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2022 Yılı 11. Cilt 2. Sayı

İçindekiler / Contents

Harran Üniv Vet Fak Derg, 2022; 11 (2): 139-145 *Research Article*
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Ameliorative Effects of Curcumin on Aflatoxin B1-Induced Nephrotoxicity in Wistar-Albino Rats*

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Abstract: Mycotoxins exposed through food can lead to organ dysfunction and even failure. The number of studies on renal failure induced by aflatoxin B1 (AFB1) is limited. This trial aimed to examine the effect of AFB1 on the kidney and whether curcumin, a traditionally used and economical antioxidant, could prevent its possible harmful effect. Thirty-eight rats were divided into five groups; group I represented the control, while the others were named D, Cur, AF, and AF + Cur, respectively. Plasma samples were taken from each group after 60 days. Then, BUN, uric acid, and creatinine levels were determined by the ELISA method. Statistical analysis has done with obtained data. Bodyweight gain at the end of the study was the least in the group administered AFB1. Also, BUN, uric acid, and creatinine levels were higher in this group than in the other groups. Concomitant administration of AFB1 and curcumin improved body weight gain and BUN, uric acid, and creatinine levels. Therefore, curcumin can be considered a low-cost, high efficacy renal protective agent in preventing renal failure caused by mycotoxins, especially AFB1.
Keywords: *Aflatoxin B1, Curcumin, Kidney Injury, Rat.*

Kurkuminin Aflatoksin B1 ile İndüklenen Wistar-Albino Ratlarda Nefrotoksisite Üzerindeki İyileştirici Etkileri

Özet: Gıda yoluyla maruz kalınan mikotoksinler, organ fonksiyon bozukluğuna ve hatta yetmezliğine yol açabilir. Aflatoksin B1'in (AFB1) neden olduğu böbrek yetmezliği ile ilgili çalışmaların sayısı sınırlıdır. Bu çalışma, AFB1'in böbrek üzerindeki etkisini ve geleneksel olarak kullanılan ve ekonomik bir antioksidan olan kurkuminin olası zararlı etkisini önleyip önleyemeyeceğini incelemeyi hedeflemiştir. Otuz sekiz sıçan beş gruba ayrıldı; grup I kontrolü temsil ederken, diğerleri sırasıyla D, Kur, AF ve AF + Kur olarak adlandırıldı. Her gruptan 60 gün sonra plazma örnekleri alındı. Daha sonra BUN, ürik asit ve kreatinin düzeyleri ELISA yöntemiyle belirlendi. Elde edilen verilerle istatistiksel analiz yapıldı. Çalışmanın sonunda vücut ağırlığı artışı en az AFB1 uygulanan gruptaydı. Ayrıca bu grupta BUN, ürik asit ve kreatinin seviyeleri diğer gruplara göre daha yüksekti. AFB1 ve kurkuminin birlikte uygulanması, vücut ağırlığı artışını ve BUN, ürik asit ve kreatinin düzeylerini iyileştirdi. Bu nedenle kurkumin, mikotoksinlerin, özellikle AFB1'in neden olduğu böbrek yetmezliğini önlemede düşük maliyetli, yüksek etkili bir böbrek koruyucu ajan olarak kullanılabileceği düşünüldü.
Anahtar kelimeler: *Aflatoksin B1, Böbrek Hasarı, Kurkumin, Rat.*

Introduction

Mycotoxins are toxigenic fungal secondary metabolites produced mainly by Aspergillus, Penicillium, and Fusarium and pose a significant threat to human and animal health worldwide (Guo et al., 2021). The Food and Agriculture Organization (FAO) reports that around 25% of agricultural raw materials worldwide are contaminated with mycotoxins, causing health problems and substantial economic losses (FAO, 2013). At least 400 mycotoxin types have been identified, including aflatoxins (AF), zearalenone, deoxynivalenol, fumonisin, patulin, T-2 toxin, and ochratoxins (Cimbalo et al., 2020). AF, a mycotoxin commonly found in the environment that seriously threatens food safety and public health, are mainly fungal metabolites produced by Aspergillus flavus and Aspergillus parasiticus (Saini and Kaur, 2012). The most important of these are aflatoxin B1 (AFB1) and aflatoxin B2 (AFB2) (McLean and Dutton, 1995). Consumption of foods or indirect exposure contaminated with AFB1 causes growth retardation, suppression of the immune system, carcinogenicity, genotoxicity, and teratogenicity (Ali et al., 2021; Ates et al., 2022; Ateş and Ortatatlı, 2020; Engin and Engin, 2019; Khan et al., 2021; Wangikar et al., 2005).

It was reported that AFB1 and its metabolites cause nephrotoxicity before being removed from the body on various parts of the nephron, which are sensitive to aflatoxins. (Sharma et al., 2011). However, it is stated that increased BUN, uric acid, and creatinine levels due to aflatoxicosis indicate protein catabolism and/or renal dysfunction (Naaz et al., 2014). Abdel-Daim et al. (2021) reported that

liver enzymes, BUN, uric acid, and creatinine levels of rats exposed to AFB1 increased significantly compared to the control group. As a result, AFB1 caused hepatotoxicity and nephrotoxicity (Abdel-Daim et al., 2021).

Health organizations, researchers, manufacturers, and governments have focused on developing effective prevention, management, and decontamination technologies to minimize the toxic effects of AF (Oguz, 2011). Especially recent studies target detoxification of mycotoxins (Ates and Ortatatli, 2021; Nasrollahzadeh et al., 2022; Yavuz et al., 2017). The addition of inert and non-nutritive non-digestible binders to the diet is a current approach for solving aflatoxicosis. These substances bind aflatoxins, reducing their absorption from the gastrointestinal tract. Enzymes that cannot be absorbed from the gastrointestinal tract such as clay and zeolites are used for this purpose (Dönmez and Keskin, 2008; Oguz and Kurtoglu, 2000). Sodium calcium aluminosilicate, activated charcoal, glucomannan, clay, zeolite, sodium bentonite, clinoptilolite, polyvinylpyrrolidone (PVPP), as antioxidants Vit-A, Vit-C, Vit-E, and amino acids containing sulfhydryl groups (Methionine, Cysteine, N-acetyl cysteine) are frequently used to prevent aflatoxicosis (Dönmez et al., 2012; Oguz and Kurtoglu, 2000; Ortatatli et al., 2005).

Turmeric is a traditional spice dating back to 600 BC and used for medicine, condiment, and flavor (Gupta et al., 2013). There are about 120 known species of curcumin, also called diferuloylmethane, and they are widely found as C. Aromatica and C. Xanthorrhiza, and Curcuma longa (Sasikumar et al., 2005; Sun et al., 2017). Pharmacokinetic and bioavailability studies of curcumin have revealed its poor absorption and rapid elimination from the body, so it is safe even at very high doses (Dei Cas and Ghidoni, 2019). In addition to being used as a spice, curcumin is used as a medicinal plant due to its antioxidant, anti-inflammatory (Ak and Gülçin, 2008), anti-mutagenic, antimicrobial (Parvathy et al., 2009), and anti-carcinogenic (Allegra et al., 2017) properties. Also, Kim et al. (2018) reported that oral administration of curcumin in rats with nephrotoxicity significantly reduced serum AST, ALT, BUN, urea, and uric acid levels (Kim et al., 2018).

Many studies have documented the effects of AF on different organs, tissues, and health parameters. However, the information about the curative effect of curcumin, which is widely used among the public, on kidney damage caused by AF is insufficient. This article provides information on the impact of AF on body weight and kidney damage and the possible amelioration of these effects with curcumin administration.

Materials and Methods

Chemicals: AFB1 (≥ 99%) was supplied by Acros Organics [Gell, Belgium (Cat. No: 227340100)]. Curcumin (≥ 99%) was purchased by Sigma Aldrich [St. Louis, MO, USA (Cat. No: C1386)]. BUN (Cat. No DF21), Uric acid (Cat. No: DF77), and creatinine (Cat. No: DF33B) kits were obtained from Siemens Medical System (Erlangen, Germany).

Animals: Thirty-eight male Wistar albino rats (34-36 g) were used in the investigation. Selcuk University Experimental Application and Research Center provided the animal material. In the beginning, the animals' general health state was examined, their body weights were determined, and they were separated into five groups based on their average body weight. The rats were kept in plastic rat cages throughout the study (60 days), with 12/12 day-night light cycles and a room temperature of 23±2 ℃ were housed ad libitum.

Experimental design: This study protocol was approved by Selcuk University Experimental Medicine Research and Application Center Ethics Committee (Report no. 2018-26). Rats were weighed before starting the experiment. They were divided into five groups, with the mean body weight of each group being equal. Group I (Control (K), n=6) animals were not applied; Group II (DMSO (D), n=6) 1 ml 10% DMSO; Group III (Curcumin (Cur), n=6) 300 mg/kg curcumin (Na et al., 2013); Group IV (AFB1 (AF), n=10) 250 µg/kg AFB1 (Tang et al., 2007); Group V (AF+Cur, n=10) 250 µg/kg AFB1 + 300 mg/kg curcumin. The trial period was ended on the 60th day, and all applications were administered orally to the animals. AFB1 and curcumin were dissolved in 10% DMSO and made ready for use. At the end of the 60th day, sufficient blood was taken from the heart of all animals under general anesthesia (Xylazine 10mg/kg and Ketamine 5mg/kg). The blood was collected in serum (BD Vacutainer SSTTM II Advance-367953) tubes and centrifuged at 4500 rpm for 10 minutes at +4 °C (Hettich Universal 32R). Serum samples were stored at -80 °C in Eppendorf tubes until analysis.

Analysis of Kidney Function Test: BUN, uric acid, and creatinine levels were measured in Siemens CentaurXP Immunoassay System following the package inserts using commercial kits from sera stored at -80^{o}C until the analysis time.

Data analysis: The SPSS 20.00 package program was used to analyze the data gathered and determine the significance of the differences between groups. We used visual and analytical methods to analyze variables for normal

distribution. All variables were reported as mean and standard deviations. The groups were compared using a one-way ANOVA test. Following the determination of variance homogeneity, where the p-value was less than 0.05, pairwise post hoc comparisons (Tukey) were employed to test the significance of the groups, and Duncan's Multiple Range Test was used in the analysis of variance.

Results

Comparison of Live Weight Averages: According to the data obtained from all groups, the average body weight of the 0, 15, 30, and 60th days of the application are shown in Figure 1. The bodyweight averages at the 60th day were statistically lower in the AF group than in the other four experimental groups (C, Cur, D, and AF+Cur) ($p<0.05$). After the curcumin application, the bodyweight average of the 60th day obtained from the AF+Cur group was significantly higher than that of the AF group ($p<0.05$). At the same time,it was statistically lower than the values in the C and Cur groups ($p<0.05$) (Figure 1).

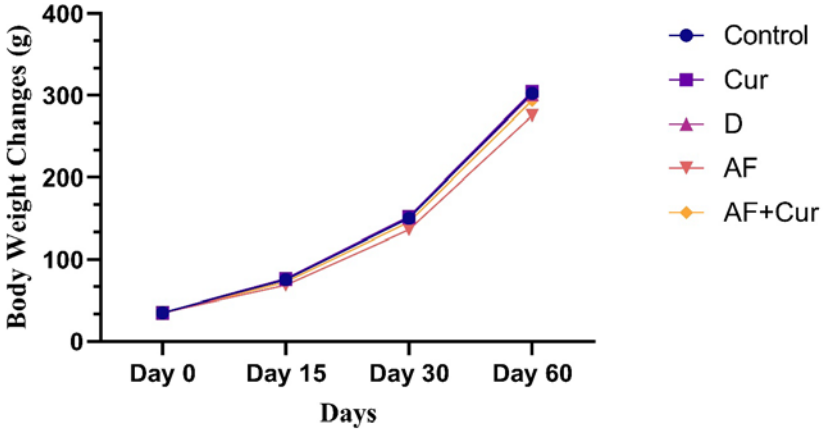

Figure 1. Intergroup average live weight changes. **C:** Control Group, **Cur:** Curcumin group, **D:** DMSO group, **AF:** Aflatoxin group, **AF+Cur:** Aflatoxin and Curcumin group

Comparison of Kidney Function Test Findings: The values of BUN, uric acid, and creatinine parameter levels obtained from all groups are shown in Figure 2. When the data obtained from the AF group were analyzed, it was observed that BUN, Uric acid, and Creatinine levels were statistically higher than the C, Cur, and D groups ($p<0.05$). Again, compared to the AF+Cur group, while no difference was observed in the creatinine level of the data obtained from the AF+Cur group compared to the AF group ($p>0.05$), it was determined that the BUN level had a decreasing trend, and the uric acid level was significantly lower ($p<0.05$). Data obtained from the Cur and D groups were similar to C. (Figure 2).

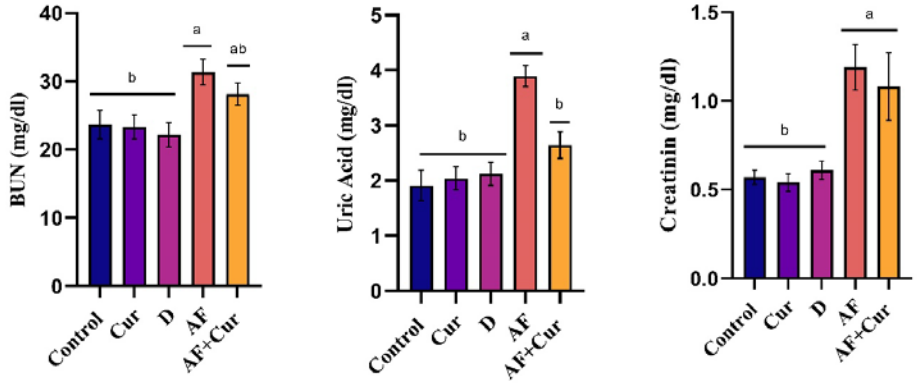

Figure 2. The effect of curcumin on renal function tests in rats administered orally Aflatoxin B_1 (X ± SEM) a,b,c; The difference between the mean values shown with different letters for the same parameter on the same graph is significant ($p<0.05$). **C:** Control Group, **Cur:** Curcumin group, **D:** DMSO group, **AF:** Aflatoxin group, **AF+Cur:** Aflatoxin and Curcumin group

Discussion

Aflatoxins are mycotoxins that cause significant economic losses due to acute-subacute and chronic toxications in farm and poultry farming and affect human health all over the world (Rai and Varma, 2010). The target organ in aflatoxicosis is the liver, followed by the kidneys. In addition to hemorrhage, cirrhosis and fatty degeneration in the liver, pancreas, gall bladder, lung and intestine are also affected (Brase et al., 2013). Removal of aflatoxin from food contaminated with aflatoxin is a critical problem. Therefore, consuming foods containing aflatoxin is still a significant public health problem for human and animal health and causes economic losses. Recently, the therapeutic effectiveness of curcumin, which is obtained from turmeric, which is readily available, easily accessible, has minimal side effects, and is a cheap therapeutic agent and widely used as a spice, has attracted attention. Curcumin has effective anti-inflammatory, antioxidant, anticarcinogenic, antimutagenic, anticoagulant, antibacterial, antiviral, and nervous system protective properties (El-Bahr, 2015; El-Mekkawy et al., 2020). Because of these properties, curcumin was preferred in this study to eliminate the damage caused by aflatoxin in the kidneys.

Urea, uric acid, and creatinine from non-protein nitrogen (NPN) fractions in the blood are used clinically to monitor kidney function (Kumar et al., 2021). It was reported that oxidative stress due to aflatoxin causes renal vasoconstriction or decreases the glomerular capillary ultrafiltration coefficient and thus leads to the formation of various vasoactive mediators that can directly affect kidney function due to reduced glomerular filtration rate (Garcia-Cohen et al., 2000).

It is known that AFB_1 causes kidney damage due to accumulation in the kidney and leads to kidney failure (Grosman et al., 1983; Karabacak et al., 2015). Oxidative stress is a significant risk factor for AFB_1-induced nephrotoxicity. AFB1 exposure increases the presence of ROS, leading to the deterioration of the antioxidant defense system and oxidative damage in the kidneys (Abdel-Hamid and Firgany, 2015; Naaz et al., 2014). In addition, AFB_1 causes an increase in the relative weight of the kidney (Quezada et al., 2000), tubular lumen obstruction and degeneration of the tubular epithelium (Asplin and Carnaghan, 1961), thickening of the glomerular basement membrane (Valdivia et al., 2001), and degenerative changes in the proximal tubule cells (Mollenhauer et al., 1989), causing structural and functional damage to the kidney. The current study observed that BUN, uric acid, and creatinine levels obtained from the AF group were statistically higher than the control groups (C, Cur, D) (Figure 2). Yu et al. (2018) reported that BUN and serum creatinine levels were higher in mice given AFB_1 than in the control group (Yu et al., 2018). Nada et al. (2010) also stated that BUN and serum creatinine levels were increased in rats given AFB1 compared to the control group. The findings obtained from this study are similar to previous studies (Eraslan et al., 2017; Nada et al., 2010; Yu et al., 2018).

According to these results, high serum creatinine level, which is an indicator of kidney toxicity and kidney damage, suggests that it may be caused by the toxic effect caused by AFB_1 (Andretta et al., 2012; Chen et al., 2014). Among the reports, the cause of high plasma urea concentrations is related to AFB_1-induced nephrotoxicity (Kubena et al., 1991). Gowda and Ledoux (2008) reported that increased urea and creatinine levels in 2–6-week-old broilers fed 3 mg/kg AFB_1 contaminated feed were associated with inflammatory and dystrophic processes in the renal tubules (Gowda et al., 2008). They suggest that higher urea and creatinine levels may indicate impaired transport function of epithelial cells of the collecting tubules and diffuse impairment of the role of the proximal tubules. It was also suggested that changes in urea and creatinine levels might be associated with necrotic changes in the kidney parenchyma (Fung and Clark, 2004). These results are accepted as an indication that AFB_1 exposure may lead to detrimental and degenerative changes in kidney tissue, leading to a decrease in the function of this organ.

Curcumin is known to have kidney protective effects against substances such as natural toxins (such as LPS) and chemical toxins (acetaminophen, cisplatin, acrylamide, gentamicin, cadmium) (García-Niño and Pedraza-Chaverrí, 2014). It is reported that curcumin significantly and dose-dependently improves creatinine and urea clearance, reducing serum creatinine and BUN levels (Hosseini and Hosseinzadeh, 2018). This study determined that BUN and creatinine levels in the AF+Cur group tended to decrease compared to the AF group.The value in this group was higher when compared to the control groups (F, Cur, D), although there was no statistical difference. It was observed that the uric acid level in the AF+Cur group was significantly lower than in the AF group. (Figure 2). Khoursandi et al. (2008) applied curcumin extract (400, 800, and 1000 mg/kg) to mice., They reported that it showed nephroprotective effects by reducing creatinine, BUN, and uric acid and lipid peroxidation levels against acetaminophen, which is used as an analgesic and antipyretic drug and causes kidney toxicity in approximately 1-2% of patients. They suggest that this effect of curcumin may be related

to its binding to acetaminophen metabolites and its decreased affinity for cellular GSH (Khoursandi and Ourazizadeh, 2008).

El-Rahman (2014) reported that curcumin administered to rats at doses of 30 and 60 mg/kg decreased serum urea and creatinine levels against cisplatin, a nephrotoxic substance, increases urea and creatinine levels (El-Rahman and Al-Jameel, 2014). It is thought that curcumin exerts its nephroprotective effect against cisplatin through its antioxidant function. Also, another study stated that a 100 mg/kg dose of curcumin decreased kidney damage by lowering MDA, serum uric acid, and creatinine levels in rats following cisplatin injection (Ugur et al., 2015). El-Bahr et al. (2015) reported that creatinine levels increased in rats given 3mg/kg AFB_1 by IP route for five weeks.The creatinine level was statistically lower in rats given 15mg/kg curcumin+3mg/kg AFB_1 orally compared to the group administered only AFB_1 for five weeks (El-Bahr, 2015). El-Mahalaway (2015) reported that serum urea, uric acid, and creatinine levels were higher in rats given AFB_1 at 250 µg/kg body weight orally five times a week for four weeks compared to the control group and that oral administration of 200 mg/kg body weight of curcumin together with AFB_1 improves the levels of these parameters (El-Mahalaway, 2015).

The data obtained in our study comply with previous studies showing that curcumin ameliorates kidney damage from exposure to AF or other toxic substances. In conclusion, curcumin is recommended to facilitate kidney damage caused by exposure to AF or other toxic substances.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical Approval

This study was approved by the Ethics Committee of Selcuk University Experimental Medicine Research and Application Center with the number 2018-26.

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Surgical Treatment of Nictitans Membrane Eversion in Dogs

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Abstract: The presented study is the first retrospective evaluation of the scrolling of the third eyelid in a total of 44 dogs referred to the Istanbul University-Cerrahpaşa, Faculty of Veterinary, Department of Surgery between 2012 and 2021 with a sudden onset of an ocular mass in the medial canthus with epiphora and conjunctival hyperemia. The clinical appearance of the patients, the treatment procedures applied, and the results were examined. A complete ophthalmological inspection revealed the cartilage eversion of the nictitating membrane in sixteen patients (24 eyes) due to the scrolling of the third eyelid and cartilage eversion accompanied by a prolapsed gland in twenty-eight patients (32 eyes). Cartilage eversion was treated by the resection of the defected cartilage portion. In cases with prolapse of the nictitans gland accompanying cartilage eversion, the Morgan pocket method was applied to reposition the prolapsed gland by resectioning the rotating cartilage. In conclusion, -taking into account the potential effect of breed, age, and gender predispositions- the therapeutic approach followed revealed satisfactory results in maintaining the functional capacity of the third eyelid in dogs with cartilage eversion of the nictitating membrane and hyperplasia of the nictitans gland.
Keywords: *Cartilage eversion, Dog, Scrolling, Third eyelid*.

