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Evaluating the Efficacy of the Treatment in Lambs with *Cryptosporidium spp. via* Saa and Haptoglobin

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ABSTRACT

Background: The purpose of this study is to evaluate the efficacy of the treatment for the diarrhea due to *Cryptosporidium*, which is prevalent in lambs, via the concentrations of Serum Amyloid A (SAA) and Haptoglobin (Hp). Although there are experimental studies in this field, there are quite a few studies on the lambs which are infected naturally.

Methods: In this study, stool samples were collected from the lambs that were affected with diarrhea and *Cryptosporidium* was detected *via* the rapid test kit (Bio-X Diagnostics S. A. Rochefort/Belgium). Following this procedure, 15 lambs were included in the study by examining same stool samples microscopically and 50 mg/kg of paromomycin sulphate (Huvepharma) was administered to the lambs in which *Cryptosporidium spp.* was detected for five days as a treatment. 10 healthy lambs formed the control group. Blood samples were taken from all lambs to measure SAA and Hp in the pre and post treatment stages. In the serums obtained from blood samples, concentrations of SAA and Hp were measured in ELISA by using commercial kits.

Result: In the lambs with *Cryptosporidium*, concentrations of SAA and HP were as (SAA; 20,62±4,83 ng/mL HP; 2,63±0,77 µg/mL) in pre-treatment and as (SAA;12,93±3,27 ng/mL, Hp; 1,59±0,73 µg/mL) in post-treatment stage while they were as (SAA;10,07±2,49 ng/mL, Hp; 1,03±0,59 µg/mL) in the control group. In the pre and post treatment stage measurements, a statistically significant difference ($p<0,05$) in the values of SAA and Hp was found between the control group and the lambs with diarrhea due to *Cryptosporidium*. It was suggested that this might have occurred as a result of inflammatory response against *Cryptosporidium*. In the light of these findings, it is asserted that routine measurements of SAA and Hp concentrations of the lambs with diarrhea due to cryptosporidiosis could be beneficial in the following up the treatment, determining the severity of the infection, choosing the right method for treatment and monitoring the efficacy of the chosen treatment method and in detecting subclinical diseases in veterinary medicine.

Key words: Cryptosporidium, Haptoglobin, Lamb, SAA.

INTRODUCTION

Neonatal diarrhea in lambs is one of the most important issues in sheep farms worldwide. Cryptosporidiosis has been reported to cause substantial economic losses such as increased labor costs, treatment and prophylaxis, susceptibility to other infections, delayed growth and even fatality in some cases (de Graaf *et al.* 1999; Olsen *et al.* 2015). The lambs infected with *Cryptosporidium spp.* start to excrete oocyst within 4-5 days after the infection and excretion lasts approximately 9 to 11 days (Bukhari and Smith, 1997; Dinler *et al.* 2017; Niine, *et al.* 2018). Cryptosporidiosis is known to be an important source of gastrointestinal diseases for vertebrates, including humans (Xiao, 2010; Ryan *et al.* 2015). Molecular studies defined around 40 species and more than 50 genotypes of *Cryptosporidium hominis* and *Cryptosporidium parvum* (*C. parvum*) that are most prevalent in humans and these cause asymptomatic or from mild to severe gastrointestinal infections (Ryan *et al.* 2014; Firoozi *et al.* 2019; Roellig and Xiao, 2020). Causing a high rate of neonatal morbidity (up to 85%) of lambs, *C. parvum* has been reported to be one of the main causes of diarrhea which leads to financial losses all around the world (Muñoz *et al.* 1996; de Graaf *et al.* 1999; Ulutaþ and Voyvoda, 2004; Robertson *et al.* 2014; Olsen *et al.* 2015). Ruminants, among the farm animals, are accepted as an important reservoir for both host-specific and zoonotic *Cryptosporidium* species because they excrete many oocytes that cause environmental contamination (Xiao, 2010; Santin, 2020). Infection and oocyst excretion last from 9 to 11 days in lambs (Bukhari and Smith, 1997). It has been acknowledged that animals infected with *Cryptosporidium spp.* start excreting oocyst within 4 to 5 days and calves pose a health risk for humans as the potential source of cryptosporidiosis (Santin, 2020). On the other hand, little is known about the infection and there is not clear information as for the role of different animal species such as sheep and goats in the epidemiology of

