Acute Phase Proteins, Clinical, Hematological and Biochemical Parameters in Dairy Cows Naturally Infected with *Anaplasma Marginale*

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Summary

The aim of the study was to evaluate acute phase response via Haptoglobin and serum amyloid-A concentrations in dairy cows naturally infected with *Anaplasma marginale*. The second aim of the study was to determine the changes in clinical, hematological and biochemical parameters in dairy cows naturally infected *Anaplasma marginale*. A total of 40 dairy cattle suffering from bovine anaplasmosis were included to the study from a dairy cattle herd. A total of 10 healthy dairy cattle were selected for control group. Analysis of acute phase proteins, hematologic analysis and biochemical analysis was performed in this study. Serum haptoglobin and serum amyloid-A concentrations significantly increased in cattle infected with *Anaplasma marginale* compared to healthy cattle. All cattle in infected group demonstrated clinical signs of anaplasmosis. Significantly decreased red blood cell count, packed cell volume, and hemoglobin concentration were observed in infected cattle compared to the control group. Serum aspartate aminotransferase, alkaline phosphatase, creatinine and bilirubin concentrations were significantly increased in infected cattle compared with the control group. In conclusion, the changes of biochemical and hematological parameters may be an indication of anemia and tissue damage in cattle with anaplasmosis. Serum haptoglobin and serum amyloid-A concentrations could be useful in evaluation of acute phase response in cattle infected with *Anaplasma marginale*.

Keywords: Anaplasmosis, Haptoglobin, Serum amyloid-A, Dairy cows

*Anaplasma marginale* İle Doğal Enfekte Sütçü İneklerde Akut Faz Proteinler ile Klinik, Hematolojik ve Biyokimyasal Parametrelerin Değerlendirilmesi

Özet


Anahtar sözcükler: Anaplasmosis, Haptoglobin, Serum amyloid-A, İnek
**INTRODUCTION**

_**Anaplasma marginale**_ is a rickettsial organism that causes bovine anaplasmosis in cattle in tropical and subtropical areas throughout the world. The disease is a major constraint to cattle production in many countries and can be seen at any age 1-2. To confirm the diagnosis, laboratory tests such as light microscopic examination of Giemsa-stained blood smears or serological/molecular diagnostic procedures are required. Infected erythrocytes are not always detectable in stained blood smears during the persistent infection 3-4 so, a variety of serologic tests are used for the detection of specific antibodies against _A. marginale_ 5. A competitive ELISA (cELISA) has been used to diagnose _A. marginale_ infection in cattle 6. This test is used to serologically detect both acute and chronic _Anaplasma_ infections in cattle 6-8.

The early protection mechanism of the host against infection, trauma or other tissue damage comprises a set of reactions known as the acute phase response (APR). During the APR, the serum concentration of the acute phase proteins (APP) changes dramatically 9-12. These proteins are synthesized mainly in the liver. The secretion of APPs is regulated by proinflammatory cytokines such as interleukin-6 (IL-6), tumor necrosis factor-a, and IL-1β 10,12. Serum haptoglobin (Hp) and serum amyloid-A (SAA) are the major APP in cattle 10,14. Many studies have indicated the significance of serum Hp and SAA as clinically useful parameters in cattle various conditions 9-12,15-17.

The symptoms of clinical disease are fever, anemia, icterus, weight loss, abortion, and lethargy. Severity and death rate increase with advancing age 2. _A. marginale_ infection causes fever and mild to marked hemolytic anemia. After infection, parasitemia increases until the hemolytic crisis, frequently with more than 50% of RBCs infected 14. The number of infected erythrocytes increases drastically and phagocytosis by reticuloendothelial cells of parasitized erythrocytes lead to development of hemolytic anemia and icterus 15. Serum biochemical parameters such as aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT), concentrations are indicators of hepatic function. Hematological and biochemical alterations are the indicators of severity of disease 16. Hornok et al. 21 have suggested that biochemical values indicate pathological changes in the liver and in muscles, but not in the kidney in cattle with anaplasmosis. Anaplasmosis may induce elevation of AST and alkaline phosphatase (ALP) concentration in cattle 22.

