Acta Veterinaria-Beograd 2022, 72 (1), 59-75
UDK: 636.2:591.111.1
636.2.09:616.24-002
636.2.09:618.19-002-085
DOI: 10.2478/acve-2022-0005

Research article

EVALUATION OF SERUM IRON AND FERRITIN LEVELS AS INFLAMMATORY MARKERS IN CALVES WITH BOVINE RESPIRATORY DISEASE COMPLEX

Şükrü DEĞİRMENÇAY[1]*, Akın KIRBAŞ[2], Hakan AYDIN[3], Ömer AYDIN[1], M. Sinan AKTAŞ[1], Ramazan KAMAN[1]

[1]Atatürk University, Faculty of Veterinary Medicine, Department of Internal Medicine, 25240, Erzurum, Turkey; [2] Yozgat Bozok University, Faculty of Veterinary Medicine, Department of Internal Medicine, 66700, Yozgat, Turkey; [3]Atatürk University, Faculty of Veterinary Medicine, Department of Virology, 25240, Erzurum, Turkey

(Received 26 November 2021, Accepted 09 February 2022)

Iron and ferritin have been used in human medicine for years to reveal the presence of inflammation. However, studies evaluating these parameters, especially in respiratory system diseases, are quite rare in veterinary medicine. We aimed to test the usability of serum Fe and Fe-related parameters [total iron-binding capacity (TIBC), unsaturated iron-binding capacity (UIBC) and transferrin saturation (TS) levels] as inflammatory and diagnostic biomarkers in calves with bovine respiratory disease complex (BRDC). To mark inflammation, some selected acute-phase proteins including serum ferritin and transferrin levels were measured because of their close relationship with iron metabolism. The material of this study consisted of 15 calves, aged 1-3 months with BRDC (Group I) and 10 healthy calves aged 1-3 months (Group II) based on the presence of respiratory clinical findings. Serum Fe, TIBC and TS levels were low and ferritin levels were high in Group I ($P \leq 0.001$). The BRDC group was separated into two subgroups based on PCR results, namely Virus+ (n=9) and Virus- (n=6). The calves in the Virus+ group had significantly lower levels of Fe (P=0.001) and significantly higher values of ferritin (P=0.002), compared to the healthy group. On the basis of inter-group comparison and ROC analysis, we concluded that Fe (primarily), ferritin, TIBC and TS levels can be used as inflammatory biomarkers and possible diagnostic markers in the BRDC as useful, practical, inexpensive substitutes. As a suggestion, these parameters which are believed to play a role in the pathogenesis of the disease, can be used as potential prognostic biomarkers in studies involving treatment.

Key words: calf, iron, ferritin, inflammatory markers, bovine respiratory disease complex

*Corresponding author: e-mail: s.degirmencay@atauni.edu.tr

INTRODUCTION

Respiratory system diseases are one of the most common diseases in cattle. Acute-phase protein analyses are the most commonly used tools in the diagnosis and prognosis of inflammatory diseases in cattle. However, these analyses are expensive and require laboratory conditions. A limited number of laboratory tests can be used in the diagnosis of diseases with inflammation by a general veterinarian under field conditions [1-3]. Therefore, it is necessary to determine reliable, easily applicable and affordable biomarkers for the diagnosis and prognosis of respiratory system infections. It has been shown that decreased serum iron (Fe) concentration and increased ferritin concentration can be used as a biomarker for inflammatory conditions [4,5]. For these reasons, we wanted to test the usability of serum Fe and Fe-related parameters [total iron-binding capacity (TIBC), unsaturated iron-binding capacity (UIBC) and transferrin saturation (TS) levels] as inflammatory and possible diagnostic biomarkers in calves with bovine respiratory disease complex (BRDC). To mark inflammation, as acute-phase proteins, we preferred ferritin and transferrin due to their close relationship with iron metabolism.

Bovine respiratory disease complex is one of the leading serious respiratory tract infections of calves with high morbidity and mortality. This disease is multifactorial given that it is caused by the combination of viral and bacterial pathogens, together with environmental risk factors such as stress and reduced immunity. *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, and *Mycoplasma bovis* are the most isolated bacterial agents in BRDC [6]. The most isolated viral agents were reported as bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus 3 (BPIV-3), bovine viral diarrhea virus (BVDV) and bovine herpesvirus 1 (BHV-1) [7-11].

Iron, an important trace element for both the host and pathogen, plays a role in many various biological processes, including immune function and metabolism, oxygen transport, cellular respiration, DNA and RNA synthesis and many enzymatic activities [12,13]. Iron is very important for the synthesis of hemoglobin and approximately 60-75% of the iron in the body is bonded to hemoglobin. The rest is bonded to transferrin (Tf) and ferritin. A very small amount of iron is bonded to myoglobin [14]. As a host defense mechanism, iron concentration decreases rapidly in response to inflammation and malignancy [15]. It is hypothesized that this deficiency develops as a defense mechanism to limit the use of serum iron (Fe) by pathogens and tumors [16-19]. IL-6 mediated hypoferremia is responsible for this deficiency [20]. Serum Fe concentration has been assessed as a biomarker of inflammation in dogs and cats [21], horses [15], and cattle [4,14,22].

Ferritin functions as a major intracellular storage molecule for iron [23]. Thus, it is used also in veterinary medicine as a useful and acceptable method to determine iron storage status [24-26]. Ferritin levels have been shown to be low in those with iron deficiency disease and high in those with an excessive iron load [27]. However, sometimes high serum ferritin levels can lead to a misdiagnosis of anemia [28].

Because in cases of inflammation, infection and malignancy, serum Fe concentration decreases while ferritin levels increase [29]. Unfortunately, these conditions complicate the interpretation of ferritin levels [30]. In this case, elevated ferritin reflects increased total body iron stores, but paradoxically, these stores are sequestered and not suitable for the process of hematopoiesis, which results in widely recognized anemia of inflammation [17]. So, serum ferritin can be used as an inflammatory biomarker of acute-phase infection [28]. High ferritin levels have been detected in the sera of cows with leukemia [31], uterine and udder inflammation [25].

Transferrin (Tf) is an important protein for binding and transporting iron [32]. Tf is a negative acute-phase protein, therefore is downregulated in inflammatory conditions [33]. It is also associated with hypoferremia during infections [34]. When iron stores are deficient, Tf levels rise in the blood [35]. Serum Ferritin and Tf concentrations are correlated with animals' iron status, especially body iron stores [36]. While transferrin carries no more than 2 iron atoms, a single ferritin molecule can hold up to 4500 iron atoms, making it a potentially very effective iron delivery actor [37].

