

***Anaplasma phagocytophilum* in Horses - Evaluation of Proinflammatory Biomarkers**

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ABSTRACT

Background: *Anaplasma phagocytophilum* is an obligate intracellular pathogen transmitted by the ticks that cause equine granulocytic anaplasmosis (EGA). This pathogen infects predominantly blood cells, principally granulocytes and especially neutrophils. *A. phagocytophilum* causes an acute febrile disease in horses accompanying with lethargy, loss of appetite, lameness and hemorrhages. In horses, this disease should be considered in all acute symptoms accompanied by thrombocytopenia and leukopenia identified by hematological test performed. Tick-borne pathogens have become increasingly threatening for both animals and also public health since ticks mostly carry numerous well-documented and undocumented pathogens, and the geographical range of ticks has expanded in the recent years. This research has aimed to evaluate the impact of *A. phagocytophilum* infection on some oxidative/nitrosative stress parameters, antioxidant enzyme activities, proinflammatory biomarkers and trace element levels in horses.

Materials, Methods & Results: The present study has been carried out using blood samples collected from 93 horses aged 1-year and older. The blood samples were centrifuged and sera were separated. Serum samples stored in the freezer (-20°C) until the day of analysis. The DNA was extracted from blood and analysed by nested-PCR technique targeting 16S rRNA gene of *A. phagocytophilum* and then positive PCR products were sequenced. *A. phagocytophilum* was found in 6 horses (6.4%) showed positive nested-PCR results. An infected group comprised of 6 positive horses according to PCR analysis results also 6 healthy horses as control were selected. Serum SOD (Horse Superoxide Dismutase(Cu-Zn)) ELISA Kit, MPO (ELISA Assay Kit Horse Myeloperoxidase) and GPx (Horse glutathione peroxidase 1 ELISA Kit Assay), IL1 (Horse Interleukin 1 Beta ELISA Kit), IL6 (Horse Interleukin 6 ELISA Kit), TNF α (Horse Tumor Necrosis Factor Alpha ELISA Kit) and IL18 (Horse Interleukin 18 (IL18) ELISA Kit) levels were determined by ELISA reader. Serum TAS, TOS Glucose-6-phosphate dehydrogenase (G6PD) levels were determined using colorimetric kit method. The determination of peroxynitrite was performed using spectrophotometry as described by Vanuffelen. The levels of Fe, Zn, Se, Cu, Mn, Ar, Cr, Co, Cd Ni and Pb elements were analyzed by ICP-OES. Total oxidant status (TOS) and peroxynitrite levels of the positive group infected with *A. phagocytophilum* were found to be higher compared with the control group ($P < 0.05$, respectively). Whereas, total antioxidant status (TAS), glutathione peroxidase (GPx), superoxide dismutase (SOD), G6PD and myeloperoxidase (MPO) levels were detected to be statistically significantly higher than the control group ($P < 0.05$, respectively). Cu, Mn, Se and Zn levels were also found to be lower in the infected group ($P < 0.05$, respectively). IL1, IL6, TNF α and IL18 from proinflammatory markers were elevated in the *A. phagocytophilum* infected group ($P < 0.05$, respectively).

Discussion: The evaluation of the proinflammatory biomarkers with respect to host-parasite interactions has been suggested as a beneficial clinical tool for determination of the infection severity and diagnosis of the disease. As a conclusion, increased oxidative stress and high levels of some proinflammatory biomarkers assessed by the multibiomarker analysis carried out for the infection in the horses caused by *Anaplasma phagocytophilum* will provide a contribution to diagnosis, treatment and clarification of the pathogenesis.

Keywords: anaplasmosis, EGA, tick-borne pathogens, pro-inflammatory cytokine, antioxidant, oxidative/nitrosative stress, equine.

