Immunity changes in healthy and coronavirus-infected bovine organisms from ecologically different districts

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The hematological and immune biomarkers of healthy and coronavirus-infected bovine organisms in ecologically different districts of Lithuania were investigated. Serological screening of bovine blood serum against coronavirus infection revealed 12.1% of positive cows in the conventionally ecologically clearer Ukmergė district (<1 t/km^2/year contaminants) and 15.9% in the conventionally ecologically contaminated Trakai district (60–62 t/km^2/year contaminants). The amounts of erythrocytes, hemoglobin, total leukocytes and segmented neutrophil were decreased, while eosinophil and methemoglobin amounts were increased in the blood of healthy and coronavirus-infected cows in the conventionally ecologically contaminated district. The immunoregulatory index (CD4/CD8) was decreased more significantly (1.5 times) in coronavirus-infected cows in Trakai district than in Ukmergė district. Natural killer (NK) cell activity decrement was determined at a target-to-effector cell ratio 1:40 in coronavirus-infected (25.3% to 19.2%) and healthy cows (29.1% to 21.7%) in the ecologically contaminated district compared with the ecologically clearer one. A significantly higher natural antibodies populational level (NAPL) against enterobacterial common antigen has been found in coronavirus-infected bovine (27.8% in Ukmergė and 32.7% in Trakai districts). The higher rates of synthesis of natural antibodies against common enterobacterial lipopolysaccharide most likely demonstrate a compensatory response of a weakened immunocompetence of the organism.

Key words: contamination, coronavirus, lymphocytes, natural killer cells, natural antibodies population level

Abbreviations: BCV, bovine coronavirus; FITC, fluorescein isothiocyanate; FLK, fetal lamb kidney; NA, natural antibodies; NAPL, natural antibodies population level; NK, natural killer; PBM, peripheral blood mononucleares; RNA, ribonucleic acid

INTRODUCTION

Harmful chemical and physical factors possess a very broad spectrum of biological effects, including altered cell proliferation and differentiation, development inhibition, allergic reactions, toxic effects. Environmental pollutants are numerous and include sources from industrial emission, fuel combustion, pesticides and herbicides, overfertilization. Nowadays reduction of overall immunity in domesticated herds of livestock are registered due continued stress from environmental pollution. Some pollutants are known to be harmful for the development of plants, animals and humans and the most prevalent symptoms observed following exposure to them are alteration in the functions of the immune, reproductive systems [1–4].

The immunity of the organism is the whole complex of biological phenomena able to maintain homeostasis against infections and other factors that are genetically foreign to it. Chemical and biological pollutants may affect the immune system locally or systemically, through antigen-dependent and antigen-independent activation pathways of the immune response. Determination of the number of variuos lymphocyte types and concentration of immunoglobulin classes in the peripheral circulation gives a relevant indication of the cellular and humoral immune responses of a person or population. It would be important to assess whether there is any relationship between exposure to pollutants and immune system biomarkers [5].

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Among mammalian and avian species, coronaviruses are widespread pathogens which cause a number of serious diseases ranging from respiratory and gastrointestinal ailments to hepatitis, encephalomyelitis, vasculitis and coagulopaties. About 30% of common colds in human beings are attributed to these viruses [6]. Several of these viruses infect domestic animals and cause significant morbidity and mortality, and the following diseases are of major economical importance: transmissible gastroenteritis and porcine respiratory coronavirus infections in pigs, diarrhea in calves, infections bronchitis in chickens, feline infectious (peritonitis), etc.

Coronaviruses constitute a large genus of spherical or pleomorphic enveloped particles and belong to the family *Coronaviridae*. Bovine coronavirus (BCV), as an enteric coronavirus, causes severe diarrhea in calves, and it is also considered to be etiologically involved in winter dysentery of adult cattle [7–9]. BCV possesses a complicated antigen spectrum in itself, which is defined by specific protein amount. Viral infections are known to be related to some alterations in cellular and / or humoral immunity chain interaction of the organism. Virus strain, dosage, environmental factors and involvement of other secondary pathogens (opportunistic infections) also influence the severity of the disease and the outcome of infection [10]. Like a majority of corpuscular antigens, viruses are rather immunogenic and cause the immune response in cellular and humoral (CD4, CD8, CD19 lymphocytes, NK and other subpopulations of cells) immunity chains. It is well known that CD4 and CD8 molecules play a critical role in T-cell responses to antigen. In general, T-cells that recognize antigen in association with class II MHC molecules express CD4 and those that recognize antigen bound to class I MHC express CD8 [11, 12]. It is some CD8 lymphocytes that directly partake in the recognition and resolution (express cytotoxicity) of virus-infected cells [13]. Also, mainly the first cellular chain in the defence of the organism against some viruses, malignant and altered cells is the natural killer (NK) cells, which act without any preliminary sensitization.

