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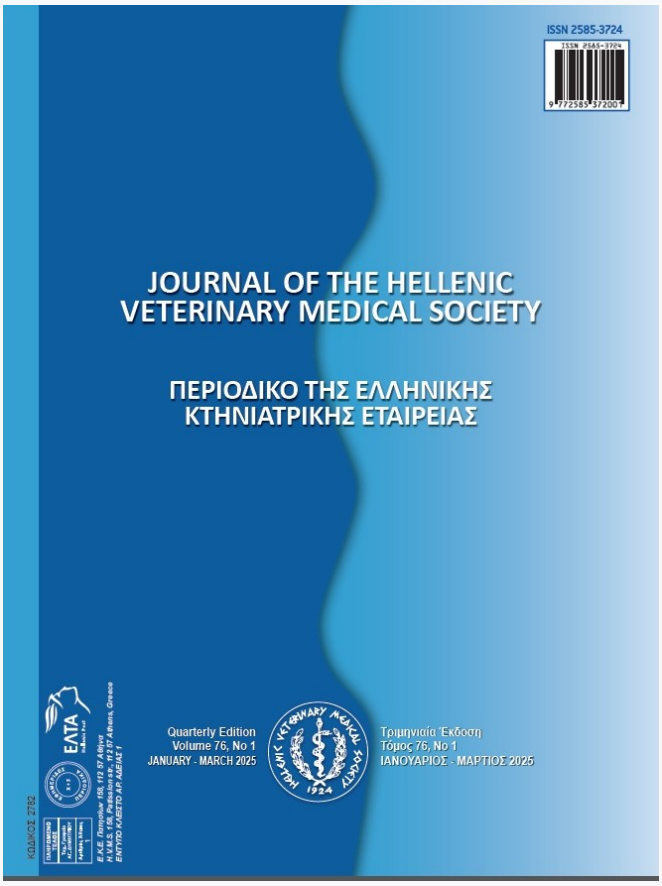

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Evaluation of Retinol, Cholecalciferol, α-Tocopherol and Phylloquinone levels in naturally infected calves with eimeriosis

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ABSTRACT: The aim of this study was to evaluate whether serum vitamin A (retinol), vitamin D (cholecalciferol), vitamin E (α-tocopherol), and vitamin K (phylloquinone) levels changed in naturally infected calves with eimeriosis. The study was conducted on a total of 70 calves, 55 with eimeriosis and 15 healthy (control) calves of different races, sexes, and ages ranging from 26 to 60 days, with complaints of bloody diarrhea, weight loss and tenesmus. As a result of statistical analysis; serum vitamin A, D, E, and K concentrations of calves with eimeriosis were significantly lower than the control group ($P<0.001$). As a result; significant decreases were detected in serum retinol (vitamin A), cholecalciferol (vitamin D), α-tocopherol (vitamin E), and phylloquinone (vitamin K) levels concentrations of calves with eimeriosis. It was concluded that the application of preparations containing vitamins A, D, E and K, in addition to the routine treatment protocol applied in cases of eimeriosis or other diarrhea, may be beneficial in the treatment and prognosis of eimeriosis.

***Keywords*:** Calf; Eimeriosis; Retinol; Cholecalciferol; α-Tocopherol; Phylloquinone

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INTRODUCTION

Eimeriosis, is caused by coccidian protozoans and widespread infectious disease of livestock (Wunderlich *et al.*, 2014). These obligate intracellular parasites strike the digestive tract of their hosts. Eimeriosis is an economically important infectious disease of livestock including cattle, rabbits, sheep, goats, and particularly, poultry (Wunderlich *et al.*, 2014; Al-Habsi *et al.,* 2020; Eğlenti *et al.,* 2020; Denizhan and Kozat, 2022). Eimeriosis can causes death, poor performance, increased susceptibility to other diseases and causes economic losses, especially in breeding animal farms and calf rearing. This disease, which causes anemia, weakness, growth retardation and decreased productivity in animals, is seen in all seasons of the year, but mostly occurs in winter months and early spring, and causes severe disease, especially in young calves up to 6 months old (Radostits *et al.*, 2007; Tufan and Çam 2009; Baydar and Özübek 2012; Eğlenti *et al.,* 2020). Calves infected with eimeriosis cause significant economic losses due to both decreased live weight gain and death of the animals due to infection. Worldwide, costs for control measures for eimeriosis in cattle and poultry alone are estimated to exceed approximately $2 billion annually (Wunderlich *et al.,* 2014). Eimeriosis can sometimes be seen in humans. Some studies have reported that the genus *Isospora*, belonging to the Eimeriidae family, has also been detected in humans (Sloper *et al.*, 1982). *Eimeria* spp are intracellular parasites of the intestinal epithelium in domestic animals (Ahmed and Hassan 2007; Ocal, 2016), and the transmission of infection begins with the oral intake of sporulated Eimeria cysts. Intestinal cytes produce four sporocysts and two sporozoites from each sporocyst and these sporozoites invade intestinal epithelial cells (Seemann *et al.,* 2012).

