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Determining Serum Amyloid-A (SAA), Haptoglobin (Hp), Tumor Necrosis Factor (TNF-α) and Interleukins-1 (IL-1β) and 6 (IL-6) Levels in Neonatal Calves with Amoebiasis

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ABSTRACT

Background: Amoebiasis is a pathogen organism which is well-known both in human and veterinary medicine**.** The aim of this study was to determine serum amyloid-A (SAA), haptoglobin (Hp), tumor necrosis factor (TNF-α) and interleukins-1 (IL-1β) and 6 (IL-6) levels in neonatal calves which were naturally infected with amoebiasis.

Methods: In the study, 40 one to four-week-old calves with amoebiasis which were clinically diagnosed with diarrhea and were non-responsive to the treatment, and 10 healthy calves were used. From the stool samples taken from the calves which were clinically diagnosed with diarrhea, *Entamoeba histolytica* was detected by using ELISA kit. Serum was obtained from the blood taken from vena jugularis from all calves. Concentrations of SAA, Hp, TNF-α, IL-1β and IL-6 in serum were measured in ELISA by using commercial kits. Also, some enzymes measurements were made from serum.

Result: Concentrations of SAA, Hp, TNF-α, IL-1β and IL-6 in calves with amoebiasis were higher ($p<0.05$) in comparison to the control group. To conclude, amoebiasis led to an increase in the concentrations of SAA, Hp, TNF-α, IL-1β and IL-6 in neonatal calves. We suggest that this might have stemmed from the inflammatory response against amoebiasis.

Key words: Cytokine**,** *Entamoeba,* Haptoglobin (Hp), Serum amyloid-A (SAA).

INTRODUCTION

Amoebiasis is a pathogen organism (Padros and Constenla. 2021). It has been reported that it affects approximately 180 million people every year, and 40.000 to 110.000 people died of this disease (Ackers and Mirelman, 2006). Depending on the morphologic findings up to now and the hosts where the parasites were found, *Entamoeba bovis (E. bovis)* was detected in the cattle and *Entamoeba ovis (E. ovis)* was detected in the sheep (Noble and Noble, 1952). In the study conducted in Japan, Entamoeba cysts were found in the environmental samples (earth and water) obtained from the cattle farms and the result revealed *E. bovis* (Matsubayashi *et al.,* 2018). Nagaraja and Ankri (2019) defined amoebiasis as a series of symptoms including acute diarrhea, dysentery, amebic colitis and hepatic abscess.

Dharmani *et al.* (2009) reported that the first step of pathogenesis consists of the adhesion of trophozoites to the mucus layer of the colon which is formed by the secreted MUC2 mucin.It was reported that in human pathogenesis, pathogenic amoebas, under dysbiosis conditions, might deteriorate the mucosa layer with glycosidases (Moncada *et al.,* 2005; Lidell *et al.,* 2006) and proteinases (Tillack *et al.,* 2007; Clark *et al.,* 2007) that they have in order to expose intestinal epithelial cells (Thibeaux *et al.,* 2013), and after the destruction of mucus layer, *E. histolytica* trophozoites adhere to the membrane of vulnerable epithelial cells using a surface lectin with high affinity for galactose (Gal) and N-acetyl-D-galactosamine (GalNAc) (Petri *et al.,* 2002; Stanley 2003; Cornick and Chadee, 2017; Leon-Coria *et al.,* 2020). Quach *et al.* (2014) demonstrated that Gal-lectin adhesion is the best protein characterized protein related to pathogenesis of *E. histolytica* and showed its ability to stimulate pro-inflammatory immune responses. Moreover, other studies found that Gal/GalNAc enhanced the expression of the proinflammatory cytokines in macrophages (IL-1α, IL-1β, TNF-α and IFN-γ) (Séguin *et al.,* 1997). It was reported that Toll Like Receptor (TLR)-2 can also adhere to amoeba's lipopeptidophosphoglycan (LPPG) (Maldonado-Bernal *et al.* 2005; Guha-Niyogi *et al.,* 2001) and induce the production of LPPG IL-1β that is recognized by TLR-2 (Maldonado-Bernal *et al.,* 2005). The activation of these TLRs lead to the activation of NF-κB in epithelial cells and

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to the production of inflammatory cytokines including IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-c and TNF-α (Sharma *et al.,* 2008). Moreover, in another study it was reported that cytokines (IL-2, IL-4 and IL-10, *etc*.) were not detected in human colon in the presence or absence of *E. histolytica* trophozoites (Labruyère *et al*., 2019).