Total iron-binding capacity is a crucial test used in the diagnosis of iron deficiency anemia and other iron metabolism disorders. Iron binding capacity is explained as the binding capacity of transferrin to iron. There are two types of iron-binding capacity: TIBC and UIBC. One-third of transferrin is saturated with iron and in the serum it has an extra binding capacity for iron. The remaining two-thirds, which are unsaturated with iron, is called UIBC. The TIBC value is the total of serum iron and UIBC. The transferrin saturation (TS), expressed as a percentage, is calculated by dividing serum Fe concentration by TIBC and multiplying the result by 100 [35]. TS indicates how much serum Fe is actually bonded to Tf [38].

Irregularity of iron homeostasis has been associated with numerous diseases including hemochromatosis, anemia, various cancers, neurodegenerative, cardiovascular and respiratory system diseases [39]. In veterinary medicine, Fe levels have been shown to be significantly reduced in cattle with septicemia [40], systemic inflammatory response syndrome (SIRS) [14], reticuloperitonitis traumatica (RPT) and mastitis [4], endotoxemia [41], local (omphalitis and arthritis) and systemic (enteritis and pneumonia) inflammation [42]. In human medicine, there is strong evidence of dysregulation of iron homeostasis in a number of major respiratory diseases, including acute respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), asthma, cystic fibrosis (CF) and lung cancer [43]. There is a handful of studies evaluating the Fe level in calves with respiratory disease [22, 42, 44]. To our knowledge, there is no study evaluating Fe, ferritin, Tf, TIBC, UIBC and TS together. Therefore, in this study, we aimed to examine whether the values mentioned above are useful as inflammatory and possible diagnostic biomarkers for calves with BRDC. Based on the conclusions of the mentioned studies, we hypothesized that serum Fe, Tf, TIBC, UIBC and TS levels would decrease as opposed to serum Ferritin levels.

MATERIALS AND METHODS

Animals and protocol design

The study material included 25 calves 1-3 months old, different breeds (Holstein and Simental) and both genders. The calves were divided into two groups as BRDC (Group I, n=15) and healthy (Group II, n=10) based on clinical examination and complete blood count findings. Based on the PCR results, subgroups in group I were divided as Virus+ (n=9), Virus- (n=6) and healthy (n=10). During the clinical examination, rectal temperature (RT), heart rate (HR) and respiratory rates (RR) of all calves were measured. Subsequently, calves were examined based on auscultation of lung sounds, respiratory type, the structure of mucous membranes, lymph nodes status, lacrimation, nasal discharge, dyspnea and cough. The study was conducted in accordance with animal welfare principles. Ethical approval of the study was not required since samples were taken as part of the routine clinical examination. An informed consent form was obtained from the owner before examining the animals.

Blood sampling

Blood samples from all the calves were taken from the *vena jugularis externa* and collected into EDTA vacutainers (Vacutainer, K2E 3.6 mg, BD, UK) and plain tubes (Vacutainer, BD, UK) for hematological and biochemical analyses. After leaving for ten minutes at room temperature for clotting, sera were obtained by centrifugation (Beckman Coulter, Allegra® X-30R, USA) at 3000 rpm for 10 minutes and stored at -80°C until being analyzed. Hematological analyses were performed immediately.

Hematological analyses

White blood cell (WBC), lymphocyte (LYM), monocyte (MON), neutrophil (NEU), eosinophil (EOS), basophil (BAS), red blood cell (RBC) and hemoglobin (HGB) counts, and hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHc) and platelet (PLT) levels of the calves were determined with a hematology analyzer (Abacus Junior Vet5, Hungary).

Biochemical analyses

In the serum samples, the concentrations of Fe, Tf and UIBC were determined by commercial kits using a biochemistry autoanalyzer (Beckman Coulter, AU5800, USA). Ferritin concentration was determined by electrochemiluminescence immunoassay (ECLIA) method [45] using an auto-analyzer (Beckman Coulter Unicel DXI 800, USA). TIBC value was obtained by the sum of serum Fe and UIBC. TS, expressed as a percentage, was calculated by dividing serum Fe concentration by TIBC and multiplying the result by 100 the formulas of which are shown below [35].

$$\text{TIBC: Fe + UIBC} \qquad \text{TS: (Fe/TIBC) x 100}$$

Molecular analysis

The nasal swabs were collected from calves for the diagnosis of BRSV, BPIV-3, BHV-1 and BVDV agents. Nasal swab samples were vortexed in 1 mL of phosphate-buffered saline and centrifuged at 3,000 rpm for 5 min. The 200µL supernatant was used for the isolation of nucleic acids with the viral nucleic acid extraction kit (Vivantis, Malaysia). All extracts were kept at −20°C until used. Nucleic acid suspensions were subjected to reverse transcription (cDNA) analyses using a cDNA synthesis kit (Thermo Fisher Scientific, Germany). All reactions were performed according to the manufacturer's instructions. cDNA samples were subject to PCR analyses using gene-specific primer pairs. The primers and estimated amplicon sizes are shown in Table 1. The PCR analyses were performed under previously described conditions [46-48]. Positive PCR amplicons were separated on a 1.5% agarose gel by gel electrophoresis and displayed under UV light (Figure 1).

Table 1. Primer pairs used in the study

Virus	Primer sequences (Forward/Reverse)	Product size (bp)	References
BVDV	ATGCCCWTAGTAGGACTAGCA TCAACTCCATGTGCCATGTAC	288	[46]
BRSV	AATCAACATGCAGTGCAGTTAG TTTGGTCATTCGTTATAGGCAT	710	[47]
PIV-3	AGTGATCTAGATGATGATCCA GTTATTGATCCAATTGCTGT	328	[47]
BHV-1	TACGACTCGTTCGCGCTCTC GGTACGTCTCCAAGCTGCCC	478	[48]