The disease is seen as subclinical, acute and peracute. Clinical findings such as bloody diarrhea, weight loss, dehydration, and tenesmus are more common in calves with acute eimeriosis, while the disease occurs in older cattle as subclinical infections. Eimeria agents multiply in intestinal epithelial cells and cause destruction of intestinal epithelial cells, and diarrhea occurs due to this (Eğlenti *et al.*, 2020). Definitive diagnosis of the disease, together with clinical findings, bloody diarrhea and examination of stool with native and/or flotation method, abundant *Eimeria spp*. It is done by seeing the oocysts (Daugschies and Najdrowski 2005; Tufan and Çam, 2009). As in most parasitic diseases, many biochemical and hematological parameters change in eimeriosis (Arslan *et al.,* 2015; Eğlenti *et al.*, 2020). As in many diseases, oxidative stress in eimeriosis causes reactive oxygen forms to be produced faster than their safe neutralization by antioxidant mechanisms. It has a negative impact on animal health and production and is seen as a significant trigger of tissue damage (Ahmed and Hassan, 2007). Eimeriosis in animal is characterized by oxidative stress, inflammation, malabsorption of nutrients, diarrhea, dehydration and increased susceptibility to bacterial pathogens such as necrotic enteritis (Alnassan *et al.,* 2014).

Fat-soluble vitamins A, D, E and K are absorbed in the form of micelles formed with bile salts together with fats. Vitamin A is necessary for regulation immune system and eye health and lack of vitamin A leads to decreased reproductive performance, mastitis and weakening of the immune system. Vitamin D is important for calcium and phosphorus metabolism. In its deficiency, growth retardation, loss of appetite, bone and joint problems and reproductive problems are seen. Vitamins A and E are reported to play a protective role against oxidative stress that occurs not only in the intestines of *Eimeria*-infected animals, but also in non-parasite-infected tissues, especially in the first-pass organ liver (Wunderlich *et al.,* 2014). The liver of newborn calves is very low in terms of vitamin A and beta carotene levels, and the offspring are particularly sensitive to vitamin A deficiency (Gazioğlu and Gül, 2002). Vitamin D is an important vitamin that can be dissolved in oil and organic solvents has different forms such as D2, D3, D4 and D5, and has prohormone properties (Mamak and Yıldız, 2016). In addition to its important role in calcium and skeletal homeostasis in human and animal health, vitamin D has rapidly gained importance in immune function (Adams and Hewson, 2010).

Vitamin E is the biochemical component of enzymes such as glutathione peroxidase (GSH-Px), which protects organism tissues against oxidation (Issı *et al.,* 2016). Reproductive problems and weakening of the immune system occur in the deficiency of vitamin E, which has an antioxidant effect. It is reported that vitamin E, which is an antioxidant that prevents oxidative damage by reducing hydroperoxide formation, prevents free radical damage, increases the immune response, and is important in the protection of many diseases such as heart diseases and cancer. It is also stated to be essential for optimal functions of the reproductive, muscular, circulatory, nervous and

immune systems (Turner and Finch, 1990; Finch and Turner, 1996; Pour *et al.*, 2011).