The aim of the study was to evaluate acute phase response via acute phase proteins in dairy cows naturally infected with anaplasmosis. The second aim of the study was to determine the change in clinical, hematological and biochemical parameters in dairy cows naturally infected with anaplasmosis.

**MATERIAL and METHODS**

**Animals**

This study was performed in southern Turkey. A total of 40 dairy cattle (Holstein) suffering from bovine anaplasmosis were included to the study from a dairy cattle herd. All animals, which had symptoms of fever, weakness, lack of appetite, and decreasing milk yields, were females ranging from 2-5 years of age. All animals were in the first lactation period. The affected cattle were selected on the basis of clinical signs and presence of anaplasma inclusion bodies in blood smears. These animals were treated with oxytetracycline (10 mg/kg, IM, Tenaline®LA, Ceva-Dif/TURKEY), which was repeated after 48 h. The control group of this study was composed of 10 dairy cattle (Holstein). Control animals were selected based on clinical examination, results of cELISA and absence of anaplasma in blood smears.

**Blood Smear Examination**

Thin blood smears were prepared from smears of each examined animal. The smears were fixed with methyl alcohol, stained with 10% Giemsa, washed under regular tap water, and dried at room temperature. Giemsa-stained thin blood smears were examined under a light microscope with immersion-oil objective.

**Blood Sample Collection**

Blood samples were taken from the vena jugularis to measure serological, biochemical, and hematological parameters. An aliquot of blood was placed into an EDTA-containing plastic tube for routine hematologic examination, and another aliquot of blood was placed into glass tubes for determination of SAA, Hp, and serum biochemical analysis and cELISA. The tubes were centrifuged after clotting, and the serum was harvested and stored at -20°C until analyzed.

**Competitive-ELISA Test**

The cELISA currently used for diagnosis of bovine anaplasmosis employs monoclonal antibody ANAF16C1, which recognizes MSP5 in _A. marginale_. The cELISA test was performed according to the test procedure of the manufacturer (Anaplasma antibody test kit, cELISA, VMRD, Inc., USA).

**Acute Phase Protein Measurement**

Hp concentrations in serum were determined using a sandwich ELISA previously used for the analysis of Hp levels in cattle 15,16,23. Serum samples were diluted according to the manufacturer’s instructions (Life Diagnostics Inc., West Chester, PA, USA). Optical density of the samples was measured by use of a microplate reader (MWT Lambda Scan 200, Biotek Instrument Inc., USA) at 450 nm using
630 nm as the reference. The manufacturer of this assay reported a limit of detection in bovine serum of 0.25 mg/L. Cutpoints for serum haptoglobin of >150 mg/L or >500 mg/L have been recommended to identify an acute phase response in postparturient dairy cows, and >670 mg/L was recommended to identify cattle with traumatic reticuloperitonitis. Estimated values for sensitivity and specificity (versus clinical examination as the gold standard) for a cutpoint of serum \([\text{haptoglobin}]\) of >150 mg/L are 0.83 and 0.58, respectively.

**SAA concentrations in serum** were measured with a commercially available ELISA kit (Tridelta Development Ltd., Maynooth, Co. Kildare, Ireland), and serum samples were diluted according to the manufacturer’s instructions. Optical density of the samples was measured by use of a microplate reader (MWGt Lambda Scan 200, Biotek Instrument Inc., USA) at 450 nm using 630 nm as the reference. The manufacturer of this assay reported a limit of detection in bovine serum of 0.3 mg/L and a reference range of 9-150 mg/L. Cutpoints for SAA of >9 mg/L or >600 mg/L have been recommended to identify an acute phase response in cattle. Estimated values for sensitivity and specificity (versus clinical examination as the gold standard) for a cutpoint of >600 mg/L are 0.79 and 0.60, respectively.

**Hematological and Biochemical Analysis**

Hematologic analysis was performed using an automated hematology cell counter (Medonic CA 530 VET, Sweden). The levels of GGT, AST, ALP, blood urea nitrogen (BUN), total protein (TP), total bilirubin, glucose, creatinine kinase and creatinine were measured using an automatic analyzer (BT 3000plus, Biotecnica Instruments SpA, Italy).

**Statistical Analysis**

Data are expressed as means ± SE. The level of statistical significance was set at \(P<0.05\). A statistical software program (SPSS 10.0) was used for statistical analysis. Comparisons of values between the two groups were analysed with the independent sample t test.