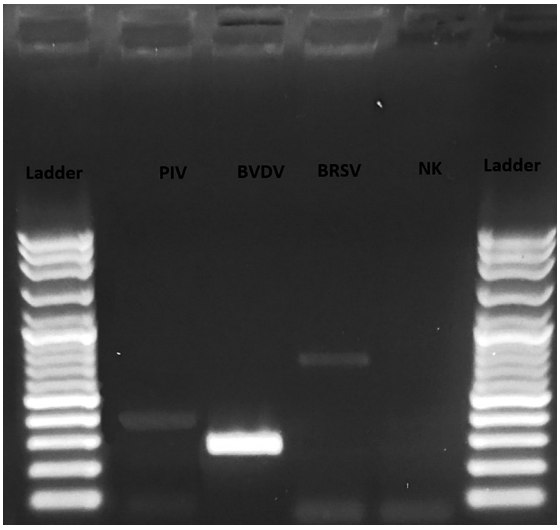

Figure 1. The appearance of positive samples under UV light. DNA fragments of PIV-3 (328bp), BVDV (288bp), BRSV (710bp) and NK (negative control)

Statistical analysis

The SPSS software program (Version 25.0, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The distribution of the data between the groups (BRDC suspected and Healthy groups) was evaluated using a Shapiro-Wilk test. Parametric variables (LYM, HGB, MCV, MCH, MCHc, PLT, UIBC, TIBC, RT, HR and RR) were compared using the T-test (Independent-Samples T-Test). Nonparametric variables (WBC, MON, NEU, EOS, BAS, RBC, HCT, Ferritin, Tf, Fe and TS) were compared using the Mann-Whitney U test. All results were presented as the mean ± standard deviation (SD) for parametric variables and the median and range (min-max) for nonparametric variables. The correlation among statistically significant parameters was measured by the Spearman Correlation test. Receiver Operating Characteristic (ROC) analysis was employed to determine sensitivity, specificity and cut-off values of statistically significant parameters. To assess the diagnostic and inflammatory potential of Fe, ferritin, TIBC, TS, NEU and EOS levels for BRDC, the areas under the ROC curves (AUC) were recorded and cut-off values were analyzed. For statistical analysis of the subgroups, Virus+, Virus-, and Healthy group, one-way analysis of variance (ANOVA) and post hoc Duncan test were used for parametric data, and Kruskal Wallis H test was used for non-parametric data. A significance level of $P < 0.05$ was used for all statistical comparisons.

RESULTS

The average age of the calves in the study was 79±29 days. Common clinical findings in the BRDC group were high fever (T:40.15±0.65°C), tachycardia (131.06±19.31 beats per minute), tachypnea (71.6±29.80 breaths per minute), dyspnea, cyanotic mucous membranes, prescapular lymphadenopathy, lacrimation, sporadic cough and serous-mucous nasal discharge. Auscultation revealed hardening in the lung sounds. The type of respiration was costo-abdominal in the majority of calves with BRDC, but very few were abdominal (Table 2). RT, HR and RR of the BRDC group were higher than the healthy group (P=0.000) (Table 3).

According to the hematologic findings, NEU (P=0.001) and EOS (P=0.026) numbers in the BRDC group were higher than that of the healthy group and MCH (P=0.004) and MCHc (P=0.000) were lower (Table 3).

In the biochemical analysis, serum Fe concentration (P=0.000), TIBC (P=0.000) and TS (P=0.001) in the BRDC group were lower than the healthy group and serum Ferritin concentration was higher (P=0.000). Tf and UIBC were statistically insignificant in the comparison between groups (Table 3).

Serum Fe concentration was negatively correlated with NEU (r=-0.720, P=0.000), EOS (r=-0.453, P=0.023), Ferritin (r=-0,582, P=0.002), but positively correlated with MCH (r=0.533, P=0.006), MCHc (r=0.608, P=0.001), TIBC (r=0.597 P=0.002) and TS (r=0.975, P=0.000). Serum ferritin concentration was positively correlated with NEU (r=0.596, P=0.002), but negatively correlated with MCHC (r=-0.543, P=0.005), Fe (r=-0.582 P=0.002) and TS (r=-0.513 P=0.009) (Table 4).

Table 2. Some clinical findings of calves with BRDC

Respiration type (n=15)	Costoabdominal 11		Abdominal 4	
Dyspnea (n=15)	Present 9		Not present 6	
Mucous membranes (n=15)	Normal 7		Cyanotic 8	
Lymp nodes (n=15)	Normal 3		Mildly swollen 12	
Lacrimation (n=15)	Present 7		Not present 8	
Cough (n=15)	Dry 11	Wet 3	Not present 1	
Nasal discharge (n=15)	Serous 1	Seromucous 9	Mucopurulent 4	Not present 1

Table 3. Comparison of haematological, biochemical and clinical examination parameters of BRDC and healthy groups

Parameters	BRDC	Healthy	Test p
WBC (x10^3/µL)	10.68 (5.61-31.97)	9.64 (7.20-11.21)	P= 0.129
LYM (x10^3/µL)	5.25±1.99	5.85±1.34	P= 0.419
MON (x10^3/µL)	0.14 (0.06-1.16)	0.30 (0.10-0.96)	P= 0.428
NEU (x10^3/µL)	5.44 (2.72-24.50)	2.90 (0.92-4.52)	P= 0.001
EOS (x10^3/µL)	0.12 (0.06-0.34)	0.08 (0.05-0.12)	P= 0.026
BAS (x10^3/µL)	0.00 (0.00-0.04)	0.00 (0.00-0.01)	P= 0.196
RBC (x10^6/µL)	10.17 (4.68-12.59)	9.88 (6.73-12.71)	P= 0.643
HGB (g/dL)	9.43±1.93	10.61±1.67	P= 0.130
HCT (%)	33.06 (14.59-37.14)	30.05 (21.35-35.33)	P= 0.428
MCV (fL)	31.13±3.66	31.80±3.04	P= 0.639
MCH (pg)	9.54±1.10	10.89±0.88	P= 0.004
MCHc (g/dL)	30.77±1.74	34.53±1.32	P= 0.000
PLT (x10^3/µL)	398.07±116.70	415.90±131.21	P= 0.725
Fe (µg/dL)	22 (1-97)	146 (66-273)	P=0.000
Ferritin (ng/ml)	2.10 (0.2-203)	0.20 (0.00-1.7)	P=0.000
Transferrin (µg/dL)	0.02 (0.00-0.06)	0.025 (0.01-0.06)	P=0.723
UIBC (µg/dL)	369.87±81.69	379.90±96.96	P= 0.783
TIBC (µg/dL)	403.40±77.01	532.40±64.69	P=0.000
TS (%)	4.75 (0.28-28.06)	28.32 (10.51-49.82)	P=0.001
RT (°C)	40.15±0.65	38.37±0.25	P=0.000
HR (beats/min)	131.06±19.31	89.60±13.49	P=0.000
RR (breaths/min)	71.60±29.80	26.40±4.69	P=0.000

WBC= white blood cell, LYM= lymphocyte, MON= monocyte, NEU= neutrophil, EOS= eosinophil, BAS= basophil, RBC= red blood cell, HGB= haemoglobin, HCT= haematocrit, MCV= mean corpuscular volume, MCH= mean corpuscular haemoglobin, MCHc= mean corpuscular haemoglobin concentration, PLT= platelet, Fe= iron, UIBC= unsaturated iron-binding capacity, TIBC= total iron-binding capacity, TS= transferrin saturation, RT= Rectal temperature; HR= Heart rate (per min); RR= Respiratory rate (per min). Data are presented as mean ± standard deviation and or median (range).