Vitamin K plays an active role in blood coagulation and in case of its deficiency, blood coagulation is delayed. In ruminant animals, the need for vitamins increases in cases of growth, pregnancy and febrile illness. Vitamin K is especially important for bone development in young people and for the continuation of bone health in adult animals. Vitamin K is required for coagulation and carboxylation of bone γ-carboxyglutamate proteins (Ferland et al. 1992; Mc Dowell, 2000). The liver, which is the synthesis site of vitamin K-dependent coagulation factors, is also the largest storage organ for vitamin K. Lipoproteins, together with vitamin E, are involved in its transport in plasma and its transport to tissues (Kaya *et al.*, 2016).

The aim of this study was to determine whether serum vitamin A, D, E and K levels were changed in calves with eimeriosis. As a result of the study, it was aimed to make a prediction about whether preparations containing vitamins A, D, E and K should be applied in addition to routine treatments of the disease by considering the changes in serum vitamin A, D, E and K levels in calves with eimeriosis.

MATERIAL AND METHOD

Animal Material

This study material consists of 55 calves with eimeriosis and 15 healthy (control) calves of different races, sexes and ages ranging from 26 to 60 days, with bloody diarrhea, weight loss and tenesmus, were brought to Van Yuzuncu Yil University Veterinary Faculty Clinic or bred in various enterprises in the Van region.

Parasitic analysis

The presence of *Rotavirus*, *Coronavirus*, *Cryptosporidium*, *E. coli K99* and *Giardia lamblia* agents from stool samples taken from sick (diarrhea) calves were determined according to the procedure specified with rapid diagnostic commercial test kits (BoviD-5 Ag Test Kit- BIONOTE). Calves with diarrhea that gave negative results in the care of *Rotavirus, Coronavirus, Cryptosporidium, E. coli K99* and *Giardia lamblia* agents according to rapid diagnostic test kits were used as study material. For parasitological examination of stool in calves with diarrhea, fresh stool samples were taken from the rectal region of the animal. The stool samples taken were examined by using the flotation method with Fülleborn's saturated salt water and a large number of coccidia oocysts were detected Mc master (OPG) is quantitative technique and its results are not reported in the discussion or in the tables. Then, the stool sample with coccidia oocyst was crushed and mixed in some tap water, filtered and transferred to the petri dish. For the detection of *Emeria* oocysts, 2.5% potassium dichromate (K2Cr2O7) was added to the petri dish and left to sporulate at room temperature. Sporulated *Eimeria spp* oocysts were examined under the microscope using the flotation technique (Denizhan and Kozat, 2022). Also, whether the calves with eimeriosis had any disease in the past, whether any treatment was applied, and the calves that had been treated for any disease in the past were not included in the study.

Vitamin (A, D, E, and K) analysis

Retinol, α-tocopherol, phylloquinone and cholecalciferol stock solutions were prepared at 500 μg/mL. To prepare a standard solution, the stock solutions were diluted suitably with methanol. To determine the proper calibration, linear regression analysis of the peak area was used to standardize the solution concentrations.

Extraction process

To minimize the sample degradation due to exposure to UV light, the samples, which were covered with plastic sleeves, were thawed at ambient temperature under fluorescent lights. Retinol, cholecalciferol, α-tocopherol, and phylloquinone in serum were extracted as follows: 100 μL serum was deproteinized by adding 100 μL ethanol and adding antioxidants such as 0.025% BHT to the extraction solvent. The samples were mixed via vortex for 1 min (Su et al., 2002). The samples were extracted twice with 600 μL n-hexane. After mixing the samples via vortex, they were centrifuged at 8000 rev/min for 10 min. A total of 500 μL of hexane layer was extracted, and it was evaporated under a nitrogen stream of 37 °C to dryness. The residue was dissolved in 50 μL tetrahydrofuran and was added to 150 μL methanol. After vortexing the samples for 1 min, 100-μL samples were autosampled using amber glass vials.

Chromatographic conditions

The chromatographic system consisted of HP Agilent 1100 with a G-1328 Diode Array Detector (DAD) and G1329 ALS autosampler (-8 °C). Agilent Technologies HP software was used to process the data. A 5-μm Gl Science C18 reversed-phase column (250 ×

4.6 mm ID) was used for separation. Then, the mobile phase of a methanol- tetrahydrofuran mixture (80:20, v/v) was modified (Siluk et al. 2007) The pump was arranged to a flow rate of 1.5 mL/min. Chromatographic analysis was performed at 40 °C using isocratic elution. The chromatogram was monitored with DAD array detection at 290, 325, 265, and 248 nm for the simultaneous measurement of α-tocopherol, retinol, cholecalciferol and phylloquinone, respectively.