**RESULTS**

All cattle in infected group demonstrated clinical signs of anaplasmosis. Anaplasmosis was confirmed by smear test for the presence of \(A.\) marginale in blood cells. The organisms, of approximately 0.5-10 µm and they were located peripherally in erythrocytes. \(Anaplasma\) marginale antibodies were found in 37 (92.5%) of 40 dairy cows. Clinical findings in all cattle with anaplasmosis included fever (>40°C), pale mucous membrane, lack of appetite, and decrease in milk yields. Most cattle demonstrated icterus, lethargy, weakness, weight loss and depression. Three cattle presented severe anemia, dehydration, and sternal recumbency and died within 3 days. Control animals were negative for antibodies to \(A.\) marginale by cELISA.

### Table 1. The levels of SAA, Hp, biochemical and hematological parameters in infected and healthy cows (X ± Sx)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Healthy Cows (n=10)</th>
<th>Infected Cows (n=40)</th>
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<tbody>
<tr>
<td>Hp (µg/ml)</td>
<td>15.90±4.19</td>
<td>203.09±19.04 *</td>
</tr>
<tr>
<td>SAA (µg/ml)</td>
<td>18.93±6.64</td>
<td>134.11±14.62 *</td>
</tr>
<tr>
<td>RBC count (10^3/µl)</td>
<td>7020.00±277.62</td>
<td>5170.10±206.41 *</td>
</tr>
<tr>
<td>WBC count (cell/µl)</td>
<td>8405.30±411.10</td>
<td>7490.13±1119.99</td>
</tr>
<tr>
<td>PCV (%)</td>
<td>28.71±0.48</td>
<td>22.91±1.07 *</td>
</tr>
<tr>
<td>HGB (g/dl)</td>
<td>10.66±0.42</td>
<td>8.19±0.29 *</td>
</tr>
<tr>
<td>PLT count (10^3/µl)</td>
<td>490.13±58.72</td>
<td>352.25±42.54</td>
</tr>
<tr>
<td>BUN mg/dl</td>
<td>20.25±2.52</td>
<td>24.26±1.58</td>
</tr>
<tr>
<td>Creatinin (mg/dl)</td>
<td>1.06±0.11</td>
<td>1.56±0.05 *</td>
</tr>
<tr>
<td>Creatine kinase (U/L)</td>
<td>150.50±25.04</td>
<td>416.98±100.87</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>64.00±2.35</td>
<td>99.38±12.80 *</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>64.63±7.62</td>
<td>122.43±6.77 *</td>
</tr>
<tr>
<td>GGT (U/L)</td>
<td>21.75±3.61</td>
<td>34.48±10.67</td>
</tr>
<tr>
<td>Bilirubin (mg/dl)</td>
<td>0.21±0.06</td>
<td>0.91±0.07 *</td>
</tr>
<tr>
<td>TP (g/dl)</td>
<td>7.55±0.20</td>
<td>8.51±0.25</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>66.87±12.63</td>
<td>72.50±4.87</td>
</tr>
</tbody>
</table>

Haptoglobin (Hp), Serum Amyloid-A (SAA), Red blood cell count (RBC), White blood cell count (WBC), Packed cell volume (PCV), Hemoglobin (HGB), Platelet (PLT), Gamma-glutamyl transferase (GGT), Aspartate aminotransferase (AST), Blood urea nitrogen (BUN), Total protein (TP), Alkaline phosphatase (ALP)

Data are presented as Mean ± SE. Asterisked (*) mean values are significantly different (*P<0.05)
The levels of SAA, Hp, and serum biochemical and hematological parameters in the infected animals and control group are presented in Table 1. Hp and SAA concentrations significantly differed between healthy cattle and *A. marginale*-infected cattle. While Hp and SAA concentrations in healthy cattle was 15.90±4.19 µg/mL, significantly decreased RBC, PCV and HGB concentration were observed in infected cattle compared to the control group. Other values were within normal range (Table 1). Serum AST, ALP, creatinine and bilirubin concentrations were significantly increased in infected cattle compared with the control group. Serum GGT, creatine kinase, total protein, and blood urea nitrogen levels were increased in infected cattle compared to the control group, but with no statistically significant difference (Table 1).