Table 4. Correlation results between haematological and biochemical parameters in BRDC and healthy groups (Spearman Correlation)

Parameters	NEU	EOS	MCH	MCHc	Fe	Ferritin	TIBC	TS
NEU	1.000	0.369	-0.395	-0.358	-0.720**	0.596**	-0.480*	-0.711**
EOS		1.000	-0.598**	-0.466*	-0.453*	0.391	-0.516**	-0.419*
MCH			1.000	0.533**	0.533**	-0.303	0.422*	0.522**
MCHc				1.000	0.608**	-0.543**	0.521**	0.558**
Fe					1.000	-0.582**	0.594**	0.975**
Ferritin						1.000	-0.378	-0.513**
TIBC							1.000	0.455*
TS								1.000

NEU= neutrophil, EOS= eosinophil, MCH= mean corpuscular haemoglobin, MCHc= mean corpuscular haemoglobin concentration, Fe= iron, TIBC= total iron-binding capacity, TS= transferrin saturation. * $p < 0.05$, and. ** $p < 0.01$

The results of ROC analyses of hematologic and biochemical biomarkers are shown in Table 5 and Figures 2 and 3. The AUC were 0.94, 0.91, 0.90, 0.88, 0.86 and 0.76 for Fe, ferritin, TIBC, TS, NEU and EOS, respectively. The cut-off values of Fe, ferritin, TIBC, TS, NEU and EOS were 72 μg/dL, 1.15 ng/mL, 471 μg/dL, 16.43%, 4.39 μL, and 0.11 μL, respectively. Sensitivity and specificity values of the proposed diagnostic cut-off point for Fe, Ferritin, TIBC, TS, NEU and EOS read as follows: (80% and 90%), (80% and 90%), (86.7% and 90%), (80% and 80%), (66.7% and 90%) and (60% and 80%), respectively.

The NEU (p=0.005) and ferritin (p=0.001) values of the Virus+ and Virus- groups were higher than the healthy group, while the Fe (p=0.001), TIBC (p=0.001) and TS (p=0.005) values were lower. The highest NEU and Ferritin values and the lowest Fe, TIBC and TS values were determined in the Virus- group (Table 6).

Table 5. Receiver operating characteristic (ROC) results of hematologic and biochemical biomarkers in BRDC and healthy groups

Parameters	NEU (μL)	EOS (μL)	Fe (μg/dL)	Ferritin (ng/mL)	TIBC (μg/dL)	TS (%)
Area	0.867	0.767	0.940	0.910	0.907	0.887
Cut-off	4.39	0.11	72	1.15	471	16.43
Sensitivity (%)	66.7	60	80	80	86.7	80
Specificity (%)	90	80	90	90	90	80
SEM	0.071	0.094	0.045	0.057	0.063	0.066
P value	0.002	0.027	0.000	0.001	0.001	0.001

NEU= neutrophil, EOS= eosinophil, Fe= iron, TIBC= total iron-binding capacity, TS= transferrin saturation.

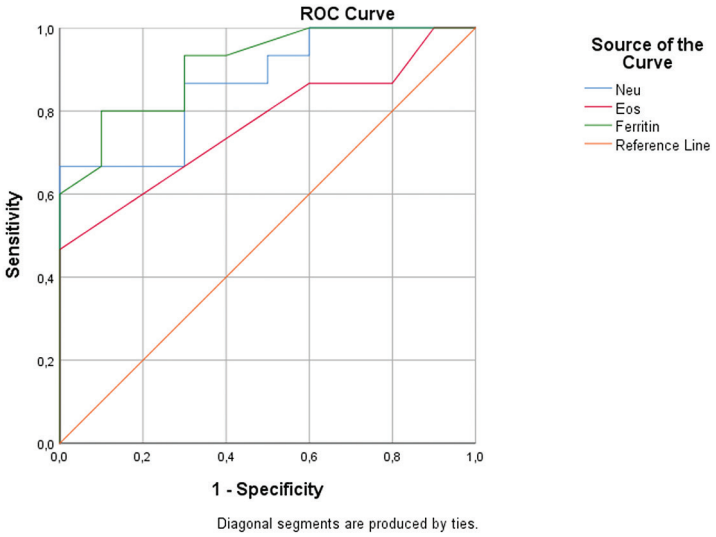

Figure 2. ROC curve analysis of Neu, Eos and ferritin levels

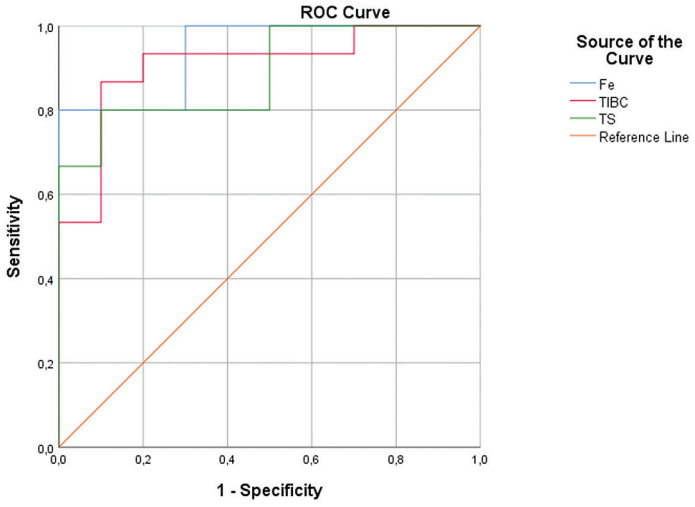

Figure 2. ROC curve analysis of Fe, TIBC and TS levels

Molecular virological analysis of nasal swab samples revealed BRSV in 26.7% (4 calves), BVDV in 13.3% (2 calves), BPIV-3 in 6.7% (1 calf) and BRSV + BVDV in 13.3% (2 calves) out of 15 calves with BRDC. The remaining 6 calves were tested negative for the viral agents. BHV-1 could not be detected in any of the nasal swab samples. Gel electrophoresis images of PCR positive samples are shown in Figure 1.

