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■ Acute Phase Response in Enzoitic Bovine Leukosis

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ABSTRACT. In the present study, we evaluated acute phase response by detecting haptoglobin (Hp), serum amyloid A (SAA) and fibrinogen in cattle with enzootic bovine leukosis (EBL). A total of 60 Holstein cattle (≤ 3 years old), comprising of 40 cattle infected with BLV, and 20 BLV-free healthy controls were enrolled in this study. Diagnosis of the BLV infection was performed by serology (ELISA and AGID) and PCR techniques. APPs were detected by commercial ELISA test kits using validated standard procedures as instructed. All the BLV-infected cattle were in good general health and had normal respiratory rates, pulse rates, body temperatures. However, 5 cattle had enlarged, hard, painless, movable superficial lymph nodes in infected group. APPs including Hp ($p < 0.001$), fibrinogen ($p < 0.001$), and SAA ($p < 0.05$) concentrations were significantly higher in cattle with EBL compared to BLV-free cattle. On hematologic examination, total leukocyte, lymphocyte and granulocytes concentrations were significantly higher in infected cattle when compared to controls. In addition, Hp and SAA ($p < 0.001$) concentrations were significantly higher in symptomatic cattle than asymptomatic. The Pearson correlation revealed significant associations between APPs and total leukocyte and granulocytes; however, there was no correlation with lymphocyte. In conclusion, the results of this study showed increased acute phase response in BLV infected cattle.

Keywords: acute phase response, BLV, cattle, enzootic bovine leukosis

INTRODUCTION

Enzootic bovine leucosis (EBL) is a virus associated lymphosarcoma (tumour) of cattle caused by bovine leukaemia virus, C-type oncovirus in the family Retroviridae. BLV affects more commonly dairy herds. Infection is spread by contact with contaminated blood or colostrum from infected animals. Cattle may be infected at any age; however, EBL is typically a disease of adult cattle and tumours develop in 0.1–10% of cattle above 3 years old

(Kahn and Line, 2010; OIE, 2012) parentheses should be big. BLV causes 3 clinical/subclinical manifestations. The clinical form is presented with lymphadenopathy (lymphosarcoma) that manifests itself by single or multiple lymph nodes of internal or external location. However, EBL is usually a subclinical disorder. There are two distinct subclinical courses that occur in most infected cattle with persistent lymphocytosis. Persistent lymphocytosis occurs in about 1-30% of all BLV-infected

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ed cows, while the remaining cows with a normal cell count. EBL causes economic losses due to reduced milk production, reproductive performance, costs associated with treatment and diagnosis, death of cattle, culling, and carcass condemnation at slaughter (Schwartz and Levy, 1994; Kahn and Line, 2010).

The detection of inflammatory processes is more difficult to assess in cattle, because the symptoms of the disease are not easily detectable and inflammation is not always followed by a leukocyte increase (Paulina and Tadeusz, 2011). APPs have been used commonly in detection of inflammation in human medicine and to a lesser extend in veterinary medicine. However, a significant progress has been made in the detection, measurement and application of APPs in both companion and farm animals in recent years (Eckersall and Bell, 2010; Schneider, 2015). APPs are blood proteins primarily synthesized by hepatocytes as part of the acute phase response. The acute phase response is a non-specific reaction of an organism in case of injury, trauma, infection, stress, inflammation, and neoplasia (Cray et al., 2009). Measurement of APPs such as Hp and SAA, often in combination, is used in the diagnosis and prognosis of mastitis, enteritis, peritonitis, pneumonia, endocarditis, and endometritis in cattle (Petersen et al., 2004). Moreover, APPs are more sensitive and specific than the routine diagnostic methods like leukocyte count (Pradeep, 2014). Based on this evidence, the present study was undertaken to evaluate acute phase response in cattle with EBL which is a mostly subclinical and virus induced lymphosarcoma.

MATERIALS and METHODS

Animals and sampling

A total of 60 Holstein-Frisian cattle (<3 years old) from two BLV-positive dairy farms (445 and 115 cattle in each farm) were enrolled in the study. According to the ELISA test, a total of 40 BLV-positive cattle (27/445 and 13/115) were selected in the study. As a control, clinically healthy, BLV-free 20 cattle were randomly selected from the rest of all. All cattle were subjected to clinical examinations and the respiratory rate, pulse rate, and body temperature were recorded. All necessary ethical guidelines were taken into consideration for randomized enrolment into the groups. Blood samples were collected from the jugular vein

into tubes with coagulation activator and anticoagulant. Serum and plasma samples were separated by centrifugation at 3000 rpm for 10 minutes at room temperature and stored at -80°C until biochemical analyses. Whole blood samples were also stored at -20°C until DNA extractions.