Köpeklerde Üçüncü Göz Kapağının Dışarı Dönmesinin Cerrahi Yöntemler ile Sağaltımı

Özet: Bu çalışma, 2012-2021 yılları arasında İstanbul Üniversitesi-Cerrahpaşa, Veteriner Fakültesi Cerrahi Anabilim Dalı Kliniği'ne epifora, konjunktival hiperemi ve medial kantusta aniden ortaya çıkan bir kitle varlığı şikâyeti ile getirilen toplam 44 köpeğin değerlendirildiği ilk retrospektif çalışmadır. Getirilen hastaların klinik görünümü, tedavisi ve sonuçları incelenmiştir. Ayrıntılı oftalmolojik muayeneler sonucunda üçüncü göz kapağının kıvrılması ile ilişkili kıkırdağın dışarı dönmesi (16 olgu, 24 göz) ve kıkırdağın dışarı dönmesi ile üçüncü göz kapağı bezinin prolapsusunun (28 olgu, 32 göz) varlığı ortaya çıktı. Kıkırdağın dışarı dönmesi olgularında bozulmuş kıkırdak bölümü uzaklaştırıldı. Kıkırdağın dışarı dönmesine eşlik eden üçüncü göz kapağı bezinin prolapsusu olgularında ise dönen kıkırdağın uzaklaştırılması ile prolabe olan bezin yeniden konumlandırılması için Morgan cep yöntemi uygulandı. Çalışma sonunda elde edilen verilere göre üçüncü göz kapağı ile kıkırdağının dışarı dönmesi ve bu duruma eşlik eden üçüncü göz kapağı bezinin hiperplazisi oluşumunda ırk predispozisyonu, yaş ve cinsiyetin etkisi dikkate alınarak, kullanılan cerrahi yöntemlerin üçüncü göz kapağının fonksiyonunun korunmasında yeterli olduğu belirlendi.
Anahtar Kelimeler*: Kıkırdak eversiyonu, Kıvrılma, Köpek, Üçüncü göz kapağı*

Introduction

The third eyelid scrolling is the rolling out or eversion of the nictitating membrane (Rezaei et al., 2019; Williams and Miller, 2006). The deformity that developed due to the rotation or folding of the T-shaped cartilage of the third eyelid (eversion) toward the lower eyelid clinically manifests itself as the nictitating membrane's partial visibility from outside on the bulbar surface (Çakmakçi, 2019; Rezaei et al., 2019; Williams and Miller, 2006). The condition is a rare ophthalmological disorder in companion animals, more common in dogs than cats (Williams et al., 2012). Although the disorder may develop at any age range, it was most frequently reported in young dogs (Ramani et al., 2010). All canine breeds may be affected; nevertheless, some breeds revealed a higher prevalence rate (Çakmakçi, 2019; Ramani et al., 2010). The etiology of the lesion, which has both unilateral and bilateral involvement in dogs, is still unclear, yet genetic predisposition and some environmental factors such as trauma have been suggested as the underlying cause (Çakmakçi, 2019; Rezaei et al., 2019). Clinical signs include epiphora, blepharospasm with relatively mild severity, sudden onset of a pinkish mass or swelling in the medial canthus (Williams et al., 2012), and even a simultaneously occurring nictitans gland prolapse (Hendrex, 2007; Williams et al., 2012).

Several surgical procedures such as application of a temporary third eyelid flap, resection of the eyelid, resection of the eyelid margin and cartilage, removal of the deformed or bent portion of cartilage, total cartilage resection, and cartilage homograft application, thermal cautery of the nictitating membrane (Allbaugh and Stuhr, 2013; Crispin, 1986; Rezaei et al., 2019), and resection of the gland and cartilage in cases with a concurrent prolapsed gland

(Allbaugh and Stuhr, 2013; Michel et al., 2020) are proposed for the surgical correction of outwardly rotated/everted nictitating membrane.

The present study aims to investigate the clinical outcomes and potential complications of the preferred surgical approach to correct the third eyelid eversion in dogs by also demonstrating the most frequently affected dog breeds with the age and gender distribution.

Materials and Methods

Dogs Involved in the Study: The study was conducted with fifty-six eyes of a total of 44 dogs, which were referred to the surgery clinics of the Istanbul University-Cerrahpaşa, Faculty of Veterinary Medicine between 2012 and 2021, with the complaints of a sudden onset of a pinkish-colored ocular swelling, blepharospasm, conjunctival hypermia, and epiphora (Figure 1a). This study is not subject to HADYEK's permission under Article 8(k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Ophthalmologic Examination and Preoperative Process: All patients underwent detailed eye examinations. Five to 10 min after applying a topical anesthetic agent, proparacaine HCl (Alcaine 0.5%, Alcon, Belgium), the rolled-out nictitating membrane was carefully examined. The everted cartilage and gland prolapse accompanying the eversion of the nictitating membrane was distinguished (Figures 1b, c,d).

All surgical procedures were performed under general anesthesia. An informed consent document was obtained from each owner, showing that they accepted all complications that may occur during and after the operation. Premedication was achieved by intravenous (IV) injection administration of xylazine hydrochloride (Rompun® 2%, Bayer, Turkey) at a dose of 0.5-1 mg/kg. Anesthesia was induced by the IV injection of 4-6 mg/kg of propofol (Propofol® 200 mg/20mL, Abbott, Turkey) and maintained by an inhalation anesthetic, isoflurane at a concentration of 2-3% (Forane®, liquid, Abbott, England) in %100 oxygen.

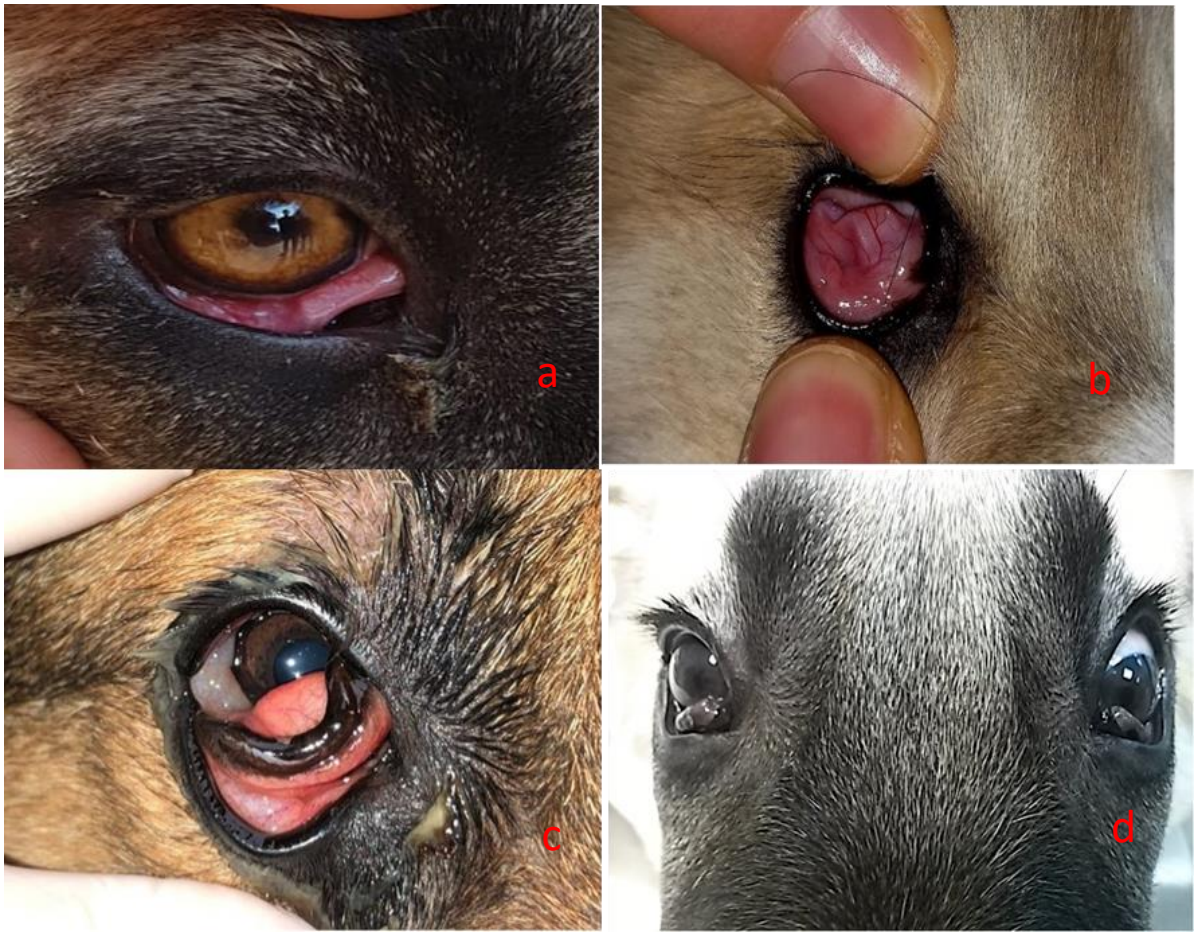

Figure 1. a) Conjunctival swelling and hyperemia in the right eye for 1 month in a one-year-old Alabai b) The appearance of cartilage eversion in a 3-months-old Alabay in case 43 c) Cartilage eversion and nictitans gland prolapse in the right eye in a 10-years-old German Shepherd d) Bilateral cartilage eversion in a 5-months-old Anatolian Shepherd.

Surgical Procedures

Resection of Cartilage: The procedure was performed in accordance with the method previously described by Ramani et al. (2010) for dogs. The surgical approach for cartilage resection was directed at the bulbar surface of the nictitating membrane. The loose end of the nictitating membrane was stabilized by two mosquito forceps. Initially, two small transverse incisions were made parallel to the long arm of the scrolled cartilage to excise the everted cartilage portion using a Stevens sharp pointed scissors. Then, a small tunnel was created by inserting the scissors into the initial incision site directed toward the latter to separate the everted cartilage portion away from the adjacent palpebral conjunctiva. Thus, the long arm of the cartilage was released from the third eyelid's palpebral conjunctiva by blunt dissection, and the scrolled cartilage was cut out. The incised conjunctival tissue was not sutured and left intact for secondary wound healing.

Resection of Cartilage and Applying Morgan Pocket Technique: When the glands prolapse accompanied the eversion of the third eyelid cartilage, the Morgan pocket technique was applied as a further intervention. The surgical procedure was performed in accordance with the combined method previously described by Georgescu et al. (2015) in a Basset Hound dog diagnosed with the relevant two conditions. Likewise, the bulbar surface of the nictitating membrane was targeted for the surgical approach. Two parallel incisions were made on the dorsal (close to the loose end of the third eyelid) (Figure 2a) and ventral (close to the cornea) (Figure 2b) aspects of the prolapsed gland on the bulbar aspect of the third eyelid. The bulbar and palpebral conjunctiva that was adjacent to and bilaterally surrounding the cartilage was bluntly dissected by Stevens scissors (Figures 2c, d) along the dorsal incision line, which was situated 2-3 mm behind the eyelid's loose end, and the bent portion of the cartilage was removed (Figures 3a, b). After resectioning the defected cartilage portion, the ventral incision line parallel to the dorsal incision was further dissected to allow the subconjunctival repositioning of the prolapsed gland (Figure 3c). Then, the prolapsed gland was inserted within the subconjunctival tissue by the Morgan pocket technique and stabilized. The incision line was closed with a continuous suture (Vicryl® 4-0, polyglactin 910, Belgium), assuring that the knots remained on the palpebral aspect of the nictitating membrane (Figure 3d).

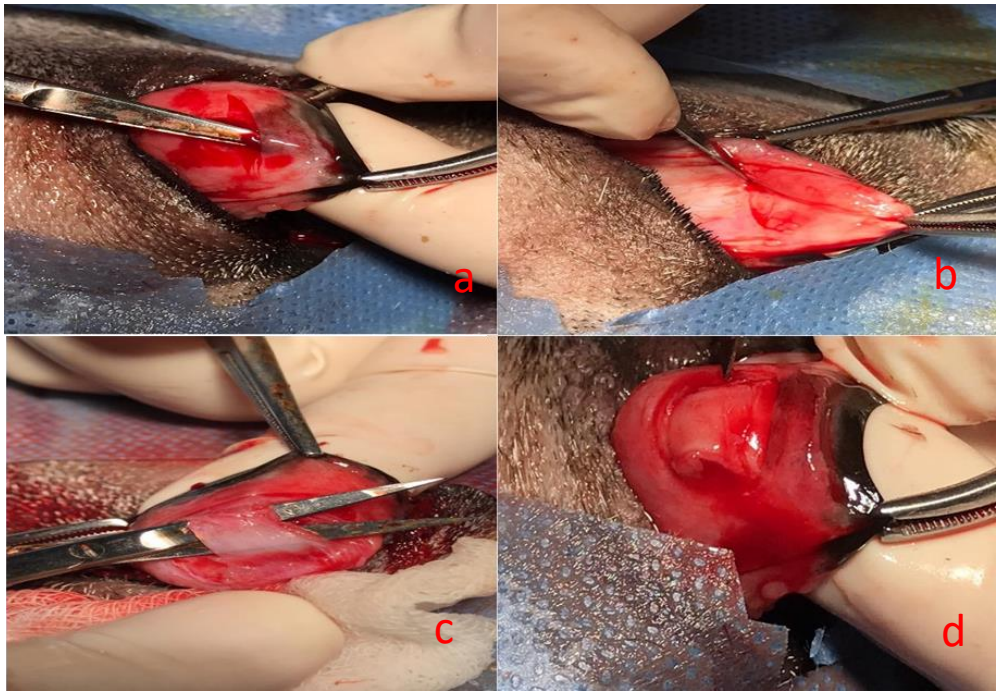

Figure 2. a) First incision close to the free edge of the third eyelid and dissection with scissors b) Second incision close to the cornea c) Blunt dissection of the scrolled cartilage portion using a Stevens pointed scissors d) Appearance of the rotating cartilage after blunt dissection from the palpebral conjunctiva.

In the postoperative period, all dogs had to wear an Elizabethan collar for two weeks to avoid self-trauma. Ofloxacin (Exocin®, Alcon, Ireland), dextran 70 and hypromellose (Tears natural II® free, Alcon, France), and diclofenac sodium (Inflased®, Bilim, Turkey) eye drops were prescribed for one

drop TID for two weeks, while carbomer gel (Thilo – Tears®, Alcon, Belgium) was topically applied twice a day. The follow-up inspections were performed on days 7, 14, 30, 45, and 90 in the postoperative period to monitor potential recurrence and complications, such as ocular surface disorders manifested by corneal injury and keratoconjunctivitis sicca.

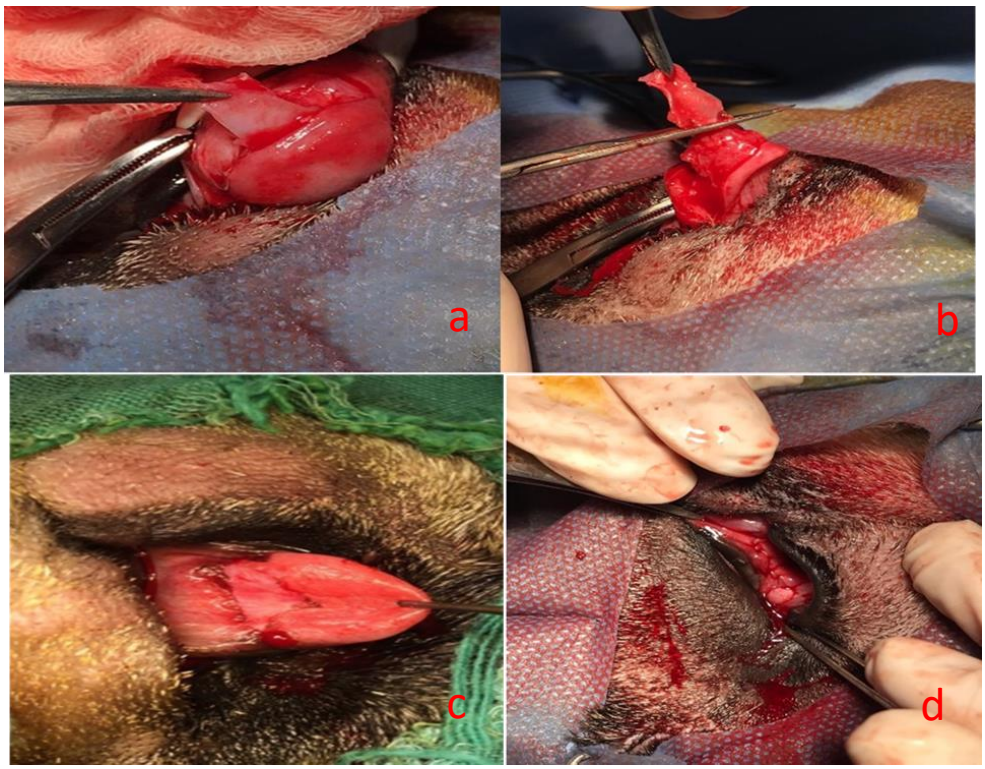

Figure 3. a) The first incision of the everted cartilage close to the nictitans gland b) Removing the rotating part of the cartilage c) Subconjunctival repositioning of the prolapsed gland through the ventral incision line after resection of the defective part of the cartilage d) Closing the incision line using of 4/0 polyglycolic acid with simple continuous suture.

Results

The distribution of the age of the dogs with the nictitating membrane eversion was as follows: Twenty (45.4%) dogs were younger than one year old, nineteen (48.1%) were between 1- 7 years old, and five (11.3%) were older than seven years, (age range from 3.5 months to 10 years, the average age was 2.5 years). The lesion showed bilateral involvement in 12 (27.2%) while unilateral in 32 (72.7%) dogs. The distribution of dogs with cartilage eversion by sex, affected eyes, and accompanying gland prolapse was shown in figure 4 and the breed distribution of dogs with cartilage eversion in figure 5.

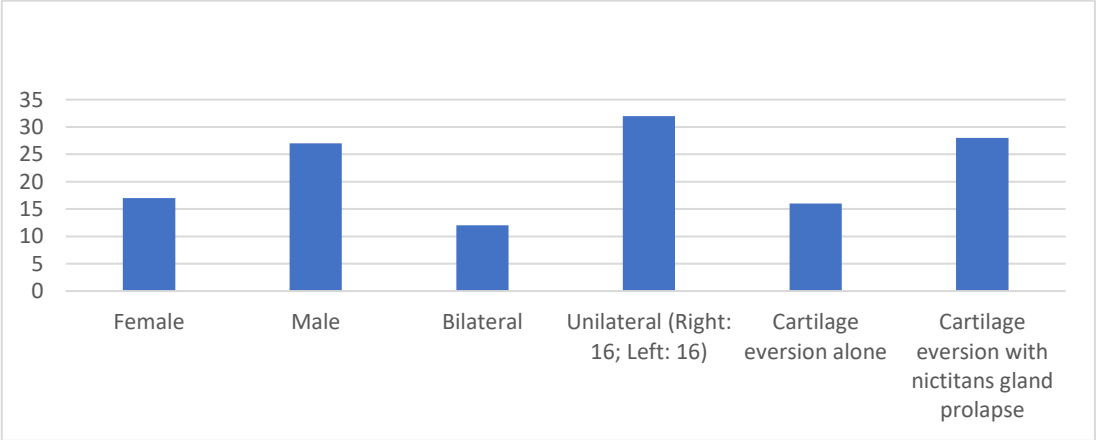

Figure 4. Distribution of cartilage eversion in dogs by sex, affected eyes, and accompanying gland prolapse.

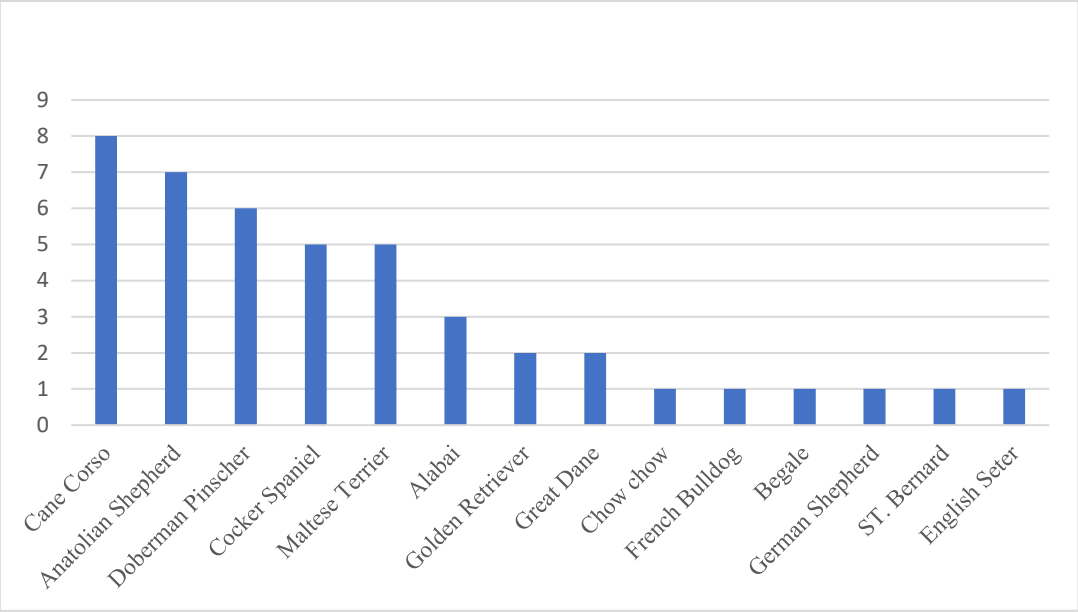

Figure 5. The breed distrubution of dogs with cartilage eversion

The period between the onset of the condition and the referral for treatment ranged from 3 days to two years (Average: 41.7 days). Seven (15.9%) dogs had an acute condition, while the disorder revealed a chronic course in thirty-seven (84.1%) patients. In 21.4% (n=12) of the cases, the eversion had developed as bending at the junction of the vertical and horizontal portions of the T-cartilage, while in a horizontal pattern overlapping the cartilage's shaft, which provides structural support for the nictitating membrane, in 78.6% (n=6, cartilage eversion per se; n=38 cartilage eversion and comorbid gland prolapse). Comorbid ocular disorders, such as keratoconjunctivitis sicca (n=4, Figure 6a), reduced tear quality and quantity (n=5), eyelid abnormalities (entropion, n=2; lagophthalmos, n=3; eyelash disorders, n=3, bulbar and orbital diseases (proptosis, n=1; enophthalmos, n=1), chronic follicular conjunctivitis (n=5), and corneal diseases (n=6) were also noted in some patients. The nictitans gland cyst that developed due to the Morgan pocket method was shown in Figure 6b. Postoperative complications were shown in figure 7.

Figure 6. a) Keratoconjunctivitis sicca, mucoprulent discharge, conjunctival hyperemia and pigmentation accompanying third eyelid diseases in one year old Anatolian Shepherd dog (Case No. 36) b) The development of nictitans gland cyst as a postoperative complication in a 6-month-old French Bulldog.

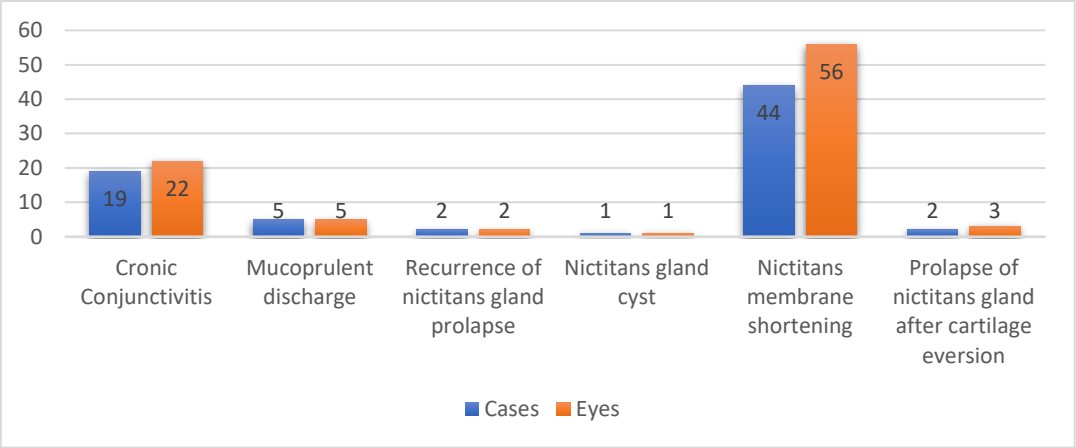

Figure 7. The observed complications after cartilage resection and/or nictitans gland replacement.