Table 6. Comparison of haematological and biochemical parameters of healthy, virus- and virus+ groups.

Parameters	Healthy	Virus -	Virus +	Test p
NEU (x10³/µL)	2.90 (0.92-4.52)[a]	10.25 (3.41-24.50)[b]	5.00 (2.72-18.35)[b]	P= 0.005
MCH (pg)	10.89±0.88[a]	9.33±1.24[b]	9.67±1.05[b]	P= 0.014
MCHc (g/dL)	34.53±1.32[a]	30.35±1.47[b]	31.05±1.93[b]	P= 0.000
Fe (µg/dL)	146 (66-273)[a]	18.50 (7-84)[b]	32.00 (1-97)[b]	P=0.001
Ferritin (ng/ml)	0.20 (0.00-1.7)[a]	4.20 (0.6-203)[b]	2.00 (0.2-69.9)[b]	P=0.002
TIBC (µg/dL)	532.40±64.69[a]	396.00±72.51[b]	408.33±83.81[b]	P=0.001
TS (%)	28.32 (10.51-49.82)[a]	4.09 (2.36-21.82)[b]	5.61 (0.28-28.06)[b]	P=0.005

NEU= neutrophil, MCH= mean corpuscular haemoglobin, MCHc= mean corpuscular haemoglobin concentration, Fe= iron, TIBC= total iron-binding capacity, TS= transferrin saturation. Data are presented as mean ± standard deviation and or median (range). Different letters in a row (a-b) indicate significant difference between groups (P<0.05). In this table, only the statistically significant (P<0.05) parameters are shown.

DISCUSSION

In this study, we investigated the usability of serum Fe, ferritin, Tf, TIBC, UIBC and TS levels as inflammation and possible diagnostic biomarkers in calves with BRDC. In line with our hypothesis, we found a decrease in serum Fe, TIBC and TS levels, and an increase in ferritin levels. Iron metabolism is affected by many conditions, including inflammation, anemia, hepatopathy, cancer, hyperadrenocorticism, and kidney failure [49]. Iron, whose concentration decreases rapidly in response to acute inflammation, has also been evaluated as a biomarker of inflammation in animals [4,14,15,21]. Besides, serum ferritin levels increase in cases of inflammation and infection, so it is used as an inflammation biomarker [28,29]. In this study, serum Fe concentrations were detected significantly low, but ferritin levels were significantly high in calves with BRDC compared to the healthy group (P=0.000). The low TIBC and TS levels in calves with BRDC compared to the healthy group were a natural result of decreased circulating Fe levels. Tsukano et al. [22] also found the serum Fe level to be quite low in cows diagnosed with respiratory disease solely on the basis of clinical findings. They reported that serum Fe concentration is a candidate inflammatory marker. As is known, BRDC is a disease in which severe inflammation generally progresses [50]. Leucocytosis and neutrophilia associated with acute respiratory inflammation have been reported in cattle with BRDC [44,51-53]. Likewise, in this study WBC (P=0.129), NEU (P=0.001) and EOS (P=0.026) values were also found to be high in calves with BRDC compared to the healthy group. A strong negative correlation between serum Fe and NEU (r= -0.720 P=0.000) while a moderate positive correlation between serum ferritin levels and NEU (r=0.596, P=0.002) were detected. Thus, we deduced that iron levels decrease and ferritin levels increase due to inflammation in cattle with BRDC [a moderate negative correlation (r=-0.582, P=0.002) was found between serum Fe and ferritin concentrations (Table 4)], and that these parameters can be used

as inflammation biomarkers. This inference is also supported by the low Tf level, a negative acute-phase protein, in the BRDC group (P=0.723). The animals were also not diagnosed with anemia indicating that the decrease in serum Fe concentrations is caused by inflammation. Fundamental aspects of serum ferritin biology are still unclear. The high ferritin levels in this study were attributed to the inflammation as hepatocytes, macrophages, and Kupffer cells have been shown to release ferritin [54]. In our study, total leukocyte, NEU and EOS values were high and Tf was found to be low in calves with BRDC compared to the healthy group, which together indicate the presence of inflammation. Therefore, this supports our thesis that ferritin levels are elevated due to inflammation and like serum amyloid A and haptoglobin, ferritin can be used as an inflammation marker in cattle with BRDC.

The virus was detected in 9 out of 15 calves in this study and the mean serum Fe, ferritin and WBC values of these calves were found as follows: BRSV (Fe: 50.25±32.27 μg/dL, ferritin: 11.12±13.00 ng/ml and WBC: 8.38±1.37 μL), BVDV (Fe: 49.5±62.93 μg/dL, ferritin: 1.1±1.27 ng/ml and WBC: 8.14±3.58 μL), BPIV-3 (Fe: 34 μg/dL, ferritin: 2.1 ng/ml and WBC:18.98 μL) and BRSV+BVDV (Fe: 4.5±4.94 μg/dL, ferritin: 35.6±48.50 ng/ml and WBC: 22.05±9.60 μL). BRSV, one of the viruses involved in the disease, is a factor that is attracted by the lower respiratory tract epithelium and directly damages the respiratory epithelium following changes created by inflammatory mediators [55]. On the other hand, BVDV is a causative agent that can lead to a direct infection in bovine lungs and combined infections with BPIV-3 and BRSV [56]. Remarkably, we found that the calves with BRSV+BVDV had the lowest Fe, but the highest ferritin and WBC levels. Therefore, it can clearly be inferred that increasing severity of the infection causes a decrease in serum Fe concentration and an increase in ferritin levels. These results further strengthen the use of serum Fe and ferritin concentrations as inflammatory biomarkers in BRDC calves.

