ORİJİNAL MAKALE / ORIGINAL ARTICLE
Balıkesir Sağlık Bilimleri Dergisi / BAUN Sağ Bil Derg
Balıkesir Health Sciences Journal / BAUN Health Sci J
ISSN: 2146-9601- e ISSN: 2147-2238
Doi: https://doi.org/10.53424/balikesirsbd.1469416

The Enzyme Histochemistry of Eimeria-Infected Lambs

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Geliş Tarihi / Received: *16.04.2024,* ***Kabul Tarihi / Accepted:*** *20.05.2024*

ABSTRACT
Objective: We aimed to investigate the effects of *Eimeria* infection on ANAE-positive lymphocyte percentages of lambs in the present study. **Materials and Methods:** For this purpose, fecal samples were taken from 200 animals into sterile containers on day 0. *Eimeria* positivity was detected in lambs according to the Fulleborn saturated saline method. Besides, oocyst load was detected by using the Mc. Master method in lambs. Forty of 200 lambs (*n*=40) aged approximately 60-75 days from a farm where Merino sheep were raised were included in this study. Experimental groups were established as *Eimeria*-positive male lambs (*n* =10), *Eimeria*-negative male lambs (*n*=10), *Eimeria*-positive female lambs (*n* =10), *Eimeria*-negative female lambs (*n*=10). Blood samples were collected to determine ANAE-positive lymphocyte percentages in lambs. **Results:** Results of the present study show that *Eimeria* positivity was detected in 10 male and 10 female lambs according to the Fulleborn saturated saline method. According to the Mc. Master method, oocyst load was determined between 10.000-15.000 in male and female positive lambs. *Eimeria*-positive male and female lambs had the greatest ANAE-positive lymphocyte percentages in our study ($p < 0.05$). Conversely, the lowest ANAE-positive lymphocyte percentages were found in *Eimeria*-negative female lambs, which were not statistically different from male lambs in the present study. It was the first report according to the detection of ANAE-positive lymphocyte percentages in *Eimeria*-infected lambs. **Conclusion:** In conclusion, ANAE-positive lymphocyte percentages can be affected by *Eimeria* positivity and oocyst load (between 10.000-15.000) both in male and also female lambs.
Keywords: Eimeria, ANAE positivity, Lymphocyte, Lambs.

Eimeria ile Enfekte Kuzuların Enzim Histokimyası

ÖZ
Amaç: Bu çalışmada, *Eimeria* enfeksiyonunun kuzularda ANAE-pozitif lenfosit oranları üzerine etkilerini araştırmayı amaçladık. **Gereç ve Yöntem:** Bu amaçla 200 hayvandan 0. günde steril kaplara dışkı örnekleri alındı. Kuzularda Fulleborn doymuş salin yöntemine göre *Eimeria* pozitifliği tespit edildi. Ayrıca Mc. Master yöntemi kullanılarak ookist yükü tespit edildi. Merinos ırkı koyun yetiştirilen bir çiftlikten ortalama 60-75 günlük 200 kuzudan 40'ı (n=40) bu çalışmaya dahil edilmiştir. Deney grupları, pozitiflik veya negatiflik durumuna göre *Eimeria* pozitif erkek kuzular (*n*=10), *Eimeria* negatif erkek kuzular (*n*=10), *Eimeria* pozitif dişi kuzular (*n* = 10), *Eimeria* negatif dişi kuzular (*n*=10) olarak oluşturuldu. Kuzularda ANAE-pozitif lenfosit oranlarını belirlemek için kan örnekleri de toplandı. **Bulgular:** Bu çalışmanın sonuçları, Fulleborn doymuş salin yöntemine göre 10 erkek ve 10 dişi kuzuda *Eimeria* pozitifliğinin tespit edildiğini gösterdi. Mc. Master metoduna göre erkek ve dişi pozitif kuzularda ookist yükü 10.000-15.000 arasında belirlendi. Negatif hayvanlarda *Eimeria* ookistlerine rastlanmadı. *Eimeria* pozitif erkek ve dişi kuzular çalışmamızda en yüksek ANAE pozitif lenfosit oranlarına sahipti ($p < 0.05$). Tersine, bu çalışmada en düşük ANAE-pozitif lenfosit oranları *Eimeria* negatif dişi kuzularda bulundu ve bunlar istatistiksel olarak erkek kuzulardan farklı değildi. Çalışmamız *Eimeria* ile enfekte kuzularda ANAE pozitif lenfosit oranlarının tespitine yönelik yapılan ilk rapordur. **Sonuç:** ANAE-pozitif lenfosit oranları hem erkek hem de dişi kuzularda *Eimeria* pozitifliğinden ve ookist yükünden (10.000-15.000 arası) etkilenebilmektedir.
Anahtar Kelimeler: Eimeria, ANAE pozitifliği, Lenfosit, Kuzular.