It was reported that as a response to pro-inflammatory cytokines such as IL-1β and IL-6 that are secreted in case of infection and damaged tissue, initially acute phase proteins (AFP) are produced by the hepatocytes in the liver (Lee and Beatty, 2021). Main cellular source for SAA which is one of these acute phase proteins is hepatocytes; however, adipocyte tissues of obese patients also produce SAA (Lee and Beatty, 2021). It was reported that Hp is synthesized mainly in liver and lungs in humans and then released in blood plasma, also, Hp mRNA has been reported to be detected in kidneys, spleen, thymus, and heart (Naryzny and Legina, 2021). Shih *et al.* (2014) asserted that Hp is functionally important to bind free hemoglobin, prevents toxic effects, and can create antioxidant and antimicrobial effects when locally synthesized.

This study aimed to determine amoebiasis caused by *E. histolytica* in calves with chronic diarrhea as well as to establish the changes that amoebiasis leads to in IL-1β, IL-6 and TNF-α, which are proinflammatory cytokines in animals, in SAA, which are acute phase proteins and in Hp.

MATERIALS AND METHODS

Entamoeba histolytica diagnosis and sample collection

In the study, 40 one to four-week-old calves which were clinically diagnosed with chronic diarrhea and were non-responsive to the treatment, and 10 healthy, which were clinically examined without diarrhea calves were used. *Entamoeba histolytica* was diagnosed in the fresh stool samples taken from calves with chronic diarrhea by using ELISA test kit, which stool samples were examined by trichrome staining for trophozoites and cysts and by immunoassay methods for specific adhesin antigens. (Techlab, Inc., Blacksburg, VA) However, stools of 10 healthy calves were also checked by ELISA and the result was negative for Entamoeba histolytica. 40 calves with diarrhea formed the study group, the control group involved 10 healthy calves.

Measurement of acute phase proteins and cytokines

In the serum procured from blood samples, measurements of Serum Amyloid A (Tridelta Development LTD, Ireland), Haptoglobin (Life Diagnostics Inc. Bovine Haptoglobin Test Kit), Tumor Necrosis Factor α (Cusabio Biotech CO., Ltd. Bovine TNF ELISA Kit), Interleukin-1β (Cusabio Biotech CO., Ltd. Bovine Interleukin 1) and Interleukin-6 (Cusabio Biotech CO., Ltd. Bovine Interleukin 6 Test Kit) were made in ELISA device (Awareness Technology, Inc. U.S.A. ChemWell) by using commercial kits.

Biochemical examinations

Blood samples that were taken into anticoagulant-free tubes for biochemical parameters were centrifuged at 5000 rpm at room temperature; Then, the serums was collected, stored at -20°C until the measurement time. In these serums, measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin (ALB), glucose (GLU), cholesterol (CHO), total protein (TP), Gamaglutamyl transferase (GGT), blood urea nitrogen (BUN), direct bilirubin (D-BIL), and total bilirubin (T.BIL) were made in autoanalyzer (Roche Cobas C111 Germany) by using Roche Diagnostics Germany commercial test kits.

Statistical analysis

Shapiro-Wilk test was applied to the data and it was seen that the data were heterogeneously distributed. Considering the number of the test subjects in each group and the heterogeneous distribution; Mann-Whitney U test was used to determine whether there was a significant difference between the groups in terms of measured parameters. A significance level of $P \leq 0.05$ was used.

RESULTS AND DISCUSSION

In our study, the concentrations of acute phase proteins and cytokines were evaluated both in the calves with diarrhea caused by amoebiasis due to E. histolytica and in the control group. From the concentrations of acute phase proteins, SAA (235.86±14.77 µg/ml) and Hp (176.62±20.75 µg/ml) were statistically ($p<0.001$) higher in amoebiasis group in comparison to the control group's SAA (9.82±1.43 µg/ml) and Hp (11.55±1.33 µg/ml) (Table 1). Besides, from the concentrations of cytokines, IL-1β (56.61±6.34 ng/ml), IL-6 (28.03±2.17 ng/ml) and TNF-α (0.42±0.03 ng/ml) in the

Table 1: Serum SAA, Hp, TNF-α, IL-1β and IL-6 concentrations (mean ± SE) of calves in amoebiasis (*E. histolytica*) and control groups.