Discussion and Conclusion

The eversion of the nictitating membrane, also known as the rotation or scrolling of the third eyelid, mainly results from the rolling out of the T-shaped hyaline cartilage at the junction of its short and long arms (Allbaugh and Stuhr, 2013). The cartilaginous tissue holds the weakest consistency or rarely from the bending of the cartilage's long arm over its shaft generating a U-shaped folding, which forces the third eyelid to move outwards away from the cornea in the affected animals (Allbaugh and Stuhr, 2013; Şaroğlu, 2010). The deformed cartilage, which might be easily distinguished beneath the conjunctival surface in the clinical inspections, exhibits a varying clinical course. Even though the bent or scrolled cartilage does not directly threaten corneal health or vision quality, malposition of the third eyelid's edge discordant with the corneal surface leads to uneven tear film distribution and impairment in its drainage. Furthermore, apart from the everted cartilage, partial or complete protrusion of the nictitans gland (Cherry eye) is one of the ocular conditions associated with the eversion of the third eyelid (Esson, 2015). In the present study, the scrolled cartilage revealed a U-shaped appearance, as was reported in previous studies (Allbaugh and Stuhr, 2013, Şaroğlu, 2010). The eversion site involved the junction of the cartilage's vertical and horizontal arms or the portion close to the junction in the dogs with cartilage eversion per se, as was previously indicated (Dehghan et al., 2012; Michel et al., 2020; White and Brennan, 2018). In contrast, the eversion occurred at the cartilage's shaft in the patients with comorbid partial or complete gland prolapses due to the localization of the gland enveloping the cartilage base, which was directly associated with the repulsive force of the protruded gland. Cartilage eversion was the sole condition in 42.8% (n=16) of the eyes that underwent the surgical procedure for repositioning the nictitating membrane. In comparison, cartilage eversion was accompanied by the nictitans gland's partial or complete prolapse in 57.2% (n=28).

Although the underlying causes of the eversion of the third eyelid are not fully understood (Allbaugh and Stuhr, 2013), several authors pointed out a genetic predisposition concerning large dog breeds, such as Great Dane, Golden Retriever, English Bulldog, St. Bernard, Weimaraner, Doberman, German Shepherd Dog, Newfoundland Dog, and Irish Setter due to the relatively increased prevalence in these breeds (Allbaugh and Stuhr, 2013; Esson, 2015; Rezaei et al., 2019). Acquired factors such as traumatic injuries, chronic conjunctivitis, and improper suturing of the conjunctival surface of the nictitating membrane during ophthalmological surgical interventions were also suggested (Hadži-Milic, 2006). Moreover, a non-equivalent growth rate of the two conjunctival surfaces or primary cartilage deformities (more rapid growth of the posterior aspect than the anterior) were hypothetically associated with the condition (Gelatt, 1972; Martin,1970; Ramani et al., 2010). Gelatt (1972) assumed that uneven forces applied on the third eyelid due to the unique orbital conformation in morphologically enophthalmic large dog breeds gave rise to the disorder. The most frequently affected dog breeds in the present study were Cane Corso (18.18%), Anatolian Shepherd (15.89%), and Doberman Pinscher (13.62%). It was considered that -apart from a genetic predisposition- the varying popularity of some dog breeds in different countries or geographical regions played a significant role in the breed-wise prevalence of the eversion of the nictitating membrane/cartilage eversion. The overall data were compatible with those of Şaroğlu (2012), indicating that the condition was relatively more prevalent in Doberman Pinschers and Anatolian Shepherd dogs, occurring as an occasional and

sporadic lesion in these breeds. Furthermore, cartilage eversion was found to have developed due to Horner Syndrome-associated secondary enophthalmos, which might be considered to have supported Gelatt's (1972) assumptions.

Unilateral/bilateral eversion of the third eyelid and cartilage eversion occurred more frequently, particularly in young animals during adolescence (most specifically in the early period of life like within the first two years) as a primary condition, while secondary to other ocular lesions in the middle-aged or older dogs (Allbaugh and Stuhr, 2013; Hadži-Milic, 2006). Allbaugh and Stuhr (2013) reported that cartilage eversion developed unilaterally in eight out of 10 dogs aged between six months and 1.7 years, while two dogs had bilateral involvement. Şaroğlu (2012) indicated an age range of three to six months. No statistically significant data is available concerning gender predisposition (Allbaugh and Stuhr, 2013). The present study comprising retrospective evaluations over nine years revealed that cartilage eversion occurred as a primary condition in 28 cases (8 dogs with bilateral involvement and 20 with unilateral involvement) between three months and nine years of age, with an average of 1.2 years. The lesion was found to have developed secondary to other ocular lesions such as corneal disorders, eyelid abnormalities, chronic keratoconjunctivitis sicca, eye globe abnormalities, and as a complication to ocular surgery in sixteen dogs (2 dogs bilaterally affected and 14 dogs unilaterally). The age range for the eversion that occurred as a secondary lesion was 3.5 months to ten years, with an average of 4.4 years. The data were consistent with the previous reports (Georgescu et al., 2015; Hadži-Milic, 2006), indicating that primary lesions were mainly encountered in young dogs, while secondary lesions more frequently affected middle-aged dogs. The gender-based evaluations revealed that 27 (61.36%) out of forty-four dogs diagnosed with the eversion of the nictitating membrane were male, while seventeen (38.63%) were female. It might be deduced that the condition was more prevalent in males.

Different techniques of varying success rates were applied in veterinary surgery for repositioning the rotated nictitating membrane, including 10 to 14 days of efficient third eyelid flapping, resection of the nictitating membrane margin and cartilage, radical excision of the nictitans gland and cartilage, excision of cartilage, and homotransplantations (Rezaei et al., 2013; Williams et al., 2012). Most of the techniques were far from offering satisfactory outcomes. Recently, thermal cautery, considered a novel, rapid and efficient technique, has been suggested to surgically correct the everted cartilage without the necessity of the defected cartilage portion's excision. However, it is inefficacious in repositioning the prolapsed gland in patients with comorbid gland protrusion (Allbaugh and Stuhr, 2013). Therefore, submucosal excision of the deformed or bent cartilage portion near the nictitating membrane's loose edge or total resection of the U-shaped cartilage and cartilage extensions (curled/scrolled portion) is most frequently preferred in the surgical management of the eversion of the nictitating membrane in dogs (Rezaei et al., 2019; Williams et al., 2012). The surgical approach is directed at the bulbar and palpebral aspects to remove the bent cartilage portion (Deveci et al., 2020; Williams et al., 2012). Some authors favor the bulbar approach due to the less potential risk for tissue adhesions, facilitating a more convenient dissection in dogs (Williams et al., 2012). Some others supported the palpebral approach to avoid potential surgical trauma-associated bulbar conjunctival injury and corneal damage due to the scarring of the dissected conjunctival tissue because of secondary healing (Deveci et al., 2020; Ramani et al., 2010; Williams et al., 2012). Redundant or insufficient cartilage resection might cause the nictitans glands to prolapse or recurrence in the third eyelid eversion (Deveci et al., 2020). In contrast, the excision of merely the bent cartilage portion might lead to a shortened or a contracted third eyelid, creating a tension force on the cartilage margins and -as a result- might cause marginal necrosis (Allbaugh and Stuhr, 2013). In the present study, the surgical correction of the everted nictitating membrane was achieved by removing the defected cartilage portion using the bulbar approach in all dogs with solely cartilage eversion due to the feasibility of the technique, allowing the gland's stabilization in terms of avoiding a potential gland prolapse, as reported in previous studies. Despite inflammation, the bulbar approach was preferred in all cases, and no complications such as corneal damage due to scarring were encountered, as reported previous study (Ramani et al., 2010). Considering the significant role of the nictitans gland in tear production removing the prolapsed gland was not considered an option; instead, the gland was inserted and repositioned by the Morgan pocket technique after resectioning the defected cartilage.

In conclusion, the overall data revealed satisfactory results in terms of feasibility and efficiency of the surgical treatment method followed in the study, which was applied as the resection of the scrolled cartilage portion accompanied by the Morgan pocket technique in the patients with a comorbid prolapsed gland in the surgical management of the eversion of the nictitating membrane in dogs. Furthermore, it might be deduced that partial or total resection of the third

eyelid cartilage, which provides structural support to the nictitating membrane, barely revealed an adverse impact on the animal's ocular health, and potentially, only a minimal decline was noted in tear drainage.

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Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical Approval

This study is not subject to HADYEK's permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

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Optimization of High Concentration Plasmid DNA for Use in COVID-19 mRNA Vaccine Development: Comparison of Between Alkaline Lysis Method and Commercial Kit Results

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Abstract: While forming the stable IVT mRNA molecule, high concentration and purity plasmid DNA must be obtained to ligase the ORF antigen sequence initially copied from the plasmid DNA with the UTR regions. In this study, in the stage of creating the mRNA molecule, which is the first step of the COVID-19 mRNA vaccine, comparison and optimization of the pDNA containing the ORF target antigen sequence were performed as a result of isolation with alkaline lysis method and commercial kit. Plasmid DNA bacteria containing the target antigen ORF sequence were grown under appropriate conditions. Plasmid DNA was isolated by commercial kit and alkaline lysis method from bacterial cultures stopped at different OD600 nm values (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5). After the obtained pDNAs were visualized on an agarose gel, their purity and concentration were measured by spectroscopic measurement. After the stab culture is resuscitated in SOC medium, bands are formed in a single form after isolation with the kit, and in multiple forms (linear, supercoiled, circular) after pDNA isolation by alkaline lysis method. The ideal OD600 nm for both methods was 0.3-0.4. As a result of isolation with the kit, higher purity on the contrary low concentration pDNA was obtained. The ideal OD600 nm value is a critical parameter that affects the concentration and purity of pDNA. The alkaline lysis method is a cheap and powerful technique that can be used as an alternative for mRNA vaccine development compared to kit isolation.
Keywords: *Alkaline lysis, Isolation, Kit, mRNA vaccine, Plasmid DNA.*

COVID-19 mRNA Aşı Geliştirilmesinde Kullanılacak Olan Yüksek Konsantrasyondaki Plazmid DNA'sının Optimizasyonu: Alkalen Lizis Metodu ve Ticari Kit Sonuçlarının Kıyaslanması

Özet: Kararlı IVT mRNA molekülü oluşturulurken başlangıçta plazmid DNA'sından kopyalanan ORF antijen sekansının UTR bölgeleri ile ligaze edilebilmesi için yüksek konsantrasyon ve saflıkta plazmid DNA'nın elde edilmesi gerekmektedir. Bu çalışmada, COVID-19 mRNA aşısının ilk basamağı olan mRNA molekülünün oluşturulması adımında, ORF hedef antijen sekansını içeren pDNA'nın alkalen lizis method ve ticari kit ile izolasyonu sonucundaki kıyaslama ve optimizasyonu yapılmıştır. Hedef antijen ORF sekansını içeren plazmid DNA bakterisi uygun üreme koşullarında çoğaltılmıştır. Farklı OD600 nm değerlerinde (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5) durdurulan bakteri kültürlerinden ticari kit ve alkalen lizis yöntemi ile plazmid DNA izolasyonu yapılmıştır. Elde edilen pDNA'lar bir agaroz jelde görüntülendikten sonra, spektroskopik ölçüme tabi tutularak saflığı ve konsantrasyonu ölçülmüştür. Stab kültür SOC medium içerisinde canlandırıldıktan sonra, kit ile izolasyon sonrasında tek formda, alkalen lizis metodu ile pDNA izolasyonu sonrasında birden çok formda (lineer, süper kıvrımlı, sirküler) bant oluşur. Her iki yöntem için de ideal OD600 nm 0.3-0.4 olarak bulunmuştur. Kit ile izolasyon sonucunda daha yüksek saflıkta ancak düşük konsantrasyonda pDNA elde edilmiştir. İdeal OD600 nm değeri, pDNA'nın konsantrasyonunu ve saflığını etkileyen önemli bir parametredir. Alkalen lizis yöntemi, kit izolasyonuna kıyasla mRNA aşı geliştirmeye alternatif olarak kullanılabilecek ucuz ve güçlü bir tekniktir.
Anahtar Kelimeler: *Alkalen lizis, İzolasyon, Kit, mRNA aşısı, Plazmid DNA.*

Introduction

Plasmid DNA (pDNA) (Liu, 2011) and mRNA vaccines that have been popular in recent years (Pardi et al., 2018) provide a platform that can be used for applications ranging from the prophylaxis to therapy and personalized medicine to global health solutions. Both vaccine methods draw attention because they have a faster production cycle and can carry the desired genetic information (Liu, 2019). In developing nucleic acid-based vaccines, creating a gene structure that encodes a recombinant antigen protein, instead of inactivating or weakening the

pathogen, prevents the study's potential live pathogen transmission risks.

Especially in the fight against COVID-19, mRNA vaccines have a critical place. mRNA vaccines are generally preferred because of their safety, high efficacy, ease of production and ability to be produced by IVT (with in vitro transcription) (Duran et al., 2021). mRNA vaccines are divided into two groups (i) self-amplifying (self-replicating) mRNA vaccines and (ii) conventional (non-replicating) mRNA vaccines. Although both vaccine types share a common mRNA structure, self-replicating mRNA vaccines additionally contain several sequences in the coding region for RNA replication (Kim et al., 2021). DNA-level modifications are also possible by using pDNA as a template.

Traditional IVT mRNA vaccines are highly similar to native mRNA, consisting of a 5' cap, 5' untranslated region (UTR), coding region (ORF), 3' UTR, and a poly (A) tail (Duran et al., 2021; Kim et al., 2021; Verbeke et al., 2019). A pDNA template for IVT contains at least one bacteriophage promoter, an ORF region, and a poly (d (A/T)) sequence transcribed into poly(A) (Duran et al., 2021; Schlake et al., 2019). After the linearized pDNA template is obtained, it is transcribed into mRNA with a mixture of recombinant RNA polymerase (T7, T3, or SP6) and free nucleoside triphosphates (dNTPs). Thus, the synthetic mRNA construction is formed, the dinucleotide regular cap analog m7G (5')-ppp-(5') G (Schlake et al., 2012; Konarska et al., 1984) or modified ARCA (5' cap form; Anti-reverse cap) with is included in the structure at the IVT stage (Verbeke et al., 2019). With the successful addition of these elements to the ORF, efficient binding of mRNA to ribosomes is achieved (Duran et al., 2021; Verbeke et al., 2019).

While forming a stable IVT mRNA molecule, high concentration and purity pDNA must be obtained to ligase the ORF antigen sequence copied from the DNA with the UTR regions. In this study, two various methods (alkaline lysis and commercial kit) were used for isolation and optimization at various OD600 nm values (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5) to obtain pDNA containing the mRNA ORF target antigen sequence in high purity and concentration.

Materials and Methods

Ethical approval

This study was approved by the KTO Karatay University Non-Pharmaceutical and Medical Device Research Ethics Committee (2021/005 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

Plasmid Selection: In the COVID-19 mRNA vaccine, the codon-optimized 6736 bp plasmid (Addgene pcDNA3-SARS-CoV-2-S-RBD-sfGFP, #141184) containing the target antigen (S-RBD) was preferred. In addition, a plasmid containing GFP (green fluorescent protein) was used, as fluorescent imaging was planned for later transfection steps.

Bacterial Adaptation and Stimulation of Stab Culture: The stab plasmid (Addgene Company, Cambridge, Massachusetts, USA) was taken from the culture with a sterile pipette tip/loop into SOC Outgrowth medium (New England Biolabs, NEB, Ipswich, MA) preheated to 37 °C and inoculated next to the Bunsen burner flames and in a laminar flow cabinet. SOC culture tubes were incubated at 1200 rpm for 2 hours in a shaking oven. Then, 100 µl of the stimulated stab starter culture was taken and inoculated into LB Miller antibiotic-containing medium (Ampicillin, 100 µg/ml, Sigma-Aldrich, USA). After incubation at 37 °C for 16-24 hours, the adapted bacteria were transferred to a solid LB Miller medium. At the time of transfer, 10x dilution was also made and inoculated culture Petri dishes. O/N cultured and pure clones were stored in cryotubes containing 1:1 glycerol at -80 °C.

Reproduce of Plasmids: Single colonies produced from adapted stab plasmids were inoculated in LB Miller broth medium with antibiotics (Ampicillin, 100 µg/ml, Sigma-Aldrich, USA). OD600 nm measurements were made by taking measurements at a wavelength of 600 nm in the spectrophotometer between 6-12 hours from the cultures that were shaken at 37 °C and high speed (200 rpm). Culture bottles taken on ice at various times were isolated at various OD600 nm values (0.02-0.05, 0.05-0.1, 0.1-0.2, 0.2-0.3, 0.3-0.4, 0.4-0.5).

Isolation of pDNA from Bacteria Using Kit: Single colony cultures were centrifuged at 2700 g for 15 min at +4 °C. Bacterial pellets were isolated with the NucleoSpin® Plasmid kit for pDNA (Macherey-Nagel™, MN) according to the manufacturer's instructions. Before the elution step, the pDNA in the spin column was washed two times with ethanol-containing wash buffer and eluted by following a two-step process. Thus, DNA suspended in the spin column was minimized.

Isolation of pDNA from Bacteria Using Alkaline Lysis Method: The following solutions were prepared for pDNA isolation by the alkaline lysis method (Green and Sambrook, 2016).

- Solution 1: 25 mM Tris-Cl (pH 8.0), 50 mM Glucose and 10 mM EDTA (pH 8.0) were mixed and filled to 100 ml with distilled water and autoclaved in 15 psi (1.05 kg/cm^2) liquid cycle for 15 minutes. Immediately after cooling, 100 mg/ml RNase A (Sigma-Aldrich, USA) was added and stored at +4 °C.

•Solution 2: 0.2 N NaOH and 1% SDS (w/v) were dissolved in 100 ml of distilled water and stored fresh at room temperature.

•Solution 3: 5 M 60 ml of potassium acetate, 11.5 ml of glacial acetic acid, and 28.5 ml of distilled water were mixed, and stored at +4 °C. It should be ice cold before use.

•STE Buffer: After mixing 10 mM Tris-Cl (pH 8.0), 0.1 M NaCl and 1 mM EDTA (pH 8.0), it was made up to 100 ml with distilled water and autoclaved in 15 psi (1.05 kg/cm^2) liquid cycle for 15 minutes. Immediately after cooling, it was stored at +4 °C. It should be ice cold before use.

All pellets were brought together and diluted with very cold (≤4 °C) STE buffer, and centrifuged at 2700 g for 10 minutes at +4 °C so that 1.5 ml of the culture remained. The supernatant was discarded and 100 µl of Solution 1 was added to the pellet. After gentle pipetting, 100 µl of freshly prepared 10 mg/ml lysozyme (Hibrigen Biotech R&D, Istanbul, Turkey) was added to the tube. Immediately after, 2 times volume (200 µl) of freshly prepared Solution 2 was added to the medium, and gently pipetting continued. After keeping the tubes at room temperature (25 °C) for 5-10 min, 300 µl of very cold (<4 °C) Solution 3 was added to the medium.

After mixing the tubes gently, they were incubated on ice for 10 min. The tubes were centrifuged at 12.000 g for 5 minutes at +4 °C. The supernatant was carefully taken into a pre-chilled Eppendorf tube, 200 µl of isopropanol (Merck & Co., Inc.) was added and incubated at room temperature for 10 minutes. Then, the tubes were centrifuged at 12.000 g for 15 minutes at 25 °C. Carefully discarding the supernatant, the pellet was washed with 70% ethanol (Merck & Co., Inc.) prepared with sterile nuclease-free water at room temperature, and centrifuged at 7500 g for 10 minutes at 25 °C. After ethanol evaporation, the DNA pellet was dissolved with 50 µl (according to pellet size) 1xTE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) buffer prepared with 2.0 µg/ml DNase-free RNase A (Sigma-Aldrich, USA) and stored at -20 °C.

Visualization in Agarose Gel: All obtained pDNAs were loaded onto agarose gel (1% agarose/0.5xTAE) containing Safe-Red (ABM, Inc.) dye. Marker and all samples were electrophoresed in 1xTAE (Tris-Acetic Acid-EDTA) Buffer at 200 Volts for 15-20 minutes. The bands were visualized under UV light with the Vilber Fusion FX gel documentation system (Vilber Lourmat, France).

Spectroscopic Measurement: Using the µdrop applicator (Thermo Fisher, Scientific, USA) of the MultiskanSky Microplate Spectrophotometer device, the concentration and absorbance values of pDNAs obtained by both methods were recorded at 260, 280, and 230 nm. All measurements were made in triplicate.

Statistical analysis: All statistical analyzes were performed using MATLAB® v.7.8.0 (The MathWorks Inc., Natick, Mass., USA). The p-value and mean standard error was calculated using Student's t-test.

Results

Stab bacteria is resuscitated in SOC medium:

Stab bacteria containing pcDNA3-SARS-CoV-2-S-RBD-sfGFP plasmid were first resuscitated in SOC Outgrowth medium. In the transfer at 10x dilution, more single colony formation was observed than in the undiluted group. One colony was taken from the single colonies formed and transferred to LB Miller medium. After incubation at 37 °C for 16-24 hours, bacterial colony growth was observed (Figure 1).

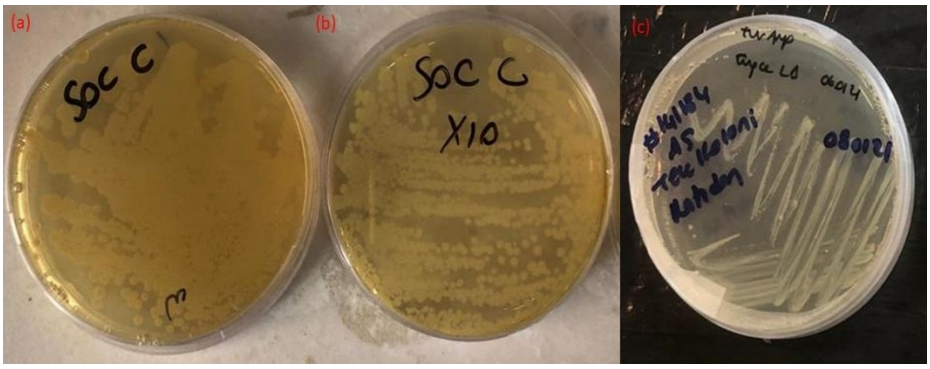

Figure 1. Image of stab bacteria containing pcDNA3-SARS-CoV-2-S-RBD-sfGFP plasmid after resuscitation in SOC Outgrowth medium. (a): Cultivation of undiluted stab bacteria. (b): Cultivation of stab bacteria at 10x dilution. (c): Single colony cultivation of stab bacteria.

Bands are formed in one form after pDNA isolation with the kit, and in multiple forms after pDNA isolation with Alkaline Lysis Method: The pDNAs isolated with the NucleoSpin® Plasmid kit and the pDNAs isolated by the alkaline lysis method were visualized on an agarose gel. After isolation with the kit, there is a single pDNA band of 6736 bp in the gel (Figure 2). However, in pDNA samples isolated by the

alkaline lysis method, more than one form (nicked, linear, supercoiled, circular) band was displayed on agarose gel (Figure 3).

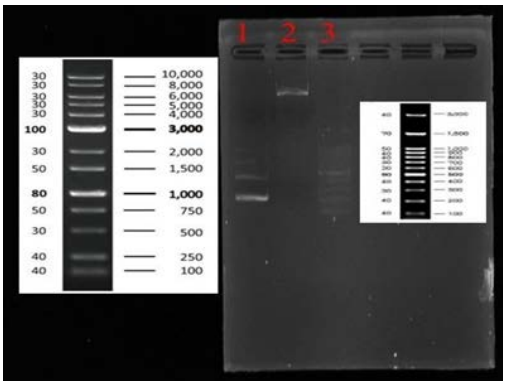

Figure 2. Single band (6736 bp) image of pDNA isolated using the NucleoSpin® Plasmid kit on agarose gel (1st and 3rd well marker, 2nd well pDNA sample).