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Bu makaleye atıf yapmak için / Cite this article: Ozuicli, M., Kisadere, I., & Aydin, M. F. (2024). The enzyme histochemistry of eimeria-infected lambs. *BAUN Health Sci J, 13*(2), 434-440. https://doi.org/10.53424/balikesirsbd.1469416

INTRODUCTION

Sheep are considered a type of potential livestock that is commonly raised in various parts of the world to generate meat and wool. Historically, the sheep industry has played an important role in the global economy. This industry still faces challenges, with parasitic infections being one of the reasons for a reduction in sheep production performance. Coccidiosis is one of these disorders that causes significant economic losses due to both clinical (diarrhea) and subclinical sickness (Martins et al., 2020a).

Eimeria infection is one of the most serious protozoal illnesses affecting several animal species. The condition, which occurs primarily in young ruminants, causes a reduction in productivity and severe economic losses through clinical infections (Filipenko & Soroka, 2023). In previous studies on this topic, there are many *Eimeria* species were detected in sheep. *E. crandallis*, *E. marsica*, *E. ovinoidalis*, *E. pallida*, *E. faurei*, *E. weybridgensis*, *E. granulosa*, *E. parva*, *E. intricata*, *E. ahsata*, and *E. ovina* (syn. *E. bakuensis*) can be given as a sample of these agents (Trejo-Huitrón et al., 2020). According to literature studies, the *E. ovinoidalis* and *E. crandallis* species are more closely associated with the disease than other disease-causing species. The first target of infection agents is intestinal cells. Disease agents are transmitted to young animals through the oral intake of sporulated *Eimeria* oocysts under appropriate humidity and temperature conditions (Alcala-Canto et al., 2020). The exogenous stage of development takes place in the external environment and necessitates the following factors: temperature, humidity, and oxygen. Additional research indicates that the ideal temperature for sporulation is 20-25 °C. The time required to sporulate to the infective stage differs amongst coccidia species and is useful for identification. Oocysts can sporulate under optimal conditions of oxygen, humidity, and temperature. Infection generally occurs when young animals consume food and water which were contaminated with feces. In addition, contamination has also been detected by licking feathers and wool-contaminated feces. Clinical signs are generally not obvious in young lambs infected with *Eimeria*. However, some animals may experience some degree of loss of appetite, weight loss, and growth retardation (Martins et al., 2020a).

Eimeria agents proliferate and develop in enterocytes, which are intestine-specific cells. This phenomenon of proliferation and development not only causes villous atrophy but also causes malabsorption in lambs (Martins et al., 2020b). Due to diarrhea, fecal stains or excessive pollution may occur in the anal areas of young animals. As the clinical prognosis in livestock worsens, copious watery diarrhea with blood streaks may occur in young lambs (Keeton & Navarre, 2017). Severe dehydration caused by diarrhea in animals can be fatal if left untreated. The subclinical course of this disease, which primarily affects young animals, and the non-specificity of clinical cases are two aspects that make identification challenging. Clinical signs, coprological exams, animal management, age, and climatic conditions should all be considered when making a diagnosis (Carneiro et al., 2022).