The aim of our studies included investigation of the influence of harmful exogenic factors on the hematological, biochemical and some immune biomarkers of healthy and coronavirus-infected cows kept in ecologically different districts.

MATERIALS AND METHODS

Collection of blood and serum samples of cows from herds were performed in Trakai (conventionally ecologically contaminated) and Ukmergė (conventionally ecologically clear) districts. For complex investigations blood samples were taken from jugular veins of Ayashire and Lithuanian red breed cows 3–6 years old. The bovine were kept in herd complexes under similar feeding and veterinary control conditions.

Coronavirus infection has been detected in blood serum samples with immunodiagnostic (immunodiffusion method) kits obtained at J. Kovalenko Experimental Veterinary Institute (Moscow, Russia) and Bio-X coronavirus ELISA Kit (Brussels, Belgium). The hematological and immune biomarkers such as the total number of erythrocytes and leukocytes, hemoglobin and methemoglobin amount, leukocyte formula, T and B populations, immunoregulatory index, NK cells activity (the target – fetal lamb kidney (FLK) $^{44}/_{2}$ culture cells) have been studied by routine immunologicaly methods [14]. The immunophenotyping of T lymphocytes CD4 and CD8 subpopulations was performed on a FACSort instrument (Becton Dickinson). Green fluorescence (fluorescein isothiocyanate (FITC)) was registered through a 530/30 nm bandpass. Fresh peripheral blood mononuclears (PBM) from bovine were isolated by centrifugation in verografin gradient. PBM labeling procedures with specific monoclonal antibodies against CD4, CD8 cells (supplied by Dr J. Naessens from ILRAD, Nairobi, Kenya) and resolution-positive cells by goat FITC-conjugated anti-mouse IgG polyclonal antibodies (Sigma, USA) were done as described [15]. The natural antibodies population level (NAPL) of enterobacterial common antigen (lipopolysaccharide component of the *B. faecalis alcaligenes* 415) were detected by immunodiffusion method [16].

The results were expressed as means ± standard deviation. The data were analysed statistically by the Student's t test with $P < 0.05$.

RESULTS AND DISCUSSION

As have been determined in 1992–1997 the air and soil pollution levels in some Lithuanian regions and districts differ considerably. We have investigated the bovine serological, haematological and immune parameters in the conventionally ecologically clearer Ukmergė district (<1 t/km^2/year of total contaminants) and the conventionally ecologically contaminated Trakai district (60–62 t/km^2/year of total contaminants). A more significant contamination of the environment with benzo(a)pyrene and nitrates has been detected in Trakai district [17].

Serological screening of bovine blood serum against coronavirus infection revealed that the bovine with a positive reaction didn't comprise a substantial part, namely 12.1% in Ukmergė (positive 283, total 2340) and 15.9% in Trakai (positive 138, total 868) districts. One can see that the percentage

of coronavirus-positive cows in the conventionally ecologically contaminated Trakai district were 1.3 times higher ($P < 0.01$) than in the conventionally ecologically clearer Ukmergė district. The reason of this phenomenon may be disturbed intestinal (mucosal) immunoprotective features of cows in the contaminated district. The mucosal immune system is structurally and functionally distinct and specific for interaction between the organism and the environment [18]. As shown earlier, the binary (corona- and rotavirus) and mixed (BLV, corona- and rotavirus) infections were more numerous in a conventionally ecologically contaminated district [19]. Our results are compatible with literature data showing that in contaminated ecological conditions a lot of people become infected with viruses [5, 20].

Typical coronaviruses are pleomorphic to rounded particles 60–220 nm in diameter with club-shaped surface projections 12–24 nm long with a lipid envelope surrounded by a fringe of surface projections termed spikes or peplomers. Internal ribonucleoprotein is present as helix or strands. Their genome consists of a large single-stranded RNA molecule comprising about 28–32 kilobases of nucleotides. This is the largest known RNA genome [21, 22].

