



TJVR 2019; 3(1): 1-8

Turkish Journal of Veterinary Research

<http://www.dergipark.gov.tr/tjvr>

e-ISSN: 2602-3695



The Influence of Geographic Area on Blood Parameters of Pramenka Sheep in the Area of Bosnia and Herzegovina

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Received: 04.12.2018

Accepted: 26.12.2018

ABSTRACT

Objectives: The aim of this study was to determine the influence of geographical area (central and southwestern part of Bosnia and Herzegovina) on hematological and biochemical blood parameters of autochthonous Pramenka sheep.

Materials and Methods: The study included 104 sheep from the Vlasic mountain (central part) (n = 52) and Livno (southwestern part) (n = 52). Blood samples were taken from the jugular vein into Vactainer tubes with EDTA anticoagulant for hematological, for glucose, analyses and BD Vacutainer® SST II gel for biochemical analyses. All hematological and biochemical analyses were performed within the next 24 hours, and until then the samples were kept at 4 °C. Hematological parameters included total Red Blood Cell (RBC), White Blood Cell (WBC), Hemoglobin concentration (Hb), Hematocrit (Hct), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Mean Platelet Volume (MPV), Red Cell Distribution Width (RDW) and White Cell Differential Count (WCDC). Analyzes are carried out using the automated veterinary hematological analyzer Advia 120 SIEMENS. Blood in the serum tubes was allowed to clot for at least 30 min prior to centrifugation. Serum samples were kept frozen at -20 °C until biochemical analyses were performed. Biochemical parameters were determined by analyzer Olympus AU400 with Beckman Coulter reagents according to the manufacturer's protocol. Parameters for biochemistry panel included: total protein (TP), albumin (ALB), globulin (GLO), urea (BUM), creatinine (CRE), glucose (GLU), aspartate aminotransferase (AST), γ -glutamyltransferase (GGT), creatine kinase (CK), cholesterol (CHO), bilirubin (BIL), calcium (Ca), phosphorus (P), sodium (Na), chloride (Cl), potassium (K) and magnesium (Mg). All analyzes were tested to spectrophotometric method, except for Na, K, Cl, which were generated by ISE method, or by ion selective method. BHBA and NEFA were constructed with reagents from Randox.

Results: The values of WBC (p<0.05), RBC (p<0.001), Hb (p<0.001), PCV (p<0.001), MCV (0.05), NEU (p<0.01) from the Livno area, while the value of LYM (p<0.05) was determined for sheep from the Vlasic area. The correlative values between RBC: Hb (P<0.001), RBC:PCV (P<0.001), WBC:NEU (P<0.001), WBC:LYM (P<0.001), WBC:BAS (P<0.001) areas. The correlative correlation at P<0.01 was established between RBC:MCH, RBC:PLT, RBC:MPV in sheep from Vlasica area, while correlative values at P<0.05 were established between RBC:MPC, WBC:MON for sheep from the Livno area. The values of BHB (p<0.001), total protein (p<0.001), albumin (p<0.001), urea (p<0.001), AST (p<0.001), cholesterol (p<0.001), magnesium (p<0.001) were determined for sheep in area Livno. The values of NEFA (p<0.001), creatinine (p<0.01), glucose (p<0.001), bilirubin (p<0.001), phosphate (p<0.001) were established for sheep in the Vlasic area. Correlative correlation (P<0.001) between total protein:chloride, calcium:phosphates, sodium:chlorides was found in animals from Vlasic area, while correlation was found (P<0.05) between sodium:chloride in animals from the Livno area.

Conclusion: It was concluded that values showed significant differences for individual haematological and biochemical parameters in sheep for both investigated areas.