The fight against *Eimeria* in sheep is carried out by adding coccidiostatics to the feed, strictly adhering to hygiene and disinfection rules. In lambs, minor mesenteric lymphadenomegaly, widespread proliferative enteritis, and mucosal thickening in the small and large intestines are observed at necropsy. Histopathological examination showed widespread proliferative enteritis as well as the appearance of intracellular coccidia at different stages of development. At the same time, treatment is ineffective in cases where severe clinical signs are observed in *Eimeria* infections in sheep; However, the use of various coccidiostats, especially toltrazuril, diclazuril, and sulfaquinoxaline, in the early stages of infection alleviates clinical findings. However, parasite resistance to treatment with chemical substances is increasing today (Olmos et al., 2020). Moreover, it has been determined that *Eimeria* infection also affects many hematological and biochemical parameters in living things in previous studies. In these studies, the decrease in erythrocytic count (RBC) and hemoglobin (Hb) concentration, as well as leukocytosis, eosinophilia, and neutrophilia, are notable (Elitok, 2020).

Alpha-naphthyl acetate esterase (ANAE) is a lysosomal enzyme predominantly generated by mononuclear leukocytes. It has been found primarily in mature, immunocompetent T cells in peripheral blood (Donmez & Sur, 2008). It has been proposed that ANAE is engaged in a variety of biochemical processes, including antigen endocytosis/degradation and the cytotoxic effects of activated T lymphocytes. ANAE expression is utilized to detect T cells, B lymphocytes, and monocytes in the peripheral blood smears of living beings (Donmez, Kisadere, & Kadiralieva, 2016). Neutrophils are exposed to sporozoites in *Eimeria* species that infect higher mammals, particularly during the cell invasion process. This presents a significant chance for the innate immune system to eradicate or lessen the infection early on, as evidenced by various species, including goats and cattle (Ruiz et al., 2014). Less is known about monocytes' function, however, there is evidence that they can cause *E. bovis* macro schizonts to degenerate both in vivo and in vitro (Hughes, Speer, Kyle, & Dubey, 1987). It has been demonstrated that both cell populations activate extracellular traps in response to various ruminant *Eimeria* stages or antigens. Conversely, numerous investigations have validated the significant function of T lymphocytes in combating *Eimeria* spp. Specifically, in animals challenged with *E. bovis*, a T-

cell-mediated immune response has been linked to a decreased excretion of oocysts, with CD4+ and CD8+ cells being the key populations involved. Lastly, it has been reported that eosinophils play a significant role in the innate and acquired responses to infections with the protozoan Apicomplexa (Pérez et al., 2016). Due to a scarcity of studies on the cellular immune response to *Eimeria* spp., we aimed to assess the impact of *Eimeria* infection on ANAE-positive lymphocyte percentages in lambs in this study.

MATERIALS AND METHODS

Animals

On day 0, 200 animals had their feces collected and placed in sterile containers. The fecal samples were promptly transported to the laboratory. The animals were selected for positive groups using the Fulleborn saturated saline method (Połozowski, Zawadzki, & Nowak, 2006). A sheep farm in Edremit/Balıkesir, Türkiye, used 40 out of 200 lambs, with an average age of 60-75 days. The experimental groups were *Eimeria* positive male lambs (n = 10), *Eimeria* negative male lambs (n = 10), *Eimeria*-positive female lambs (n = 10), and *Eimeria*-negative female lambs (n = 10) based on positivity or negativity. Feeds were provided ad libitum before fecal and blood sample collection. The lambs' feeds are changed based on their daily demands. The lambs were fed with alfalfa hay as roughage together with the mixed feed. Lambs had free access to water and shade.

Determination of the *Eimeria* positivity and oocyst load

Fecal samples (approximately ~ 5 grams) were collected from the rectum of 200 lambs into sterile carrier bags. Then, they were transferred to the parasitology laboratory for the analysis. The Fulleborn saturated saline method was used to determine *Eimeria* positivity in lambs. Some of the lambs had specific Eimeriosis symptoms including diarrhea, weight loss, and less daily feed consumption. These animals did not receive any anti-coccidial or coccidiostat treatment before the administration. According to the Mc. Master method, oocyst load was determined in lambs (Gokbulut et al., 2016).