Laboratory analyses

Serologic assays

Blood samples were tested for the detection of antibodies against BLV by a commercial ELISA kit (IDEXX Laboratories, Inc. USA) as instructed by producer. All ELISA positive samples were confirmed by Agar Gel Immune Diffusion (AGID) test (Institute Pourquer, France) and PCR.

DNA Extraction and Nested RT-PCR Assays

Proviral DNA was extracted directly from suspected blood leukocyte using the QiaAmp viral DNA extraction kit (Qiagen Sciences, Hilden, Germany) as described by manufacturer. The outer and nested primers (region of envelope) were used for amplification. Primers (OBLV1A, OBLV6A, OBLV3 and OBLV5) were selected from published sequence of the gp51 (*env*) gene ((Table 1).

Biochemical and hematologic examination

Serum APPs; Hp (Tridelta, Ireland), SAA (Tridelta, Ireland), and fibrinogen (Cusabio Biotech, China) concentrations were measured using commercially available ELISA kits as described by the manufacturer. CBC was measured by automated blood cell counter (Abacus Junior Vet).

Statistical analysis

First, Shapiro-Wilk test was used for evaluating the normal distribution of the variables. According to results, the two-sided Student's t-test using computer software, SPSS Version 15.0 for Windows, was used to test for significant differences between the groups for each parameter. Association between APPs and WBC was investigated using Pearson's correlation coefficients. $P \leq 0.05$ were considered as significant. The cut-off points for Hp, SAA, fibrinogen, total leukocyte, lymphocyte, and granulocytes were calculated using receiver operating characteristic (ROC) curves.

RESULTS

All cows were in good general health with normal appetite, body condition, respiratory rate, pulse rate and body temperature, and had no other inflammatory problems such as pneumonia, enteritis, mastitis or metritis. However, 5 cattle (12.5%) in BLV infected group had enlarged, hard, painless, movable superficial lymph nodes (up to 8 cm in diameter).

Descriptive statistics and results of APPs and CBC of EBL and BLV-free cattle are presented in Table 2 and Table 3, respectively. As shown in Table 2, Hp ($p < 0.001$), fibrinogen ($p < 0.001$), and SAA ($p < 0.05$) concentrations were significantly higher in cattle with EBL compared to BLV-free cattle. The diagnostic cut-off points of SAA ≥ 66.18 ng/ml (sensitivity 50%, specificity 45%, $p > 0.05$), Hp ≥ 0.95 mg/ml (sensitivity 90%, specificity 95%, $p < 0.001$), and fibrinogen ≥ 161.45 mg/dl (sensitivity 95%, specificity 95%, $p < 0.001$) were detected for EBL. In addition, Hp ($p < 0.001$) and SAA ($p < 0.001$) concentrations were significantly higher in symptomatic cattle than asymptomatic.

On hematologic examination; total leukocyte ($p < 0.001$), lymphocyte ($p < 0.001$), and granulocytes ($p < 0.05$) concentrations were significantly higher in infected cattle compared to controls. When evaluating total leukocyte in BLV-infected cattle, 62.5% (25/40) lymphocytosis, 50.0% (20/40) leukocytosis and 25.0% (10/40) granulocytosis were observed. Moreover, all the BLV-infected cattle below 1 year old (6 cases) revealed lymphocytosis ($> 7.5 \times 10^3/\mu\text{L}$) and leukocytosis ($> 12.0 \times 10^3/\mu\text{L}$). However, WBC, lymphocyte, and granulocytes values were not statistically different ($p > 0.05$) in symptomatic and asymptomatic cattle. Correlations between APPs and WBC in BLV-infected cattle were evaluated and significant positive correlations between SAA and total leukocyte ($r = 0.400$, $p < 0.05$), SAA and granulocytes ($r = 0.537$, $p < 0.001$), Hp and total leukocyte ($r = 0.369$, $p < 0.05$), Hp and granulocytes ($r = 0.394$, $p < 0.05$) were recorded; however, there was no correlation between lymphocyte and APPs ($p > 0.05$). The diagnostic cut-off points of WBC $\geq 9.30 \times 10^3/\mu\text{L}$ (sensitivity 87.5%, specificity 97.5%, $p < 0.001$), lymphocyte $\geq 5.54 \times 10^3/\mu\text{L}$ (sensitivity 90%, specificity 90%, $p < 0.001$), and granulocytes $\geq 3.16 \times 10^3/\mu\text{L}$ (sensitivity 65%, specificity 65%, $p < 0.01$) were detected for EBL.