Keywords: Blood, Pramenka, Hematology, Biochemistry, Sheep

INTRODUCTION

An autochthonous breed of sheep in Bosnia and Herzegovina is the Pramenka sheep, or its strains originated on different geographic and macro-climatic conditions. The growing area is the wider region of the Vlasic mountain and Livno fields. Vlasic is a mountain that dominates the area of central Bosnia at elevation of 1300m. In the narrower part of Vlasic, the climate is mountainous, and in the lower parts and river valleys it's moderate continental. In the areas of higher altitudes (1000 m and above), sheep farming is the only significant agricultural activity. The Livno field is located in the southwestern part of Bosnia and Herzegovina with an area of 405 km² and at an average height of 730m. Long and cold winter months full of snow, with characteristic northern wind bushes, replaced mostly rainy winters, with slightly reduced rainfall and long, hot summers. A few years ago, the sheep-breeding regime on mountain pastures was characterized by extensive farming, often followed by transhumance. Transhumance was exercised in the summer pastry of sheep on mountain pastures, while winter herds moved to flatland areas in search of food. However, by adopting a ban on making such a manner of sheep breeding, transhumance is minimized (FMPVŠ, 2018). Hematological and biochemical parameters in healthy sheep show several variations in relation to breed, age, reproductive status, environmental factors and management conditions. Therefore, there is a need to investigate these factors and how they affect the animal's hematology. Sheep are small ruminants that have special attributes over other livestock resources. They are more adapted to broad ranges of environment, have short generation cycles and reproductive rate which lead to high production efficiency. Hematological, biochemical and mineral profiles are important to be determined because they provide valuable information about the breed, sex and animals health status. There is considerable information about the normal parameters of blood of the domestic animal species, but the values are expected to vary according to the breeds, different environmental factors and the different methods of management. The physiological adaptation and the systemic relationship are widely determined using the hematological values. The biochemical profile shows some changes and the blood plasma components which varies according to the growth requirements, breed, ages, environmental factors,

management conditions, sexual maturity and the productivity of the animals. Biochemical parameters are responsible for various body functions and its deficiency results in impairment of functions induce structural and physiological abnormalities (American Diabetes Association, 2009). Knowing the physiological values of hematological parameters is of paramount importance for the understanding of physiological processes in the body. Determination and monitoring of metabolic profile parameter values may show whether homeostatic mechanisms can maintain blood composition in physiological limits under different conditions of animal husbandry (Prodanović et al. 2012). The research that aims at exploring and understanding the specifics of any hematological and biochemical parameters of indigenous breeds of sheep in Bosnia and Herzegovina (B&H), in order to contribute to the identification and preservation of this important resource. The values of key hematology parameters will provide an objective information about the health and production of animals at the time of sampling, displaying nutritional status, any illness or the effects of stress which the animals were exposed to. Reference ranges of hematological parameters are the basis of laboratory analysis and have practical application in distinguishing healthy from sick animals. Animal health care includes early detection, monitoring, protection of appearance and control of infectious and parasitic diseases, including combating zoonosis. For these reasons, monitoring the health status of animals is of crucial importance because at any moment we can monitor the health status of animals within Bosnia and Herzegovina. We think that one of the biggest problems farmers are facing with is illegal imports of animals through illegal border crossings that threaten both the health status of the existing livestock, and health of the population because these animals have not undergone the official control at the border, nor veterinary sanitary examination was performed.

The research would be a continuation of the research on preservation of the health status, completing of the so-called "Database" on the metabolism of Dubska Pramenka sheep breed, as well as continuation of affirmation B&H indigenous breeds and products.

MATERIALS AND METHODS

The research was conducted on a total of 104 sheep (Vlasic n = 52, Livno n = 52) from the site of the Vlasic mountain and Livno fields. The sheep were fed by free grazing with the addition of hay and grain. The study was conducted in June 2018. Blood samples were taken from the jugular vein into Vactainer tubes with EDTA anticoagulant for hematological, for glucose, analyses and BD Vacutainer® SST II gel for biochemical analyses. All hematological and biochemical analyses were performed within the next 24 hours, and until then the samples were kept at 4 °C. Hematological parameters included total Red Blood Cell (RBC), White Blood Cell (WBC), Hemoglobin concentration (Hb), Hematocrit (Hct), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Mean Corpuscular Volume (MCV), Mean Platelet Volume (MPV), Red Cell Distribution Width (RDW) and White Cell Differential Count (WCDC). Analyzes are carried out using the automated veterinary hematological analyzer Advia 120 SIEMENS. Blood in the serum tubes was allowed to clot for at least 30 min prior to centrifugation. Serum samples were kept frozen at -20 °C until biochemical analyses were performed. Biochemical parameters were determined by analyzer Olympus AU400 with Beckman Coulter reagents according to the manufacturer's protocol. Parameters for biochemistry panel included: total protein (TP), albumin (ALB), globulin (GLO), urea (BUM), creatinine (CRE), glucose (GLU), aspartate aminotransferase (AST), γ -glutamyltransferase, (GGT), creatine kinase (CK), cholesterol (CHO), bilirubin (BIL), calcium (Ca), phosphorus (P), sodium (Na), chloride (Cl), potassium (K) and magnesium (Mg). All analyzes were tested to spectrophotometric method, except for Na, K, Cl, which were generated by ISE method, or by ion selective method. BHBA (β -hydroxybutyrate) and NEFA (non-esterified fatty acids) were constructed with reagents from Randox (UK).