Percentage of ANAE positive-lymphocytes

Blood samples were drawn from lambs' jugular veins with a needle and transferred to heparinized tubes. Each sample yielded two blood smears, which were air-dried at 20 °C. To detect ANAE activity, materials were fixed for three minutes at -10°C in a 1:1 glutaraldehyde: acetone solution with a pH of 4.8. To prevent crystallization, samples were air-dried at 20°C and incubated with a solution made by gradually dissolving 20 mg of α-naphthyl-acetate substrate (N-8505; Sigma, Steinheim, Germany) in 0.8 ml acetone (Merck, Darmstadt, Germany) drop by drop. The solution had a pH of 5.0. After mixing 2.4 ml of 4% sodium nitrite (S-3421; Merck) and 2.4 ml of pararosanilin (P-3750; Merck) solution (1 g pararosanilin, 20 ml distilled water, 5 ml concentrated HCl) for two minutes, a hexazotized pararosanilin solution was created. The combination was then promptly added to a buffered phosphate solution, totaling 4.8 ml. After adding 1 N NaOH to reduce the pH of the incubation solution to 5.8, it was filtered. The resulting solution was used to incubate the smears for two hours at 37 °C. Following three distilled water rinses, the smears were counterstained for ten minutes with 1% methyl green (Merck) in 0.1 M acetate buffer (pH 4.2) (Maiti, Saini, & Sharma, 1990). Cells with 1 to 5 reddish brown cytoplasmic patches were recognized as ANAE positive, whereas all other lymphocytes were negative. Each specimen was examined using a light microscope (Leica DM 2500; Leica Microsystems, GmbH, Wetzlar, Germany) (Aydin, Celik, & Sur, 2012). Two hundred lymphocytes were counted for each specimen, and the percentage of lymphocytes stained with ANAE was detected, as shown in Figure 1-3.

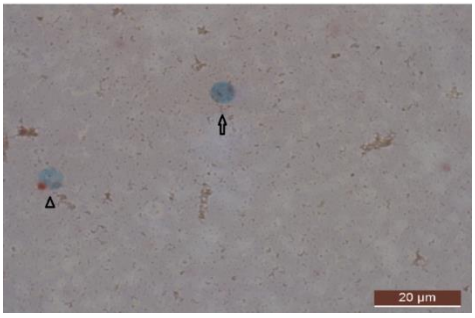

Figure 1. ANAE demonstration in lambs. Arrow: ANAE negative lymphocyte. Arrowhead: ANAE positive lymphocyte.

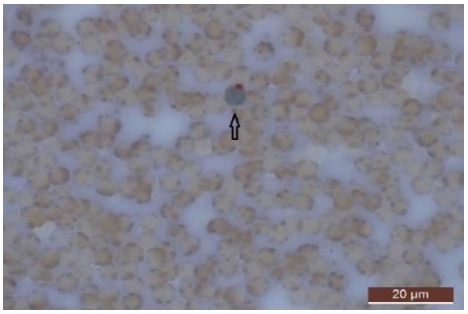

Figure 2. ANAE demonstration in lambs. Arrow: ANAE positive lymphocyte.

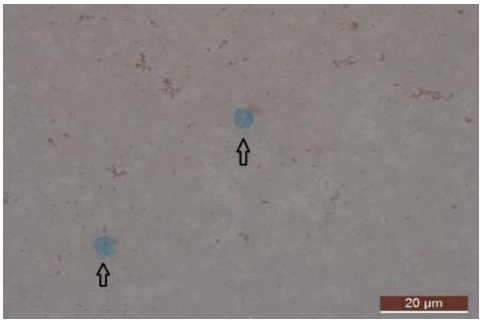

Figure 3. ANAE demonstration in lambs. Arrows: ANAE negative lymphocytes.

Statistical analysis

The SPSS 22.0 program (SPSS, Inc, Chicago, IL) was used to examine differences between and within groups using the independent samples T-test and variance (ANOVA), followed by Duncan's test. P-values below 0.05 were considered significant.

Ethics

The Balıkesir University Animal Care Committee gave its approval for the experimental applications (Reference Number: 2021/10-3).