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human infections as a route for transmission (Ryan *et al.* 2005; Broglia *et al.* 2008; Cacciò and Ryan, 2008; Xiao, 2010). Also, there are other studies indicating that the sheep and goats host the zoonotic species or genotypes of protozoa (Geurden *et al.* 2008; Mueller-Doblies *et al*. 2008). It has been reported that cryptosporidium infection begins within 1-2 days following the oocyst excretion in lambs and Acute Phase Response (APR) accompanied with severe diarrhea and reduced milk consumption around 15-20 days (Quílez *et al.* 2002; Dinler *et al.* 2017). Acute phase proteins (APPs) have been used as early biomarkers for general health screenings in both veterinary and human medicine in order to determine ongoing diseases, severity and the presence of prognoses (Eckersall and Bell, 2010; Ceciliani *et al.* 2012; Iliev and Georgieva, 2018). APR is a response that develop following infections, inflammatory reactions, immunologic problems and traumatic or neoplastic issues in living bodies and this response has been reported to develop due to systemic and metabolic changes (Petersen *et al.* 2004; Gruys *et al.* 2005). Most of the APPs have been examined in detail in human medicine and today, they are routinely used in the diagnosis and prognosis of many diseases. It should be borne in mind that APPs might have important uses in animal health as well. Regarding these facts, it is also true that because APPs have varying significances among different animal species, enough studies could not be conducted in this field; therefore, APPs could not be used within the range of routine tests in the field of animal health (Gökce *et al.* 2009; Eckersall and Bell, 2010). Recent studies have proven that in ruminants, Hp and SAA are among significant acute phase proteins while α1 acid glycoprotein has a moderate importance (Eckersall and Bell, 2010; Ceciliani *et al.* 2012; Dinler *et al.* 2017; Niine, *et al.* 2018). The ratios of APPs in plasma concentrations depend on the severity and activity of inflammatory reaction; thus, determining the number of APPs in the bloodstream can provide information on the current inflammatory reaction. Therefore, APP measurements can limit mortality incidence. Moreover, for an early and accurate diagnosis of the disease, APP concentrations should be determined in healthy neonatal and sick lambs. The aim of this study is to determine the changes *Cryptosporidium* causes in SAA and Hp concentrations in neonatal lambs, to establish the changes in APPs during the treatment and to interpret the efficacy of the treatment *via* SAA and Hp.

MATERIALS AND METHODS

Clinic examination and diagnosis of *cryptosporidium spp.* with rapid test kits

In this study, 15 lambs with clinical diarrhea and 10 healthy lambs between 3-15 days of age were used. 15 lambs with diarrhea formed the study group and 10 healthy lambs formed the control group. 2 of the lambs with diarrhea died in the course of study. In the fresh stool samples taken from the lambs with diarrhea, *Cryptosporidium spp.* was diagnosed by using a test kit (Bio-X Diagnostics S. A. Rochefort/Belgium). As a treatment, the lambs with *Cryptosporidium spp.* were administered with 50 mg/kg of paromomycin sulphate (Huvepharma) for five days.

Staining stool samples and detecting oocysts

The stool samples in which *Cryptosporidium spp.* was found with test kits in the field were brought to the laboratory on the same day. In order to detect the oocysts of *Cryptosporidium spp.,* stool samples were stained by using Kinyoun Acid Fast staining method (Turgay, 2011). Stained samples were examined under Olympus CX31 trinocular research microscope at magnifications of 10x and 40x. The oocysts detected in the samples were imaged with Olympus LC30 digital camera system (Fig 1).

Measurement of acute phase proteins

For the measurement of acute phase proteins, blood taken from the vena jugularis of lambs in both groups was centrifuged at 5000 rpm and room temperature to obtain serum. Samples of serum were stored at -20°C until the time of measurement. Measurements of Serum Amyloid A (Cusabio Biontech CO., LTD. China) and Haptoglobulin (Cusabio Biontech CO., LTD. China), were made in ELISA device by using commercial kits.

Statistical analysis

In the statistical analysis of this study, data were tested by T-Tukey test and significance level was set as $p<0.05$. Table values were given as mean±standard error.

RESULTS AND DISCUSSION

In this study, SAA concentrations in lambs with *Cryptosporidium* were found as (20,62±4,83 ng/mL) in pre-treatment and (12.93±3.27 ng/mL) in post-treatment stage while in the control group, SAA concentration was determined as (10.07±2.49 ng/mL) (Table 1). In the light of these findings, SAA measurements of lambs showed statistically significant differences ($p<0,05$) between pre-treatment (20.62±4.83 ng/mL) and post-treatment (12.93±3.27 ng/mL) and the control group (10.07±2,49 ng/mL) while there was no statistically significant difference

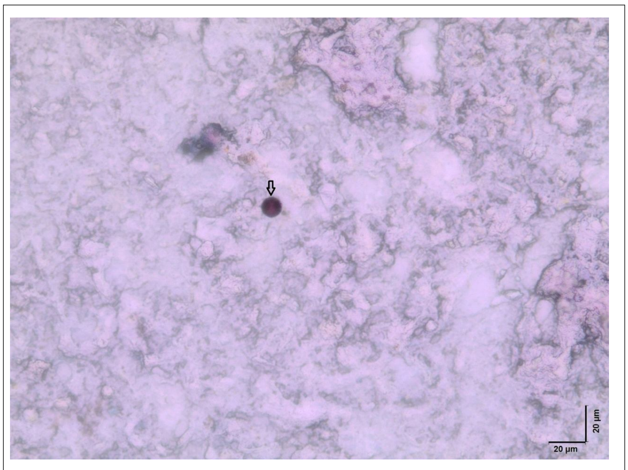

Fig 1: The image of *Cryptosporidium spp.* oocyst in stool sample from kinyoun acid fast staining method.