Statistical Analysis

Descriptive statistics for the results were expressed as mean and standard deviation. Independent samples t-test was used to compare two groups (Eimeriosis and healthy). Pearson Correlation was used to some variables in calves with diarrhea. SPPS 21 package program was used for necessary statistical analysis.

RESULTS

Vitamin A, D, E, and K levels of the calves with eimeriosis and control are given in Table 1, Figure1-4. Pearson correlation for both groups is given in Tables 2. *Rotavirus, Coronavirus, E. coli K99* and *Giardia lamblia* were not detected in the stool samples of the calves included in the study. However, *Eimeria* spp species were detected in the stool examination.

Clinical findings

As a result of the clinical examination of the calves included in the study, clinical findings such as sluggish, stagnant, mixed hair, slanting, dehydrated abdomen, perineum region and hind legs contaminated with feces, tenesmus and increased heart rate were detected.

Biochemical Findings

The changes in the biochemical parameters of the eimeriosis and control groups are given in Table 1. As a result of statistical analysis; Vitamin A, D, E, and K concentrations of calves with eimeriosis were significantly lower than the control group (P<0.001) in Table 1 and in Figure 1. In Pearson correlation for eimeriosis group, between serum Vitamin A, D, E, and K respectively; Positive correlations were found at the levels of 0.996, 0.996 and 0.990 in Figure 2.

Table 1. Serum vitamin A, D, E, and K levels in calves with eimeriosis and healthy

Parameter	Control X±SD n=15	Eimeriosis X±SD n=55	P <
Vitamin A (µg/dL)	0.228±0.021	0.090±0.04	0.001
Vitamin D (µg /dL)	0.060±0.048	0.013±0.01	0.001
Vitamin E (µg /dL)	0.234±0.02	0.087±0.03	0.001
Vitamin K (µg/dL)	0.164±0.014	0.053±0.03	0.001

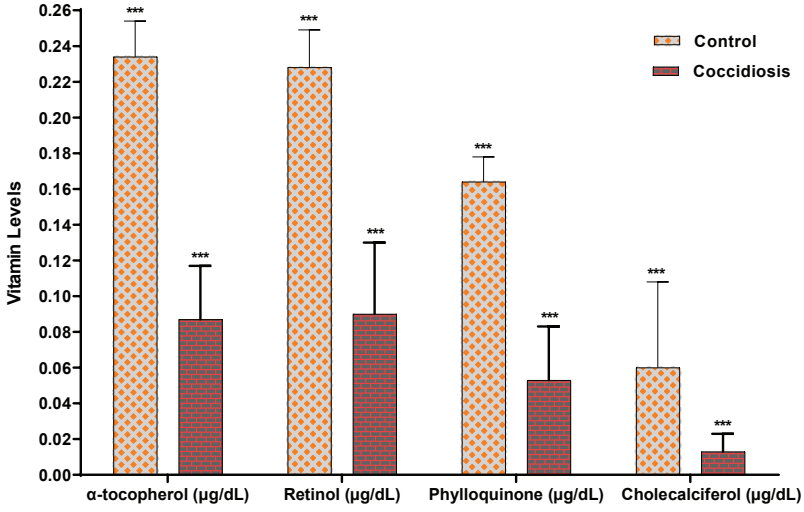

Figure 1. Serum α-tocopherol, retinol, phylloquinone and cholecalciferol levels in calves with eimeriosis and healthy control.