According to the results of the ROC analysis which was performed in the BRDC and healthy groups, the cut-off value for Fe was 72 μg/dL and for ferritin was 1.15 ng/mL. Sensitivity and specificity values of the proposed diagnostic cut-off point for Fe and ferritin were 80% and 90%, respectively. According to these numbers, Fe levels below 72 μg/dL and/or ferritin levels above 1.15 ng/mL point out to inflammation with 80% sensitivity. Vice versa Fe levels above 72 μg/dL and/or ferritin levels below 1.15 ng/mL indicate that there is not any inflammation with 90% specificity. As known, ROC curves may be useful for assessing the diagnostic power of a candidate marker by comparing its effectiveness with other known markers. A powerful diagnostic ability can be revealed by a higher AUC value. We found the AUC for Fe and ferritin to be 0.94 and 0.91, respectively. Both of these values are satisfactory for Fe and ferritin as being inflammatory and possible diagnostic markers, however serum Fe is slightly superior to ferritin in that regard. Similarly, Tsukano et al. [22] reported the AUC of serum Fe level was 0.97 in cows with respiratory system disease and concluded that serum Fe level was a satisfactory substitute for plasma haptoglobin and serum amyloid A.

The decrease in Fe level is not only observed in bacterial infections [15,57]. Similar findings have also been reported in cases of inflammation of non-infectious origin like dehorning [58], laryngeal neuropathy [59] etc. and of viral origin, such as live attenuated measles vaccine in newborn babies [60], cytomegalovirus (CMV) [61], and COVID-19 [62]. In veterinary medicine, there are almost no studies conducted on changes in serum Fe levels in viral diseases [63]. In line with this, we found the Fe levels of Virus+ calves are significantly lower than that of the healthy group (P=0.001) (Table 6). The lack of bacteriological analysis could be one of the shortcomings of our study. However, since Virus+ calves had higher Fe levels and lower NEU than Virus- calves (Table 6), we can infer that Virus+ calves might not be co-infected with bacterial agents. Besides, it is clear that a probable detection of bacterial agents would have further reduced the serum iron concentration.

The decrease in serum Fe levels in viral diseases can be explained by the following mechanisms. While some viruses infect iron-receiving cells by binding to the transferrin receptor type 1, other viruses target the Human Homeostatic Iron Regulator Protein (HFE) genes and hepcidin to induce iron overload at the cellular level to encourage their survival and reproduction [64]. Increased IL-6 in the case of an inflammation elevates hepcidin production and release, which is necessary for systemic Fe homeostasis [20]. On the other hand, hepcidin alleviates serum Fe levels by increasing iron uptake [65]. Therefore, in this study comprised of BRDC calves with viral etiology, we think that the decrease in serum Fe levels is due to inflammation, as can be understood from high ferritin level (Table 6). In addition, the immunosuppressive effects of BRSV and BVDV agents predispose to respiratory tract infections in animals [8,66,67]. Therefore, we deduced that the decrease in Fe level can be advantageous in preventing secondary bacterial infections that might occur due to immunosuppression caused by viral agents. On the other hand, low Fe levels may be disadvantageous as it may worsen tachypnea observed in respiratory diseases. We detected lower concentrations of HGB, MCH and MCHC in the BRDC group due to low iron levels. Accordingly, RR and HR numbers were higher which together indicates that low Fe levels have a negative impact on tachypnea. However, these inferences should be supported with genetic and biochemical studies on the mechanisms underlying the reduction of Fe levels in viral respiratory diseases in cattle.

CONCLUSION

We detected a statistically significant decrease in serum Fe, TIBC and TS levels and an increase in ferritin levels in BRDC calves. To simply put into words, in the case of inflammation while ferritin levels are elevated, serum Fe levels are declined. Additionally, a decrease in serum Fe would also result in a decrease in the levels of TS, TIBC, Tf, Hb, MCV, MCH and MCHc. Based on strong, promising statistical outcomes of serum Fe and ferritin levels, these parameters can be used as inflammatory and possible diagnostic markers for BRDC. Since these parameters are useful, easily

measured, inexpensive and sensitive, they have a strong potential for a wider use in veterinary medicine. Serum Fe and ferritin may also play a role in the pathogenesis of the disease, therefore the mechanisms behind their changes in cattle with viral diseases should be fully elucidated and further studies including genetic and biochemical analyses are needed.

Acknowledgements

The study was self-funded.

The part of this study was presented as a poster proceeding in the 2nd International-13th National Veterinary Internal Medicine Congress held in Ankara, TURKEY, October 11-13, 2019.

Authors' contributions

ŞD conceived the study, participated in its design and coordination, performed the statistical analysis, interpreted the data and wrote the manuscript. AK conceived the study, participated in its design and coordination. HA carried out molecular genetic studies. ÖA, MSA and RK participated in the coordination of the study. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

REFERENCES

1. Ceciliani F, Ceron JJ, Eckersall PD, Sauerwein H: Acute phase proteins in ruminants. J Proteomics 2012, 75:4207-4231.
2. Petersen HH, Nielsen JP, Heegaard PM: Application of acute phase protein measurements in veterinary clinical chemistry. Vet Res 2004, 35:163-187.
3. Murata H, Shimada N, Yoshioka M: Current research on acute phase proteins in veterinary diagnosis: an overview. Vet J 2004, 168:28-40.
4. Baydar E, Dabak M. Serum iron as an indicator of acute inflammation in cattle. J. Dairy Sci 2014, 97:222-228.
5. Orino K, Watanabe K: Molecular, physiological and clinical aspects of the iron storage protein ferritin. Vet J 2008, 178:191-201.
6. Welsh RD, Dye LB, Payton ME, Confer AW: Isolation and antimicrobial susceptibilities of bacterial pathogens from bovine pneumonia: 1994--2002. J Vet Diagn Invest 2004, 16:426-431.
7. Snowder GD, Van Vleck LD, Cundiff LV, Bennett GL: Bovine respiratory disease in feedlot cattle: environmental, genetic, and economic factors. J Anim Sci 2006, 84:1999-2008.