The obtained results were statistically analysed using Microsoft Excel 2010, module Data Analysis. The differences between the mean values of the groups were tested with t- Test. Differences were considered statistically significant at the level of significance and correlation coefficient ($p < 0,05^*$; $p < 0,01^{**}$, $p < 0,001^{***}$).

RESULTS AND DISCUSSION

Evaluation of the blood parameters has become an essential tool for diagnostic purposes in medical problems. Interpretation of the hematology results with clinical findings and the results of other paraclinical tests may suggest a specific differential diagnosis or prognosis (Ahmadi-Hamedani et al., 2015). The unique characteristic of the Pramenka sheep is that they are adaptive to different geographic and climatic conditions in B&H. Other authors suggest that haematological variables in sheep may be affected by various internal and external factors such as age, gender, season, breed, lactation and nutrition (Tibo et al., 2004; Jenko, 2009; Soch at al. 2010; Addass, 2011; Vojta et al., 2011; Antunovic et al., 2014; Antunovic et al., 2017; Shek-Vugrovečki et al., 2017). The obtained results of hematological parameters of the sheep's body are shown in Table 1.

Table 1. Hematological parameters in blood sheep

Parameters (unit)	Area Livno	Area Vlasic	P
WBC ($10^9/L$)	7.97	9.18	*
RBC ($10^{12}/L$)	8.83	7.41	***
Hb(g/L)	93.37	76.56	***
PCV (%)	27.94	22.14	***
MCV (fl)	31.81	30.17	*
MCHC (g/L)	339.51	345.02	ns
MCH (pg)	10.78	10.91	ns
RDW (%)	15.46	18.56	***
PLT ($10^9/L$)	657.59	652.02	ns
MPV (fl)	15.35	14.61	ns
WCDC			
Lymphocytes (%)	57.94	63.49	*
Neutrophils (%)	30.35	22.97	**
Eosinophils (%)	5.27	4.78	ns
Basophilis (%)	1.78	1.64	ns
Monocytes (%)	3.74	4.58	ns

* ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$),

The values of RBC, Hb, PCV ($p < 0.001$) and MCV ($p < 0.05$) were statistically significantly higher among Livno area sheep (Table 1). The difference of 570 in altitude between the sites of Livno and Vlasic can affect the partial oxygen pressure in the blood of sheep and the analyzed haematological parameters show a statistically significant difference at $p < 0.001$. However, this statistical significance is likely to be due to the influence of the physiological status of sheep from the Livno area and less likely the influence of altitude because the number of erythrocyte significantly