Epithelial cells are an important target for coronavirus infection. Coronaviruses replicate in the cytoplasm and assemble, bud at membranes of the intermediate compartament, located between the endoplasmic reticulum and Golgi complex. Like other enveloped viruses, a coronavirus assembly is presumably dependent on protein localization and protein–protein as well as protein–RNA interactions [23].

BCV represents one of the better characterized coronaviruses with hemagglutinin properties. Four major structural proteins are associated with infectious BCV. The hemagglutinin as well the integral membrane glycoprotein M (23–26 kDa) are associated with the viral envelope, while the phosphorylated N protein (50–54 kDa) functions as a nucleocapsid. Proteolytic cleavage of the S glycoprotein precursor into S1 and S2 of 100 and 110 kDa is required for cell fusion activity. The S1/S2 glycoproteins facilitate virus attachment to susceptible cells and also binding to erythrocytes, cell fusion, and induction of neutralizing antibodies. The exact functions of hemagglutinin and S1/S2 and their interplay in infectious processes *in vitro* and *in vivo* are not fully elucidated [24–28].

Hematological investigations revealed that in the conventionally ecologically contaminated Trakai district the erythrocyte, hemoglobin, leukocytes, segmented neutrophil levels were lower, while eosinophil and methemoglobin levels (2–4% by total hemoglobin) higher in the blood of healthy bovine, and in coronavirus-infected cows decreased hemoglobin and increased methemoglobin levels were found (Table 1, $P < 0.05$) than in the conventionally ecologically clearer Ukmergė district. A comparison of biomarkers revealed an increase in erythrocyte and segmented neutrophil levels, as well as a decrease in eosinophil level ($P < 0.05$) among coronavirus-infected bovines in the contaminated Trakai district. The decrease in eosinophil levels was recorded among infected bovines ($P < 0.05$) both in Ukmergė and Trakai districts. The level of blood serum nitrates in healthy and infected cows was also markedly increased ($P < 0.05$) in Trakai district as shown previosly [19].

It is well know that high levels of nitrates in the foliage and water are the main reason for their reduction to increased concentracions of nitrites. Nitrites in the blood oxidize the hemoglobin to Fe^{3+} form. It may be supposed that the process of into-

Table 1. **Haematological biomarkers of healthy and coronavirus-infected bovine from ecologically different districts**

Biomarker	District			
	Ukmergė		Trakai	
	I	II	I	II
Erythrocytes (10^{12}/l)	6.4 ± 0.4	6.8 ± 0.4	4.8 ± 0.3[1]	6.3 ± 0.5[2]
Hemoglobin (g%)	11.7 ± 0.9	11.0 ± 0.9	9.6 ± 0.4[1]	9.7 ± 0.2[2]
Methemoglobin	0–1%Hb	0–1%Hb	2–4%Hb[1]	1–3%Hb[3]
Leukocytes (10^9/l)	6.6 ± 0.3	6.4 ± 0.3	4.8 ± 0.8[1]	6.3 ± 0.3
Leukocyte formula (%)				
Neutrophils:				
stick	4.3 ± 0.2	4.6 ± 0.2	3.7 ± 0.2	5.1 ± 0.3
segmented	25.3 ± 0.7	25.4 ± 0.4	23.5 ±0.7[1]	25.2 ± 0.5[2]
eosinophils	6.2 ± 0.4	4.0 ± 0.3[2]	11.1 ± 0.5[1]	4.8 ± 0.4[2]
basophils	0.7 ± 0.1	0.8 ± 0.2	1.2 ± 0.2	1.2 ± 0.2
lymphocytes	60.5 ± 1.6	62.1 ± 1.4	56.1 ± 1.8	60.2 ± 1.2
monocytes	2.5 ± 0.3	2.7 ± 0.4	2.8 ± 0.2	2.8 ± 0.2
Other cells	0.5 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.4 ± 0.1

I – healthy, n= 63; II – coronavirus infected, n = 78; [1, 2, 3] – $P < 0.05$, comparison: 1 – healthy – healthy (different districts)
2 – healthy–infected (in the same district), 3 – infected–infected (different districts).

xication with a weak supply of the tissues with oxygen negatively influences the immunity of the organism and develop allergization reactions (higher eosinophil amounts). Prolonged accumulation of nitrates and their metabolites may disturb and weaken the immunity of the organism and cause susceptibility to viral infections [29, 30].