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INTRODUCTION

Anaplasma phagocytophilum is a Gram negative obligate intracellular pathogen that reproduces in neutrophils and causes febrile diseases in the humans and animals due to transmission by ticks from Ixodes spp. [10]. The oxidative stress index changes in parasitic diseases [7]. *Anaplasma phagocytophilum* infection causes down regulation of host granulocyte defense genes including myeloperoxidase (MPO) [14]. Peroxynitrite has direct harmful effects on proteins. NO, plays a role in the host immun system mechanism against many intracellular parasitic diseases [1,4]. G-6-P-D catalyzes the key step in penthose phosphate pathway, during which NADPH molecules are produced. NADPH is used to reduce the oxidized glutathione formed during detoxification reactions. In case of NADPH depletion, hemolysis occurs as a result of loss of membrane integrity by inhibiting the ROS induced lipid peroxidation [8]. Microminerals are essentially used in the synthesis of antioxidant enzymes. The assessment of elements may contribute to the evaluation of oxidant/antioxidant status because of their oxidative characteristic [13]. Pro-inflammatory cytokine production plays an important role in immunology and pathogenesis of the disease in the animals [6].

In the present study, we aimed to provide contribution to the researches on the relationship between oxidative/nitrosative stresses and equine granulocytic anaplasmosis (EGA), as well as, their role in the pathogenesis of anaplasmosis. Moreover, we aimed to reveal the clarification of the fact whether cytokines, peroxynitrite and free radicals have an impact on the progression of dysfunction.

MATERIALS AND METHODS

Animals and samples

The present study has been carried out using blood samples collected from 93 horses aged 1-year and older in the region of Muş Province between June-August 2017 [20]. The study was conducted in accordance with the regulations issued by Animal Experiments Local Ethics Committee of Van Yüzüncü Yıl University (2021/09-07). Blood samples drawn from vena jugularis of the horses according to the appropriate technique and collected in the vacuum gel biochemistry and EDTA tubes were brought to the laboratory under cold chain. Plasma and serum samples were obtained from the EDTA and biochemistry tube

blood samples centrifuged at 322 g for 15 min, respectively, and stored at -80°C to be used in the analysis.

The control group consisted of 6 clinically healthy horses having a similar age the infected horses. The horses were considered healthy on the basis clinical examination and laboratory data. The infected group consisted of 6 horses with positive *A. phagocytophilum* as a result of PCR analysis.

Parasitological examination

Genomic DNAs were obtained from the complete blood samples of the horses using a commercial blood kit¹. The obtained DNA samples were stored at -20°C until PCR analysis. Genomic DNAs obtained from blood samples at the first stage. PCR products were obtained utilizing forward primer EC9:5'-TAC CTTGTTACGACTT and reverse primer EC12A: 5'-TGATCCTGGCTCAGAACGAACG of which common conserved regions in all *Ehrlichia/Anaplasma* species were amplified at the first stage, and 1 µL of the PCR product was taken for the nested PCR procedure and subjected to PCR with specific primers SSAP2-F:GCTGAATGTGGGGATAATTTAT and SSAP2-R: ATGGCTGCTTCCTTTTCGGTTA that amplify a 641 bp fragment of the 16S rRNA gene for *A. phagocytophilum* [18]. *A. phagocytophilum* DNA (GenBank Accession number: MW642479) confirmed by Genekam Biotechnology Company (Germany) sequencing was used as the positive control while sterile water was utilized as the empty control. PCR products were appropriately packaged and sent to Sentebiolab Company (Ankara) for DNA sequence analysis. The nucleotide series obtained in the study were recorded in GenBank with accession numbers of MW642476-MW642478.

Biochemical analysis

Serum TAS and TOS levels were determined using colorimetric kit² method. TAS/TOS ratio was calculated and accepted as oxidative stress index (OSI).

Serum SOD (Horse Superoxide Dismutase(Cu-Zn), ELISA Kit³, MPO (ELISA Assay Kit Horse Myeloperoxidase)³ and GPx (Horse glutathione peroxidase 1 ELISA Kit Assay)³ levels were measured by using commercial ELISA kits following methods described in the kits. The optical density was read at 450 nm and results of serum SOD, MPO and GPx were extrapolated from their respective standard curves.