Table 1: Pre and Post treatment concentrations of SAA and Hp in the control group and in lambs with *Cryptosporidium*.

Group	Pre-treatment	Post-treatment	Control	P
SAA	20.62±4.83[a]	12.93±3.27[b]	10.07±2.49[b]	0.001
Hp	2.63±0.77[a]	1.59±0.73[b]	1.03±0.59[b]	0.001

*p<0,05 **p<0,001 (Differences in pre and post treatment stages were indicated by lower cases (a and b).

(p>0,05) between the post-treatment (12.93±3.27 ng/mL) and the control group (10.07±2.49 ng/mL) (Table 1). Whereas Hp concentration was determined as (2.63±0.77 µg/mL) in pre-treatment and as (1.59±0.73 µg/mL) in post-treatment stage, it was found as (1.03±0.59 µg/mL) in the control group (Table 1). These findings indicated a statistically significant difference (p<0.05) between pre-treatment (2.63±0.77 µg/mL) and post-treatment (1.59±0.73 µg/mL) and the control group (1.03±0,59 µg/mL); even though there was a numerical difference between the control group (1.03±0.59 µg/mL) and the post-treatment group (1.59±0.73 µg/mL), there was not a correlation that would sum up to statistical difference (p>0,05). Regarding the pre-treatment group (2.63±0,77 µg/mL), the measured values were higher than both in the control group (1.03±0,59 µg/mL) and in post-treatment group (1.59±0.73 µg/mL); however, this significantly increased value in lambs returned to normal levels with the applied treatment (Table 1).

It has been reported that the concentrations of antibody (IgG and IgM) and APPs significantly increase in neonatal lambs with *Cryptosporidium* (Ortega-Mora *et al.* 1993; Dinler *et al.* (2017); Niine, *et al.* 2018). Dinler *et al.* 2017 reported that following the experimental infection of lambs with *Cryptosporidium spp.,* serum concentrations of SAA and Hp increased significantly. The same study demonstrated that serum concentrations of both SAA and Hp were much higher in the lambs infected with Cryptosporidium than healthy control group (Dinler *et al.* 2017). In the study where experimental infection was developed with Cryptosporidium, there was a moderately positive correlation between serum Hp concentration and oocyst excretion number while no meaningful correlation was observed with serum SAA concentration (Dinler *et al.* 2017). Similarly in our study it was determined that SAA and Hp concentrations were high in 3 to 15-day-old lambs with *Cryptosporidium* in pre-treatment stage. We suggest that this could be related to acute phase reaction against oocyst excretion. Another study reported that serum SAA concentration increased from the first day of life to the fifth day, the highest level was on the day after birth and then decreased until the second week of life (Kilpi, 2015). In some studies which were conducted on sheep and goats, the researchers found that the SAA and Hp concentrations in healthy animals were quite low in the APP examination (Iliev and Georgieva, 2018; Dinler *et al.* 2020). Similarly in our study, SAA and Hp concentrations of post-treatment and control groups were lower than the concentrations of pre-treatment. Hence, it has been concluded that the therapy is effective. Also, the fact that SAA and Hp concentrations were in close values to each other in control and post-treatment groups is another indicator of the success of the treatment. To conclude, the fact that SAA and Hp concentrations were found to be low in healthy animals in some studies (Iliev and Georgieva, 2018; Dinler *et al.* 2020) supports the hypothesis.

In the literature, although there were a few studies on lambs naturally infected with *Cryptosporidium*, we generally found prevalence studies (Walter *et al.* 2021; Zhang *et al.* 2020). On the other hand, in the experimental *Cryptosporidium* studies on lambs, it was reported that APP measurements were made and SAA and Hp values increased due to acute phase reaction (Dinler *et al.* 2017). It was also reported that similar results were derived in the studies on calves (Rulutaº *et al.* 2011; Albayrak and Kabu, 2016; Niine *et al.* 2018). We had two priorities in our study; firstly, to determine the acute phase response in lambs naturally infected with *Cryptosporidium* and secondly, to evaluate the treatment for *Cryptosporidium* with clinical findings as well as acute phase response. Similar to previous studies, SAA and Hp values in lambs naturally infected with *Cryptosporidium* were found to be high in our study, as well (Dinler *et al.* 2017; Niine *et al.* 2018). Thus, it was associated to inflammation caused by *Cryptosporidium*. After the treatment, SAA and haptoglobin concentrations decreased almost to the values of the control group which consisted of healthy animals. The study proves the efficacy of treatment.

CONCLUSION

To conclude, evaluation of acute phase response in labs naturally infected with Cryptosporidium is crucial for the follow-up of the disease in a cellular level. This suggests that it will be essential for veterinary doctors to monitor acute phase response as much as clinical data in the follow-up of the treatment of the disease in terms of the evaluation of the treatment. Further study is required to assess *Cryptosporidium* in lambs.

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Ethical approval

This study has received permission with, Afyon Kocatepe University HADYEK number AKÜHADYEK-46-18 and 25.04.2018 date.

Conflict of interest

The authors declared that there is no conflict of interest.

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