Our investigations of bovine peripheral blood lymphocytes and their populations (CD4 and CD8 cells) demonstrated a tendency of increment of the amount of CD8 lymphocytes in healthy and coronavirus-infected cows in the conventionally ecologically contaminated Trakai district (Table 2, $P < 0.05$). The immunoregulatory index (the ratio CD4/CD8 cells) was lower in coronavirus-infected cows 1.5 times in the conventionally ecologically contaminated Trakai district ($P < 0.05$), compared with the conventionally ecologically clearer Ukmergė district. The tendency of increment in the CD8 (suppressor/cytotoxic) cells proportion possibly demonstrates not only the known fact of their involvement in the clearance of virus and infected cells, but also a shift of immunoregulatory functions when the organism is affected by unfavourable ecological conditions.

Table 2. **Distribution of immune biomarkers in healthy and coronavirus-infected bovine from ecological different by districts**

Biomarker	District			
	Ukmergė		Trakai	
	I	II	I	II
	(n = 65)	(n = 25)	(n = 54)	(n = 27)
Leukocytes (10^9/l)	6.2 ± 0.4	6.4 ± 0.3	4.9 ± 0.9[1]	6.3 ± 0.4[2]
Lymphocytes (%)	58.9 ± 2.9	57.2 ± 2.8	56.7 ± 2.1	55.9 ± 2.4
T	45.8 ± 0.7	44.3 ± 2.2	44.0 ± 2.3	42.7 ± 1.9
B	21.8 ± 1.6	20.0 ± 1.8	23.9 ± 1.1	24.1 ± 2.4
CD4	21.2 ± 1.6	21.1 ± 1.9	24.9 ± 2.1	18.9 ± 2.0
CD8	10.5 ± 0.7	10.9 ± 1.3	12.5 ± 1.5	13.4 ± 2.8
CD4/ CD8	2.2	1.9	2.0	1.3[1,3]

I – healthy, II – coronavirus-infected, [1, 2, 3] – $P < 0.05$, comparison: 1 – healthy – healthy (different districts), 2 – healthy – infected (in the same district), 3 – infected – infected (different districts).

The coronavirus S, N and M-proteins are relevant targets for recognition by cellular immunity [31]. N-protein is frequently involved in T-cell responses to coronaviruses [32–34]. Collaboration between B and T-cell antigenic determinants may lead to induction of optimum immune responses to coronavirus [35].

Some virus-infected and malignant cells are recognized and lysed by NK cells, large granular lymphocytes with $CD16^+$, $CD\ 56^{\pm}$ phenotypes, in the blood they comprise 10–16% of all lymphocytes [36]. We measured the functional activity of NK cells *in vitro* from healthy and coronavirus-infected animals using three different target and effector cell ratios (Figure). Only at the ratio 1:40 a decreased ($P < 0.05$) functional activity of NK cells of healthy and coronavirus-infected cows was demonstrated in the conventionally ecologically contaminated district. The functional activity of NK cells from coronavirus-infected cows was decreased ($P < 0.05$) also in conventionally ecologically clearer Ukmergė district. This assay reveals a lowered potency of NK cells during coronavirus infection. Harmful ecological factors weaken these functions even more (the decrement from 25.3% to 19.2%).

Apparently some harmful ecological factors are capable of suppressing the differentiation and proliferation processes in NK cell lineage. The functional activity of NK cells seems to be a functional index of the immune status, related to the stage and outcome of viral diseases.

A significantly higher populational level of natural antibodies against enterobacterial common antigen has been found in healthy cows in the conven-