The determination of peroxynitrite was performed using spectrophotometry as described by

Vanuffelen [29]. Peroxynitrite-mediated nitration of phenol leads to formation of nitrophenol, formed the basis of peroxynitrite measurement. In summary, 10 μ L of serum was placed in a test tube and 5 mM phenol in 50 mM sodium phosphate buffer. The final volume of 2 mL and mixed well. It was incubated for 2 h at 37°C, followed by an additional 15 μ L of 0.1 NaOH and mixed. The absorbance of the sample at 412 nm was measured in a spectrophotometer⁴. Nitrophenol yield was calculated: ($\epsilon = 4400 \text{ M}^{-1}\text{cm}^{-1}$)

Glucose-6-phosphate dehydrogenase (G6PD) activity was measured using colorimetric kit⁵ at 340 nm and 37°C.

Commercial ELISA kits were used to measure the levels of IL1 (Horse Interleukin 1 Beta ELISA Kit)³, IL6 (Horse Interleukin 6 ELISA Kit)³, TNF α (Horse Tumor Necrosis Factor Alpha ELISA Kit)³ and IL18 (Horse Interleukin 18 (IL18) ELISA Kit)³. The analysis was carried out according to the instructions of the manufacturer. Standard curves were drawn and the logarithmic calculations were carried out.

Serum samples were prepared according to Papageorgiou *et al.* [22] method for trace element analysis. One mL of each serum sample was taken into glass tubes and 1 mL of 3% HNO₃ solution prepared from 65% HNO₃ solution was added to the tubes, and centrifuged at 322 g. After removal of residual particles by leaching, 1 mL 1% Triton-X was added onto the tubes and the volume was completed to 10 mL by adding deionized pure water. The levels of Fe, Zn, Se, Cu, Mn, Ar, Cr, Co, Cd Ni and Pb elements were analyzed by ICP-OES⁶ (inductively-coupled plasma-optical emission spectroscopy, 0.005 ppm detectable limit) device.

Statistical analysis

Statistical analysis were performed using SPSS 22 program. The normality of data distribution for each parameter was evaluated using a Shapiro-Wilk test. The independent sample t-test was used to determine the difference between the groups. The obtained results were given as $\bar{X} \pm \text{SE}$. The value of $P < 0.05$ was accepted as statistically significant.

RESULTS

Anaplasma phagocytophilum DNA was detected by nested PCR procedure in 6 blood samples

(Figure 1). Six PCR-negative horses without previous history of anaplasmosis were used as negative controls. Mean TAC, TOS, SOD, GPx, MPO, G6PD and peroxynitrite levels of the control and anaplasma positive groups were presented in Table 1. Accordingly, TOS and peroxynitrite levels were found to be statistically significantly higher than the control group ($P < 0.005$). TAS, SOD, GPx, MPO and G6PD level of the equines infected with *A. phagocytophilum* was found to be lower than the control group ($P < 0.005$). TNF- α , IL-6, IL-1 and IL18 levels in both control and infected horses were presented in Table 2. TNF- α , IL-6, IL-1 and IL18 levels showed significant increased in infected group ($P < 0.005$). Comparison of serum levels Ar, Cd, Co, Cr, Ni, Pb, Cu, Mn, Se, Fe and Zn levels is demonstrated in Table 3. Serum levels of Ar, Cd, Co, Cr, Ni, Pb, did not differ among groups. Nevertheless, serum Cu, Mn, Se ($P < 0.05$) and Zn ($P < 0.05$) levels were significantly lower in group infected group as compared with control group. It was observed that the serum iron level increased in the infected group ($P < 0.05$).