Group	SAA (µg/ml)	Hp (µg/ml)	TNF (ng/ml)	IL-1(ng/ml)	IL-6 (ng/ml)
Control	9.82±1.43	11.55±1,33	0.16±0.02	25.15±2.12	17.22±2.93
	min: 4.25	min: 6.48	min: 0.08	min: 15.32	min: 4.59
	max: 17.31	max: 17.03	max: 0.27	max: 35.55	max: 32.67
Amoebiasis	235.86±14.77	176.62±20.75	0.42±0.03	56.61±6.34	28.03±2.17
	min: 103.29	min: 67.43	min: 0.21	min: 20.55	min: 10.02
	max: 352.65	max: 385.78	max: 0.77	max: 127.76	max: 46.11
p	0.001**	0.001*	0.001**	0.001*	0.013*

*p<0.05 **p<0.001 (Differences in pre and post treatment stages were indicated by lower cases (a and b).

calves with amoebiasis were statistically higher ($p<0.05$) than the control group's IL-1β (25.15 ± 2.12 ng/ml), IL-6 (17.22 ± 2.93 ng/ml) and TNF-α (0.16 ± 0.02 ng/ml) (Table 1). In our study, the levels of AST, ALT, ALB, GLU, CHO, TP, GGT, BUN, D-BIL, and T. BIL were assessed in both groups (Table 2). In the group with amoebiasis, serum AST (U/L), D-BIL (mg/dL), GGT (U/L), and BUN (mg/dL) concentrations were statistically higher ($p<0.05$) in comparison to the control group. However, no statistical difference was found between the amoebiasis groups and the control group regarding ALT (U/L), GLU (mg/dL), ALB (g/L), CHO (mg/dL), TP (g/L), and T.BIL (mg/dL) values (Table 2).

SAA and Hp concentrations have been proven to be highly significant in calves (Mohammadi *et al.* 2021). Acute phase proteins (APPs) are blood proteins that synthesized primarily by liver as a part of acute phase response (APR). Their concentrations may increase (positive APPs) or decrease (negative APPs) (Eckersall *et al.,* 2001). Evaluation of inflammatory processes in cattle is more challenging than in other species due to the fact that the symptoms of the disease cannot be easily spotted and inflammation is not always accompanied by leukocytosis (Hunter and Jones, 2015). Hence, more accurate and useful bioindicators for inflammation must be searched urgently. SAA and Hp are important acute phase proteins in cattle infections (Cray, 2012). Choi *et al.* (2021) found a positive correlation between acute phase proteins and diarrhea. *Entamoeba histolytica* is a major cause of amebic diarrhea. In their study on mice, Burgess *et al.* (2014) found that when they transplanted the feces containing segmented filamentous bacteria to recipient mice, the mice showed resistance to *E. histolytica* and that SAA increased along with intestinal concentrations of dendritic cells and neutrophils after infection. During rotavirus, coronavirus and *E.coli* infections, a significant increase in SAA concentrations as well as diarrhea were observed in calves (Balikci and Al 2014). In case of naturally occurring rotavirus or coronavirus coinfection with Cryptosporidium versus mono-infection, significantly higher SAA concentration increase has been previously reported (Eckersall *et al.,* 2001; Kabu *et al* 2016). In a previous study Molina *et al.* (2014) established that SAA concentration significantly increased in animals infected with bovine viral diarrhea virus. In our study, SAA concentration in calves with diarrhea caused by Amoebiasis (235.86±14.77 µg/ml) were found to be statistically higher ($p<0.001$) than in the control group (9.82±1.43 µg/ml). We are of the opinion that the increase in SAA, which is the major acute phase protein for the cattle, stems from systemic inflammation.

It was reported that serum Haptoglobin concentration due to calf diarrhea was in rise (Albayrak and Kabu, 2016; Chae *et al.,* 2019; Choi *et al.,* 2021; Kabu and Uyarlar, 2022). Chae *et al.* (2019) reported that in diarrhea due to bovine coronavirus in calves, serum HP concentration was high. Another study reported that in experimental respiratory system infections induced by bovine herpes virus 1 and *Pasteurella haemolytica* serotype A1, Hp concentration

Table 2: Serum AST, ALT, ALB, GLU, CHO, TP, GGT, BUN, D-BIL, T.BIL concentrations (mean ± SE) of calves in amoebiasis (*E. histolytica*) and control groups.

