirty four AGID-positive cows the proviral DNA was absent. Leukocytosis ($> 20\times10^9$ blood leukocytes per liter) was revealed only in six AGID- and PCR-positive cows. The only common feature of BLV-infected animals with moderate and severe leukocytosis was thrombocytosis, as well as disruption of correlational relationships between the number of agranulocytes and granulocytes in peripheral blood. The detected disruption of the network relationships between different leukocyte populations reflects deep changes in the immune system functioning induced by retroviral infection. We observed a deficiency of neutrophils in cows with leukocytosis, which is in consistency with the data on neutropenia in milk of BLV-infected cows with leucosis (M. Nishiike et al., 2016). Considering the absence of BLV diagnostic tests that are able to reliably exclude the false-positive or false-negative results, it seems that the most effective approach for herd sanitation may consist in a simultaneous quantification of viral load (the number of BLV RNA in peripheral blood cells) and estimation of leukocytosis severity.

Keywords: anaplasmosis, bovine leukemia virus, viral load, leukocytosis, neutropenia, erythrocyte and leukocyte characteristics

A global key problems of modern livestock is the loss from infectious diseases [1, 2]. Substantial change in the situation is not achieved, despite the improvement of vaccines and antibiotics and their wide use. It is assumed that the formation of groups of animals with increased resistance to the most common pathogens can be more promising. However, this requires in-depth understanding of the development of infection and the key links in the interaction between a pathogen and a host organism.

In dairy cattle breeding, the most common cause of economic losses is

due to enzootic bovine leukemia caused by *Bovine leukemia virus*, BLV) [3, 4]. In some countries of South America, for example in Argentina, 90.9 % of bovine cattle herds are BLV-infected [5]. Due to the complex effect on the host's immune system, methods for effective animal vaccination protecting against this retrovirus have not yet been developed [6, 7]. Control of the spread of BLV is complicated by the fact that, as a rule, the host's immune system performs a negative selection against B lymphocytes producing mature viral particles [8]. In order to prevent the spread of BLV, various programs are proposed to detect infected animals based on identification of antibodies to BLV in peripheral blood and (or) proviral DNA in genomes. In addition to immunological and genetic tests, counts of lymphocytes in peripheral blood of animals are used. The combination of all three methods is most effective and allows increasing the reliability of detection of infected cows [1]. However, it should be taken into account that the BLV infection in animals is often accompanied by an increase in the sensitivity to bacterial infectious agents, such as *Mycobacterium bovis* [9], to *Escherichia coli* [10], and by an increase in mastitis [11], which may, in turn, lead to changes in the number of peripheral blood leukocytes.

Anaplasmosis in cattle caused by *Anaplasma marginale* (*Rikskettsiales*: *Anaplasmatacea*) is another reason of significant economic losses in livestock [12-14]. The frequency of infected cattle depends geographically on the region. Thus, in the states of North Africa, for example, in Morocco and Tunisia, it is 25.4 and 29.1 % [15, 16], respectively, while in Central and South Africa this index ranges from 38 to 100 % [17, 18]. In the Russian Federation, according to veterinary reports, anaplasmosis is mostly recorded in the southern regions, Bryansk, Kaluga, Ryazan, Kaliningrad, Saratov, Tyumensk, Vladimir, Nizhny Novgorod, Novosibirsk and Ulyanovsk regions, Altai territory [19]. Thus, 21.4-56.0 % of cattle are infected by *A. marginale* in the south of the Tyumen region [20]. In different climatic regions, lethality at cattle anaplasmosis is also not the same and ranges from 10-30 % [21, 22] to 100 % [23. *A. marginale* refers to erythrocyte parasites. The proportion of infected erythrocytes in the acute stage of anaplasmosis can be 70 % or more [24, 25], and parasitaemia in the blood can reach more than 10^9/ml [26-28]. The main clinical manifestations of anaplasmosis are anemia with a decrease in the amount of erythrocytes up to 1.5×10^6/mm^3 and hemoglobin up to 4-2 g% [21], and jaundice which develop due to the destruction of red blood cells by cells of the reticuloendothelial system [29, 30]. Other symptoms may include fever, weight loss, cardiovascular disorders (arrhythmic pulse) and gastrointestinal disorders (forestomach impaction, constipation); in severe form, abortions, muscle tremors and convulsions are noted [12, 19]. The minimal infecting dose of *A. marginale* leading to the development of clinical signs, is 1.5×10^5 [21], however in *A. marginale* strains this value may vary. The ill animals become lifelong anaplasmic carriers, for which the rickettsemia ration is 10^{2}-10^7 anaplasm per 1 ml of blood [28].