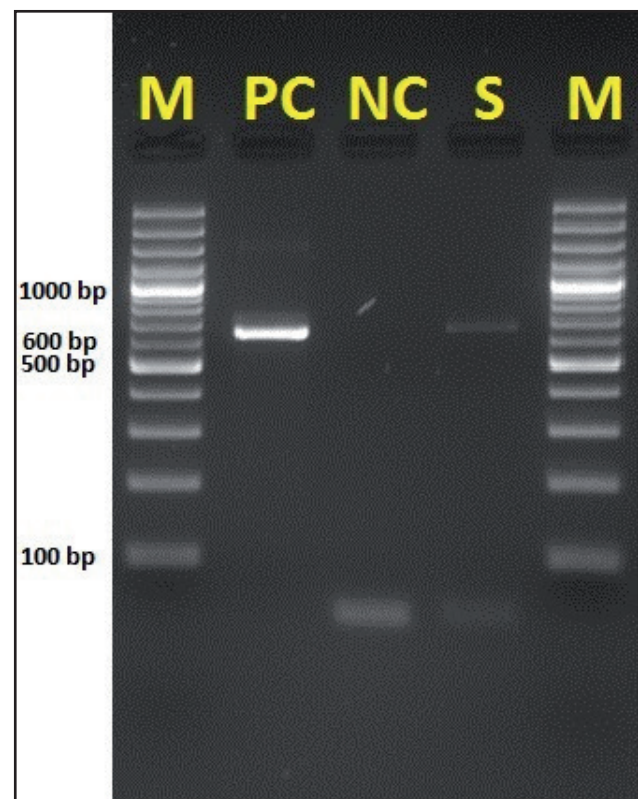


Figure 1. *Anaplasma phagocytophilum* positive sample on agarose gel at the second stage of nested PCR analyses: M: Marker (100bp); S: Positive sample; PC: Positive control; NC: Negative control.

Table 1. Comparison of serum TAS, TOS, OSI, SOD, GSH-Px, MPO, G6PD, peroxynitrite levels between control and infected groups.

Parameters	Control group (n = 6)	Infected group (n = 6)
TAS (mmol Trolox Eq/L)	2.37 ± 0.18*	1.58 ± 0.19
TOS (µmol H ₂ O ₂ Eq/L)	3.79 ± 0.48*	6.26 ± 0.93
OSI (arbitrary unit)	0.17 ± 0.02*	0.43 ± 0.09
SOD(ng/mL)	4.98 ± 0.80*	3.816 ± 0.94
GPx (ng/mL)	42.85 ± 6.08*	22.74 ± 0.38
MPO(ng/mL)	29.4 ± 0.30*	20.1 ± 0.27
G6PD (mU/mL)	375.50 ± 44.02*	352.23 ± 53.21
Peroxyntirite (µmol/L)	0.69 ± 0.03*	0.82 ± 0.26

Values are presented as mean ± SD; n, number; TAS, total antioxidant status; TOS, total oxidant status; OSI, oxidative stress; SOD (superoksidge dismutaz); GPx (Glutatyon peroksidaz); MPO (Myeloperoxidaz); G6PD (Glukoz-6-fosfat dehidrojenaz) Significance: * $P < 0.05$.

Table 2. Comparison of serum IL6, IL1, TNFα, IL18 levels between control and infected groups of horses.

Groups	IL1 (ng/L)	IL6(ng/L)	TNF- α (ng/L)	IL18 (ng/L)
Control (n=6)	23.23 ± 1.0	30.5 ± 0.01	135.4 ± 0.12	128.85 ± 4.88
Infected (n=6)	26.95 ± 1.20*	31.9 ± 0.04*	141.2 ± 0.10*	133.82 ± 2.58*

Values are presented as mean ± SD. N: number. IL1: Interleukin-1. IL-6: Interleukin 6. TNF-α: tumor necrosis factor. IL-18: Interleukin 18. Significance: * $P < 0.05$

Table 3. Comparison of serum Ar, Cd, Co, Cr, Ni, Pb, Cu, Mn, Se, Fe, Zn levels between control and infected groups of horses.

Parameters	Control group (n = 6)	Infected group (n = 6)
Ar (µmol/L)	0.1291 ± 0.0162	0.1092 ± 0.0331
Cd (µmol/L)	0.0124 ± 0.0004	0.0135 ± 0.0005
Co (µmol/L)	0.0568 ± 0.0028	0.0454 ± 0.0106
Cr (µmol/L)	0.5852 ± 0.1218	0.5454 ± 0.1609
Ni (µmol/L)	0.1955 ± 0.0719	0.1420 ± 0.0326
Pb (µmol/L)	0.1135 ± 0.0311	0.0742 ± 0.0175
Cu (µmol/L)	9.4216 ± 0.7992*	5.3316 ± 1.5444
Mn (µmol/L)	2.3543 ± 0.2936*	1.1007 ± 0.0906
Se (µmol/L)	0.6503 ± 0.1825*	0.1950 ± 0.0837
Fe (µmol/L)	4.224 ± 0.1083*	22.992 ± 0.5828
Zn (µmol/L)	9.224 ± 0.1776*	3.704 ± 0.0966

Values are presented as mean ± SD. N: number. Ar: arsenic. Cd: cadmium. Co: cobalt. Cr: chromium. Ni: nickel. Pb: lead. Cu: copper. Mn: manganese. Se: selenium. Fe: iron. Zn: zinc. Significance: * $P < 0.05$.