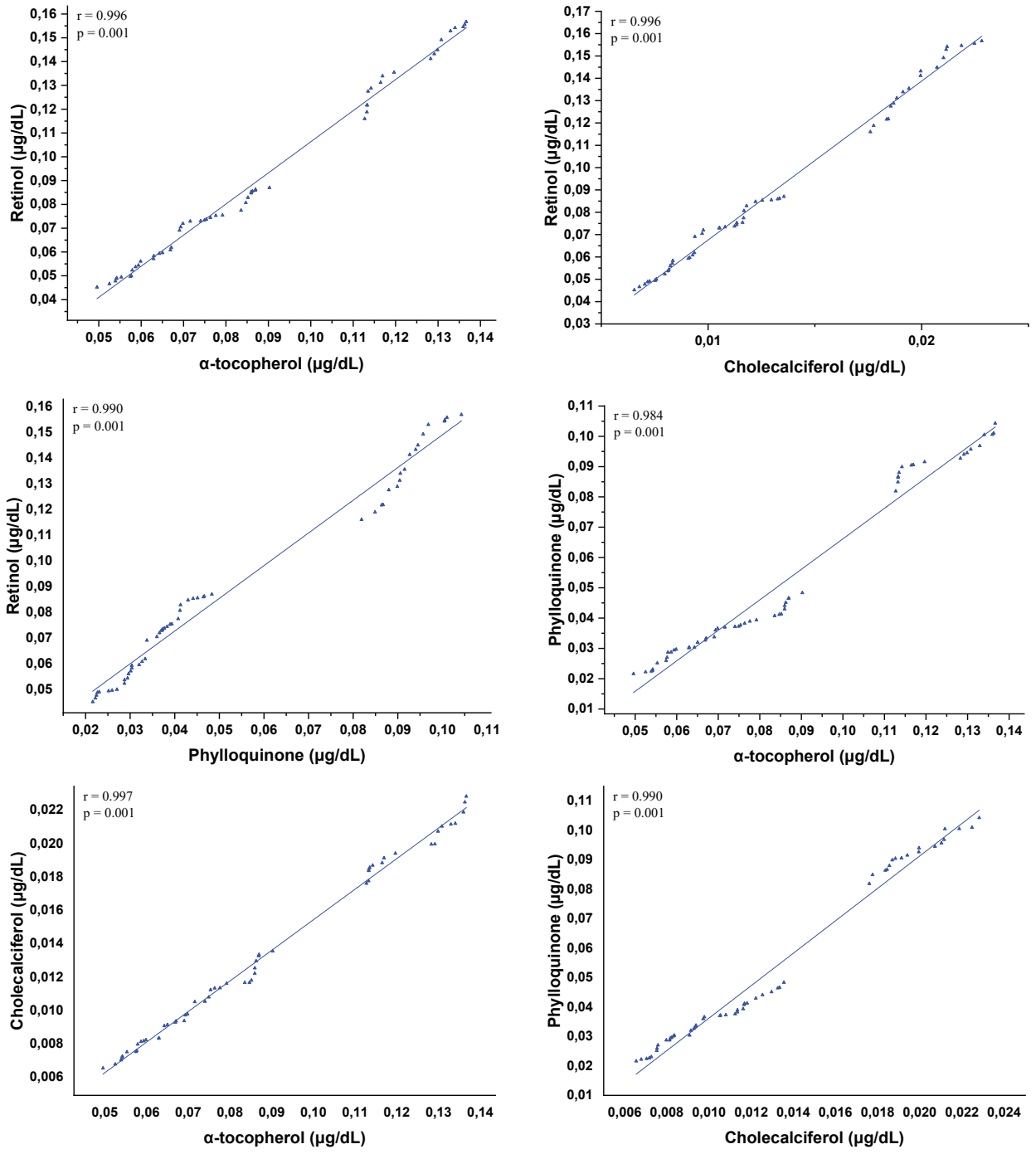

Figure 2. Positive linear correlations in naturally infected calves with eimeriosis among retinol-α-tocopherol, retinol- cholecalciferol, retinol- phylloquinone, α-tocopherol- phylloquinone, α-tocopherol- cholecalciferol and cholecalciferol- phylloquinone.

DISCUSSION

Eimeriosis causes great economic losses for livestock as a result of reduced feed efficiency in young offspring, slowed weight gain, and increased susceptibility to other diseases (Thomas, 1994). In severe eimeriosis, acute bloody diarrhea occurs as a result of extensive damage to the intestinal epithelium (Eğlenti *et al.,* 2020; Denizhan and Kozat, 2022). Damage induced by *Eimeria* spp is also limited to villous atrophy, crypt hyperplasia, and cellular infiltration, and

loss of the absorbent surface area of the intestine leads to severe diarrhea and dehydration as a result of impaired absorption of liquids and nutrients (Davoodi and Kojouri, 2015). Vitamin A, D, E, and K deficiencies can cause many problems in animals, such as decreased productivity, slow growth, and low weight gain, as well as animals being more susceptible to diseases. Vitamin and mineral supplementation may vary according to age, species, breed and individual characteristics (Kozat, 2006; Cazarotto *et al.*, 2018). Since there is not enough data on vitamin A, D, E, and K levels in calves with eimeriosis in the current literature, this study was designed to shed light on eimeriosis disease and vitamin A, D, E, and K levels in calves.