The ideal OD600 nm value is an important parameter that affects the concentration and purity of pDNA: The concentrations of the samples were also measured after the isolations were made separately at different OD600 nm values. Accordingly, for both isolation methods, the OD600 nm value was in the range of 0.3-0.4, and the maximum concentration of pDNA was obtained. The OD600 nm value was in the range of 0.3-0.4, followed by the concentration value of 0.4-0.5, 0.2-0.3, 0.1-0.2, 0.05-0.1, 0.02-0.05, respectively. The lowest concentration value was found in the range of 0.02-0.05 for both methods (Figure 4). Looking at the A260/280 values, values close to 1.8 were found for both methods, and the OD600 nm value was the closest in the range of 0.3-0.4 (Figure 5). When the A260/230 values were examined, values closer to 2.0-2.2 values were found as a result of the isolation with the kit (Figure 6).

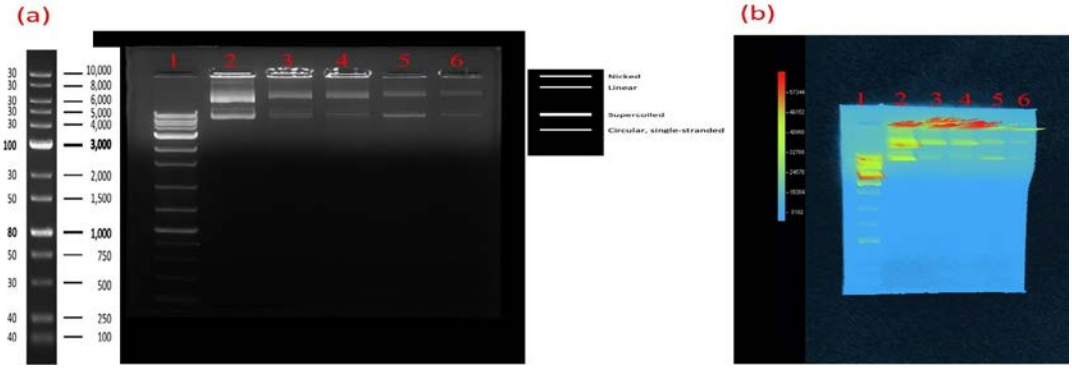

Figure 3. Multiband (nicked, linear, supercoiled, circular) image of pDNA isolated using alkaline lysis method on agarose gel (1st well marker, all following wells are samples of pDNA in different band forms). (a): Two-dimensional gel imaging. (b): Three-dimensional gel imaging.

Table 1. Spectrophotometric measurement results were obtained as a result of plasmid DNA isolation with a plasmid miniprep kit and alkaline lysis method at ideal OD600 nm 0.3-0.4.

Method	Measure	Concentration (ng/µl)	$A_{260/280}$	$A_{260/230}$
Kit	Measure 1	110,76	1,82	2,15
	Measure 2	111,25	1,81	2,16
	Measure 3	110,89	1,82	2,16
	Mean	110,96±0,146	1,81±0,003	2,15±0,003
Alkaline Lysis	Measure 1	2560,56	1,72	1,92
	Measure 2	2565,78	1,71	1,92
	Measure 3	2568,57	1,72	1,91
	Mean	2564,97±2,347	1,71±0,004	1,91±0,004
	p-value	<0.001	<0.001	<0.001

Isolation with the kit results in lower concentration but higher purity pDNA:

At 0.3-0.4, which is the ideal OD600 nm value, both the alkaline lysis and A260/280 purity values of the pDNAs isolated with the kit were found to be close to 1.8, according to the spectrophotometric measurement results. However, the pDNA isolated with the kit was found to be closer to the ideal DNA A260/280 ratio of ~1.8. When A260/230 values are analyzed, the pDNA isolated only with the kit is in the range of ~2.0-2.2 values, which is the ideal A260/230 ratio (Table 1). In contrast, pDNA isolated by the alkaline lysis method has an almost ~20-fold higher concentration (Figure 7). Comparisons made with the parameters specified between the methods were found to be statistically significant (p<0.001).

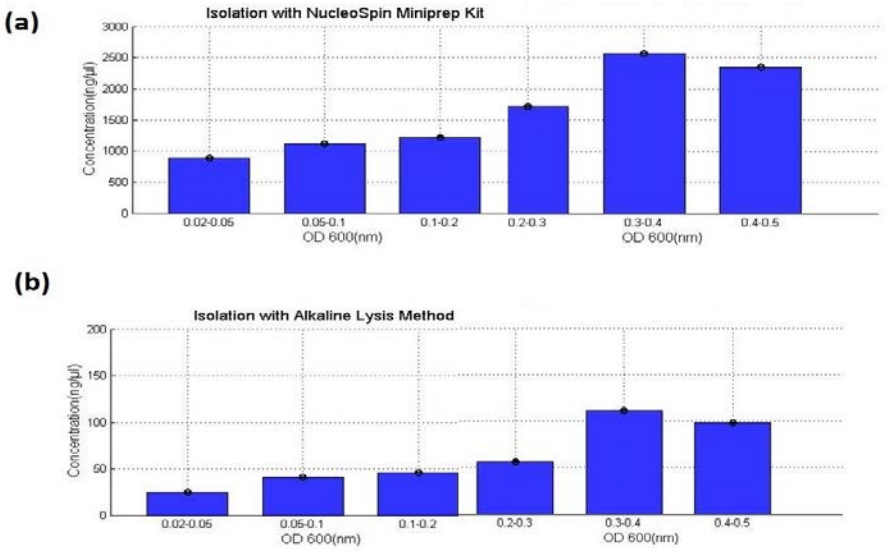

Figure 4. Graph of pDNA concentrations isolated by kit and alkaline lysis method at different OD600 nm values. (a): NucleoSpin® Plasmid kit concentration values. (b): Concentration values of alkaline lysis method.

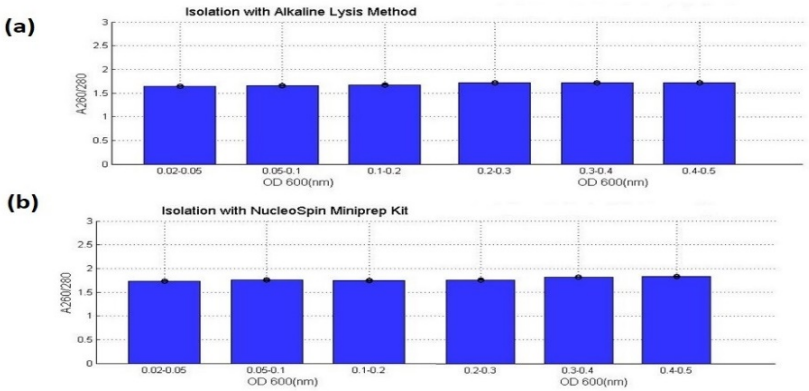

Figure 5. Graph of pDNA A260/280 values isolated by kit and alkaline lysis method at different OD600 nm values. (a): NucleoSpin® Plasmid kit A260/280 values. (b): Alkaline lysis method A260/280 values.

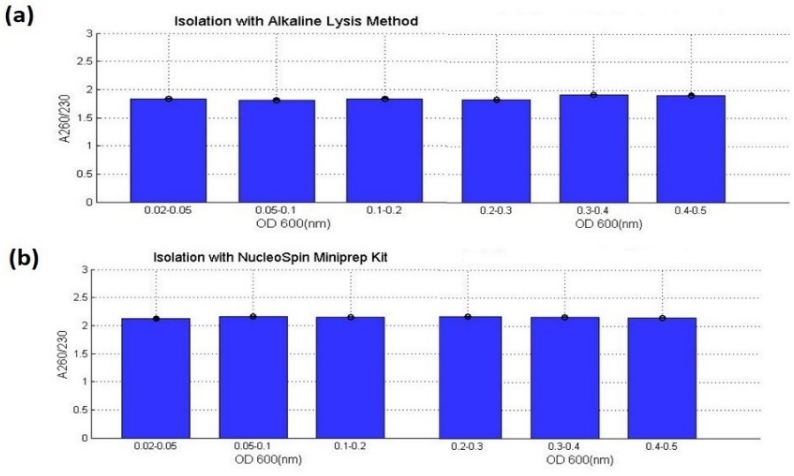

Figure 6. Graph of pDNA A260/230 values isolated by kit and alkaline lysis method at different OD600 nm values. (a): NucleoSpin® Plasmid kit A260/230 values. (b): Alkaline lysis method A260/230 values.

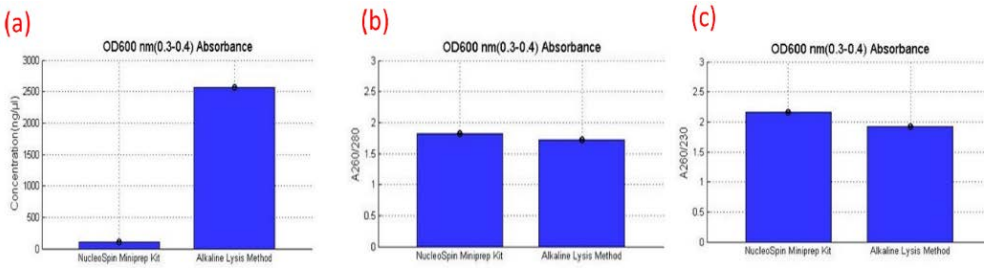

Figure 7. Graph comparing (a): concentration, (b): A260/280, (c): A260/230 values of pDNA isolated by the kit and alkaline lysis method at ideal OD600 nm (0.3-0.4).

Discussion and Conclusion

Since the ’90s, vaccine studies with nucleic acids targeting cancer and infectious diseases have been ongoing (Villarreal et al., 2013; Yang et al., 2014). While mRNA vaccines are developed *in vitro*, the target antigen is frequently obtained on pDNA (Pardi and Weissman, 2017). In particular, using pDNA templates offers researchers a safe option to minimize the risk of infection (Anand and Stahel, 2021). Synthetic mRNA can typically be obtained by in vitro transcription (IVT) of plasmid DNA (pDNA) using a bacteriophage RNA polymerase (Krieg and Melton, 1984; Pascolo, 2006). Thus, the first step in mRNA production is the preparation of pDNA (Schlake, 2012), and it may also seem like the production of the mRNA molecule requires more effort than the production of pDNA. This is undoubtedly related to the quick and easy obtaining of high-quality pDNA.

pDNA contains tiny amounts of bacterial genomic DNA and varying proportions of three forms of pDNA (supercoiled, loose circle, or linear). Therefore, reproducible preparation, ie optimization, of pDNA in pure and invariant form is required to develop a reliable mRNA vaccine (Schlake, 2012). In this study, supercoiled pDNA, which is the most optimized form of pDNA isolated with the alkaline lysis method and kit, was obtained by the alkaline lysis method. However, obtaining pDNA in other forms besides the supercoiled form requires recovery of the supercoiled DNA from the agarose gel. Looking at the literature, the supercoiled form of pDNA resulted in higher transfection activity than nicked circular or linear DNA (Hirose et al., 1985). Moreover, the amount of supercoiled pDNA form in the first step of the mRNA preparation step indicate pDNA stability and activity (Chancham and Hughes, 2001). However, during DNA isolation, the DNA topology can be altered by shear stress to transform the supercoiled plasmid into open circular (single-stranded notched), linear, or even fragmentary (Adami et al., 1998). This conformational change may affect the transfection efficiency of pDNA. During recovery from the gel, care must be taken not to damage the supercoiled pDNA.

Another parameter that determines the quality and purity of pDNA is the OD600 nm value. In the early log stage, the OD600 nm range of 0.3-0.4 is generally accepted as the ideal value (Zou et al., 2012). Considering the results, the ideal OD600 nm value was found to be 0.3-0.4 in this study, which is consistent with the literature. Increased bacterial count in the late exponential growth stage of bacteria and higher concentration were directly related, resulting in a low number of bacteria and low concentration in the early log phase. However, the OD600 nm value at which growth is stopped and isolation is started must be optimized for each plasmid and bacterial species. Because the ideal OD600 nm value is related to the structure of the origin of replication in the plasmid, the plasmid copy number, and the growth rate of the host cell.

At ideal OD600 nm and all other values, the alkaline lysis method resulted in a very high concentration of pDNA (2564.97±2.87 ng/µl) compared to the kit. The alkaline lysis method has been preferred for many years in molecular biological studies with its easy, cheap, and high-concentration product efficiency. However, its purity is slightly lower than that of mass-isolated pDNA. The purity ratio of DNA samples at 260 and 280 nm is used to determine the purity of the DNA (Glasel, 1995) and the A260/280 ratio of ~1.8 is considered “pure” DNA (Hassan et al., 2015). If the ratio of A260/280 is significantly low (≤1.6), it is possible to speak of contamination of proteins and phenol (Lucena-Aguilar et al., 2016). The A260/280 ratio (1.71±0.04) of the pDNA obtained by the alkaline lysis method was found to be lower than the purity of the pDNA isolated with the kit (1.81±0.04), however, it is not at or below the limit of phenolic contamination. Therefore, there is a tolerable relative impurity. On the contrary, the A260/280 value is far from the pure RNA value of ~2.0 (Nouvel et al., 2021), and also indicates the absence of RNA contamination.

In addition, A260/230 is the secondary spectrophotometric parameter commonly used to measure DNA purity (Aleksić et al., 2012; Usman et al., 2014), and DNAs in the ∼2.0-2.2 range are generally considered pure (Lucena-Aguilar et al., 2016). This value was found as 1.91±0.04 and 2.15±0.04 for the pDNA obtained by the alkaline lysis method and the kit, respectively. Since the rate for pDNA isolated by the alkaline lysis method is lower than the expected value, the effectiveness of contaminants such as salts, guanidine HCl, EDTA, lipids, carbohydrates or phenol can be mentioned (Stulnig and Amberger, 1994). However, this value is not a stable DNA quality indicator for our study due to the saline elution buffer used in the final isolation stage with the kit. Because in the isolation method with the kit, there may be a higher salt concentration increase than the DNA concentration in the sample. Consequently, out of two pDNA samples of the same purity, the less concentrated sample will show a lower 260/230 ratio due to the absorbance of the salts at 230 nm. Therefore, the fact that the finding obtained by the alkaline lysis method is slightly far from the ideal value does not mean it is of poor quality.

The target antigen of the recently popular immune weapon mRNA vaccines can be obtained using pDNA. Amplifying target antigen from pDNA provides a safe option for investigators and targeted specific therapies. Especially when working with infectious or zoonotic infectious agents such as COVID-19, plasmids containing the relevant gene present should be primarily preferred. On the other hand, the supercoiled form of pDNA is ideal for vaccine and molecular biology applications. The alkaline lysis method can be preferred if it is desired to obtain cheap/easy pDNA in high concentration and supercoiled form. According to our study, the alkaline lysis method resulted in pDNA of relatively less purity, but a very high concentration than the commercial kit. Before using pDNA samples, DNA purity and quality must be determined by conventional methods. It should also be noted that the excellent OD600 nm value is also essential for pDNA quality.

Similarity Rate

We declare that the similarity rate of the article is 14% as stated in the report uploaded to the system.

Ethical Approval

This study was approved by the KTO Karatay University Non-Pharmaceutical and Medical Device Research Ethics Committee (20.12.2021, 2021/005 Number Ethics Committee Decision). In addition, the authors declared that Research and Publication Ethical rules were followed.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

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Author Contributions

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Control/Supervision: TD, NC, NK
Data Collection and / or Processing: TD, NC, NK, SK
Analysis and / or Interpretation: TD, NC, NK, SK
Literature Review: TD, NC, NK
Writing the Article: TD
Critical Review: TD, NC, NK

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Improvement of Enzyme-Linked Immunoelectrotransfer Blot Assay by Quantitative Approach for Foot-and-Mouth Disease diagnosis

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Abstract: Foot-and-mouth disease (FMD) is a highly contagious animal disease that causes devastating economic losses. The trade of live animals and derived products is only possible if the exporting country is free from disease, according to the World Organization for Animal Health (WOAH) code for FMD. One of the most important ways to prove disease-free is to measure the levels of non-structural protein antibodies (NPS) of FMD virus in the target population sampled. For detection of the disease status of a herd, mass screening and assays such as Enzyme-linked immunosorbent assay (ELISA) and Enzyme-linked immunoelectrotransfer blot assay (EITB) were developed and described in the WOAH diagnostic manual. In this study, recombinant FMDV NS proteins were produced and tested with sera panels collected from uninfected and naturally infected animals using a quantitative Western blot assay as an improved EITB, which enables numerical documentation and statistical analysis. NSP band intensities were used to determine the cut-off values, differentiating infected from non-infected animals and revealing variable sensitivity among the different NSPs. The quantitative EITB results also showed a correlation with the NSP-ELISA results.
Keywords: *Enzyme-Linked Immunoelectrotransfer Blot Assay, Foot-and-Mouth Disease Virus, Non-Structural Proteins, NSP-ELISA, Quantitative Computerised Western blot (QCWB).*

Şap Hastalığı Teşhisi için Enzim Immunotransfer Blot Testinin kantitatif bir yaklaşım ile iyileştirilmesi

Özet: Şap hastalığı büyük ekonomik kayıplara neden olan çok bulaşıcı bir hayvan hastalığıdır. Dünya Hayvan Sağlığı Örgütü Şap Hastalığı koduna göre canlı hayvanların ve bunların ürünlerinin ticareti, ancak, ihraç eden ülkenin hastalıktan ari olması şartıyla mümkündür. Hastalıktan ariliğin gösterilmesi için en önemli yöntemlerden biri örneklenen hedef popülasyonun Şap virüsü yapısal olmayan proteinlerine (YOP) karşı oluşan antikor seviyelerinin ölçümü temeline dayanır. Bir sürünün hastalık durumunun belirlenmesinde yaygın tarama ve Enzim bağlı Immunosorbent test (ELISA) ve Enzim bağlı Immunotransfer testi (EITB) geliştirilmiş ve WOAH (Dünya Hayvan Sağlığı Örgütü) teşhis el kitabında tanımlanmıştır. Bu çalışmada Şap virüsünün yapısal olmayan proteinleri rekombinant olarak üretilmiş, enfekte olmayan, aşılı ve doğal olarak enfekte olan hayvanlardan toplanan serumlardan oluşan panellerle, sayısal belgelemeye ve istatistik analize olanak veren, iyileştirilmiş, kantitatif bir Western blot kullanılarak test edilmiştir. YOP bant yoğunlukları, hasta ve enfekte hayvanların birbirinden ayırt edecek eşik değerlerin ve farklı YOP'lerin bu teşhiste farklı hassasiyet gösterdiğinin belirlenmesinde kullanılmıştır. Kantitatif EITB sonuçları aynı zamanda YOP-ELISA sonuçları ile korelasyon göstermiştir.
Anahtar Kelimeler: *Enzim-Bağlı İmmunoelekrotransfer Blot Testi (EITB), Şap Virüsü, Yapısal Olmayan Proteinler (YOP), YOP-ELISA, Kantitatif Western blot.*

Introduction

Foot-and-mouth disease (FMD) is one of the most contagious and economically important diseases of cloven-hoofed animals. The causative agent is a virus that belongs to the family *Picornaviridae*. Like other picornaviruses, the virus has an approximately 8 kb RNA genome that encodes a single polyprotein split into 12 different proteins. Among these proteins, there are structural virus capsid proteins, VP1-4, and non-structural proteins (NSPs), L, 2A, 2B, 2C, 3A, 3B, 3C, and 3D. FMD virus infection elicits antibodies against both structural and non-structural proteins. Theoretically, purified FMD vaccines containing inactivated virions would be expected to elicit antibodies against structural proteins but not against NSPs (Kitching, 2002; Kweon et al., 2003).

FMD threatens the livestock industry of developed countries due to their non-vaccination policy (Orsel and Bouma, 2009). The disease is one of the most critical factors causing poverty in developing countries (Perry and Rich, 2007). Vaccination has been successfully utilised to control and eradicate the disease in many countries in Europe and South America. FMD was eradicated from large areas without any animal culling (Bergmann et al., 2005). Vaccination has become a more profitable strategy for future European outbreaks (Orsel and Bouma, 2009). However, vaccination can mask clinical symptoms, and infected animals may become carriers (defined as having the infectious virus in the pharynx more than 28 days post-infection). The role of FMD carriers is unclear in transmission, but they still pose a risk for FMD-free parts of the world (Davies, 2002).

An effective surveillance system has to be set up, and not only the clinical disease but also the risks must be controlled to gain FMD-free status in a formerly endemic country or region. In an FMD-free zone where vaccination is practised, serological assays must be able to discriminate between previously infected and vaccinated animals, as described in the WOAH code (Anonymous, 2011). Many ELISA-based tests have been developed for differentiating between infected and vaccinated animals (DIVA) by targeting antibodies to the NSPs. Among these tests, a 3ABC recombinant protein has been found to be the most successful protein thus far for this purpose (Clavijo et al., 2004).

Due to some NSPs within FMD vaccines (if not purified), some sera from vaccinated animals can give positive reactions in the absence of infection by NSP ELISA tests (Lee et al., 2006). Therefore, follow-up studies supported by confirmatory DIVA assays are critical in surveillance studies to reveal false positive animals having no FMD history.

A highly sensitive enzyme-linked immunoelectrotransfer blot (EITB) test that utilizes recombinant NS proteins (2C, 3A, 3B, 3ABC, and 3D) produced in *Escherichia coli* (*E. coli*) has been developed as a confirmatory assay (Bergmann et al., 1993; 1998; 2000; 2003; 2005). Although the assay has diagnostic potential as it is sensitive, reliable, rapid, and economical for FMD (Bergmann et al, 1993), there are some drawbacks to limiting its use, such as it is not as simple as ELISA and it needs more laboratory equipment. Besides, *E. coli* infections of calves or vaccinations against *E. coli* generate anti-*E. coli* antibodies in the cattle sera may produce several non-specific bands with recombinant antigens produced in *E. coli* expression system in Western blot assays (Shen et al., 1999). Another complication of this assay is that the molecular weight and strength of the bands may change slightly depending on the test conditions. Qualitative and empirical solutions to overcome these problems increase Western blot assay subjectivity. Hence, standardization of the assay becomes difficult.

This study aimed to improve the known EITB by a quantitative approach. Improving the EITB will facilitate the screening of FMD infection in cattle populations and identify actual NSP antibody-positive animals.

Materials and Methods

The cattle sera used in this study are not subject to the ethical guidelines for using animals in research according to the current regulation in Türkiye.

Cattle Sera:

Group 1: Cattle sera from animals with a history of clinical disease were collected one month after an A Iran/2005 FMD outbreak at Samsun Karaköy State Farm in the Black Sea Region of Türkiye in 2008. The animals were vaccinated multiple times against FMD previously.

Group 2: Cattle imported from a disease-free country to a private dairy farm located at Aydın Germencik in the Aegean Region of Türkiye were vaccinated twice with oil-adjuvanted FMD bivalent vaccine (Turvac Oil, O_1 Manisa, and A_{22} Iraq) at one-month intervals, intramuscularly. The sera were collected one month after the second vaccination.

Group 3: Sera from non-infected and non-vaccinated cattle collected from the farm from which the animals of Group 2 were obtained. The sera were collected from imported animals before vaccination (Table 1).

Table 1. Number of animals, vaccination and disease status.