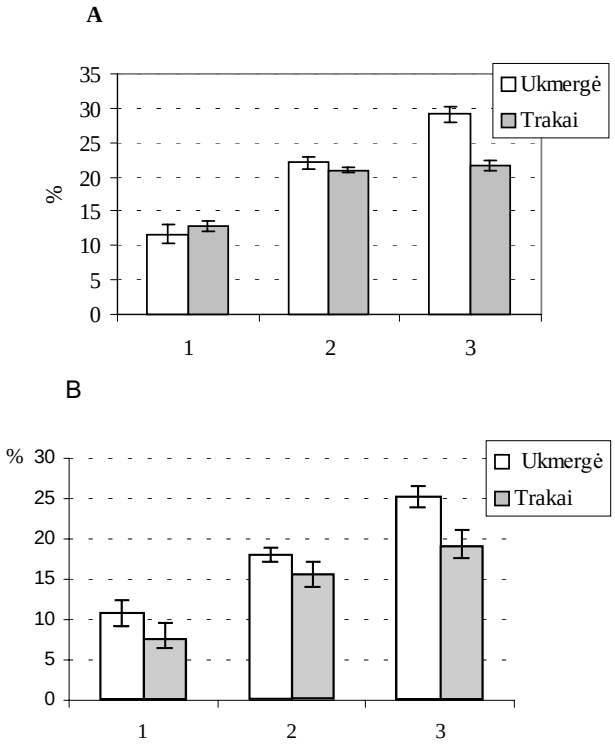

Figure. Functional activity of bovine blood natural killer cells. A – healthy, B – coronavirus-infected. Target and effector cells ratio: 1 – 1:5, 2 – 1:20, 3 – 1:50

tionally ecologically contaminated Trakai district (24.5% (13 from 53) *versus* 12.7% (8 from 63) in the conventionally ecologically clearer Ukmergė district ($P < 0.05$). Coronavirus-infected animals with positive NAPL indices were even more numerous – 32.7% (18 from 55) in Trakai *versus* 27.8% in Ukmergė (17 from 61) districts.

As is known, low quantities of natural antibodies against the gram-negative bacterial outer membrane lipopolysaccharide component are present in all organisms without deliberate immunisation with antigen [37, 38]. These antibodies, mainly of IgM class, recognize and neutralize a wide spectrum of antigens of the patogenic and saprofitic microbes. NA are one of the earliest chains of the organism's immunity system. It is possible that the coronavirus infection mobilizes the immunity, and the increased levels of NA in the blood of bovine demonstrate the compensatory reaction of weakened humoral immunity.

In conclusion, the results obtained show that the contaminated environment initiates disturbances in immune homeostasis in the organism and predisposes to transmission of bovine coronavirus infection.

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SVEIKŲ IR KORONAVIRUSAIS INFEKUOTŲ GALVIJŲ IMUNITETO POKYČIAI EKOLOGIŠKAI SKIRTINGUOSE RAJONUOSE

S a n t r a u k a

Buvo tirti sveikų ir koronavirusais infekuotų galvijų hematologiniai ir imuniniai biožymenys ekologiškai skirtinguose Lietuvos rajonuose. Atlikus koronavirusinės infekci-

jos serologinį patikrinimą nustatyta, kad koronavirusais infekuoti galvijai sudarė 12,1% sąlygiškai ekologiškai švariame Ukmergės rajone (< 1 t/km^2/metus teršalų) ir 15,9% sąlygiškai ekologiškai užterštame Trakų rajone (60–62 t/km^2/metus teršalų). Sveikų ir koronavirusais infekuotų galvijų kraujyje sąlygiškai ekologiškai užterštame rajone eritrocitų, hemoglobino, leukocitų ir segmentuotų neutrofilų kiekis buvo sumažėjęs, tuo tarpu eozinofilų ir methemoglobino – padidėjęs. Imunoreguliacinis indeksas (CD4/CD8) Trakų rajono koronavirusais infekuotų galvijų organizme buvo sumažėjęs (1,5 karto) palyginus su Ukmergės rajono koronavirusais infekuotų galvijų. Natūralių „kovotojų“ funkcinio aktyvumo sumažėjimas, esant taikininių ir efektorinių ląstelių santykiui 1:40, nustatytas koronavirusais infekuotų (nuo 25,3% iki 19,2%) ir sveikų galvijų organizme (nuo 29,1% iki 21,7%) sąlygiškai ekologiškai užterštame rajone. Išaugęs natūralių antikūnų prieš enterobakterinį bendrąjį antigeną populiacinis lygis nustatytas koronavirusais infekuotų galvijų organizme (27,8% Ukmergės ir 32,7% Trakų rajonuose). Padidėjusi natūralių antikūnų sintezė prieš bendrąjį enterobakterinį lipopolisacharidą gali atspindėti susilpnėjusios bendros organizmo imunokompetencijos kompensacinę reakciją. Gauti rezultatai rodo, jog nepalankūs ekologiniai veiksniai daro įtaką imunodeficitinės būsenos formavimuisi organizme ir virusinių infekcijų paplitimui.