Vitamin A and its precursor beta-carotene effect on vision, normal cell growth, epithelial cells and thus mucosal surface integrity and stability have been known for a long time. Vitamin A deficiency has been reported to cause night blindness, rough dry skin, loss of appetite, weight loss, diarrhea and dry eyes. Many studies have reported that vitamin A can strengthen antioxidant defense systems against oxidative stress (Palace et al., 1999; Kleczkowski *et al.,* 2004; Ma *et al.,* 2005). At the same time, many studies indicate that adding vitamin A and beta-carotene improves immune function (Jin *et al.,* 2014). In many studies on calves with diarrhea, it has been reported that injections of vitamin A preparate will be beneficial due to the low serum and liver vitamin A and beta carotene levels of calves with diarrhea. It is stated that feeding animals rich in beta carotene will reduce the morbidity and mortality of calf diseases (enteritis, neonatal septicemia) (Gazioğlu and Gül, 2002; Gül and Issi, 1989). In another study, it is reported that in calves with eimeriosis, the disease is a disease characterized by diarrhea with an acute course, as it causes the destruction of the intestinal mucosa (Baydar and Özübek, 2012). Studies have reported that clinical eimeriosis is more common in animals younger than one year of age, and there is a negative correlation between the infection rate of cattle and age (Reddy *et al.,* 2015). While there are many studies on serum or plasma vitamin concentrations in calves with diarrhea, there are not studies on the changes in serum or plasma vitamins concentrations in calves with eimeriosis. Another study reported that Retinol concentrations in plasma ranged from 0.033 to 0.532 mg/L, and in serum from 0.043 to 0.360 mg/L in cattle (HPLC method) (Raila *et al.,* 2017). Colostrum milk to be given to newborn calves should be approximately 10% of the calf's live weight and this amount should be given in the first 6 hours after birth as much as possible (Kozat 2019). It is recommended to prophylactically give 30,000- 50,000 IU vitamin A peros or intramuscularly to on the 1st and 3rd days of life of calves of cows fed carotene-poor diet during pregnancy (Gül and İssi, 2016). It is stated that blood plasma vitamin A level is 0.8-6.5 µg/dl in healthy calves (Radostits *et al.,* 2008). In animals, vitamin A deficiency has been shown to reduce local intestinal immune responses, including reduction of intraepithelial CD4+-T cells. In poultry, chickens deficient in vitamin A have increased susceptibility to *E. acervulina* infections, as evidenced by increased oocyst count and impaired spleen cells' capacity to respond to mitogens (Wunderlich *et al.,* 2014). In this study, serum Vitamin A of control (healthy) calves: 0.228±0.021 µg/dl and serum vitamin A of calves with eimeriosis: 0.090±0.04 0.001 µg/dl were detected. In the comparison of serum vitamin A levels between groups, serum vitamin A levels of calves with eimeriosis were found to be significantly lower than the control group ($P<0.001$ and Figure 1). It can be interpreted that the decrease in vitamin A concentrations of calves with eimeriosis is known to be due to the reasons suggested by researchers (Gül, 1989; Gazioğlu and Gül, 2002; Gül and İssi, 2016).

Vitamin D takes an important role in calcium homeostasis and in modulating innate and adaptive immunity (Mamak and Yıldız, 2016). In a study conducted in human medicine, it was reported that serum 25(OH)D3 was found to be significantly lower in patients with Rotaviral diarrhea compared to the healthy control group (Bucak *et al.,* 2016). An another study, multiple epidemiologic studies in young children indicate an association between vitamin D deficiency and an increased risk of respiratory infection (McNally *et al.,* 2009; Battersby *et al.,* 2012). Many studies have been conducted to determine whether there is a relationship between different diseases seen in the neonatal period and 25 (OH) D3 levels in calves (Reis et al., 2009; Reid *et al.,* 2011). It has been reported that especially vitamin D can limit the occurrence of diarrhea and increase resistance to diarrhea. It has also been reported that vitamin D protects against intestinal surface infections and prevents leaky gut syndrome and regulates the inflammatory response and activates immune cells in this process (Shehta *et al.,* 2022). In this study, Vitamin D levels of calves with eimeriosis were found to be lower than those of the control (healthy) group ($P<0.001$). The reason for