Groups	Number	Country of origin	Vaccination status	Clinical Infection in herd
Group 1	119	Türkiye	Multiple	Yes
Group 2	79	New Zealand	Twice	No
Group 3	62	New Zealand	Unvaccinated	No

Production of recombinant proteins: Oligonucleotide primers were designed to target the FMDV O_1 Manisa strain (GenBank® Accession Number: AY593823.1) NSP coding regions; 2B, 2C, 3A, 3B, 3C, 3AB, and 3ABC. BamHI and HindIII restriction endonuclease recognition sites, initiation and termination codons were added near to the 5' and 3' ends of each primer, respectively. RT-PCR and purification of PCR products were performed as previously described (Clavijo et al., 2006). The pET30c vector (Novagen, Madison, WI, USA), which carries an N-terminal His-Tag sequence, was used for cloning the target sequences. The ligation reaction was performed according to the manufacturer's protocol following the restriction digest of both the PCR products and the vectors. *E. coli* JM109 cells (Promega, Madison, WI, USA) were used for cloning, and *E. coli* BL-21 (DE3) pLysS (Novagen, Madison, WI, USA) cells were used for the expression of these proteins.

All protein extractions and purifications were performed using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices (QIAexpressionist kit, Qiagen, Germantown, MD, USA) under denaturing conditions. The eluted protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Design of multiplex assay system: The multiplex assay system used two gels containing 2C-3B or 3AB-3A recombinant protein mixtures. To provide equal spread, running, and transfer conditions and to prevent within-gel variation, the protein mixtures were loaded onto the gel without a comb. Each serum was tested in both the 2C-3B and 3AB-3A antigen systems. Antibody probing was performed in a 20-lane multiscreen apparatus (Miniprotean II multiscreen, Bio-Rad Laboratories, Hercules, CA, USA). One of the lanes was probed with anti-his MAb (monoclonal anti-polyhistidine, H1029, Sigma-Aldrich, St. Louis, MO, USA) to standardise the band densities and used as molecular weight markers. Other lanes were probed with the bovine test sera.

Quantitative computerised Western blot test (QCWB): According to the sizes of the recombinant proteins, two sets of protein mixtures have been designed. The serum samples have been tested in the assay using two different gels for Mixture 1 (2C and 3B) and Mixture 2 (3AB and 3A) (Figure 1).

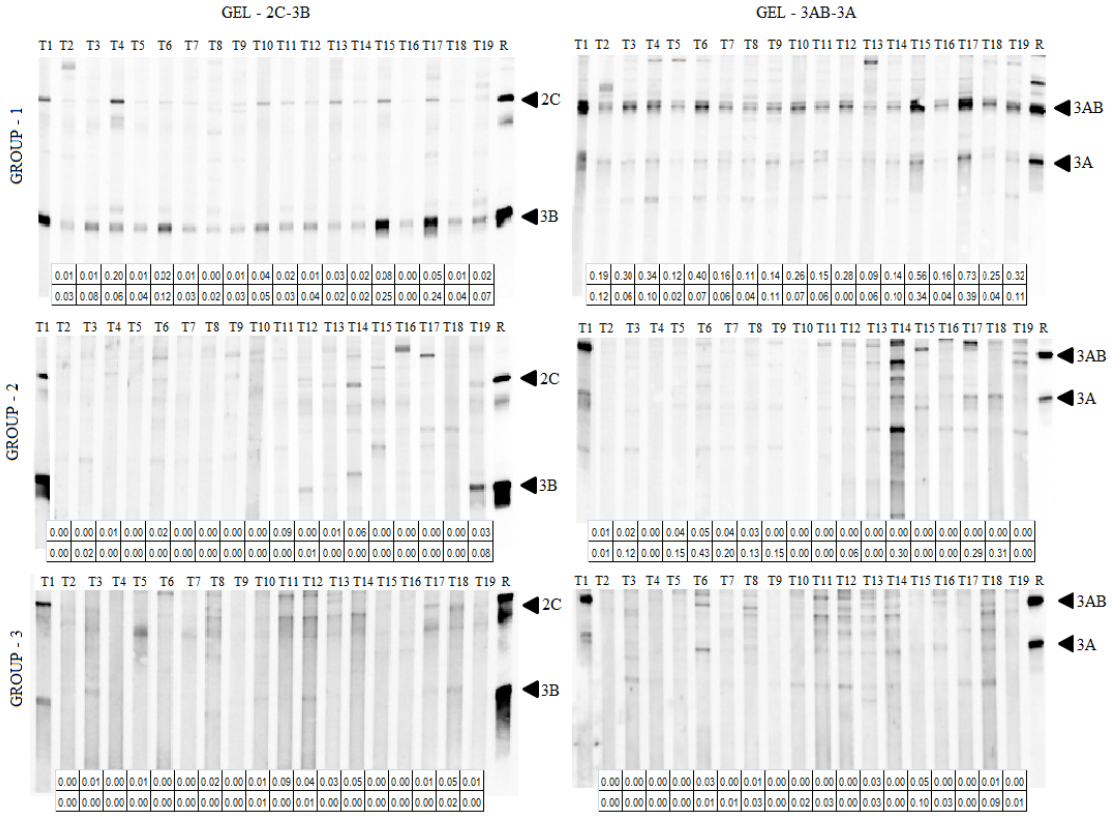

Figure 1. Multiplex assay format and calculating the relative intensity values (RTI). Lane R, probed with monoclonal anti-polyhistidine, is a reference lane. The other lanes, T1-T19, were probed with bovine sample sera. The RTI value of each band was shown under the lane of each serum.

Purified recombinant NS proteins were electrophoresed in 4-12% polyacrylamide gels (acrylamide/bis-acrylamide 40% solution) to evaluate their reactivities to MAb Anti-polyhistidine and bovine sera in QCWB. The separated proteins were transferred to nitrocellulose membranes by electrophoresis at 25 V for 25 minutes with a semi-dry blotter (Scie-Plas, Cambridge, UK). The blots were first blocked with TBS-T-M, which contains 5 mM Tris, 30 mM NaCl with pH 7.6, and 0.1% Tween-20 (0777-1L, Amresco, USA) and 5% dried skimmed milk (Marvel, UK) in a vertical motion shaker. After five minutes washing step with TBS-T (TBS-0.1% Tween-20), the membranes were probed with primary antibodies (anti-his MAb or bovine sera at a dilution of 1:750 in TBS-T-M, respectively) in the shaker. After three times five minutes shaking with TBS-T, the membranes were probed with secondary antibodies, which are secondary goat anti-mouse (31430, Thermo Fischer Scientific, Waltham MA, USA) or rabbit anti-bovine HRP conjugates (A5295, Sigma-Aldrich, St. Louis, MO, USA), at a dilution of 1:1000 in TBS-T-M. After three times five minutes shaking with TBS-T, the blots were incubated with the chemiluminescent substrate (SuperSignal West Pico Substrate Thermo Fisher Scientific, Waltham MA, USA). The blots were then imaged using an imaging station (Gel Logic 1500, Kodak, Rochester, NY, USA). Specific band densities were acquired as the net intensity and converted to the relative intensity values (RTI), which were processed using the software (KODAK Molecular Imaging Software v 4.0.5) provided with the image station. Bands of interest were identified by their exact weight by matching with the bands probed with anti-his MAb using the software tools. Therefore, both non-specific bands and the background colour were excluded to improve the interpretation of the test, which might be affected due to the impurity of the recombinant proteins.

Lane R, probed with monoclonal anti-polyhistidine, is a reference lane. The other lanes, T1-T19, were probed with bovine sample sera. Band images were digitised by Kodak Molecular Imaging Software as the net intensity (T_N or R_N). T_N for each NSP band in the bovine lanes was converted to RTI as a percentage of the R_N of the same NSP band in the reference lanes according to the formula ($RTI=1xT_N/R_N$). The RTI value of each band was shown under the lane of each serum.

Enzyme-linked immunosorbent assay (ELISA): NSP ELISA tests (PrioCHECK FMDV-NS, Prionics, Switzerland) were performed according to the manufacturer's instructions for all sera samples. The percentage inhibition (PI) values were categorised into two groups, 50–69 and 70–100, based on validation criteria for PI values of weak positive and positive controls indicated in the manufacturer's instruction manual. The weak positives were repeated twice and tested with another 3ABC ELISA kit (AniGen FMD NSP Ab ELISA, Bionote Inc. Gyeonggi-do, Republic of Korea) according to the manufacturer's instructions as well.

Statistical Analysis: Statistical analysis was performed using two different software suites (SPSS Statistics 20, IBM, New York, NY, USA, and Medcalc V.13.2.2.0 Ostend, Belgium). Comparisons between the groups were performed using receiver operating characteristic curve (ROC) and Student's t-test analyses. The ROC analysis is the most common method to evaluate the performance of a diagnostic test. This analysis allows the comparison of different test and operator efficiencies, determining appropriate cut-off values, and monitoring the laboratory results quality (Greiner et al., 2000). ROC curves that display the diagnostic performances of the proteins were drawn. The optimal cut-off values of band intensities for each protein were determined using the ROC curves. The diagnostic sensitivity and specificity of the test were also established with the help of these cut-off values. A comparison of the areas under the ROC curves for sensitivity and specificity calculations has been made by using Medcalc software for the simultaneous use of protein results for the overall performance of the test. Correlations between EITB and ELISA results were computed using the SPSS software mentioned above using Pearson's correlation coefficient. P values of less than 0.01 were considered significant. Significant differences between the groups were examined by Student's t-test for independent samples, and P values < 0.05 were considered significant.

Results

Quantitative computerised Western blot test (QCWB): FMD Virus O_1 Manisa reference strain NSPs 2B, 2C, 3A, 3AB, 3ABC, 3B, and 3C recombinant proteins were expressed in *E. coli*. The 2B, 3ABC, and 3C proteins could not be used for further work and were excluded from the tests due to the degradation or low yield of these proteins.

The 2C, 3B, and 3AB proteins did not react with sera from the non-infected groups (Groups 2 and 3). From the infected group, detection of all of the FMDV proteins tested was achieved. The band densities, read by the software, were transformed into numerical values, and the RTI values were given under each lane (Figure 1).

ELISA: NSP ELISA (PrioCHECK FMDV NS) results from the groups are shown in Figures 2a, b, and c. All 119 animals in the infected group (Group 1) tested positive. Four of these 119 animals gave values

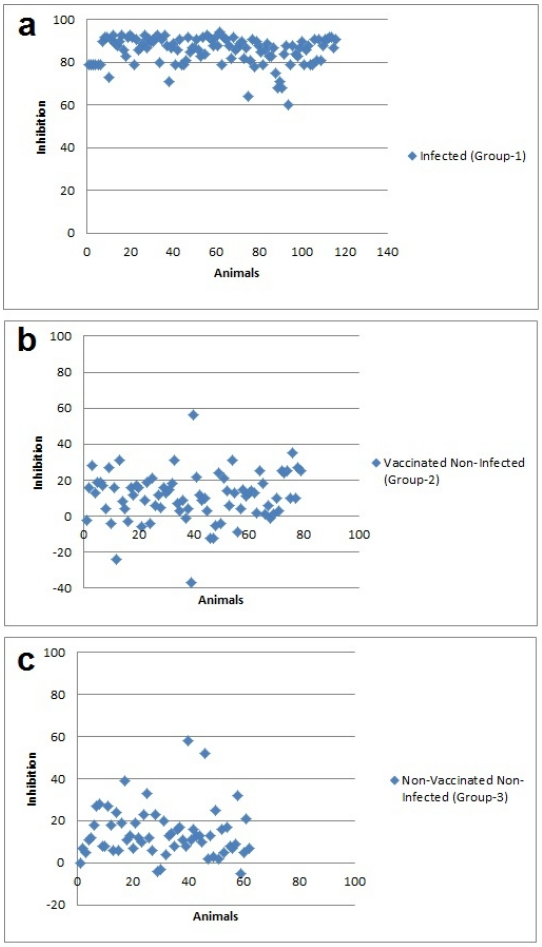

Figure 2. NSP ELISA percentage inhibition values of Group 1 (a), Group 2 (b) and Group 3 (c) Inhibition value < 50% is defined as negative, while an inhibition value of ≥ 50% is positive for the infection.

between 50 and 70% inhibition; in other words, these four were weak positives while the other 115 were strongly positive. One animal was determined to be a weak positive (56% inhibition) in the vaccinated but non-infected group (Group 2). In contrast, 2 out of 62 animals in the non-vaccinated and non-infected group (Group 3) were determined to be weak positives (52% and 58% inhibition). The weak positives obtained by NSP-ELISA were retested, and the results showed that sequential tests detected all infected animals (Group 1). Only one animal also tested positive in PrioCHECK had false reactivity in the vaccinated non-infected group (Group 2). The repeated seven weak positive sera results are given in Table 2.

Table 2. Percentage Inhibition and Relative Intensity values of the weak positive sera.

Ear Tag	Category	NSP-ELISA				Quantitative EITB			
		PrioCHECK	PrioCHECK 2nd test	PrioCHECK 3rd test	AniGen	2C	3B	3AB	3A
4276	Group 3	**58**	39	31	19	0	0	0	**0.12**
4268	Group 3	**52**	34	22	28	0	0	0	0
6421	Group 2	**56**	**55**	48	48	**0.02**	**0.08**	0	0
6672	Group 1	**64**	**67**	**67**	**83**	**0.03**	**0.02**	**0.08**	**0.06**
6538	Group 1	**68**	**81**	**78**	**85**	0	0	**0.10**	**0.38**
6531	Group 1	**68**	**86**	**82**	**74**	**0.02**	0	**0.29**	**0.45**
6557	Group 1	**60**	**65**	**67**	**81**	0	0	**0.07**	**0.79**

-The percentage inhibition and relative intensity values above the cut-off levels marked as bold characters. The AniGen test interpretation method was similar to the PrioCHECK ELISA.

Statistical Analysis: The derived minimum, maximum, mean, standard error, and standard deviation values are evaluated by statistical analysis. Statistical analysis of the data in Table 3 showed that

Table 3. The Pearson correlation of the Relative Intensity (RTI) values of the bands.

	ELISA	RTI 2C	RTI 3B	RTI 3AB	RTI 3A
ELISA	1	0.233*	0.366*	0.552*	0.265*
RTI 2C	0.233*	1	0.577*	0.615*	0.430*
RTI 3B	0.366*	0.577*	1	0.742*	0.205*
RTI 3AB	0.552*	0.615*	0.742*	1	0.571*
RTI 3A	0.265*	0.430*	0.205*	0.571*	1

* Correlation is significant at the 0.01 level (2-tailed).

the reaction against the proteins correlated well with each other and ELISA results. The highest correlation was detected between 3AB and 3B (r: 0.742), followed by that between 3AB and 2C (r: 0.615). Briefly, the highest correlation with the ELISA results was detected with the 3AB protein (r: 0.552), followed by 3B, 3A, and 2C. The area under the curve was calculated by ROC curve analysis; the largest area under the curve was for 3AB, followed by 3B, 3A, and 2C (Figure 3).

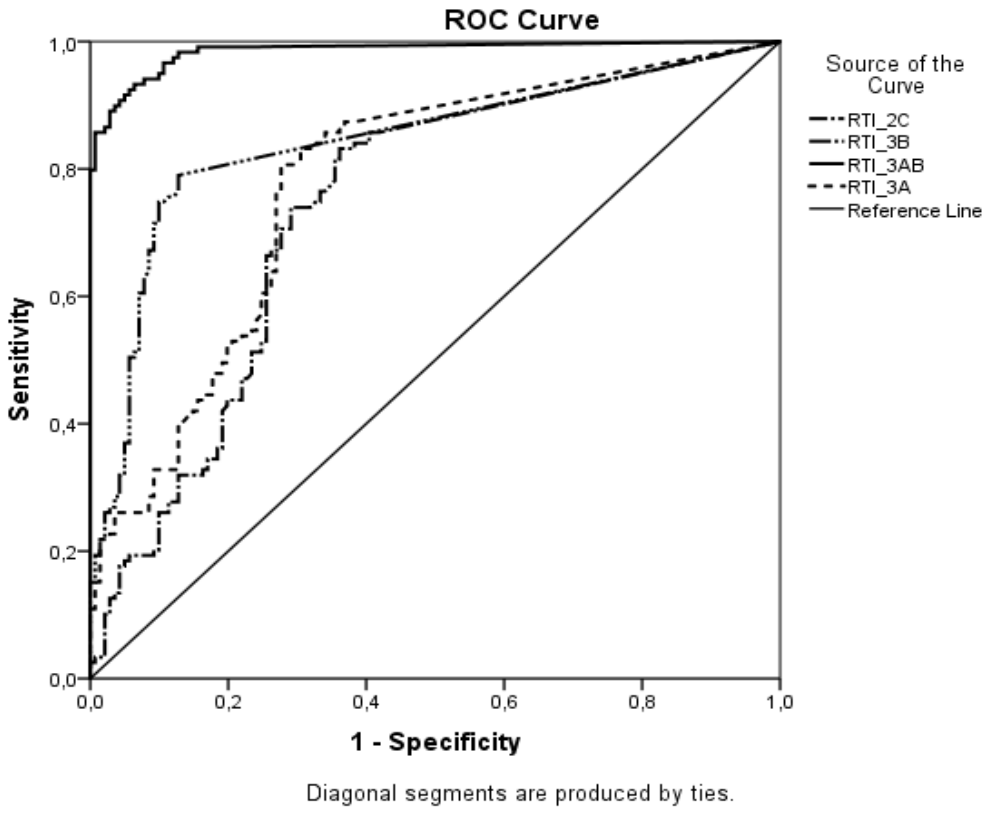

Figure 3. The Receiver Operating Characteristic (ROC) curve analysis of Relative Intensity (RTI) values of individual recombinant proteins in quantitative EITB assay.

The optimum cut-off values for the RTI of the bands were determined by using the ROC curve to obtain the maximum sensitivity and specificity values for the test. (Table 4). A statistically significant difference was found among the RTI values of the 3A protein between the vaccinated (Group-2) and the non-vaccinated, non-infected groups (Group-3), as assessed by independent Student's t-tests ($p<0.05$).

Table 4. Cut-off, sensitivity and specificity values of the proteins and protein combinations.

Proteins	Cut-Off (RTI)	Sensitivity	Specificity
2C	0.010	0.739	0.709
3B	0.002	0.790	0.872
3AB	0.044	0.924	0.936
3A	0.035	0.807	0.723
3AB and 3B	*	0.756	0.993
3AB and 3A	*	0.781	0.964
3B and 2C	*	0.655	0.929
3AB, 3B and 2C	*	0.621	0.993
3AB, 3B, 2C and 3A	*	0.546	0.993

*Cut-off values for single use of the proteins are also valid for multiple use of the proteins.

Discussion

The detection of infection is essential for the control and eradication of FMD. In some countries where widespread vaccination is used, when animals have antibodies to the structural proteins, then clinical disease may not be apparent, and detection of previously infected animals can only be possible using NSP ELISA tests. According to the WOAH code (Anonymous, 2011), NSP-seropositivity of animals in which NSP-free FMD vaccine is applied should be confirmed by retesting with a confirmatory test and follow-up investigation for the presence of ongoing infection where the clinical disease is absent. To evaluate the NSP ELISA test results, some alternative tests such as Dot blot, Luminex-based multiplex assay, or another ELISA have been developed (Clavijo et al., 2004; Fu et al., 2011; Paton et al., 2006).

This study was designed to improve the well-known EITB assay, which is generally used to confirm of NSP-ELISA results. Recombinant proteins are used in both tests. Production of recombinant NSPs of FMDV can be difficult because of their toxic effect on the bacterial expression system (Lewis et al., 1991). Moreover, purification of the proteins can also be troublesome because some proteins may be lost during the extraction or purification steps (Bergmann, 2000). According to our experience, the production of some FMDV NS proteins, such as 2B and 3C, was difficult, probably, the 2B was a too small, and the 3C was a protease. Despite choosing a special expression system, BL-21 (DE3) pLysS, which is suitable for expressing toxic proteins, the 2B, 3C, and 3ABC proteins could not be produced in sufficient amounts for the assay. In addition, the 3C protein was degraded during its storage.

The number of sera used in a single test was increased using of a multichannel Western blot apparatus so that 38 samples could be tested simultaneously compared to 24 stripes of conventional EITB assay (Anonymous, 2011). The subjectivity of the test was also reduced by using Kodak Molecular Imaging software, which converts band densities into numerical RTI values.

In a similar study (Mackay 1998), it was found that there was a high correlation between indirect ELISA and EITB for reactive and non-reactive sera results. According to their indirect ELISA results, 3A, 3B, and 3ABC had the best results. Another study (Bronsvoort et al., 2006) showed that infected animals could be differentiated from vaccinated animals by detecting of antibodies against the 2C, 3A, and 3ABC proteins.

In this study, the RTI values correlated well with each other and with the ELISA results. The most concordant result with ELISA was obtained for the 3AB protein (Table 3). Furthermore, it was found that the 3AB, 3B, 3A and 2C proteins could be used for differential diagnosis by ROC analysis (Figure 3). According to the curve, the highest diagnostic sensitivity was obtained for 3AB, followed by 3B, 3A, and 2C. These findings agree with the first study's results (Mackay, 1998), except for 2C. Our findings showed that the 2C proteins could also be used as another indicator of infection in the EITB assay.

It was reported that using several different NS proteins increases the test's specificity (Bergmann, 2005). In this study, when the 3AB and 3B proteins were evaluated together, and both results were

positive, the sensitivity and the specificity were measured at 75.63% and 99.29%, respectively. Similarly, when the reactions to all three or four proteins were simultaneously positive, the specificity values were above 99%. Consequently, combining two or more proteins improved the test's specificity compared to using single proteins. Conversely, depending on the combinations chosen, the sensitivities were reduced compared to the use of single proteins. Specificity should take the highest priority here because the quantitative EITB is designed as a confirmatory test following ELISA and it is desired to have a low false positivity rate. The NSP ELISA tests have specificity problems and should be complemented by an alternative assay such as EITB (Espinoza et al., 2004). In our study, a few weak positives detected in the first NSP test were evaluated together with the sequential tests and quantitative EITB. The results are consistent with the animal groups when the 3AB-3B pair is considered (Table 4). The results of this study also revealed that not all of the proteins have to be used for evaluation. The best combination was the 3AB-3B pair for optimum specificity and sensitivity.

There is a continuing debate about the presence of trace amounts of NSPs in the FMDV vaccine formulations (Espinoza et al., 2004). An early study (Lubroth, 1996) showed that 2C was removed entirely during the vaccine preparation process, and therefore repeated vaccination could not elicit antibodies against 2C. On the other hand, another study (Dekker and Gijsen, 1998) found 3A in the supernatant of virus cultures. Our data support these findings, as there were significantly more animals reactive to 3A protein in Group 2 than in Group 3 ($p<0.05$).

In conclusion, quantitative EITB can contribute to evaluating the results obtained by NSP-ELISA tests as an essential complementary tool. False positives and negatives can be detected more precisely with the help of this computerized assay. Using this test may lead to better estimations of the true prevalence and incidence of the disease. However, this quantitative EITB should be validated with standard sera panels against various strains provided by international laboratories and with some controls for linearity, gel-to-gel variation, and reproducibility. In addition, because the assay utilises anti-bovine conjugates, sheep sera and anti-sheep conjugates should also be studied to validate of the assay.

Conflict of Interest

We did not have any real, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

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Similarity Rate

We declare that the similarity rate of the article is 13% as stated in the report uploaded to the system.