Mechanisms of the immune response to *A. marginale* invasion in cattle have not been fully studied, neveretheless, it is known that γ-interferon secreting CD4+ T-lymphocytes and the production of IgG1 and IgG2, mainly to the immunodominant and hypervariable surface protein MSP2, as well as to the proteins MSP1, MSP3, MSP4 and MSP5 of *A. marginale*, are activated [31-35]. It is suggested that the effect of antibodies is directed at neutralizing *A. marginale* cells prior to their introduction into erythrocytes and/or opsonization followed by phagocytosis by macrophages [22).

In recent years, the neutrophils/lymphocytes ratio, the number of platelets and morphological variability of erythrocytes [36] are widely used as markers of chronic inflammation, including that associated with pre-neoplastic state in

various diseases. These parameters can be determined on an automatic hemoanalyzer, which allows obtaining fairly objective data on hemopoiesis modifications in the development of pathology.

In order to assess the sensitivity of BLV-infected animals to bacterial infections and the accompanying changes in blood morphological parameters, in this study we first tested of black-and-white Holstein cows for the presence of BLV proviral DNA in genomes and *A. marginale* in erythrocytes with regard to counts of cell populations in peripheral blood, i.e. the number of erythrocytes and leukocytes, lymphocytes, monocytes, neutrophils, eosinophils, basophils. In addition, the mean volume and heterogeneity of erythrocytes in diameter (anisocytosis) was compared as indicators reflecting the change in health, for example, when taking antimicrobial drugs, and the development of pathologies, including hormonal abnormalities, bone marrow abnormalities in leukocytosis, and certain malignant diseases. It was found that the infection of animals with BLV does not promote coinfection with anaplasma, moreover, of *A. marginale* free cows, more than a half are infected with BLV and almost $^1/_3$ have leukocytosis.

The aim of the paper was to study the variability of erythrocyte and leukocyte characteristics in specialized dairy cattle, naturally infected with *Anaplasma marginale* and the *Bovine leukemia virus*.

Technique. Blood for the study was taken from the jugular vein of 67 black-and-white Holstein cows aged 2-5 years (ZAO Mozhaiskoye, Moscow Region).

Erythrocyte and leukocyte profiles and erythrocyte characteristics were determined individually for each animal on an automatic hematological analyzer Abacus junior Vet5 (Diatron, Austria, the principle of operation is based on the Coulter method) using 100 μl of EDTA-stabilized fresh whole peripheral blood.

Carriers of BLV proviral DNA were detected using Mancini radial immunodiffusion (RID) and PCR protocol developed by us earlier [37]. Methods for assessing the infection of *A. marginale* and the rickettsemia are described in detail earlier [38, 39].

The data was analyzed in Statistica 6.0 software (StatSoft Inc., USA). Differences were considered significant at $P < 0.05$. The tables show the arithmetic mean (X) and the errors of the arithmetic mean (x).

Results. Of 20 individuals free of BLV proviral DNA in two tests (RID and PCR), 13 ones (65 %) were infected with *A. marginale*, and of 22 BLV proviral DNA carriers *A. marginale* were detected in 11 ones (50 %). That is, in the group of animals infected with BLV, there was no increased sensitivity to *A. marginale*.

When analyzing involvement of different cell populations of peripheral blood in the development of *A. marginale* infection, we assessed their presence in infected *A. marginale* cows and those free from infection (Table 1). According to the manufacturer's protocol, all the parameters studied on the hematological analyzer corresponded to the physiological norm for *Bos taurus*, with the exception of the clearly increased leukocyte counts in the cows not infected by the bacterial pathogen. The fact that in the animals infected by *A. marginale* the number of red blood cells also remained within the normal range could be explained by the persistent stage of the infection, which was indicated by the rickettsemia values of 1.58×10^5 to 2.31×10^6/ml blood characteristic of anaplasmosis [28].

Statistically significant differences ($P < 0.05$) between infected and free from *A. marginale* individuals appeared only in leukocytes and lymphocytes, and was directed towards a decrease in infected animals (see Table 1). Obviously, these differences are due to the fact that BLV-infected animals with a high leukocytosis were infected with a bacterial pathogen (see Table 1). Of 6 individuals with high leukocytosis ($> 20\times10^9$/l), only one cow was infected with *A. marginale*, and 5 cows entered the group of 18 individuals free of anaplasma. Conse-

quently, changes in the profiles of leukocyte populations induced by BLV did not increase the likelihood of infection with *A. marginale*.