RESULTS

Eimeria positivity and oocyst load

Eimeria positivity was detected in 10 male and 10 female lambs according to the Fulleborn saturated saline method. According to the Mc. Master method, oocyst load was found between 10.000-15.000 in male and female positive lambs. No *Eimeria* oocysts were found in negative animals.

Percentage of ANAE positive lymphocytes

Eimeria-positive male and female lambs had the greatest ANAE-positive lymphocyte ratios in our study ($p < 0.05$). Conversely, the lowest ANAE-positive lymphocyte ratios were found in *Eimeria*-negative female lambs, which were not statistically different from male lambs in the present study, shown in Table 1.

Table 1. Percentage of ANAE-positive lymphocytes in the experimental groups.

Experimental Groups	*n*	ANAE positivity (%) Mean±SE
Eimeria Positive Female Lambs	10	48.20±0.41**a**
Eimeria Negative Female Lambs	10	45.90±0.84**b**
Eimeria Positive Male Lambs	10	49.50±0.34**a**
Eimeria Negative Male Lambs	10	46.00±0.44**b**

[a-b]Values within a column with no common superscripts are different, $p < 0.05$

DISCUSSION

Changes in hematological parameters recorded with Eimeriosis generally included a reduction in RBCs and lymphocyte count (lymphocytopenia) as well as Hb levels**.** Furthermore, the condition is associated with leukocytosis, eosinophilia, and neutrophilia (Ghanem & Abd el-raof, 2005). Leukocytosis is the result of the body's general reaction, or inflammatory reaction, to the parasites that typically penetrate the small intestines and severely harm animals' digestive systems, particularly in lambs and also calves (Taubert, Hermosilla, Sühwold, & Zahner 2008). The lymphocytopenia may be linked to lymphocyte depletion and atrophy in follicles of the ileal Peyer's patch caused by infection (Matos et al., 2018). On the other hand, previous studies on the subject have found that parasite infestation also causes important changes in lymphocyte numbers and percentages. ANAE enzyme activity is commonly used to identify T cells, B lymphocytes, and monocytes in the circulation (Donmez, Kısadere, & Undag, 2019). The total leucocyte count and granulocyte percentage were significantly enhanced in slightly infected lambs. Besides, lymphocyte percentages were determined the highest in case of heavy *Eimeria* infections by the same researchers, interestingly (Abdulmageed & Mohamed, 2022). On the other hand, a sharp decrease in lymphocyte infection in the blood of *Eimeria*-infected lambs compared to non-infected animals was recorded (31.6% in lymphocytes and 12.4% in monocytes) (Filipenko & Soroka, 2023). Significant elevation of WBCs and also percentages of neutrophils along with a significant reduction in lymphocyte percentages were observed in lambs infected with *Eimeria* (Abdel-Saeed & Salem, 2019). On day 28 after infection with *E. ahsata*, the infected group had a higher mean number of lymphocytes and neutrophils than the control group (Baberi, Razmi, Karimi, Nourani, & Azizi, 2021). In another study, healthy and infected with *Eimeria* lambs had different hematological parameters as follows; WBC: 9.231 ± 3.79.3/µL and 13.707 ± 5.62.8/µL, lymphocytes: 55.8 ± 1.27/mL and 43 ± 1.46 /µL, neutrophils: 30.6 ± 0.76/mL and 40.6 ± 2.25 /µL, monocytes: 0.9 ± 0.10/µL and 2.2 ± 0.30/µL, eosinophils: 1.9 ± 0.27/µL and 5.2 ± 0.64/µL, basophils: 0.3 ± 0.05/µL and 0.3 ± 0.07/mL, respectively (Al-dujaily & Al-mialy, 2017). They also informed that infected lambs had leukocytosis with a significant increase in neutrophils and eosinophils. In a previous study, a significant decrease in lymphocyte percentages, while a significant increase in WBCs in sheep infected with intestinal protozoa (*Eimeria*, *Cryptosporidium*) and nematodes were found (Aziz & Mahmoud, 2022). Using diverse animal breeds, ages, rations, parasite loads, and *Eimeria* species could all be contributing factors to disparate outcomes. In addition, we intended to investigate the characteristics of variable circulatory cell responses during experimental