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Author Contributions

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Control/Supervision: FÖ
Data Collection and / or Processing: ÜP, BS
Analysis and / or Interpretation: AB, AB
Literature Review: CÇ
Writing the Article: CÇ
Critical Review: FÖ

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Timokinon Uygulamasının Akciğerler Üzerine Antioksidan Etkisinin İncelenmesi

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Özet: Bitkiler uzun yıllardan bu yana lezzet verici özelliklerinin dışında, hastalıkların tedavilerinde kullanılmıştır. Fitoterapi amaçlı olarak kullanılan bitkiler oldukça fazladır ve en yaygın kullanılanlarından biri de Çörek otudur. *Nigella sativanın* en önemli ve en yaygın kullanılan etken maddesi tohumlarından elde edilen timokinon (thymoquinone)'dur. Yapılan çalışmaların büyük bir çoğunluğunda ise timokinon ya direkt olarak kullanılmış ya da tohumdan elde edilen yağlar kullanılmıştır. Çalışmamızda farklı hastalıklar üzerine etkisi olan timokinon'un antioksidan etkisinin ağız yoluyla ve intraperitoneal yolla uygulamaları sonrasında akciğerler üzerine olan olası etkilerinin invivo olarak incelenmesi amaçlanmıştır. Çalışmada 35 adet Sprague Dawley soyu rat kullanıldı. Ratlar, bir kontrol ve dört deney grubu olmak üzere toplam 5 gruba ayrıldı ve deney 42 gün sürdürüldü. Deney gruplarına her gün düzenli olarak 1mg/kg, 2 mg/kg (intraperitoneal); 10 mg/kg, 20 mg/kg (oral gavaj) timokinon uygulaması yapıldı. Tüm gruplardaki ratların akciğer dokularında farklı şiddetlerde immun reaksiyonlar gözlendi. Sonuç olarak timokinon'un farklı dozlarının hem ağız yoluyla hem de intraperitoneal yolla uygulamaları sonrasında akciğerler üzerine olan olası immunomodülasyon etkileri karşılaştırmalı olarak incelenmiştir. Antioksidan mekanizmada oldukça önemli belirteçler arasında olan iNOS ve SOD-1'in akciğerlerdeki lokalizasyon ve ekspresyonları in vivo olarak gösterilmiş ve timokinonun sistemdeki antioksidan etkileri gösterilmiştir. Tüm gruplarda farklı immun reaksiyonların gözlenmesi, timokinonun sitokin türüne, uygulama şekillerine ve doza göre farklılıklar olduğu sonucuna varılmıştır.
Anahtar kelimeler: *Akciğer, Antioksidan etki, İmmunohistokimya, Rat, Timokinon.*

Investigation of Antioxidant Effect of Thymoquinone Application on Lungs

Abstract: Plants have been used in treating diseases for many years, apart from their flavoring properties. The plants used for phytotherapy are quite numerous. One of the most commonly used herbs is black cumin (Nigella sativa). The most essential and common ingredient in Nigella sativa is thymoquinone, which is produced from its seeds. In most studies, thymoquinone was used directly, or the oils derived from the seeds were used. We aimed to study the potential effects of thymoquinone on the lungs, affected by numerous diseases, after oral and intraperitoneal administration in vivo. In our study, 35 Sprague Dawley rats were used. The rats were divided into five groups, a control group, and four test groups, and the experiment lasted 42 days.1 ml/kg, 2 mg/kg (intraperitoneal) and 10 mg/kg, 20 mg/kg (oral gavage) thymoquinone were administered to the experimental groups on a daily basis. Different intensities of immune reactions were observed in the lung tissues of rats in all groups. In conclusion, this study investigated the possible immunomodulation effects on the lungs after oral and intraperitoneal administration of different doses of thymoquinone. The localization and expression of iNOS and SOD-1, which are among the most important markers in the antioxidant mechanism, in the lungs have been demonstrated in vivo, and the antioxidant effects of thymoquinone in the system have been demonstrated. The observation of different immune reactions in all groups led to the conclusion that there were differences in thymoquinone cytokine type, application methods and dose.
Keywords: *Antioxidant effect, Immunohistochemistry, Lung, Rat, Thymoquinone.*

Giriş

Bitkiler insanların ve diğer canlıların beslenmesinde oldukça önemli yer tutmaktadır. Özellikle aromatik ve tıbbi bitkiler hem lezzet artırıcı olarak hem de tedavi amaçlı olarak günlük hayatta oldukça fazla kullanılmaktadır. *Nigella sativa* (Çörek otu), bu tür bitkiler arasında yer alan ve uzun geçmişe dayanan bitkidir. Yapılan birçok çalışmada Nigella sativanın antioksidan, antiinflamatuar, antidiyabetik, antikanserojen ve immunmodülasyon etkileri olduğu gösterilmiştir. Timokinon, timohidrokinon ve ditimokinon bitkinin aktif bileşenleri arasında yer almaktadır (Randhawa ve Alghamdi, 2011). Bilimsel çalışmalarda en sık kullanılan etken madde ise timokinon'dur. Timokinonun birçok kanser hücresine karşı sitotoksik etkili olduğu ve kanser hücrelerine spesifik antikor üretiminde aktif rol oynadığı yapılan çalışmalarda gösterilmiştir. Salemi ve Hossainb (2000) yaptıkları çalışmalarında çörek otu tohumunun özünün ve yağının viral ve bakteriyel enfeksiyonlara karşı etkili olduğunu göstermiştir

(Salemai ve Hossainb, 2000). Timokinon ile yapılan çalışmalarda etken maddenin belli nörofarmakolojik etkileri de gösterilmeye çalışılmış ve timokinon'un hafif epilepside antikonvülzan olarak kullanabileceği ortaya konulmuştur (Abuharfeil ve ark., 2001; Hosseinzadeh ve Parvardeh, 2004; Rahmani ve Aly, 2015).

Organizmada reaktif oksijen türevleri (ROT) ile enzimatik ve enzimatik olmayan anti-oksidan savunma mekanizmaları arasında bir düzen bulunmaktadır. Bu düzenin ROT lehine bozulması oksidatif stres olarak tanımlanır ve bu durum bazı değişikliklere sebep olur (Kahraman ve ark., 2003). Oksidatif stres, oksijene ihtiyaç duyan tüm canlı sistemlerde, çeşitli basamaklarda oluşabilen doğal bir süreçtir (Turna ve ark., 2011). Çörek otu uçucu yağı, sabit yağlara kıyasla fazla miktarda antioksidan özelliğe sahiptir (Sultan ve ark., 2009). Rasheed ve ark.nın (2018) yapmış oldukları çalışmalarında timokinonun peroksinitrit (ONOO-) ile indüklenen histon-A2 hasarını önemli ölçüde azalttığı ve tirozin, lizin, arjinin, prolin ve treonin aminoasitlerinin oksidatif hasarını önlediği bildirilmiştir. Timokinonun antioksidan etki potansiyelinin, molekül yapısındaki kinonun redoks özellikleriyle ve fizyolojik bariyerlerden kolay geçmesi ile ilişkili olduğu bildirilmiştir (Darakhshan ve ark., 2005). Karaciğerde yüksek düzeyde bulunan glutatyon molekülü hücresel mekanizmalarda önemli role sahiptir. Glutatyonun tükenmesi ve bunun ardından artan oksidatif stres; protein inaktivasyonuna, protein oksidasyonuna, lipit peroksidasyonuna, toplam tiyol bileşenin azalmasına ve hücre canlılığının kaybına neden olabilmektedir. Timokinon uygulamaları sonucunda malondialdehitin azaldığı, toplam tiyol bileşen ve glutatyon seviyesinin arttığı ve böylece oksidatif stresin azaltıldığı bildirilmiştir (Mollazadeh ve Hosseinzadeh, 2014). İndüklenebilir nitrik oksit sentaz (iNOS) düşük miktarda salındığında antioksidan etki gösterirken yüksek miktarda salındığında oksidatif stres oluşturan oksidatif stres belirteçlerinden bir tanesidir. Yapılan çalışmalarda timokinonun, oksidatif streste önemli fonksiyonu olan iNOS ekspresyonunu inhibe edebildiği ve süperoksit dismutaz (SOD) gibi antioksidan enzimlerin ekspresyonunu indükleyebildiği gösterilmiştir (Khalife ve Lupidi, 2007). Timokinonun aynı zamanda da sisplatin, doksorubisin, gentamisin, vankomisin ve civa klorürün neden olduğu böbrek toksisitesine; karbon tetraklorür, siklofosfamid, asetaminofen ve aflatoksin B1 ile indüklenen hepatotoksisiteye ve sikloffosfomid ve doksorubiisinin kalp toksisitesine karşı koruyucu etkilere sahip olduğu bildirilmektedir (Faroooqui ve ark., 2017). Ayrıca siklofosfamid toluen ve bleomisin kaynaklı akciğer hasarını azalttığı, benzopiren kaynaklı mide tümörlerini engellediği ve gentamisin ototoksisitesini engelleyerek koruyucu rol aldığı bilinmektedir (Darakhshan ve ark., 2015). Timokinonun, katalaz (catalaz CAT) aktivitesini arttırdığı ve timokinon tedavisinin iskemi-reperfüzyon (I/R) yaralanmasına karşı karaciğer dokusunu koruyabileceği de yapılan çalışmalarda bildirilmektedir (Yildiz ve ark., 2008). Çalışmamızın amacı; timokinon'un farklı dozlarının hem ağız yoluyla hem de intraperitoneal (ip) yolla uygulamaları sonrasında akciğerler üzerine olan olası antioksidan etkilerinin karşılaştırmalı olarak incelenmesidir.

Materyal ve Metot

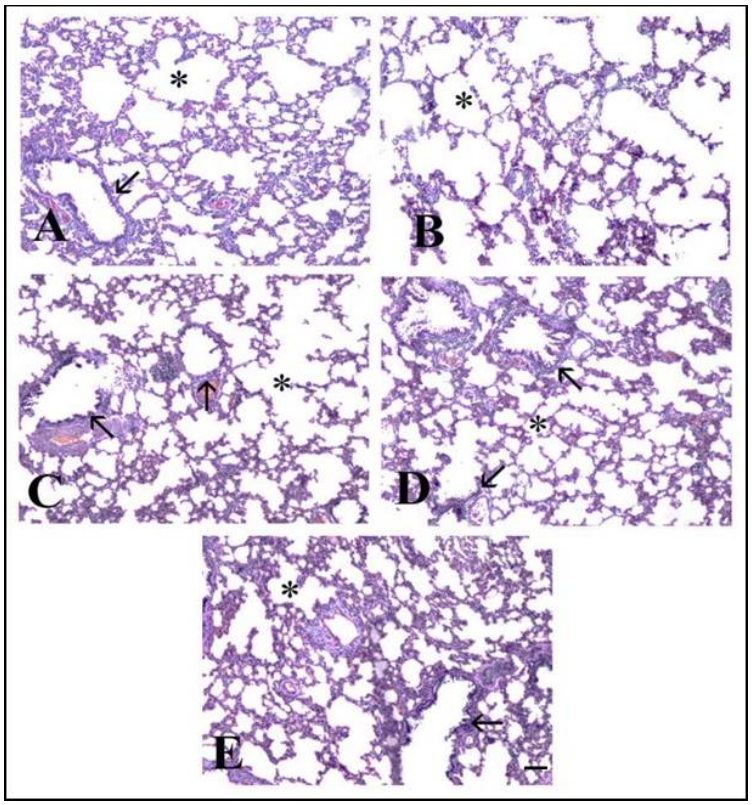
Çalışma Ondokuz Mayıs Üniversitesi Deney Hayvanları Uygulama ve Araştırma Merkezi, Ondokuz Mayıs Üniversitesi Veteriner Fakültesi Histoloji ve Embriyoloji Anabilim Dalı'nda yürütüldü (Ondokuz Mayıs Üniversitesi Hayvan Deneyleri Yerel Etik Kurulu, Deneysel Onay No: 2015/51). Çalışma materyali olarak 35 adet Sprague Dawley ırkı erişkin dişi rat kullanıldı. Ratlar, Deney 1 (1mg/kg timokinon, IP), Deney 2 (2mg/kg timokinon, IP), Deney 3 (10 mg/kg, gavaj), Deney 4 (20 mg/kg, gavaj) ve Kontrol (herhangi bir uygulama yapılmayan) olarak 5 gruba ayrıldı ve her grup 7'şer rattan oluştu (Abdelmeguid ve ark., 2011; Al-Asoom ve ark., 2014). Çalışma sonunda akciğer dokuları alınarak %10'luk tamponlu formaldehit solüsyonunda tespit edildi. Rutin histolojik doku takibi prosedürleri uygulanarak elde edilen kesitlerde histolojik yapının incelenmesi için Crossmon'ın üçlü boyama tekniği uygulandı. Ayrıca iNOS ve SOD-1 ekspresyonunu göstermek için immunohistokimyasal yöntemlerden streptavidin-biotin-kompleks yöntemi kullanıldı. Elde edilen preparatlar Nikon E-80İ araştırma mikroskobu ve Nikon digital-sight DS-Fi1 görüntüleme sistemi ile fotoğraflandı. İmmunohistokimyasal değerlendirmeler boyanmama (-), zayıf boyanma (+), orta şiddette boyanma (++) ve şiddetli boyanma (+++) özelliklerine göre 0'dan 3'e kadar değerler verilerek yapıldı (Kayhan ve ark., 2013; True, 1990).

İmmunohistokimyasal Boyama: Parafin bloklardan alınan 5µ'luk kesitlerde iNOS (1/200) ve SOD-1 (1/750)'in varlığı immunohistokimyasal yöntemlerden streptavidin-biotin-kompleks yöntemi kullanılarak gösterildi (True, 1990). Primer antikor olarak tavşan poliklonal iNOS (Abcam, ab3523) ve tavşan poliklonal SOD-1 (Bioss, bs-10216R) kullanıldı. Sekonder antikor olarak fare ve tavşana specific HRP/DAB (ABC) Detection IHC kit (ab64264) kullanıldı. Kesitler deparafinize edildikten sonra proteoliz için sitrat buffer (pH:6) solüsyonu içerisinde, 700 watt'lık devirde mikrodalga fırında ısıtma işlemine tabi tutuldu. Endojen peroksidaz aktivitesini önlemek için, kesitler %3'lük hidrojen

peroksit solüsyonunda inkübe edildi. Fosfat tampon solüsyonu (PBS) ile yıkamayı takiben kesitlerde spesifik olmayan protein bağlanmalarını önlemek amacıyla, kit içerisindeki serum damlatıldı. Daha sonra kesitlere 1/200 (iNOS) ve 1/750 (SOD) dilüsyonlarında primer antikor damlatılarak +4 ^{0}C'de 1 gece bekletildi. Yıkama işlemini takiben kesitlere biotinlenmiş sekonder antikor damlatıldı ve yıkama işleminden sonra streptavidin-HRP komplekste inkübe edildi. Son aşamada kromojen olarak 3,3'-diaminobenzidine (DAB) kullanıldı ve hematoksilen ile zıt boyama yapılarak preparatlar entellan yapıştırıcı ile kapatıldı.

Bulgular

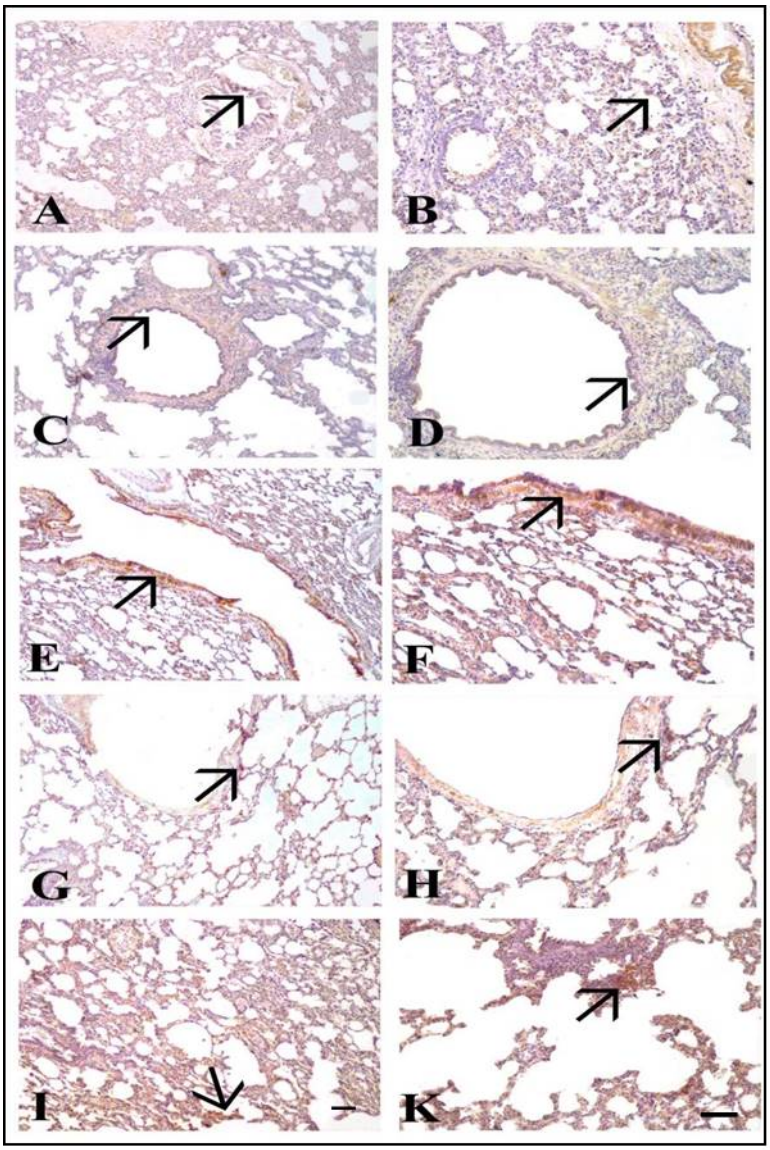
Histolojik Bulgular; Akciğerler incelendiğinde, organın dıştan fibröz bir kapsülle sarılı olduğu, alveoler yapı, bronş, bronşiyol ve damarlar net olarak belirlendi. Organ; bronş, bronşiyol ve alveoller yönünden incelendiğinde gruplar arasında bazı morfolojik farklılıklar tespit edildi. Tüm deney gruplarında akciğerlerdeki lenfosit infiltrasyonlarının ve dejenerasyonlarının kontrol grubuna kıyasla azaldığı belirlendi (Şekil 1).



Şekil 1. Akciğer trikrom (üçlü) boyama; 1mg/kg timokinon ip (A), 2mg/kg timokinon ip (B), 10mg/kg gavaj timokinon (C), 20mg/kg gavaj timokinon (D), kontrol (E), akciğer genel görünüm, ok: bronşiyoller, *: alveoller, Bar 50µm

İmmunohistokimyasal Bulgular: İmmunohistokimyasal olarak iNOS ve SOD-1 ekspresyonunları, tüm gruplara ait akciğerlerin bronş ve bronşiyollerinin epitel hücrelerinin, bronş ve bronşiyollerinin duvarındaki hücrelerin ve alveol duvarında bulunan hücrelerin reaksiyon yoğunluğuna bakılarak yapıldı. Tüm gruplarda bronş ve bronşiyollerin epitel hücrelerinde, bronş ve bronşiyol duvarındaki hücrelerde ve alveol duvarında bulunan hücrelerde farklı şiddette boyanma reaksiyonları gözlendi.

iNOS; Gruplara ait tüm preparatlar değerlendirildiğinde immun pozitif reaksiyonların genellikle bronş ve bronşiyollerin epitel hücrelerinde, bronş ve bronşiyol duvarındaki hücrelerde ve alveol duvarında farklı şiddetlerde olduğu gözlendi. En yoğun reaksiyonların kontrol grubunda olduğu, diğer gruplarda ise daha hafif olduğu belirlendi. Kontrol grubunda bronş ve bronşiyol epitellerinde orta şidette immun reaksiyonlar gözlendi. 10mg/kg gavaj uygulanan gruptaki reaksiyonlar kontrol grubuna benzer iken diğer üç grupta reaksiyonların azaldığı tespit edildi. Alveol duvarlarındaki reaksiyonlara baktığımızda ise, kontrol grubunda orta şiddette reaksiyonlar gözlenirken, deney gruplarında zayıf şiddette reaksiyonların varlığı tespit edildi (Şekil 2) (Tablo 1).



Şekil 2. Akciğer iNOS ekspresyonu, Ok:İmmun pozitif hücreler: 1mg/kg timokinon ip (A), 2mg/kg timokinon ip (C), 10mg/kg gavaj timokinon (E), 20mg/kg gavaj timokinon (G), kontrol (I) x10; 1mg/kg timokinon ip (B), 2mg/kg timokinon ip (D), 10mg/kg gavaj timokinon (F), 20mg/kg gavaj timokinon (H), kontrol (K) Bar 50µm

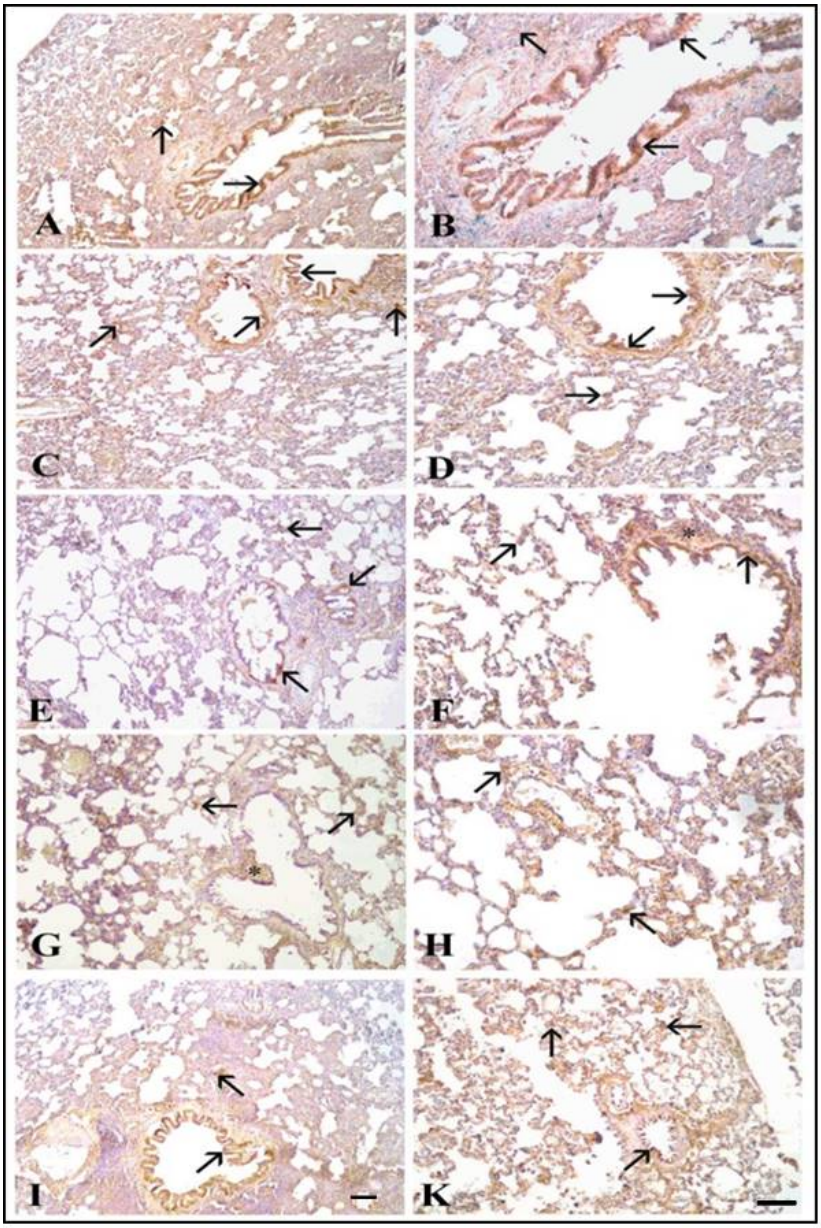
Tablo 1. iNOS'un ortalama immunohistokimyasal reaksiyon şiddetleri.