DISCUSSION

This study results support that *A. phagocytophilum* infection stimulate proinflammatory cytokine response in horses. Acute phase response (APR) is activated mediated by some proinflammatory cytokines (IL-6, TNF-α and IL-1) as a consequence of tissue damage caused by parasitic infections. The levels of some plasma proteins increase in acute phase response.

Healing is achieved via inhibition of tissue damage by the activation of these proteins. The cytokines are the essential effective mediators in all these metabolic changes [3]. The infected cells are the inhibitor of hematopoietic stem cell proliferation. It has been demonstrated in the in vitro studies that production of proinflammatory chemokines and cytokines is stimulated as a result of *A. phagocytophilum* infection [19].

Grab *et al.* [16] have reported that IL-6 level increases in *A. phagocytophilum* infection. It has been reported in the studies that proinflammation markers play an important role in the control of EGA (*A. phagocytophilum*) infections in horses [6]. It has been detected that IL-6 level increased in anaplasmosis caused by *A. phagocytophilum* [13]. It has been exhibited in the previous studies on horses that cytokines such as TNF- α , IL-1, IL-6 and IFN- γ , increase in the infections caused by *Trypanosoma vivax* [25], *Babesia caballi* [17] and *Theileria equi* [21]. According to our study, IL 1, IL6, TNF α and IL18 levels increased in the group infected with *A. phagocytophilum*. The increase in the level of IL-6 may be resulting from increased proinflammatory cytokine synthesis due to stimulation of the macrophages by the anaplasma agent. This increase in IL1, IL6, IL18 and TNF- α levels may be due to the important roles of the immune system in the occurrence of clinical symptoms against parasitic infections.

It has been stated that reactive nitrogen species are effective in the pathogenesis of many disease. It rapidly reacts with nitric oxide (NO $\cdot$) when excessively produced. Reticuloendothelial cells or polymorphonuclear leukocytes may produce high concentrations of nitric oxide and superoxide when activated by cytokines. NO react with superoxide to form peroxynitrite (ONOO $\cdot$) which is a more toxic oxidant via a radical termination reaction at a high and constant speed. Underlying pathogenesis seems to be altered bioavailability of NO $\cdot$ and tissue damage caused by increased reactive oxygen and nitrogen species. Increased production of ROS, RNS and reduced antioxidant mechanisms leads to oxidative and nitrosative stress [27]. Enzymatic and non-enzymatic antioxidants eliminate ROS and protect the tissues against oxidative damage. On the other hand, *A. phagocytophilum* reduces antioxidant activity. Infection with *Anaplasma* spp. promotes the production of various proinflammatory cytokines by the mononuclear cells. These cytokines activate lipid peroxidation by increasing the release of reactive oxygen species by the phagocytic cells [30]. In a study, the plasma NO level of cattle with anaplasmosis was found to be statistically higher at $P < 0.001$ significance level than healthy cattle [11]. According to the our findings, peroxynitrite level increased in the infected group. The reason for this increase may be due to the fact that *A. phagocytophilum* stimulates the production and release of NO in macrophages.

Myeloperoxidase is an enzyme stored in the azurophilic granules of the polymorphonuclear neutrophils. Its levels increase in infections. Since *A. phagocytophilum* localizes also in the neutrophils; defects may occur in the pathways of neutrophil enzymes such as MPO elastase. *A. phagocytophilum* infection impairs the phagocytic ability of neutrophils [15]. The activities of SOD, catalase and glutathione have decreased in naturally acquired babesiosis, anaplasmosis and hepatozoonosis coinfections in dogs [24]. Among the goats with anaplasmosis, SOD catalase activity increased whereas MDA levels decreased in the group administered Vitamin E- selenium combination together with treatment [9]. It has been reported in the different studies conducted in horses that TOS level increased whereas TAC level decreased in the horses with equine piroplasmosis infection [2] while GSH and CAT concentrations decreased in *Theileria equi* and *Babesia caballi* infections [7,21]. In this study, TOS level was found to be higher in horses infected with *A. phagocytophilum* than in healthy horses. This increase may be due to the continuous and increased production of free radicals in parasitic diseases and the reduction of antioxidant defense systems. In this study, SOD, GPx, MPO enzyme activity and TAS levels were significantly decreased in infected horses. This reduction may be due to its use to eliminating increased lipid peroxidation products. Our results are similar with the studies of Ujjwal *et al.* [28] and Radwan *et al.* [23]. The presence of a significant correlation between parasitemia and decrease in antioxidant enzyme activities including SOD and GPx indicates the exposure of erythrocytes to oxidative substances in blood parasitic diseases. In the light of this study result, oxidative stress occurs in the horses found positive for *A. phagocytophilum* infection.