Group	AST (U/L)	ALT (U/L)	GLU (mg/dL)	ALB (g/L)	D-BIL (mg/dL)	CHO (mg/dL)	TP (g/L)	GGT (U/L)	BUN (mg/dL)	T-BIL (mg/dL)
Control	57.92±2.84	15.00±0.97	65.52±3.58	33.15±1.54	0.02±0.01	94.36±10.08	66.88±2.22	15.26±0.36	16.57±1.20	0.11±0.01
	min: 49.70	min: 12.20	min: 54.48	min: 25.09	min: 0.01	min: 68.54	min: 55.00	min: 14.00	min: 10.00	min: 0.00
	max: 78.20	max: 20.20	max: 85.62	max: 39.26	max: 0.06	max: 153.77	max: 73.10	max: 17.40	max: 20.71	max: 0.20
Amoebiasis	42.26±2.55	12.73±1.29	57.03±4.30	32.14±0.62	0.03±0.00	103.34±15.20	68.18±1.76	30.63±5.86	32.87±2.57	0.10±0.00
	min: 21.50	min: 3.90	min: 26.32	min: 23.81	min: 0.00	min: 23.10	min: 51.10	min: 11.30	min: 17.91	min: 0.10
	max: 67.00	max: 24.50	max: 100.89	max: 38.08	max: 0.08	max: 297.31	max: 87.80	max: 153.20	max: 68.11	max: 0.10
p	0.002*	0.228	0.134	0.177	0.022*	0.306	0.770	0.037*	0.001**	0.578

*p<0.05 **p<0.001 (Differences in pre and post treatment stages were indicated by lower cases (a and b).

increased (Petersen *et al.* 2004). Choi *et al.* (2021) reported that Hp concentrations in calves with diarrhea significantly increased and there was a positive correlation between a 2-globulin and diarrhea. These results provide valuable information for clinicians who might use serum protein profiles and Hp to evaluate the diagnosis and prognosis of calves with diarrhea. Our study also established higher ($p<0.001$) Hp concentration in calves with diarrhea due to amoebiasis (176.62±20.75 µg/ml) than in the control group (11.55±1.33 µg/ml). Nevertheless, during hereditary diseases or infections haptoglobulin binds free hemoglobin that is secreted during various autoimmune diseases (Cray, 2012). Now that haptoglobulin has that feature, we think that the rise in its concentration increases in proportion to the hemoglobin released after *Entamoeba* lyses erythrocytes.

Studies reported an increase in HP and SAA in dairy calves as a response to *Cryptosporidium* infections, especially in diarrhea (Enemark *et al.,* 2003a,b; Pourjafar *et al.,* 2011). *Entamoeba histolytica*, like *Cryptosporidium*, settles in the gastrointestinal tract. We suggest that this drastic difference was caused by the nature of the *E. histolytica* infection and probably due to the fact that localized damage to the small intestine was more prone to trigger an immunological response that increased HP rather than SAA concentrations.

TNF-α is one of the main cytokines which partake in systemic inflammation and contribute to acute phase reaction. Even though it is synthesized in and secreted from neutrophil, eosinophiland mast cells, its main source is activated macrophages (Ankri, 2015). Additionally, TNF-α attracts entamoeba and enable them to penetrate interstitial extracellular matrix. Moreover, some intestinal cells act as a reservoir for TNF-α and might be active in the earlier stages of the infection (Labruyère *et al.,* 2019). Peterson *et al.* (2010) demonstrated the correlation between high TNF-α concentration and *E. histolytica* in the study they conducted on the children with diarrhea caused by *E. histolytica*. Our study similarly determined that serum TNF-α concentration (0.42±0.03 ng/ml) in the calves with diarrhea caused by amoebiasis was higher ($p<0.001$) than in the control group (0.16±0.02 ng/ml). IL-1β is a potent pro-inflammatory cytokine that plays an important role in inflammation and the host defense against tissue damage; although it is produced and released in various cell types, it is produced by monocytes and macrophages, which are cells that initiate the immune response (Lopez-Castejon and Brough, 2011). Macías-Pérez *et al.* (2019) demonstrated thatIL-1β significantly increased in hamsters with amoebic hepatic abscess and decreased after the treatment. Similarly in our study, serum IL-1β concentration in the calves with diarrhea caused by amoebiasis (56.61±6.34 ng/ml) was higher ($p<0.05$) than in the control group (25.15±2.12 ng/ml).