Grup	Kontrol Grubu	1.Grup (1ml/mg,ip)	2.Grup (2ml/mg,ip)	3.Grup (10mg/kg,gavaj)	4.Grup (20mg/kg,gavaj)
Bronş ve bronşiyol epiteli	±	+	++	+++	++
Alveol Duvarı	±	+	+	++	++

Boyanmama (-), zayıf boyanma (+), orta şiddette boyanma (++) ve şiddetli boyanma (+++)

SOD-1; Preparatlar incelendiğinde, bronş ve bronşiyollerin epitel hücrelerinde, bronş ve bronşiyol duvarındaki hücrelerde ve alveol duvarlarında farklı şiddetlerde immun pozitif reaksiyonlar gözlendi. Grupları kendi aralarında ayrıntılı olarak değerlendirdiğimizde; bronş epitellerinde en yoğun immun pozitif reaksiyonların 1mg/kg ip timokinon uyguladığımız birinci deney grubunda, en zayıf reaksiyonun ise 20mg/kg gavaj grubunda olduğu tespit edildi. Alveol duvarında immun reaksiyon şiddetlerine baktığımızda kontrol grubu, 2mg/kg ip grubu ve 20mg/kg gavaj gruplarında reaksiyon

şiddetlerinin birbirine benzer ve orta şiddette olduğu belirlendi. 1mg/kg ip ve 10mg/kg gavaj gruplarındaki immun reaksiyonların ise birbirine benzer ve zayıf şiddette olduğu tespit edildi (Şekil 3) (Tablo 2).



Şekil 3. Akciğer SOD-1 ekspresyonu, Ok: İmmun pozitif hücreler: 1mg/kg timokinon ip (A), 2mg/kg timokinon ip (C), 10mg/kg gavaj timokinon (E), 20mg/kg gavaj timokinon (G), kontrol (I) x10; 1mg/kg timokinon ip (B), 2mg/kg timokinon ip (D), 10mg/kg gavaj timokinon (F), 20mg/kg gavaj timokinon (H), kontrol (K) Bar 50µm

Tablo 2. SOD-1'in ortalama immunohistokimyasal reaksiyon şiddetleri

Grup	Kontrol Grubu	1.Grup (1ml/mg,ip)	2.Grup (2ml/mg,ip)	3.Grup (10mg/kg,gavaj)	4.Grup (20mg/kg,gavaj)
Bronş ve bronşiyol epiteli	++	+++	++	++	+
Alveol Duvarı	++	+	++	+	++

Boyanmama (-), zayıf boyanma (+), orta şiddette boyanma (++) ve şiddetli boyanma (+++)

Tartışma ve Sonuç

Timokinon içerdiği fenolik bileşikler ve faydalı farmakolojik etkileri nedeni ile geleneksel tıpta tedaviye destek olarak yaygın kullanımı söz konusudur. Timokinon yüksek antioksidan özelliğe sahip ana aktif fenolik bir bileşik içermesinden dolayı birçok çalışmada antienflamatuar, antimikrobiyal ve antikanserojen etkilere sahip olduğu ileri sürülmektedir. Timokinonun oksidatif hasara karşı böbrek, karaciğer, kalp, akciğer ve mide üzerinde koruyucu etkilere sahip olduğunu gösteren çok sayıda çalışma mevcuttur (El-Sheikh ve ark., 2015). Ancak literatür taramaları sonucunda antioksidan etkisinin karşılaştırmalı olarak farklı doz ve uygulama şekline göre değişiklik gösterip göstermediğini bildiren çalışmalara rastlanılmamıştır. Bu çalışma, antioksidan etkide doz ve uygulama yöntemlerinin karşılaştırılarak hangi uygulama yönteminin ve dozun daha etkili olduğunu değerlendirmek amacıyla yapılmıştır.

Timokinon ve akciğerler üzerine yapılan çalışmalarda; timokinonun siklofosfamid, toluen ve bleomisin kaynaklı akciğer hasarını azalttığı, benzopiren kaynaklı mide tümorlerini engellediği ve gentamisin ototoksisitesini engelleyerek koruyucu rol aldığı yapılan çalışmalarda bildirilmektedir (Güzelsoy ve ark., 2018). Çalışmamızda tüm akciğer dokuları ayrıntılı olarak değerlendirildi. Timokinon'un ip ve oral gavaj yolları ile farklı dozlarda uygulanmasının akciğerlerin histolojik yapısında belirgin bir farklılık tespit edilemedi. Çalışmamızın bulguları Yüncü ve ark. (2013)'nın yapmış oldukları çalışmanın bulguları ile paralellik göstermektedir. Ayrıca yapılan immunohistokimyasal boyamalar sonucunda, timokinon'un ip ve gavaj uygulamaları sonucunda iNOS ekspresyonunun gavaj uygulanan iki deney grubunda da kontrol grubuna göre arttığı gözlendi. Beydilli ve ark. (2015) rat beyin dokusunda timokinon ile yaptıkları çalışmalarında, deney grubunda iNOS seviyesinin kontrol grubuna göre azalmış olduğunu bildirmişler. Usta ve ark. (2018) karaciğer ve böbrek dokusu ile yaptıkları çalışmalarında timokinon uygulamasının kontrol gruplarına göre azalmış olduğunu tespit etmişler. Bu azalışı ise timokinonun nitrik oksit ve nitrik oksit sentetaz inhibisyonu ile antioksidan etki oluşturduğunu söylemişlerdir. Çalışmamızın bulguları bahsedilen çalışmaların (Beydilli ve ark., 2015; Usta ve ark., 2018) bulgularından farklılık göstermektedir. Yapılan literatür taramaları da göz önüne alındığında, bu farklılığın nedeninin; uygulama şekli, doz ve organa göre değişiklik gösterdiğini bildiren (Hojna ve ark., 2010) literatür bulguları ile uyumluluk gösterdiği görülmektedir.

Sunulan çalışmada SOD-1'in immun ekspresyonun 20mg/kg gavaj uygulanan grupta diğer gruplara göre azaldığı, ip uygulama gruplarında ise kontrol grubuna benzer şekilde artmış olduğu gözlendi. Aslankoç ve ark. (2019) oksidatif stres durumu ve antioksidan mekanizma üzerine yaptıkları çalışmalarında SOD düzeylerinin artmış olduğunu bildirmişler. Beydilli ve ark. yaptıkları çalışmalarında timokinon uygulanan grupta SOD seviyelerinde artış olduğunu bildirmişlerdir. Desai ve ark. (2015) tarafından yapılan farklı bir çalışmada timokinon uygulamasının SOD seviyelerini arttırdığını, bunu da lipid peroksidasyonunu azaltarak yapmış olabileceğini söylemişler. Çalışmamızda üç deney grubunun bulguları bahsedilen çalışmalarla paralellik göstermektedir. Parlar ve ark. (2019) akciğerde yaptıkları çalışmalarında antioksidan belirteçlerin timokinon uygulanan grupta azaldığını bildirmişler. Mevcut çalışmamızda yüksek doz gavaj uygulanan grubun bulguları Parların ve Arslan (2019)'ın bulguları ile uyumluluk göstermektedir.

Mevcut çalışmamızda timokinon uygulanan deney gruplarında hem iNOS hem de SOD-1 ekspresyonlarında farklı şiddetlerde immun ekspresyonlarında azalmaların gözlenmesi, bize timokinon uygulamalarının antioksidan mekanizma üzerine olumlu etkisi olduğunu düşündürmüştür. Ayrıca sunulan bu çalışma ile timokinon'un farklı dozlarının hem ağız yoluyla hem de intraperitoneal yolla uygulamaları sonrasında akciğerler üzerine olan olası antioksidan etkileri karşılaştırmalı olarak incelenmiştir. Antioksidan mekanizmada ve oksidasyonda etkili olan iNOS ve SOD-1'in akciğerlerdeki lokalizasyon ve ekspresyonları *in vivo* olarak gösterilmiş ve timokinonun sistemdeki antioksidan etkileri gösterilmiştir. iNOS ve SOD-1'in ekspresyonları tüm gruplarda alveollerde, bronş ve bronşiyollerde gösterilmiştir. Tüm gruplarda farklı immun reaksiyonların gözlenmesi, timokinonun farklı uygulama şekillerinin antioksidan mekanizmayı inaktive etmediğini; etki mekanizmasının ise uygulama şekline ve dozuna bağlı olarak değişiklikler gösterdiği çalışmamızda gösterilmiştir. iNOS için en etkili dozun 1mg/kg ip uygulamasının; SOD-1 için ise gavaj uygulamalarının daha etkili olduğu sonucuna varılmaktadır. Sonuçlarımızı desteklemek için, daha fazla histokimyasal ve biyokimyasal teknikleri içeren çalışmaların yapılması gerektiğini düşünmekteyiz.

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Benzerlik Oranı

Makalenin benzerlik oranının sisteme yüklenen raporda belirtildiği gibi %13 olduğunu beyan ederiz.

Teşekkür

Bu çalışmada desteklerini veren Ondokuz Mayıs Üniversitesi Proje Yönetim Ofisi'ne teşekkürlerimizi sunarız.

Açıklama

Makale 1. Yazarın yüksek lisans tezinden özetlenmiştir.

Yazar Katkıları

Fikir/Kavram: ŞT
Tasarım: ŞT
Denetleme/Danışmanlık: ŞT
Veri Toplama ve/veya İşleme: ŞT, KT
Analiz ve/veya Yorum: ŞT, KT
Kaynak Taraması: ŞT, KT
Makalenin Yazımı: ŞT, KT
Eleştirel İnceleme: ŞŞT

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Investigation of the Skull Basally in Honamli, Hair, Kilis and Saanen Goats Using Geometric Morphometric Methods

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Abstract: The study aimed to analyze the skulls of Honamli, Hair, Kilis, and Saanen goats from the basal aspect using the geometric morphometric method. In the study, 48 adult goat skulls were used for each breed in six males and females. After the skulls were photographed from the basal aspect, 11 homologous landmarks were marked. In the study, based on the breed, male and female Honamli, Hair, Kilis, and Saanen goat skulls were significantly separated from each other. The most prominent clusters were seen between female Kilis and Saanen's skulls and male Honamli and Kilis goats. As a result, it is thought that the information obtained could create a reference for the skull remains of ruminants obtained from archaeological excavations.
Keywords: *Geometric morphometric, Goat, Landmark, Principal component analysis.*

Honamli, Kıl, Kilis ve Saanen Keçilerinde Kafatasının Basal Yönden Geometrik Morfometrik Metot Kullanılarak İncelenmesi

Özet: Çalışmada geometrik morfometri yöntemi ile Honamli, Kıl, Kilis ve Saanen keçisine ait kafataslarının basal yönden analizi amaçlandı. Çalışmada her ırk için 6'şar adet erkek ve dişi olmak üzere toplamda 48 adet ergin keçi kafatası kullanıldı. Kafatasları basal yönden fotoğraflandıktan sonra 11 adet homolog landmark işaretlendi. Çalışmada ırk baz alındığında dişi ve erkek Honamli, Kıl, Kilis ve Saanen keçi kafatasları birbirinden önemli düzeyde ayrıldı. En belirgin kümelenmeler dişi Kilis ve Saanen, erkek Honamli ve Kilis keçisine ait kafatasları arasında görüldü. Sonuç olarak elde edilen bilgilerin arkeolojik kazılardan elde edilen geviş getirenlere ait kafatası kalıntıları için referans oluşturabileceği düşünülmektedir.
Anahtar Kelimeler; *Geometrik morfometri, Keçi, Landmark, Temel bileşenler analizi.*

Introduction

The domestic goat (Capra hircus) is a domesticated species of goat generally for livestock. The goat is a member of the Bovidae family (Ansell, 1972; Payne and Wilson, 1999). The hair goat is a combined productive goat breed resistant to Anatolia's challenging climate, can be evaluated for weak pastures, and raised in mountain villages (Gunlu and Alasahan, 2010; Sengonca and Kosum, 2005). Kilis goat is a crossbreed of Damascus goat with Hair goat and is a goat breed with a very high milk yield. Kilis goat is a goat breed widely cultivated in the Southeastern Anatolia region, especially around Hatay, Gaziantep, and Sanliurfa. These goats are a goat breed with durable body structure, long walking ability, high milk and reproductive efficiency (Gunlu and Alasahan, 2010; Kaymakcı and Askin, 1997; Sengonca and Kosum, 2005; Yalcin, 1986). The Honamli goats have been purely bred in the central and western Taurus by the Honamli Yoruks for centuries. According to Communiqué No. 2005/8503 on the Protection of Animal Genetic Resources, this species was included in the scope of protected native breeds in 2005 by the General Directorate of Agricultural Research and Policies (Karadağ and Soysal, 2018). Saanen goats are bred in Europe, in the Saanen valley of Switzerland. Since it is a goat breed with high fertility and milk yield, it has a widespread breeding area (Ceyhan and Karadag, 2009).

Geometric morphometry is a method that allows the detection of shape differences that cannot be detected with the naked eye through landmark coordinates. It, therefore, measures the amount of shape change by exploiting the differences in the position between the objects of the coordinates (Kimmerle et al., 2008; Viscosi and Cardini, 2011; Zelditch et al., 2012). This method allows the magnitude and direction of movement of coordinates between different populations or samples to be mapped and the result interpreted (Bigoni et al., 2010; Frost et al., 2003). In the literature, there are studies of small Ruminantia revealed by geometric morphometrics (Demiraslan et al., 2021; Demircioglu et al., 2021; Yalcin et al.,

2010). The literature review on goats shows a limited number of geometric morphometric method studies (Casanova and Miquel, 2015; Demiraslan et al., 2021; Haruda, 2017; Pares-Casanova and Domènech-Domènech, 2021). Therefore, the study aimed to analyze the skulls of four different goat breeds by geometric morphometric methods in the basal aspect.

Materials and Methods

This study is not subject to HADYEK permission by Article 8 (k) of the 'Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees.'

The study used six males and females for each breed, totaling 48 adult goat skulls. The skulls were collected from slaughterhouses in the Mediterranean, Middle, and Southeastern Anatolia regions. The materials were macerated first by boiling. After the maceration process, the skulls were photographed from the basal, from a distance of 30 cm, focusing on the sutura maxillopalatina. The photographs were converted to a Tps file using the TpsUtil (Version 1.79) program. We marked 11 homologous landmarks on the basal of the skulls using the TpsDig2 program (Version 2.31) (Figure 1). Thus, the Cartesian coordinates of the landmarks were determined. A homologous landmark verification test was performed with the TpsSmall (Version 1.34) program (Rohlf, 2017). Since there are differences in skulls, such as size, position, and direction, General Procrustes Analysis (superimposition) was performed on the skulls to eliminate these variables (Slice, 2005). PAST (Version 4.02) was used for this analysis (Hammer et al., 2001). Principal component analysis was performed with the same program on the new coordinates obtained as a result of superimposition. Thus, the degree of separation of the samples according to breed or sex was determined by using covariance analysis among the factors (Zelditch et al., 2004). In addition, the MorphoJ program was used to show which LMs and in which direction the shape difference is (Klingenberg, 2011). In the study, consensus graphs of the groups were created by performing Relative Warp Analysis with the TpsRelw (Version 1.70) program. The distribution of the groups on the graph was also tested with this analysis (Rohlf, 2017). Statistical analysis of LM coordinate values according to groups was performed with an ANOVA test in the PAST (Version 4.02) program.

Results

The results of the principal components analysis are shown in Table 1. Accordingly, the first principal

Table 1. Results of principal component analysis of the basal aspect of the skulls of male and female Honamli, Hair, Kilis and Saanen goats.

	Female		Male	
	PC Eigenvalue	**%Variance**	**PC Eigenvalue**	**%Variance**
1	0,00094486	30,319	0,000796745	28,164
2	0,000671452	21,546	0,000576305	20,371
3	0,000464533	14,906	0,00037422	13,228
4	0,000300546	9,6442	0,000317092	11,209
5	0,00021416	6,8721	0,000188696	6,6701
6	0,000131522	4,2204	0,000148862	5,262
7	0,000109768	3,5223	0,000112827	3,9883
8	9,10536E-05	2,9218	9,95847E-05	3,5202
9	5,02777E-05	1,6134	7,08039E-05	2,5028
10	4,47753E-05	1,4368	4,4781E-05	1,5829
11	3,22615E-05	1,0352	2,94083E-05	1,0395
12	2,47946E-05	0,79563	2,07973E-05	0,73515
13	1,35685E-05	0,4354	1,81443E-05	0,64137
14	9,39521E-06	0,30148	1,11181E-05	0,39301
15	7,19158E-06	0,23077	9,47571E-06	0,33495
16	4,64459E-06	0,14904	5,30567E-06	0,18755
17	9,23531E-07	0,029635	2,83117E-06	0,10008
18	5,22829E-07	0,016777	1,92067E-06	0,067893
19	1,00104E-07	0,0032122	6,11772E-08	0,0021625
20	7,33543E-17	2,3539E-12	4,66223E-17	1,648E-12
21	3,93401E-17	1,2624E-12	2,20207E-17	7,784E-13
22	5,34831E-18	1,7162E-13	1,57276E-18	5,5595E-14

component explained 30.319% and 28.164% of the total shape difference in female and male goat skulls, respectively.

The distribution graph of individuals according to the first principal component and Relative Warp analysis are shown in Figure 2. According to this, while significant differences were observed between breeds, the most significant separation among females was between Kilis and Saanen goats, and the most significant separation among males was between Honamli and Kilis goats.

The graph showing the points where the shape difference is concentrated in males and females according to PC1 is presented in Figure 3. Accordingly, LM1, LM9, and LM10 in females, LM5, LM6, LM7, and LM8 in males were the homologous points causing the most variation.

The level of LMs at which directional differences between individuals by breed and sex occurred are shown in Figures 4 and 5. Directional differences were observed in LM3 in all females, and in LM5 in all males.

Graphics of canonical variance analysis results are shown in figure 6. Accordingly, male goats were grouped more clearly than females in the breed.

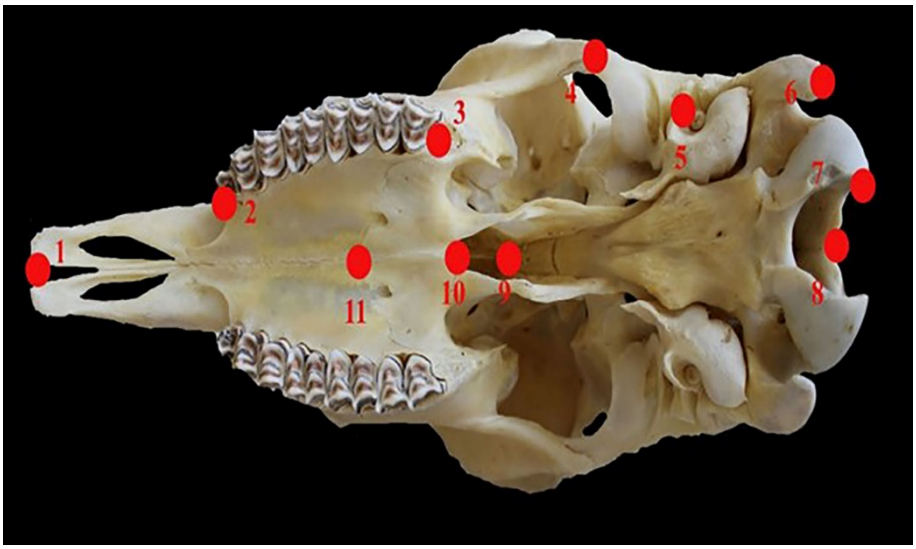

Figure 1. Landmarks marked from basal on the skulls of female Hair.

1. The most rostral of the fissura interincisiva, 2. rostro-oral corner of the margo alveolaris of II. premolar tooth, 3. caudo-oral corner of the margo alveolaris of III. molar tooth, 4. midpoint of the arcus zygomaticus, 5. meatus acusticus externus, 6. Processus jugularis, 7. Condylus occipitalis 8. Caudo-median point of foramen magnum, 9. Caudal of vomer, 10. Base of choana, 11. Median point of sutura maxillopalatina).

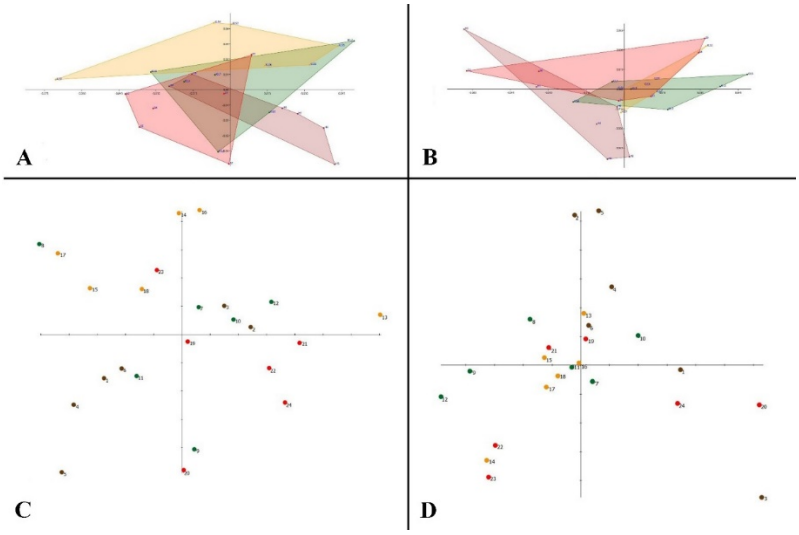

Figure 2. The Scatter plots of principal component (PCA) and relative warp analysis (RWA) of male and female Honamli, Hair, Kilis and Saanen goats.

A. PCA plot for female, B. PCA plot for male, C. RWA plot for female, D. RWA plot for male. 1-6 or brown: Honamli, 7-12 or green: Hair, 13-18 or orange: Kilis, 19-24 or red: Saanen.