It is documented that cytokines elevate the production of superoxide anion and iNOS [5]. If cytokines continuously keep high concentration, SOR that has a short half-life is continuously produced and supports permanence of cell damage. With this respect, antioxidants may be effective against cell damage induced by SOR. Such studies may leading to the use of the inhibitors of cytokines or superoxide anion production and nitric oxide as the progressive inhibitor agents in the various diseases.

To our knowledge, there are no published reports on the G6PD activity in *A. phagocytophilum*

infection in the literature. Glucose-6-phosphate dehydrogenase (G6PD) enzyme is the first and regulatory key enzyme of the pentose phosphate pathway and plays a role in the protection of NADPH, sulfhydryl groups formed as a result of the reaction, and detoxification of free radicals and peroxides. It also stimulates protective systems against free radicals in cells without mitochondria, like erythrocytes [8]. There are the role of erythrocytic deformation in the pathogenesis of hemoparasitic infection [24]. An increase in G6PD activity would be expected in case of increased oxidative stress. However hemolysis caused by anaplasma infection may have decreased the G6PD. It can be thought that the decreased G6PD contributes to the increase of oxidative stress. In addition to this, low G6PD activity has been reported in the bovines and ovines with piroplasmiasis [12] and *A. marginale* infection [13]. The decreased G6PD activities that were found to be low in also present study may be related with formation of hemolysis.

In the current study, serum iron levels were increased in infected horses, while serum manganese, copper, selenium, and zinc levels were decreased. It may be assumed that reduced trace element levels may represent an antioxidant role coordinately with antioxidant enzyme activities during infection. Similar results have been identified also in the bovines infected with *Anaplasma marginale* [13]. This results demonstrate that protozoan hemoparasites probably impair the activities of antioxidant enzymes by decreasing the levels of the necessary trace elements [21]. In addition, reduced TAC levels may be partially associated with the decreased levels of these critical microelements. In the present study, high serum Fe levels in the infected equines may be most probably due to hemolytic anemia. Abnormal RBCs are identified by the macrophages in the bone marrow, spleen and/or liver, and following phagocytosed by macrophages. Macrophages cause an increase in serum iron levels by reducing he-

moglobin to globin, heme and iron. The decrease in the zinc levels may be resulting from the indirect impact of host-parasite interaction or increased need for zinc of the parasite itself [25] as well as hormonal changes [7]. Zaeemi *et al.* [31] have identified that both zinc and copper levels decreased. Studies have shown that serum calcium, phosphorus, magnesium and potassium concentrations decreased [26,31], sodium and chloride concentrations [26] has been reported to be increased in horses with hemoparasitic infections.

CONCLUSIONS

The evaluation of proinflammatory biomarkers according to host-parasite interactions is important for determining the severity of infection. As a conclusion, *A. phagocytophilum* infection induces oxidative/nitrosative stress. Additionally, the levels of the proinflammation markers that may lead to functional impairments were found to be elevated. Taking these results into consideration, anti-inflammatory drugs and antioxidant supplements may be preferred to antibiotics in future studies. The results this study in *Anaplasma phagocytophilum* infection, which we think is neglected, may help future research on diagnosis, treatment, prognosis and immunosuppressive treatment.

MANUFACTURERS

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Ethical approval. This study was approved by the Animal Experiments Local Ethics Committee of Van Yüzüncü Yıl University (2021/09-07).

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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