differed between the sheep of both areas. The RBC value were statistically higher in sheep at a lower altitude. The RBC and Hb values of Livno blood samples are in line with the reference values mentioned by other authors (Jackson and Cockcroft, 2002; Antunović et al., 2017). Comparing our results with the results of Shek Vugrovečki et al. (2017), the values of erythrocytes, hemoglobin and hematocrit were similar or the same for the sheep in the Livno area, while the values of the mentioned parameters for sheep from the Vlasic area were reversed (Softić et al., 2018.), which explains the population in the other geographic area and in different strain of Pramenka sheep, different ages, different percentage of protein in the diet of sheep, or the health status of the animal. The increased level of Hb concentration is attributed to the ability to undergo infection, good nutritional status as well as animal breeding (Anwar et al., 2012), whereas the reduced concentration can be interpreted by the causes of different etiology, consequences of regeneration or anemia formation (Awodi et al. 2005). By comparing the results of hematological values with the results (Comba et al., 2017) our results show somewhat lower values for RBC, Hb, PCV, WBC whereas score values for MCV, MCHC, PLT, MPV, LYM, MON were higher. Shek Vugrovečki et al., (2017) carried out the researches at Lička Pramenka, which by the physical frame (Ivanković et al., 2009) is considerably underdeveloped compared to the strain used in our research, the values of erythrocyte indices for both groups of sheep were also similar or the same. Erythrocyte indices (MCV, MCH, MCHC) give us information on the average cell size, hemoglobin content, and the concentration of hemoglobin concentrations in erythrocytes. A higher value of the MCV can be a sign of the cell's regenerative response. We have to emphasize that above mentioned authors used other types of animals in different experimental treatments. According to the results of Vojta et al. (2011), determined on the Dalmatian Pramenka using the bootstrap method, the values of MCV, MCH and MCHC are in line with the results of our research, except for a slightly higher platelet count of compared to the research of Vojta et al. (2011), Antunović et al. (2014), Shek Vugrovečki et al., (2017). Differential blood count of sheep from both areas (Table 1), did not deviate from the reference values of Weiss and Wardrop (2010), except for the values of lymphocytes. The high lymphocyte

share in the blood of the sheep from both areas indicates the immunomodulatory response of the organism, but a significantly higher lymphocyte count in the bloodstream from the Vlasic area was found ($P < 0.001$). It is known that lymphocytes are responsible for the humoral and cellular immune response and their increase in blood can be an indicator of the immunomodulatory response of the organism (Qureshi et al., 2001).

Table 2. Correlation factors (r) between some haematological parameters

Ratio	Correlation with P-value	
	Area Vlasic	Area Livno
RBC:Hb	0.893***	0.636***
RBC:PCV	0.882***	0.949***
RBC:MCV	0.469***	0.273 ns
RBC:MCH	0.404**	0.648***
RBC:MCHC	0.353*	0.535***
RBC:RDW	0.603***	0.151 ns
RBC:PLT	0.419**	0.134 ns
RBC:MPV	0.375**	0.346*
WBC:NEU	0.673***	0.556***
WBC:LYM	0.823***	0.642***
WBC:MON	0.561***	0.292*
WBC:EOS	0.322*	0.154 ns
WBC:BAS	0.651***	0.535***

* ($P < 0.05$), ** ($P < 0.01$), *** ($P < 0.001$)

The correlation coefficient values showed statistically justified on the level of very high significance of differences ($p < 0.001$) between RBC and Hb, RBC and PCV, WBC and NEU, WBC and LYM, WBC and BAS for both geographic areas, while significant justification of differences $p < 0.01$ and $p < 0.05$ established between RBC and MCH, respectively RBC and MCHC, RBC and PLT, and RBC and MPV for sheep from the Vlasic area. In the Livno area a correlation between RBC and MPV ($p < 0.05$), as well as for WBC and MON ($p < 0.05$) was determined (Table 2). According to available literature (Hrković-Porobija et al. 2013; Antunović et al., 2017; Softić et al. 2018), justified significant correlations have been established. We have to emphasize that the authors mentioned in their researches used the same or different animal species with different experimental designs.

Biochemical parameters

The serum biochemical values of sheep obtained in this study are presented in Table 3.

In combination with hematology, a biochemical profile represents a database important for