DISCUSSION

EBL is usually a subclinical disease and BLV infected cattle remains infected for life. Typically, EBL is a disease of adult cattle, although juvenile form of lymphosarcoma can occur in younger animals. BLV infection most often does not cause any clinical signs; however, in about 30% of them develop abnormal increases in lymphocytes (persistent lymphocytosis) in three to five years. Moreover, cattle with persistent lymphocytosis may progress to fatal lymphoproliferative disease in less than 5% of infected animals after 3 to 10 years (Kahn and Line, 2010; OIE, 2012). In this study, we evaluated BLV infected 40 cattle between 6 months and 3 years old. All the BLV-infected cattle were in good general health, normal appetite, body condition, respiratory rates, pulse rates, and body temperatures. However, we found lymphosarcoma manifested by enlarged, hard, painless, movable superficial lymph nodes (up to 8 cm in diameter) in 5 cases (12.5%). In this study, total leukocyte, lymphocyte, and granulocytes counts were significantly higher in infected cattle compared to healthy controls, and lymphocytosis was detected as 62.5% in BLV-infected cattle. Similarly, Batmaz et al. (1995) and Swenson et al. (2013) reported high total leukocyte and lymphocyte counts in BLV infected cattle.

APPs, blood proteins primarily synthesized by hepatocytes, are non-specific reactions of acute phase response (Schneider, 2015). Serum APPs levels have been detected to be more sensitive and exhibit a faster response to inflammatory diseases than white blood cells in animals (Pradeep, 2014). Hp and SAA are the major APPs of cattle. Major APPs are virtually undetectable in the blood of healthy animals, but their concentration can increase 10-1000 times following stimulation (Ceron et al., 2005). In this study, Hp, SAA, and fibrinogen concentrations were significantly higher in cattle with EBL compared to BLV-free cattle. To the authors' knowledge, there was no detailed research about acute phase response in EBL except for the report by Nagahata et al. (1989) which reported highly elevated mean serum acid soluble glycoproteins as acute phase proteins, in cattle with EBL compared to BLV-free cows. However, there are many results reported for dog with malignant lymphoma (Mischke et al., 2007; Nielsen et al., 2007). Mischke et al. (2007) also reported that APPs such as CRP and Hp concentrations increase in neoplastic lymphoma.

phatic disorders like malignant lymphoma, multiple myeloma, and acute lymphoblastic leukemia in dogs. In the present study, Hp and fibrinogen concentrations in cattle with BLV significantly elevated compared to SAA. Moreover, significant positive correlations were detected between APPs and total leukocyte and granulocytes; however, no correlation between lymphocyte and APPs were detected. Bovine SAA increases rapidly during the very early stages

of inflammatory stimuli; however, Hp increases later in extended inflammatory period. So, SAA has maximum clinical sensitivity, while Hp is known to be a marker of chronic inflammatory process (Horadagoda et al., 1999).

In conclusion, the results of this study revealed increased acute phase response in cattle with virus associated lymphosarcoma and positive correlation between APPs and total leukocyte and granulocytes.

Table 1. The primers used to n-PCR for detection of BLV.

Genomic region	Sequence	Nucleotid position
OBLV1	(5'-CTT-TGT-GTG-CCA-AGT-CTC-CCA-GAT-ACA-3')	5029
OBLV6	(5'-CCA-ACA-TAT-AGC-ACA-GTC-TGG-GAA-GGC-3')	5442
OBLV3	(5'-CTG-TAA-ATG-GCT-ATC-CTA-AGA-TCT-ACT-GGC-3')	5065
OBLV5	(5'-GAC-AGA-GGG-AAC-CCA-GTC-ACT-GTT-CAA-CTG-3')	5376

PCR-product size: 440 bp; PCRII-product size: 341 bp.

Table 2. Concentrations of acute phase proteins in cattle with EBL and BLV-free controls (Mean±SEM).

Parameters	BLV (n=40)	Control (n=20)	p
Haptoglobulin (mg/ml)	1.39±0.08	0.43±0.06	0.001
SAA (ng/ml)	124.90±17.60	60.29±14.64	0.033
Fibrinogen (mg/dl)	382.86±4.47	81.88±6.59	0.001

Table 3. Hematologic parameters in cattle with EBL and BLV-free controls (Mean±SEM).

Parameters	BLV (n=40)	Control (n=20)	Normal value	p
WBC (x10 ³ /μL)	13.68±0.95	7.51±0.39	4.0-12.0	0.001
Lym (x10 ³ /μL)	9.11±0.60	4.22±0.24	2.5-7.5	0.001
Gran (x10 ³ /μL)	4.20±0.48	2.76±0.19	0.6-4.0	0.046
Mon (x10 ³ /μL)	0.38±0.03	0.52±0.07	0-0.8	0.070
RBC (x10 ⁶ /μL)	7.55±0.17	6.98±0.13	5.0-10.0	0.036
Hb (g/dL)	8.98±0.08	9.58±.19	8.0-15.0	0.002
HCT (%)	27.40±0.38	27.72±0.67	24-46	0.659
PLT (x10 ³ /μL)	298.25±21.61	212.65±21.19	100-800	0.012

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