coccidiosis in goats. In this mentioned study, blood samples were collected by the researchers at 0, 3, 7, 14, 17, 21, 24, 28, and 35 days post-infection. Results of the study showed that the percentage of circulatory neutrophils increased from 7 to 24 days. Unlike neutrophils, lymphocytes decreased significantly from 7 to 24 days. In addition, a study was carried out to determine the effects of *Eimeria* species on hematological parameters in calves with coccidiosis. The study found a considerable reduction in the average number of lymphocytes and eosinophils compared to healthy animals. On the other hand, there was a significant increase in the average total leukocyte and neutrophil count (Rakhshandehroo et al., 2013). The lymphocyte percentages were found to be 52.10 ± 4.00 and 38.30 ± 5.00 for the control group animals and the infected group, respectively (Elitok, 2020). Researchers investigated the metaphylactic effect of minerals on immunological, hematological, biochemical, and antioxidant responses, weight gain, and coccidiosis reduction in newborn lambs. Results of the above-mentioned study showed that decreased lymphocyte counts due to *Eimeria* infection significantly increased over time in treated lambs (Cazarotto et al., 2018). A previous study evaluated the reaction of age-related appearance to the cleaner with *Eimeria ninakohlyakimovae* in their capricorns. These researchers observed that all age groups (1-5 days old) exhibited a major local immune-inflammatory response with significant increases in eosinophils, lymphocytes, neutrophils, spherical leukocytes, and mast cells. Furthermore, lymphocytes increased significantly more in the challenged groups; This confirms the role of the T response process in the data obtained against *Eimeria* infection in ruminants (Matos et al., 2018). In our study, ANAE-positive lymphocyte percentages were found higher in *Eimeria* positive male and also female lambs than *Eimeria* negative in both male and female lambs. Besides, the lowest ANAE-positive lymphocyte percentages were found in *Eimeria*-negative female lambs. In the literature, we could not find any information about the influences of *Eimeria* infection on ANAE-positive lymphocyte ratios in lambs. It was the first report according to the detection of ANAE-positive lymphocyte percentages in *Eimeria*-infected lambs. It was reported that the establishment of protection against challenge infections is largely dependent on the highly specific cellular immune responses against *E. bovis* (Taubert et al., 2008). However, detailed assessments of cellular immune responses in animals with clinical coccidiosis are unusual. There have been few data on calves' cellular immune responses to *E. bovis* infection. There were conflicting findings about the timing of the T cell response during primary infection. Previously, multiple researchers established that exposure to a specific antigen stimulated lymphocyte growth. During primary infection, peripheral CD4+ and CD8+ T cell subpopulations increased, whereas the proportion of γδ-TCR+ T cells remained stable (Hermosilla, Bürger, & Zahner, 1999). Furthermore, after a first infection, gut lymph nodes had higher levels of IL-2 gene transcripts but not IL-4 gene transcripts. In comparison, immunological animals' peripheral blood mononuclear cells (PBMC) and antigen-dependent T cell line/clone were unable to increase IL-2 production following stimulation with *E. bovis* oocyst antigen (Hughes, Thomas, & Speer, 1988). Nonetheless, few cytokine studies that cover the length of primary or challenge *E. bovis* and other *Eimeria* infections.

CONCLUSION

In conclusion, *Eimeria* positivity and oocyst load (between 10.000 and 15.000) affect ANAE-positive lymphocyte percentages in both male and female lambs. As a result, future scientific research on this topic will be useful in explaining the aforementioned major relationships.

Acknowledgement

The authors sincerely thank everyone who made a contribution to this research.

Conflict of Interest

With regard to the research, writing, and/or publication of this work, the author disclaims any potential conflicts of interest.

Author Contributions

Plan, design: MO, IK; **Material, methods, and data collection:** MO, IK, MFA; **Data analysis and comments:** IK; **Writing and corrections:** MO, IK, MFA.

Funding

None

Ethics

The University of Balıkesir Animal Care Committee gave its approval for the experimental applications (Reference Number: 2021/10-3).

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