diagnostic methods. Generally, serum concentrations of BHBA have been used to determine hyperketonemic state and subclinical ketosis, but there are different ideas about cut-off point of BHBA. This rise in serum BHBA is a compensatory mechanism and a reflectionary response to carbohydrate deficiency and inhibition of Krebs's cycle (Reece WO, 2004). Biochemical parameters for assessing energy status of pregnant ewes are mainly serum concentrations of glucose, BHBA (β -hydroxybutyrate) and NEFA (non-esterified fatty acids). Generally, researchers believe that BHBA concentration is a golden marker for diagnosis of pregnancy toxemia and/or ketosis in ewes and cattle (Kaneko et al., 2008). It is the most stable ketone and accounts for approximately 85% of the total ketones in sheep with pregnancy toxemia (Bostedt and Hamadeh, 1990) but there are different ideas about cut-off point of BHBA. As parameters in which variations are most often reflected in the regularity of sheep's diet are glucose and cholesterol. These parameters represent indicators of nutritional status and the possibility of using glucose by ruminants. Ruminants appear to be well adapted to a carbohydrate economy based on their ability of gluconeogenesis. But while fetal glucose demands increase with increasing body size, it can increase susceptibility to pregnancy toxemia. A fetus of sheep in late pregnancy utilizes about one-third to one-half of the daily glucose turnover of 100 g (Kaneko et al., 2008). The glucose level in the Vlasic area (3.34 mmol / l) was statistically significantly higher ($p < 0.001$) than the established value in Livno (2.97 mmol / l). The values found in both sampling areas were within the range of values stated by Kaneko et al. (1997), but are higher than the values given by other authors in their work (Hrković et al., 2009; Shek-Vugrovečki et al., 2017), which indicates good energy supply, before the sampling was carried out at the beginning of the past period when plants are at the beginning of their vegetation. Despite the many influences on the values of haematological variables in the blood of sheep, in most cases the values are within relatively narrow limits which usually correspond to the wide reference ranges published in the relevant textbooks (Feldman et al., 2000; Kaneko et al., 2008; Pugh and Baird, 2012).

The value of cholesterol in Livno's livestock was statistically ($p > 0.001$) significantly higher than in the area of Vlasic, but also in relation to the values reported by Kramer (2000) and Kaneko (2008).

According to the results of Hrković-Porobija (2011) found on the Pramenka sheep from Livno and Travnik, cholesterol values are approximate to the results of our research.

Table 3. Biochemical parameters in sheep blood

Parameters (unit)	Area Livno	Area Vlasic	P
BHBA (mmol/L)	0.59	0.44	***
NEFA (mmol/L)	0.37	0.49	***
TP (g/l)	75.27	70.66	***
ALB (g/l)	32.78	29.53	***
GLO (g/l)	42.48	41.17	ns
BUM (mmol/l)	5.69	5.31	*
CRE (μ mol/L)	45.61	48.29	**
GLU (mmol/l)	2.97	3.34	***
AST (IU/L)	172.24	132.12	***
GGGT (IU/L)	43.57	43.89	ns
CK (IU/L)	412.60	429.75	ns
CHO (mmol/l)	2.23	1.74	***
BIL (mmol/l)	0.57	2.60	***
Ca (mmol/l)	2.67	2.47	***
P (mmol/l)	1,48	2.18	***
Na (mmol/l)	142.31	142.58	ns
Cl (mmol/l)	103.59	104.46	ns
K (mmol/l)	0.71	0.33	ns
Mg (mmol/l)	1.19	1.02	***

*Statistical significance difference ($p < 0.05$), **Statistical significance difference ($p < 0.01$), *** Statistical significance difference ($p < 0.001$).

The values of total protein and albumin were statistically significantly ($p > 0.001$) higher in Livno sheep and in line with the results obtained at Dubrovnik and Lička Pramenka (Antunović et al., 2011; Shek-Vugrovečki et al., 2017), but greater than the values quoted by Stevanović et al. (2105). Hrković-Porobija et al., (2011) also found lower values of total protein and albumin at the locality of Travnik at the same sites. Calcium and Magnesium were statistically ($p < 0.001$) significantly higher in Livno's sheep (Table 3), while phosphate concentration was statistically ($p < 0.001$) significantly higher in sheep from the Vlasic area (Table 3). Other authors have published different reference values - Ca 2.88 - 3.20 mmol/l, - Mg 0.90-1.31 mmol/l, - P 1.62-2.36 mmol l (Kaneko, 1997) and - Ca 2.53 and - Mg 1.01, - P 2.10 (Dias et al., 2010). The level of minerals in the blood depends on the diet, which indicates that the calcemia may be directly correlated with the category and diet of sheep (Hrković et al., 2009). The levels of the main serum minerals, such as Ca, P, and Mg, were determined for the evaluation of

the mineral status of the organism, bearing in mind that the diagnosis of mineral deficiencies highly depends on the homeostatic control level of each mineral and on the time that the basic problem lasts (Dias et al., 2010). Sodium, chloride and potassium did not show statistically significant differences between sampling areas (Table 3).