**DISCUSSION**

Anaplasmosis, formerly known as gall sickness, traditionally refers to a disease of ruminants caused by obligate intra-erythrocytic ricketsia of the genus *Anaplasma* [27]. Producers in endemic areas often suspect anaplasmosis based on a history of previous disease outbreaks in that locality. Birdane et al. have indicated of a local outbreak in Turkey [8]. Clinical outbreaks occur most frequently during warm, wet seasons when vector-borne transmission is more prevalent. Naïve cattle in non-endemic areas may become infected with anaplasmosis when higher than 200 µg/mL, severe inflammation at the plasma and thus reduces the oxidative damage associated with hemolysis, whereas SAA mainly modulates the immune response. SAA is a valuable APP in diagnosing cattle with inflammation [10]. An effect of free hemoglobin in serum samples towards reduction of measured Hp concentration has also been found [31,32]. In contrast to those results, Nazifi et al. [33-35] observed increased in cattle infected theileriosis and anaplasmosis. Our present study is consistent with Nazifi’s findings, with Hp concentration increased in *A. marginale*-infected cattle compared to healthy cattle. This significant increase indicates an inflammation in cattle with anaplasmosis. Nazifi et al. [32] indicated that SAA demonstrated more obvious changes than Hp during different levels of parasitemia in *A. marginale*-infected cattle. However, the current study demonstrated that both Hp and SAA concentrations could be good indicators of inflammation in cattle with *A. marginale*-associated parasitemia. In inflammation following Anaplasma infection causes to important stimulation of the synthesis of APP. Thus, the evaluation of acute phase response in cattle with *A. marginale* is important for the determination of inflammation.

All animals in this study had high rectal temperature (>40°C), mild icterus, reduced milk yield, and restlessness. Three of 40 anaplasmosis-infected cattle died after oxytetracycline treatment following diagnosis. Clinical symptoms progressed from mild to severe anaplasmosis in cattle, suggesting that carrier cows in advanced pregnancy and/or lactation may relapse and develop signs of acute infection. Such events may be related to immunosuppression associated with the periparturient period in cows [36,37]. Pecurate anaplasmosis, characterized by a high mortality rate within a few hours of clinical signs developing, is most frequently encountered in purebred animals and high-producing dairy cows [38]. In the current study, peracute anaplasmosis was not observed in any cattle. The severity of anaplasmosis may have been due to immunosuppression associated with the postparturient status of the cattle.

After erythrocytic infection is detected, the number of infected erythrocytes increases geometrically. Bovine anaplasmosis often results in development of mild to severe anemia and icterus without hemoglobinemia or hemoglobinuria, which arises from phagocytosis of these infected erythrocytes by bovine reticuloendothelial cells [5,2]. The decrease in RBC, HGB, and PCV resulted in anemia in the infected group (Table 1). Hematological analysis indicated pronounced decrease in platelets, but this value was not statistically significant. Other values were within normal range. Decrease in RBC, PCV, and HB may be indicative of the severity of anemia as parasitemia progressed.

Serum AST, GGT, and ALT concentrations are indicators of hepatic function [20]. In the present study, an increase in...
AST, ALP and GGT concentration was observed in infected cattle compared with healthy cattle, indicating hepatic dysfunction. The rise of serum AST and creatine kinase concentration in cattle may have been caused by muscular trauma as a result of recumbency due to anaplasmosis. The increased serum bilirubin concentration may be attributable to hemolysis of parasitized erythrocytes. Biochemical analysis revealed pronounced elevation of GGT, creatine kinase, total protein, and blood urea nitrogen plasma levels, but these increases were not statistically significant. Non-significant increase of total protein, and blood urea nitrogen levels in infected cattle may have resulted from dehydration observed in 5 dairy cows.

In conclusion, serum Hp and SAA concentrations could be also useful in evaluate of acute phase response in cattle with *Anaplasma marginale*-associated parasitemia. Changes of biochemical and hematological parameters may be indicate of anemia and tissue damage in cattle with *Anaplasma marginale*. These parameters may be helpful to understanding the disease pathogenesis and could be used as tools for diagnosis.

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