As the parasite invasion process develops, the epithelium responds with a protective inflammatory response. However, as epithelial cell lysis occurs, pro-inflammatory cytokines such as pro-IL-1β are released.This cytokine is rendered to its active form by cysteine proteases, some of which are of parasite origin (Sharma *et al.,* 2005). A series of regulatory events may occur at this stage.These may include the interaction of surface and soluble parasite-derived molecules (Yu and Chadee, 1997; Kammanadiminti *et al.,* 2004; Maldonado-Bernal *et al.,* 2005) with the host cell, resulting in the release of epithelium cytokines/ chemokines such as IL-6, granulocyte/macrophage colony stimulating factor TNF-α. (Espinosa-Cantellano and Martinez-Palomo, 2000). We suggest that the increase in the concentrations of TNF-α, IL-1β and IL-6 are related to this mechanism. Our results indicated that *E. histolytica* impact on the production of APP and associated clinical signs could be attributed to the *E. histolytica* infection.

In our study, there was no statistical difference in terms of alanine aminotransferase (ALT, U/L), glycose (GLU, mg/ dL), albumin (ALB, g/L), cholesterol (CHO, mg/dL), total protein (TP, g/L), and total bilirubin (T.BIL, mg/dL) between the groups with Amoebiasis and the control group. In the group with Amoebiasis, serum aspartate aminotransferase (AST, U/L), direct bilirubin (D-BIL, mg/dL), Gamaglutamyl transferase (GGT, U/L), and blood urea nitrogen (BUN, mg/ dL) were statistically higher ($p<0.05$) than the control group; these enzymatic parameters should not be disregarded while assessing hepatic damage.While AST is present in many tissues including brain, kidneys and muscles (heart, skeleton, face) and the presence of ALT in high concentration in hepatocytes and in low concentrations in other tissues indicates hepatic damage specifically (Woreta and Alqahtani, 2014). It was reported that after trophozoites adhere to and lyse intestinal epithelium, they spread hematologically to peritoneum, liver, lung or brain through the vein (Kantor *et al.,* 2018). We suppose that serum AST concentration increases due to the hematologic spread of trophozoites to peritoneum, liver, lungs and brain as a result of the presence of AST in several tissues (brain, kidneys, muscles) and the damage this creates in the tissues and organs. D-BIL was reported to have increased during cholestatic diseases with impaired bile flow and hepatocellular infections (Kwo *et al.,* 2017). We assume from the increase in D-BIL value that *E. histolytica* might affect bile ducts. Moreover, GGT is a potent indicator for damage to bile ducts and liver and GGT has been reported to be mainly localized in biliary epithelial cells and the apical membrane of hepatocytes (Woreta and Alqahtani, 2014). We suggest that the increase in GGT together with D-BIL supports the fact that bile ducts are affected. BUN parameter is often used in the diagnosis and follow-up of kidney diseases (Hokamp and Nabity, 2016). Rodrigues Ferreira-Filho *et al.* (2011) reported in a conducted study that the prevalence of amoebiasis caused by *E. histolytica*/*dispar* was approximately 8%. In the light of this study and measured BUN value, we assert that *E. histolytica* damages kidneys.

CONCLUSION

As far as the literature is concerned, there are no natural studies where potential *E. histolytica* infection has been demonstrated in calves. Therefore, we believe that our study will pave the path in this field. APPs can be employed as a potential diagnostic tool in veterinary medicine. Our results indicated that *E. histolytica* effect on the production of APP and cytokine and contracted calves' diarrhea could be attributed to the *E. histolytica* infection. We are of the opinion that it would be beneficial for veterinary doctors to consider to *E. histolytica* related amoebiasis diarrhea in calves diarrheas and design their treatments accordingly. Moreover, it would be rewarding to evaluate acute phase proteins and cytokines in order to control the efficacy of the treatment of amoebiasis related diarrhea in calves. To conclude, we believe that the study we conducted will pave the path for further studies on amoebiasis in calves diarrhea.

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Ethical approval

This study has received permission with, Afyon Kocatepe University HADYEK number AKÜHADYEK-145-12 and 14.08.2012 date.

Conflict of interest

The authors declared that there is no conflict of interest.

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