1. Erythrocyte and leukocyte profiles of peripheral blood in black-and-white Holstein cows infected with *Anaplasma marginale* and free from infection($X \pm x$, ZAO Mozhayskoe, Moscow Province)

Parameter	Permissible limits for *Bos taurus*	Not infected by *A. marginale*		Infected by *A. marginale* (n = 23)
		total (n = 18)	free of *Bovine leukemia virus* (n = 7)	
Cell population:				
erythrocytes, $\times 10^{12}$/l	5-10	6.53±0.18	6.83±0.18	6.23±0.18
leucocytes, $\times 10^9$/l	4-12	15.21±1.63*	10.18±0.67	10.87±0.98*
lymphocytes, $\times 10^9$/l	2.5-7.5	9.85±1.77*	4.85±0.83	5.62±1.00*
monocytes, $\times 10^9$/l	0-0.84	0.74±0.17	0.52±0.11	0.34±0.08
neutrophils, $\times 10^9$/l	0.6-6.7	4.07±0.69	4.54±0.48	4.41±0.50
eosinophils, $\times 10^9$/l	0.1-1.0	0.51±0.09	0.45±0.05	0.49±0.06
basophils, $\times 10^9$/l	0-0.5	0.0089±0.0011	0.0110±0.0010	0.0104±0.0020
thrombocytes, $\times 10^9$/l	100-800	87.72±35.23	10.29±6.32	86.91±33.94
Morphology of erythrocytes:				
average volume, fl	40-60	45,56±0,89	44,86±0,82	45,00±0,73
variability in diameter, %		19,66±0,33	20,83±0,40	19,63±0,38

* Differences between infected and non-infected individuals are statistically significant at $P < 0.05$.

In the erythrocyte component of uninfected animals, there were statistically significant ($P < 0.05$) correlations between erythrocytes and eosinophils ($r = -0.5$), erythrocytes and platelets ($r = -0.5$), as well as between erythrocyte heterogeneity in diameter and the number of leukocytes ($r = -0.5$), lymphocytes ($r = -0.5$), platelets ($r = -0.6$) and basophils ($r = +0.6$). That is, in uninfected animals, there was correlation between an increase in the number of erythrocytes and a decrease in eosinophils and platelets, the markers of inflammation, and higher morphological variability of erythrocytes correlated with decreased levels of leukocytes and lymphocytes. Several other reliable correlations revealed in infected animals were between the number of erythrocytes and neutrophils ($r = +0.5$), erythrocytes and basophils ($r = +0.5$), between the heterogeneity of erythrocytes in diameter and the number of basophils ($r = +0.4$), platelets ($r = -0.7$) and the mean volume of erythrocytes ($r = -0.6$). Positive correlation of the number of erythrocytes and neutrophils allows us to assume the activation of a nonspecific immune response in *A. marginale* infected cows.

Thus, the generally accepted view that the infection of animals with one pathogen, accompanied by a change in immunoreactivity, contributes to an increase in sensitivity to another pathogen possessed no confirmation for *A. marginale* and BLV infections. In general, this is consistent with the conclusion of several authors that each infectious agent specifically interacts with the host's immune system, and for such pathogens these mechanisms often do not overlap [40].

Infection with the retrovirus BLV, regardless of anaplasma, since its presence, as we showed, did not change the numerical ratio of blood cell populations) (see Table 1) was evaluated in the traditional test for the presence of antibodies to BLV envelope proteins in the peripheral blood (RID) and by integration of the BLV proviral DNA into the host genome. Of 67 cows examined, 33 were seronegative in RID, but one cow contained BLV proviral DNA in the genome. In 7 of 34 RID positive animals no BLV proviral DNA was found. These data are consistent with the findings of previous studies, in which there was also no complete agreement between estimates of animal infection in the RID and the BLV proviral DNA detection [41]. It should be noted that this discrepancy was observed when different viral genes (*env*, *gag*, *pol*) were used to identify BLV proviral DNA integration. The data obtained are consistent with the recent report of M. Nishlike et al. [1]. After examining 774 cows, they showed that in 7 % of the animals with antibodies to BLV in blood the BLV

proviral DNA in the genome, when estimating by viral gene *tax* nucleotide sequences, was not detected.

It is not excluded that this difference can be based on the differences between animals according to the number of B-lymphocytes infected by BLV and the immune response to infection. In this connection, we compared the distribution of peripheral blood cell populations in BLV-infected and non-infected cows (Table 2).