The serum enzyme activities of aspartate aminotransferase and gamma-glutamyl transferase for sheep are shown in Table 3. Concentration of AST was statistically significant ($P < 0.001$) higher in Livno's sheep, while GGT concentration did not statistically differ significantly between sampling areas but was higher in value than other authors (Ramos et al., 1994; Akgul et al., 2000). Higher activity of ALT could be a consequence of intensification in metabolic processes with increase in age (Antunović et al., 2008). AST is not specific only for liver; skeletal muscles are another great source of AST in animals. Because of huge difference in concentration of AST in tissues and blood serum, in processes where tissues that are rich in AST have lesions, this enzyme exceeds in circulation, which is reflected in increase of AST activity in blood serum (Hrković et al., 2017). The obtained urea concentrations, 5.69-5.31 mmol / l, do not differ from the given physiological values of 2.86-7.14 mmol / l (Kaneko, 1997) and are in accordance with the values we encounter during the summer sampling period (Ašimović, 2005). The results obtained for the total protein, urea, bilirubin indicate higher values of our study in both study areas, while the values of creatine and albumin in the Vlasic region were lower (Comba et al., 2017). Urea concentration is used as a indicator of renal function. Bilirubin values were statistically significantly higher ($p < 0.001$) in Vlasic sheep (Table 3). Ašimović (2005) states that the influence of the breed and breeding sites is not statistically justified. The statistically justified differences are primarily the effect of the season (summer), nor the sheep diet. Animals are fed to small amounts of creatine by consuming food containing animal tissue. Creatine is well absorbed from the intestinal tract. Creatinine values in this study were saturated ($p < 0.01$) between sampling areas (Table 3) and were lower than other authors (Njidda et al., 2014). Particularly important is the interaction of urea and creatinine, the effect of the season and the association with nutrition. A special aspect is the involvement of

microorganisms of the rumen in these processes (Ašimović, 2005).

Correlation coefficients of our research between some biochemical elements are shown in Table 4. Positive correlation was established between Total Protein and Chloride, Total Protein and Phosphate as well as BHBA and Total Protein at the level $p < 0.05$, while a very high correlation coefficient ($p < 0.001$) between Sodium and Chloride, and Calcium and Phosphate ($r = 0.522$) was found among sheep in Vlasic, which represents a higher value compared to Gonzalez et al. (2009). The only interconnection that was found, was the 0.293 ($p < 0.05$) between Sodium and Chloride in the area of the Livno. The research of Anoushepour et al. (2014) showed a significant ($p < 0.05$) negative correlation between glucose and BHBA ($r = -0.549$, $p < 0.05$), which is consistent with our research ($r = -0.380$, $p < 0.01$) for the Vlasic area. BHBA and glucose concentrations are mainly used to assess the energy status of pregnant sheep.

Table 4. Correlation factors (r) between some biochemical parameters

Ratio	Correlation with P-value	
	Area Vlasic	Area Livno
TP:Cl	0.315*	ns
TP:P	0.305*	ns
Ca:P	0.522***	ns
Na:Cl	0.621***	0.293*
BHBA:TP	0.312*	ns

*($P < 0.05$), *** ($P < 0.001$), beta-hydroxybutyrate - (BHBA)

CONCLUSION

Analyzing hematologic indicators in sheep breeds there were no major deviations from the reference values for sheep, except lower hemoglobin for sheep in the Vlasic area and lower values of RBC in sheep of both areas. Higher values of lymphocytes were found in sheep of both areas. Higher lymphocyte counts and very high statistical significance for Vlasic' sheep may be a signal for cell regeneration response. Almost all of the biochemical parameters showed variations between the sampling areas that could be caused by the influence of different geographic areas and floristic composition, as well as the current health status of the examined individuals. Correlative values showed significant differences for individual haematological and biochemical parameters in sheep for both investigated areas.

ACKNOWLEDGMENTS

Conflict of Interests: The authors declared that there is no conflict of interests.

Financial Disclosure: The authors would like to thank the Faculty of Landscape and Society, Ås, NMBU, Noragric, for their financial support and kind cooperation in conducting this valuable study.

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