8. Valarcher JF, Taylor G: Bovine respiratory syncytial virus infection. Vet Res 2007, 38:153-180.
9. Jones C, Chowdhury S: A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. Anim Health Res Rev 2007, 8:187-205.
10. Ellis JA: Update on viral pathogenesis in BRD. Anim Health Res Rev 2009, 10:149-153.
11. Divers TJ: Respiratory diseases. In: Divers TJ, Peek SF, editors. *Rebhun's Diseases of Dairy Cattle*. 2 nd ed. St. Louis, Missouri: Saunders; 2008, 79-127.
12. Ong ST, Ho JZ, Ho B, Ding JL: Iron-withholding strategy in innate immunity. Immunobiology 2006, 211:295-314.
13. Beard JL: Iron biology in immune function, muscle metabolism and neuronal functioning. J Nutr 2001, 131:568-579.
14. Aydoğdu U, Coşkun A, Yıldız R, Güzelbekteş H, Şen İ: Changes of hematological parameters and serum iron levels in calves with systemic inflamatory response syndrome. Eurasian J Vet Sci 2018, 34:56-59.
15. Borges AS, Divers TJ, Stokol T, Mohammed OH: Serum iron and plasma fibrinogen concentrations as indicators of systemic inflammatory diseases in horses. J Vet Intern Med 2007, 21:489-494.
16. Zandman-Goddard G, Shoenfeld Y: Ferritin in autoimmune diseases. Autoimmun Rev 2007, 6:457-463.
17. Ganz T, Nemeth E: Iron sequestration and anemia of inflammation. Semin Hematol 2009, 46:387-393.
18. Hintze KJ, Theil EC: Cellular regulation and molecular interactions of the ferritins. Cell Mol Life Sci 2006, 63:591-600.
19. Weinberg ED, Miklossy J: Iron withholding: a defense against disease. J Alzheimers Dis 2008, 13:451-463.
20. Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T: IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 2004, 113:1271-1276.
21. Neumann S: Serum iron level as an indicator for inflammation in dogs and cats. Comp Clin Path 2003, 12:90-94.
22. Tsukano K, Fukuda T, Ikeda K, Sato K, Suzuki K: Serum iron concentration is candidate inflammatory marker for respiratory diseases in beef cows. J Vet Med Sci 2021, 83:824-828.
23. Zhang C: Essential functions of iron-requiring proteins in DNA replication, repair and cell cycle control. Protein Cell 2014, 5:750-760.
24. Miyata Y, Furugouri K, Shijimaya K: Developmental changes in serum ferritin concentration of dairy calves. J Dairy Sci 1984, 67:1256-1263.
25. Furugouri K, Miyata Y, Shijimaya K: Ferritin in blood serum of dairy cows. J Dairy Sci 1982, 65:1529-1534.
26. Furugouri K, Miyata Y, Shijimaya K, Narasaki N: Developmental changes in serum ferritin of piglets. J Anim Sci 1983, 57:960-965.
27. Jacobs A, Miller F, Worwood M, Beamish MR, Wardrop CA: Ferritin in the serum of normal subjects and patients with iron deficiency and iron overload. Br Med J 1972, 4:206-208.

28. Lorier MA, Herron JL, Carrell RW: Detecting iron deficiency by serum tests. Clin Chem 1985, 31:337-338.
29. Wang W, Knovich MA, Coffman LG, Torti FM, Torti SV: Serum ferritin: Past, present and future. Biochim Biophys Acta 2010, 1800:760-769.
30. Jacobs A, Worwood M: Ferritin in serum. Clinical and biochemical implications. N Engl J Med 1975, 292:951-956.
31. Orino K: Biochemical studies on bovine ferritin. Jpn J Vet Res 1998, 46:96-97.
32. Gomme PT, McCann KB, Bertolini J: Transferrin: structure, function and potential therapeutic actions. Drug Discov Today 2005, 10:267-273.
33. Koc M, Taysi S, Sezen O, Bakan N: Levels of some acute-phase proteins in the serum of patients with cancer during radiotherapy. Biol Pharm Bull 2003, 26:1494-1497.
34. Graziadei I, Kaserbacher R, Braunsteiner H, Vogel W: The hepatic acute-phase proteins alpha 1-antitrypsin and alpha 2-macroglobulin inhibit binding of transferrin to its receptor. Biochem J 1993, 290:109-113.
35. Faruqi A, Mukkamalla SKR. Iron Binding Capacity. StatPearls. Treasure Island (FL)2020.
36. Hunter JE: Variable effects of iron status on the concentration of ferritin in rat plasma, liver, and spleen. J Nutr 1978, 108 (3):497-505.
37. Li JY, Paragas N, Ned RM, Qiu A, Viltard M, Leete T, Drexler IR, Chen X, Sanna-Cherchi S, Mohammed F, Williams D, Lin CS, Schmidt-Ott KM, Andrews NC, Barasch J: Scara5 is a ferritin receptor mediating non-transferrin iron delivery. Dev Cell 2009,16:35-46.
38. Elsayed ME, Sharif MU, Stack AG: Transferrin saturation: a body iron biomarker. Adv Clin Chem 2016, 75:71-97.
39. Gozzelino R, Arosio P: Iron homeostasis in health and disease. Int J Mol Sci 2016, 17:130.
40. Kirbas A, Kandemir FM, Celebi D, Hanedan B, Timurkan MO: The use of inflammatory markers as a diagnostic and prognostic approach in neonatal calves with septicaemia. Acta Vet Hung 2019, 67:360-376.
41. Tsukano K, Shimamori T, Suzuki K: Serum iron concentration in cattle with endotoxaemia. Acta Vet Hung 2020, 68:53-58.
42. Aydoğdu U, Yurdakul İ: The effects of local and systemic inflammatory status on iron metabolism and lipid profile in calves. Eurasian J Vet Sci 2020, 36:121-126.
43. Ali MK, Kim RY, Karim R, Mayall JR, Martin KL, Shahandeh A, Abbasian F, Starkey MR, Loustaud-Ratti V, Johnstone D, Milward EA, Hansbro PM, Horvat JC: Role of iron in the pathogenesis of respiratory disease. Int J Biochem Cell Biol 2017, 88:181-195.
44. Šoltésová H, Nagyová V, Tóthová C, Nagy O: Haematological and blood biochemical alterations associated with respiratory disease in calves. Acta Vet Brno 2015, 84:249-256.
45. Seitz WR: Immunoassay labels based on chemiluminescence and bioluminescence. Clin Biochem 1984, 17:120-125.
46. Timurkan MO, Aydin H: Increased genetic diversity of BVDV strains circulating in Eastern Anatolia, Turkey: first detection of BVDV-3 in Turkey. Trop Anim Health Prod 2019, 51:1953-1961.
47. Timurkan MO, Aydin H, Sait A: Identification and molecular characterisation of Bovine Parainfluenza Virus-3 and Bovine Respiratory Syncytial Virus - first report from Turkey. J Vet Res 2019, 63:167-173.