2. Erythrocyte and leukocyte profiles of peripheral blood in black-and-white Holstein cows free from infection and infected by bovine leukemia virus at different development of leukocytosis ($X \pm x$, ZAO Mozhayskoe, Moscow Province)

Parameter	RID^-, BLV^- (n = 21)	RID^+, BLV^+	
		without high leukocytosis (n = 18)	with maximum leukocytosis (n = 6)
Cell population:			
Erythrocytes, $\times 10^{12}$/l	6.59±0.19	6.15±0.17	6.34±0.36
Leucocytes, $\times 10^9$/l	10.00±0.56	11.73±0.88	25.49±1.01
Lymphocytes, $\times 10^9$/l	4.14±0.24	6.12±0.73*	21.70±1.08**
Monocytes, $\times 10^9$/l	0.35±0.06	0.28±0.09*	1.61±0.24*
Neutrophils, $\times 10^9$/l	5.10±0.44	4.63±0.83**	1.66±0.36**
Eosinophils, $\times 10^9$/l	0.43±0.06	0.59±0.09	0.51±0.18
Basophils, $\times 10^9$/l	0.0119±0.0011	0.0088±0.0027	0.0067±0.0021
Thrombocytes, $\times 10^9$/l	12.90±9.91	160.81±47.55**	122.33±78.28**
Neutrophils:Lymphocytes	1.26±0.10	0.88±0.21*	0.08±0.02*
Morphology of erythrocytes:			
average volume, fl	44.19±0.83	46.12±0.86	46.50±1.09
variability in diameter, %	20.84±0.27	18.55±0.34	18.83±0.31

N o t e. RID — radial immunodiffusion, BLV — bovine leukemia virus (proviral DNA detected in the genome by PCR method).
*, ** $P < 0.05$ and $P < 0.01$, respectively, for thresholds of statistical significance of the differences between infected and BLV-free individuals.

3. Distribution of peripheral blood cells in black-and-white Holstein cows with antibodies to bovine leukemia virus in the absence of BLV proviral DNA (n = 7, $X \pm x$, ZAO Mozhayskoe, Moscow Province)

Cell population:	Indeces
Erythrocytes, $\times 10^{12}$/l	6,40±0,19
Leucocytes, $\times 10^9$/l	8,11±0,47
Lymphocytes, $\times 10^9$/l	3,57±0,13
Monocytes, $\times 10^9$/l	0,31±0,10
Neutrophils, $\times 10^9$/l	3,59±0,61
Eosinophils, $\times 10^9$/l	0,64±0,13
Basophils, $\times 10^9$/l	0,0042±0,0020

N o t e. The presence of antibodies was determined by radial immunodiffusion (RID), BLV provirus DNA in the genome was detected by PCR.

In animals with high leukocytosis, there were a statistically significant ($P < 0.05$) increase in the number of lymphocytes, monocytes and platelets, a decrease in neutrophils and, correspondingly, a decrease in the neutrophils and lymphocytes ratio. BLV-infected cows without high leukocytosis significantly differed from uninfected ones only in the number of platelets (see Table 2). In this case, in 7 cows with RID^+, but without the insertion of BLV proviral DNA, the distribution of cellular populations in the peripheral blood corresponded to the physiological norm (Table 3). The peculiarity of this group was an unusually high statistically significant ($P < 0.05$) correlation between the number of leukocytes and neutrophils ($r = 0.871178$).

In the cows free from BLV, as it resulted from RID and lack of proviral DNA, there were numerous positive correlations between the counts of agranulocytes and granulocytes, as well as platelets (Table 4), i.e. between the number of lymphocytes and neutrophils, lymphocytes and basophils, monocytes and eosinophils, neutrophils and basophils, eosinophils and basophils, monocytes and platelets, eosinophils and platelets (see Table 4). That is, in the animals free from infection, the changes in the profiles of the peripheral blood cells of white root were closely interrelated, whereas the BLV infection detected by RID^+ and the insertion of proviral DNA was, in fact, accompanied by the apparent de-

struction of all these positive correlations. In the infected individuals there were positive correlations between the number of lymphocytes and monocytes, and negative correlations between basophils and platelets, lymphocytes and monocytes, lymphocytes and neutrophils. In infected cows with relatively low leukocytosis, only two correlations were statistically significant, a positive one between lymphocytes and monocytes, and a negative one between basophils and platelets, and when high leukocytosis ($> 20\times10^9$/l) was developed, only one statistically significant negative correlation was found between lymphocytes and basophils.