the low vitamin D levels in the eimeriosis group can be interpreted as the reasons stated by the researchers (Reis *et al.,* 2009; Reid *et al.,* 2011; Bucak *et al.,* 2016).

Vitamin E plays important role performance, reproduction, and health is well known for livestock (Baldi, 2005). Beneficial effects of vitamin E on health properties are due to improvement of the immune system and/or the antioxidant system (Goodier *et al.,* 2012). An improvement in immune response and growth performance of calves fed vitamin E supplement was reported. Since there is a synergistic relationship between selenium and vitamin E, selenium also reduces the excessive use of vitamin E in the organism. In their deficiency, degeneration occurs in striated muscles as cell membranes cannot be protected (Smith, 2009). Selenium is important for the proper regulation of the immune system in inflammation and infection. Se status influences the activation, differentiation and proliferation of immune cells, including T and B cells and macrophages (Huang *et al.,* 2012; Rayman, 2012). It has been reported that the concentrations of essential microelements such as serum Se and Fe in Eimeria-infected sheep are lower than in healthy sheep (Davoodi and Kojouri, 2015). Denizhan et al (2022) reported that the Fe, Cu, Zn, Se, and Co concentrations of calves with eimeriosis were significantly lower than in the control group ($P<0.001$). In this study, vitamin E concentration levels of calves with eimeriosis were found to be significantly lower than the control group ($P < 0.001$). It can be interpreted that the decreases in Selenium concentrations in calves with eimeriosis may be due to the reasons stated by the researchers (Davoodi and Kojouri, 2015; Smith, 2009).

Vitamin A, D, E, and K are essential vitamins for the growth, development and healthy maintenance of young and fast-growing animals. The diagnosis of deficiencies of these vitamins is revealed by measuring their concentrations in serum or plasma (Yatoo *et al.,* 2013). Vitamin K in preruminant animals synthesized by intestinal microorganisms. Researches have shown that intestinal microorganisms synthesize menaquinone 4 at a sufficient level to meet the vitamin K needs of calves before rumen development (Mc Dowell, 2000). Since microorganisms (*E. coli*) in the rumen synthesize large amounts of vitamin K in adult animals, vitamin K deficiency is rarely seen in such animals (Kaya *et al.*, 2016). In the studies, it is emphasized that the need for vitamin K increases in eimeriosis depending on the severity of the disease, therefore, 8 mg of vitamin K per kilogram should be added to the feeds in addition to the etiological treatment (Vermeer, 1984; Kaya *et al.,* 2016). The main causes of vitamin K deficiency are the lack of adequate levels of vitamin K in feed, disruption of microbial synthesis in the digestive system, malabsorption (insufficiency of bile secretion, bile duct obstruction) and liver diseases. In addition, the need for vitamin K increases in hemorrhages resulting from ulcers in the digestive system (Mc Dowell, 2000; Kaya *et al.,* 2016). In our study, it can be interpreted that low vitamin K levels in calves with eimeriosis may cause bloody diarrhea due to eimeriosis.

In result, it was determined that serum Vitamin A, D, E, and K levels were significantly lower in calves with eimeriosis. Decreases in serum vitamin A, D, E and K concentrations in calves with eimeriosis may be due to diarrhea, intestinal damage and anorexia due to malassimilation. Based on these evidences, we conclude that the subcutaneous or intramuscularly use of vitamin complex based on Vitamin A, D, E and K in neonatal calves increases their concentration in the blood, and indirectly these vitamins mediate the activation of the antioxidant system and enhance their immune responses. As a consequence, it is recommended to vitamin complex applied subcutaneously or intramuscularly to calves may be beneficial in addition to the routine treatments in cases of eimeriosis or other diarrhea and may favor the metabolism of proteins and carbohydrates, improving the clinical signs and leading to increased body weight gain.

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