48. Fuchs M, Hübert P, Detterer J, Rziha HJ: Detection of bovine herpesvirus type 1 in blood from naturally infected cattle by using a sensitive PCR that discriminates between wild-type virus and virus lacking glycoprotein E. J Clin Microbiol 1999, 37:2498-2507.
49. Kaneko JJ. Iron metabolism. In: Clinical biochemistry of domestic animals. New York: Academic press; 1980, 649–669.
50. Kirchhoff J, Uhlenbruck S, Goris K, Keil GM, Herrler G: Three viruses of the bovine respiratory disease complex apply different strategies to initiate infection. Vet Res 2014, 45:20.
51. Dörtkardeş AB, Şahinduran Ş: Determination of serum amyloid A, haptoglobin and hepcidin levels in calves with endemic viral pneumonia. Ankara Üniv Vet Fak Derg 2020, 67:127-131.
52. Gökçe G: Investigations on Clinic,Haematology,Biochemistry,Oxidative Stress, Acute Phase Proteins in Infectious Respiratory Disease Complex (BRDC) in Cattle. Ataturk Univ Vet Bil Derg 2017, 12:34-44.
53. Hanedan B, Kirbas A, Dorman E, Timurkan MO, Kandemir FM, Alkan O: Cardiac troponin-i concentration in weaned calves with bovine respiratory disease. Acta Vet-Beograd 2015, 65:454-462.
54. Wesselius LJ, Nelson ME, Skikne BS: Increased release of ferritin and iron by iron-loaded alveolar macrophages in cigarette smokers. Am J Respir Crit Care Med 1994, 150:690-695.
55. Yaman T, Büyükbayram H, Özyıldız Z, Terzi F, Uyar A, Keles ÖF, Özsoy ŞY, Yener Z: Detection of Bovine Respiratory Syncytial Virus, Pasteurella Multocida, and Mannheimia Haemolytica by Immunohistochemical Method in Naturally-infected Cattle. J Vet Res 2018, 62:439-445.
56. Fulton RW, Purdy CW, Confer AW, Saliki JT, Loan RW, Briggs RE, Burge LJ: Bovine viral diarrhea viral infections in feeder calves with respiratory disease: interactions with Pasteurella spp., parainfluenza-3 virus, and bovine respiratory syncytial virus. Can J Vet Res 2000, 64:151-159.
57. Ward CG, Bullen JJ, Rogers HJ: Iron and infection: New developments and their implications. J Trauma 1996, 41:356-364.
58. Tsukano K, Shimamori T, Fukuda T, Nishi Y, Otsuka M, Kitade Y, Suzuki K: Serum iron concentration as a marker of inflammation in young cows that underwent dehorning operation. J Vet Med Sci 2019, 81:626-628.
59. Jacobsen S, Nielsen JV, Kjelgaard-Hansen M, Toelboell T, Fjeldborg J, Halling-Thomsen M, Martinussen T, Thoefner MB: Acute phase response to surgery of varying intensity in horses: a preliminary study. Vet Surg 2009, 38:762-769.
60. Olivares M, Walter T, Osorio M, Chadud P, Schlesinger L. Anemia of a mild viral-infection - the measles-vaccine as a model. Pediatrics 1989, 84:851-855.
61. Drakesmith H, Prentice A: Viral infection and iron metabolism. Nat Rev Microbiol 2008, 6:541-52.
62. Zhao K, Huang J, Dai D, Feng Y, Liu L, Nie S: Serum iron level as a potential predictor of coronavirus disease 2019 severity and mortality: a retrospective study. Open Forum Infect Dis 2020, 7:ofaa250.
63. Schnell SA, Ohtsuka H, Kakinuma S, Yoshikawa Y, Watanabe K, Orino K: Iron and ferritin levels in the serum and milk of bovine leukemia virus-infected dairy cows. Front Vet Sci 2015, 2:12.
64. Payne SM: Iron acquisition in microbial pathogenesis. Trends Microbiol 1993, 1:66-69.

65. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T: Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003, 101:2461-2463.

66. Potgieter LN, McCracken MD, Hopkins FM, Walker RD: Effect of bovine viral diarrhea virus infection on the distribution of infectious bovine rhinotracheitis virus in calves. Am J Vet Res 1984, 45:687-90.

67. Yaman, T and Aydemir, C: Respiratory Syncytial Virus Infection Induces Expression of Inducible Nitric Oxide Synthase, CD3, and CD8 in Naturally Occurring Pneumonia in Lambs. Acta Vet-Beograd 2021, 71:170-188.

PROCENA NIVOA SERUMSKOG GVOŽĐA I FERITINA KAO MARKERA INFLAMACIJE KOD TELADI SA KOMPLEKSOM RESPIRATORNIH BOLESTI GOVEDA

Şükrü DEĞİRMENÇAY, Akın KIRBAŞ, Hakan AYDIN, Ömer AYDIN,
M. Sinan AKTAŞ, Ramazan KAMAN

Gvožđe i feritin se godinama koriste u humanoj medicini kako bi se otkrilo prisustvo inflamacije. Međutim, studije koje procenjuju ove parametre, posebno kod bolesti respiratornog sistema, prilično su retke u veterinarskoj medicini. Cilj nam je bio da testiramo upotrebljivost serumskog Fe i parametara vezanih za Fe [ukupni kapacitet vezivanja gvožđa (TIBC), nezasićeni kapacitet vezivanja gvožđa (UIBC) i zasićenost transferinom (TS)] kao inflamatorni i dijagnostički biomarkeri kod teladi sa kompleksom respiratornih bolesti goveda. (BRDC). Da bi se označila upala, izmereni su odabrani proteini akutne faze, uključujući nivoe feritina i transferina u serumu, zbog njihove bliske veze sa metabolizmom gvožđa. Materijal za ovu studiju se sastojao od 15 teladi, starosti 1-3 meseca sa BRDC (I grupa) i 10 zdravih teladi uzrasta 1-3 meseca (Grupa II) procenjenih na osnovu prisustva respiratornih kliničkih nalaza. Nivoi Fe, TIBC i TS u serumu su bili niski, a nivoi feritina visoki u grupi I ($P \leq 0,001$). BRDC grupa je podeljena u dve podgrupe na osnovu PCR rezultata, naime Virus+ (n=9) i Virus- (n=6). Telad u grupi Virus+ imala su značajno niže nivoe Fe (P=0,001) i značajno veće vrednosti feritina (P=0,002), u poređenju sa zdravim životinjama. Na osnovu međugrupnog poređenja i ROC analize, zaključili smo da se nivoi Fe (prvenstveno), feritina, TIBC i TS mogu koristiti kao inflamatorni biomarkeri i mogući dijagnostički markeri u BRDC kao korisne, praktične i jeftine zamene. Kao sugestija, ovi parametri za koje se veruje da igraju ulogu u patogenezi bolesti, mogu se koristiti kao potencijalni prognostički biomarkeri u studijama koje uključuju lečenje.