4. Correlation coefficients between the number of agranulocytes, granulocytes and platelets in peripheral blood of black-and-white Holstein cows free from bovine leukemia virus (n = 20, CJSC "Mozhayskoe", Moscow Province)

Cell population	Lymphocytes	Monocytes	Neutrophils	Eosinophils	Basophils	Platelets
Lymphocytes	1.000000	0.514220*	0.507795*	0.382689	0.591109*	0.064150
Monocytes	0.514220*	1.000000	–0.244767	0.675024*	0.147069	0.488814*
Neutrophils	0.507795*	–0.244767	1.000000	0.151900	0.626046*	–0.285535
Eosinophils	0.382689	0.675024*	0.151900	1.000000	0.464138*	0.558992*
Basophils	0.591109*	0.147069	0.626046*	0.464138*	1.000000	–0.055497
Platelets	0.064150	0.488814*	–0.285535	0.558992*	–0.055497	1.000000

N o t e. Antibodies to the bovine leukemia virus in radial immunodiffusion and proviral DNA in PCR were not detected.

* Correlations are statistically significant at $P < 0.05$.

In general, with regard to the physiological norm for different populations of peripheral blood cells, the marked differences between BLV-infected animals and the control group were manifested in thrombocytosis and at leukocytosis in a sharp drop in the number of neutrophils.

Neutrophils form the first line of cell defense against pathogens, largely providing innate immunity against microorganisms. When the pathogen is phagocytized, neutrophils produce free radicals that destroy it. It was found that in milk of cows infected with BLV, neutrophils are significantly reduced [11], while for individuals with leukocytosis and high load of BLV proviral DNA a reduced expression of γ-interferon (IFN-γ) by peripheral blood monocytes is characteristic. IFN-γ promotes phagocytosis and production of free radicals by neutrophils, which can, in particular, explain the decrease in neutrophil function along with the decrease in their number in BLV infected cows. Earlier publications also noted the fact that blood serum of BLV infected cows suppresses the phagocytic activity of neutrophils [42].

A large amount of data has been accumulated on the pronounced effect of expression of BLV proviral DNA, in particular *tax* gene, on stress reactivity in cells, apoptosis, cell renewal rates, and immune system of infected animals [7, 43, 44]. We have previously shown that it is in animals with high leukocytosis that expression of BLV RNA can be detected by RT-PCR [4]. These data coincide with the reports of many researchers, in particular Japanese ones, who found the greatest amount of BLV RNA in the peripheral blood of cows with the highest leukocytosis [1]. Obviously, the increased expression of BLV proviral DNA accompanied by leukocytosis will inevitably lead to changes in the network relationships between different populations of leukocytes which we observed in our study.

Thus, the prevalence of the combined infection of BLV and *A. marginale* and the changes in peripheral blood cell populations, revealed by us, do not reflect a mutual increase in sensitivity to these pathogens. The profiles of the leukocyte population mostly correspond to the norm, however a feature which distinguishes infected *A. marginale* cows from uninfected, is a statistically significant positive correlation between the number of erythrocytes and neutrophils.

As to BLV, the obtained data indicate that none of the currently available methods of confirming infection with this virus, i.e. detection of antibodies

to BLV in RID, PCR analysis for BLV proviral DNA, excess of the number of leukocytes above physiological norm of $10\text{-}12\times10^9$/l, does not allow to avoid false positive or false negative results. The most distinguishing feature of BLV infection, even at low leukocytosis, is the disappearance of the normally correlated links between the counts of different populations of leukocytes, indicating the destruction of the network relationships between them. Importantly, in animals with high leukocytosis, marked neutrophilopenia is observed, which coincides with the literature data on the change in the neutrophil content in milk at BLV infection and high leukocytosis [11]. Given the need for a simpler method for identifying the most infectious animals in industrial herds, it seems appropriate to simultaneously evaluate the viral load (the amount of BLV RNA in the peripheral blood) and the severity of leukocytosis.

So, infection by bovine leukemia virus (BLV) does not contribute to *Anaplasma marginale* coinfection. Statistically significant correlations ($P < 0.05$) for the erythrocyte component are not the same in uninfected and anaplasma-infected animals, and the positive correlation between the number of erythrocytes and neutrophils suggests the activation of a nonspecific immune response in *A. marginale* infection. The BLV infection leads to the destruction of positive correlations between the sizes of white blood cell populations observed in uninfected individuals. These significant differences result in the development of thrombocytosis, and in individuals with leukocytosis in a sharp drop in the number of neutrophils. When detecting BLV infection in herds, it is most expedient to evaluate leukocytosis in combination with the viral load estimated by the amount of BLV RNA in the peripheral blood.

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