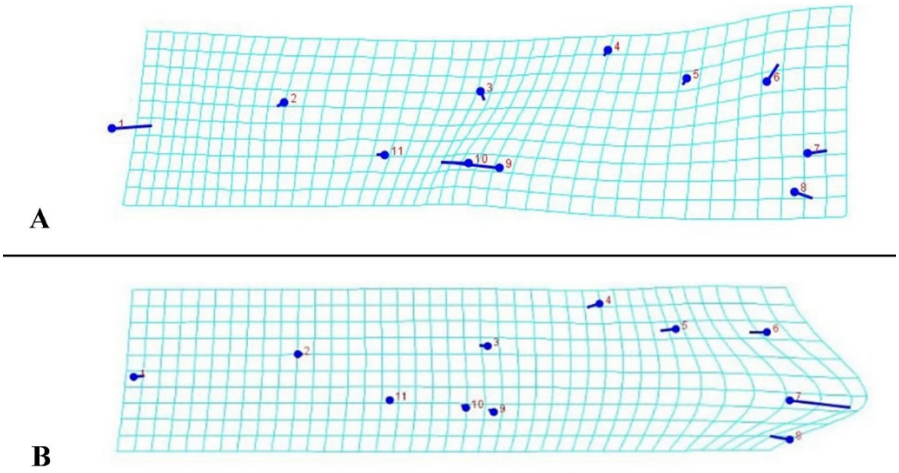

Figure 3. Transformation grid model of Honamli, Hair, Kilis and Saanen goat skulls according to the PC1. A. female, B: male

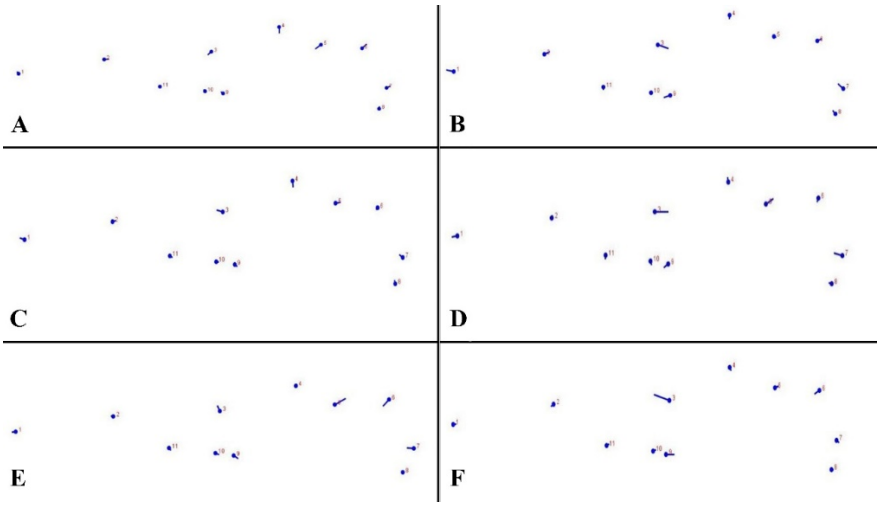

Figure 4. Graphs of discriminant function analysis of shape variation according to breed for female goats. A. Hair-Honamli, B. Hair-Kilis, C. Hair-Saanen, D. Honamli-Kilis, E. Honamli-Saanen, F. Kilis-Saanen.

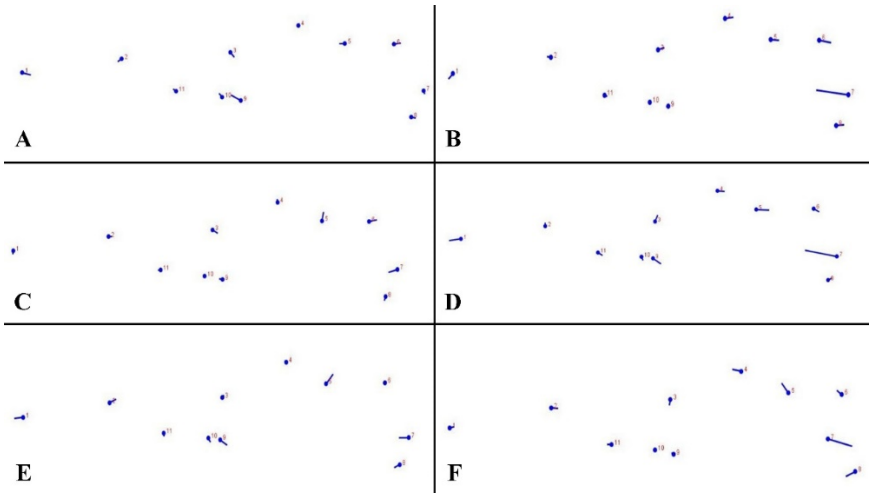

Figure 5. Graphs of discriminant function analysis of shape variation according to breed for male goats. A. Hair-Honamli, B. Hair-Kilis, C. Hair-Saanen, D. Honamli-Kilis, E. Honamli-Saanen, F. Kilis-Saanen.

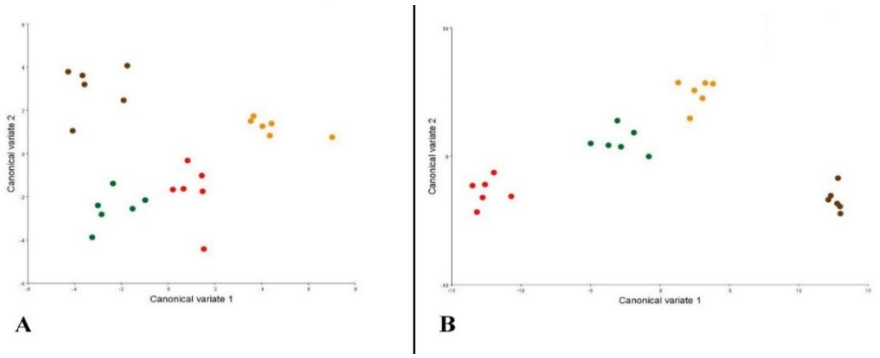

Figure 6. Graphical distribution of individuals according canonical variance analysis. A.Female, B. Male. Brown: Honamli, Green: Hair, Orange: Kilis, Red: Saanen.

Discussion

In this study, the skulls of Honamli, Hair, Kilis, and Saanen goats were analyzed by a geometric morphometric method based on the basal- to- breed factor. Few studies have been found in the literature on goat skulls using the geometric morphometric method. This situation constituted the most apparent limitation of the study as it largely prevented the discussion of the findings.

It has been reported that critical macro-anatomical changes occurred in the morphology of the cranial bones, as well as some general morphological features of sheep and goats, with domestication (Schaffer and Reed, 1972). Our study examined goats' skulls living in different geographical regions, whose breed was registered, by basal geometric morphometric methods. The study' overarching goal was to determine whether racial dimorphism in general body appearance or body part is also reflected in the basal skull. The first findings in this study, which we conducted with a limited number of materials, point to racial and morphological dimorphism in the basal of the goat skull.

Casanova and Miquel (2015) reported that in the geometric morphometric examination of the dorsal skulls of White Rasquera goats, sex discrimination could be made, and the sagittal points

of the viscerocranium provided the greatest contribution to this distinction. In our study, the skulls of Hair, Honamli, Kilis, and Saanen goats were analyzed geometric morphometrically, and it was found that it was possible to distinguish between goat breeds. The most important points where the shape variation was evident were caudo-oral corner of the margo alveolaris of molar tooth III in females and the meatus acusticus externus in males. It is thought that the caudo-oral corner of the margo alveolaris of molar tooth III in females may be distinctive due to differences in feeding habits. The fact that the meatus acusticus externus is distinctive in males suggests that it may be due to the variability of auricle lengths according to breeds.

Demiraslan et al. (2021), in their geometric morphometric study on the mandible of Honamli and Hair goats, reported that Hair goats showed a very distinct sex difference compared to Honamli goats and that male goats were clustered compared to female goats in terms of breed factor. In our study, the basal of the skull was distinctly separated according to goat breeds.

In conclusion, in this study, using geometric morphometric methods, Honamli, Hair, Kilis, and Saanen goat skulls were analyzed for the first time in basal racial dimorphism. The study provides the first inference that racial distinction between goats can be possible from the basal skull. It can be suggested that the study' findings should be elaborated with more material and by including different breeds of goats. Thus, the geometric morphometric findings obtained from the skull base can have a prominent place in the taxonomy of the goat breed. Despite all these, it is thought that the data presented in this study will contribute to morphometric studies on the skull of Ruminantia.

Conflict of Interest

We did not have any real, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

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Sığırlarda Mastitisin Teşhisinde Çiftlikte Kültür ve Konvansiyonel Kültür Yöntemlerinin Karşılaştırılması

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Özet: Bu çalışmada, bakteriyel nedenli inek mastitislerinin teşhisinde çiftlikte kültür ve konvansiyonel kültür yöntemlerinin karşılaştırılması, izole edilen bakterilerin bazı anibiyotiklere *in vitro* duyarlıklıklarının ortaya konulması amaçlandı. Çalışmada kanlı agar, MacConkey agar, Edwards medium ve Columbia CNA agar (CNA) kullanıldı. *İn vitro* antibiyotik duyarlılığının belirlenmesinde neomisin/basitrasin/tetrasiklin (30 µg/10 IU/30), sefapirin (30 µg), amoksisilin/klavulanik asit (2/1) (30 µg), penisilin (10 IU), tetrasiklin (30 µg), klindamisin (2 µg), kanamisin (30 µg) ve sefalotin (30 µg) diskleri kullanıldı. Konvansiyonal izolasyon ve identifikasyon yöntemlerinin uygulandığı çalışmada, örneklerin %90,3'ünden kültür pozitif sonuç alınırken, %9,7'sinde ise herhangi bir aerobik bakteri üremedi. Kanlı agarda yapılan izolasyonda kültür pozitif örneklerinin %17,9'unda (n: 14) *Staphylococcus aureus*, %61,6'sında (n: 48) *Streptococcus* spp., %7,7'sinde (n: 6) *E. coli*, %6,4'ünde (n: 5) *Enterococcus* spp., %3,8'inde (n: 3) maya ve %2,6'sında (n: 2) koagulaz negatif stafilokok (KNS) saptandı. MacConkey agara yapılan ekimlerin %60'ında (n: 6) *E. coli*, %30'unda (n: 3) *Enterococcus* spp. ve %10'unda (n: 1) maya belirlendi. Edwards mediuma ait ekimlerin %91,5'inde (n: 43) *Streptococcus* spp., %6,4'ünde (n: 3) *Enterococcus* spp., %2,1'inden (n: 1) maya ve KNS görüldü. CNA'a yapılan ekimlerin %20'sinden (n: 14) *S. aureus*, %68,6'sından (n: 48) *Streptococcus* spp., %4,3'ünden (n: 3) *Enterococcus* spp., %4,3'ünden (n: 3) maya ve %2,8'inden (n: 2) ise KNS izole edildi. Sonuç olarak, mastitisli inek sütlerinde bazı aerobik bakteriyel patojenlerin saptanmasına yönelik uygulanan çiftlikte kültür yönteminin, konvansiyonel kültür yöntemine benzer sonuçlar verdiği ve bu yöntemin enfeksiyona yönelik koruma ve kontrol programlarında kullanılabileceği kanısına varıldı.
***Anahtar Kelimeler**: Antibiyogram, Çiftlikte kültür yöntemi, İnek, Mastitis.*

Comparison of On-farm Culture and Conventional Culture Methods in the Diagnosis of Mastitis in Cattle

Abstract : This study aimed to compare the microbiological culture and farm-culture methods used to diagnose mastitis in cattle. In the study, blood agar, MacConkey agar, Edwards medium, and Columbia CNA (CNA) agar were inoculated in the media. The samples were incubated under appropriate conditions to perform microbiological analysis of milk samples. While bacterial isolation and identification were performed in 90.3% (75) of the milk samples examined in the study, it was determined that there was no growth in 9.7% (8) of the milk samples, and two different types of pathogens were found in some samples. In blood agar isolation, 17.9% (n: 14) of the culture positive samples were Staphylococcus aureus, 61.6% (n: 48) Streptococcus spp, E. coli in 7.7% (n: 6), Enterococcus spp. in 6.4% (n: 5), yeast in 3.8% (n: 3) and coagulase-negative staphylococci (CNS) in 2.6% (n: 2). *E. coli* was detected in 60% (n: 6)*, Enterococcus spp.* in 30% (n: 3), and yeast in 10% (n: 1) of the inoculations on MacConkey agar. Streptococcus spp. was observed in 91.5% (n: 43), Enterococcus spp. in 6.4% (n: 3), and yeast and CNS in 2.1% (n: 1) of the cultures of Edwards medium. S. aureus was isolated from 20% (n: 14), Streptococcus spp. from 68.6% (n: 48), Enterococcus spp. from 4.3% (n: 3), yeast from 4.3% (n: 3) and CNS from 2.8% (n: 2) of CNA inoculations. As a result, it was concluded that the on-farm culture method for detecting some aerobic bacterial pathogens in mastitic cow milk gave similar results to the conventional culture method. This method can be used in infection prevention and control programs.
***Keywords**: Antibiogram, On-farm culture, Cow, Mastitis.*

Giriş

Mastitis, tüm memeli hayvanlarda görülebilen ve meme bezlerinde çeştili mikroorganizmalar (*Staphylococcus, Streptococcus, Escherichia coli, Arcanobacterium, Corynebacterium* gibi) tarafından oluşturulan yangılı ve irinli bir meme hastalığıdır. Süt veren hayvanların en önemli sağlık sorunlarından biri olan mastitislerde, süt veriminde azalma, sütün bileşiminde bozulma, tedavi edilmediği bazı vakalarda ise ölümler dahi görülebilmektedir. Süt inekçiliğinde endemik seyreden hayvan hastalıkları içerisinde en fazla ekonomik kaybın, mastitisten kaynaklandığı bildirilmiştir (Dufour ve ark., 2019; İlhan, 2018; Sharun ve ark., 2021).

Hastalığın kesin tanısı, hastalığın nedeni ve tedavisi arasındaki en önemli aşamadır. Hastalık ne kadar erken teşhis edilirse, neden olduğu olumsuzluklar o derece az olacaktır. Bu nedenle, çiftlikte kullanılmak üzere güvenilir teşhis araçlarına ihtiyaç bulunmaktadır. Mastitisin primer nedeni, bakteriyel etkenlerdir (Cheng ve Han, 2020). Bu bakteriler arasında en fazla *Staphylococcus aureus, Staphyloccoccus* spp., *Streptococcus* spp. ve *E. coli* bulunmaktadır. Mastitisin tanısında PCR'a dayalı bakteriyel identifikasyon sistemleri geliştirilmiştir (Hiitiö ve ark., 2015). Ancak, bu tür sistemler önemli laboratuvar altyapısı ve uzmanlık gerektirmekte; ayrıca, numune taşıma, analiz ve raporlama zaman almaktadır. Selektif besiyeri kullanılarak oluşturulan çiftlikte-kültür yöntemi, klinik mastitise neden olan patojenleri hızlı bir şekilde tanımlamak için kullanılmıştır (Lago ve Godden, 2018). Çiftlik kültür yöntemi, uygulama, kullanım ve yorumlama kolaylığına sahiptir (Neeser ve ark., 2006). Bu yöntem, mastitise neden olan bakterilerin hızlı identifikasyonunu sağlayarak, antimikrobiyal tedavi kararının alınması veya değiştirilmesine karar verilmesine yardımcı olmaktadır (Lago ve ark., 2011). Bu yöntemin uygulanması ile süt ineği sağlığı ve performansı olumsuz etkilenmeden, antimikrobiyal kullanımını azalttığı bildirilmiştir (Vasquezve ark., 2017; McDougall ve ark., 2018).

İlk hazırlanan çiftlikte kültür yönteminde, MacConkey agar ve Kanlı agar kullanılmıştır. Bu testle etkenler Gram pozitif ve Gram negatif olarak 24-32 saat içinde belirlenebilmektedir. Bu yöntemin mikrobiyolojik kültür metotlarından daha düşük maliyetli olduğu ifade edilmiştir. Diğer yandan kromojenik besiyeri kullanılarak yapılan çiftlikte kültür testlerinde, mastitis patojenleri çıplak gözle daha kolay bir şekilde identifiye edilebildiğine dikkat çekilmiştir (Ferreira ve ark., 2018). MacConkey agar ve Factor agar kullanılarak yapılan çiftlikte kültür sisteminin, mikrobiyolojik kültüre göre değerlendirilmesinde sırasıyla %78 ve %83 duyarlılık ve özgüllüğe sahip olduğu belirtilmiştir (Lago ve ark., 2011). Benzer şekilde, üç seçici besiyerinden oluşan bir çiftlikte kültür sisteminin, *S. aureus* tespit etme duyarlılığı ve özgüllüğünü sırasıyla %97.9 ve %81.8, *Streptococcus* spp. için ise sırayla %92,6 ve %89,5 olarak tespit edilmiştir (McCarron ve ark., 2009). Çiftlikte kültür sistemlerinin kullanılmasıyla Gram pozitif patojenlerin ürediği durumlarda tedaviye gerek olduğu, ancak Gram negatif patojenlerin veya üreme görülmeyen durumlarda tedaviye gerek olmadığı ya da iki günlük antimikrobiyal tedavi kullanımının uygun karar olacağı bildirilmiştir (Pinzon-Sanchez ve ark., 2011).

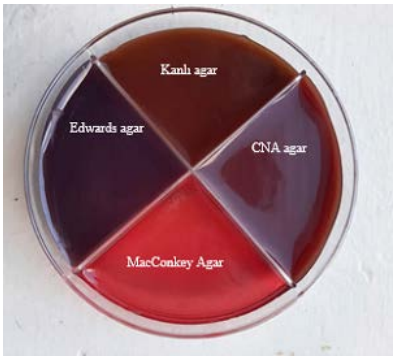
Bu çalışmada, laboratuvarımızda hazırlanan çiftlikte kültür yönteminin etkinliği ve tanısal doğruluğu, standart mikrobiyolojik kültür metodu ile karşılaştırmalı olarak değerlendirilerek, çiftlikte kültür sisteminin etkinliğini saptamak amaçlanmıştır.

Materyal ve Metot

Bu çalışma için Harran Üniversitesi Hayvan Deneyleri Yerel Etik Kurulundan (Harran Üniversitesi-HADYEK) 03/06/2020-E.20027 numara ile izin alınmıştır.

Çalışmada meme bölgesinde şişkinlik, kızarıklık, ağrı ve süt örneklerinde pıhtılaşma saptanan toplam 83 adet inekten alınan 83 adet süt örneği mikrobiyolojik olarak incelendi. Süt örnekleri National Mastitis Council (NMC, 2022) tarafından bildirilen şartlarda aseptik şartlarda toplandı. Süt örnekleri alınmadan önce meme başı %70'lik etil alkolle silinerek temizlendi. İlk süt örnekleri atılıp, test edilecek sütler steril falkon tüplerine alınarak, soğuk zincirde ve kısa sürede Harran Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı Laboratuvarına en kısa sürede ulaştırıldı.

Çiftlikte kültür besiyeri: Bu amaçla, MacConkey agar (Merck, Germany), %5-7 koyun kanlı CNA agar (Oxoid, UK), Edwards medium (Oxoid, UK) ve %5-7 koyun kanlı agar (Oxoid, UK) hazırlanarak, 4 gözlü petrilere döküldü (Şekil 1). Gram-negatif bakterilerin MacConkey agarda, *Streptococcus* spp. şüpheli bakterilerin Edwards mediumda, Gram pozitif bakterilerin ise CNA agarda izolasyonu amaçlandı. Laboratuvara getirilen süt örnekleri vorteksle homojenize edildikten sonra, steril svaplarla hazırlanan besiyerine ekildi. Petri kutuları 37 °C'de, aerobik ortamda, 24-48 saat inkübe edildi (NMC, 2022). Etkenler makroskopik ve mikroskobik morfolojileriyle birlikte, çeşitli konvansiyonel testlere göre identifiye edildi (Quinn ve ark., 2011). Değerlendirmede üç ve daha fazla sayıda farklı bakteri türünün ürediği örnekler kontaminant olarak değerlendirilirken, bir ya da daha fazla sayıda saf kolooninin görüldüğü örnekler ise kültür pozitif olarak kabul edildi (Verbeke ve ark., 2014). Selektif besiyerlerinde üreyen koloniler kanlı agar temel alınarak değerlendirildi.

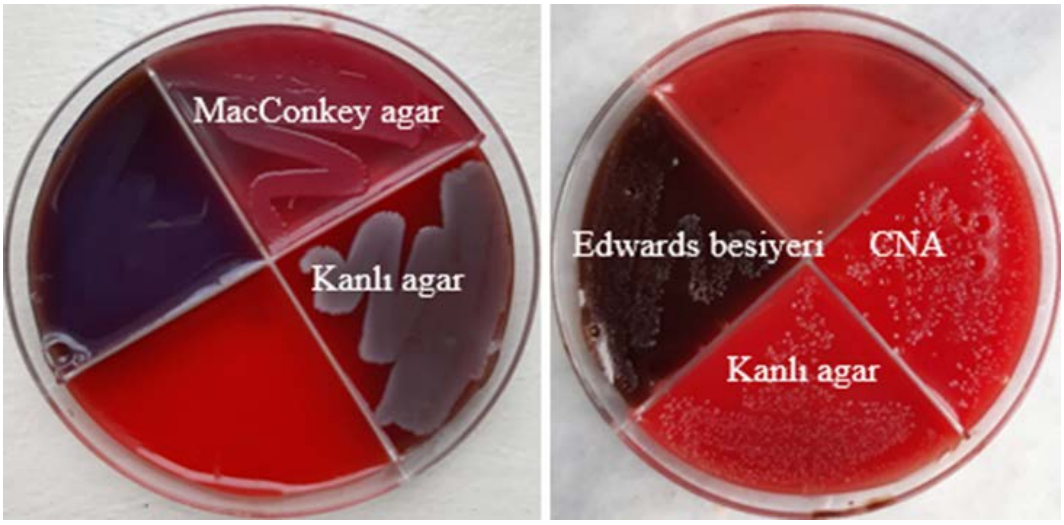


Şekil 1. Çiftlikte kültür yöntemi için hazırlanan besiyeri.

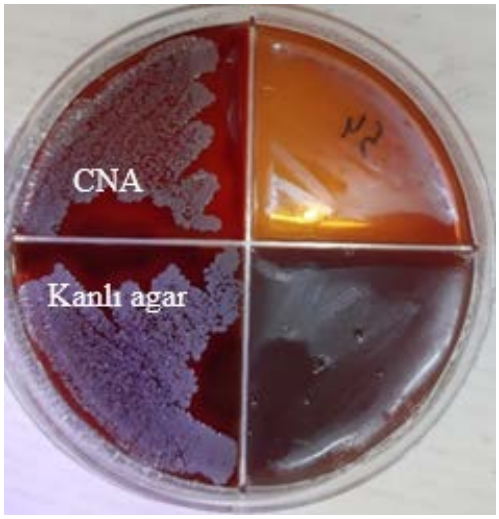
Antibiyotik duyarlılık testi: İzole edilen bakteriler, mueller hinton agarda (Merck, Germany) *in vitro* antibiyotik duyarlıklık testiyle değerlendirildi (CLSI, 2017). Bu amaçla neomisin/basitrasin/tetrasiklin (30 µg/10 IU/30 µg, Mast Diagnostic), sefapirin (30 µg, Mast Diagnostic), amoksisilin/klavulanik asit (2/1) (30 µg, Oxoid), penisilin (10 IU, Oxoid), tetrasiklin (30 µg, Oxoid), klindamisin (2 µg, Oxoid), kanamisin (30 µg, Oxoid) ve sefalotin (30 µg, BBL) diskleri kullanıldı. CLSI standart manuelde bulunmayan neomisin/basitrasin/tetrasiklin için ≤8 dirençli, 9-12 orta duyarlılıkta ve ≥13 duyarlı; sefapirin için ise <14 dirençli, 14-18 orta duyarlılıkta ve ≥19 duyarlı olarak belirlenen zon çapları dikkate alındı.

Bulgular

İzolasyon: Bu araştırmada Harran Üniversitesi Veteriner Fakültesi Mikrobiyoloji Anabilim Dalı Rutin Teşhis Laboratuvarına getirilen klinik mastitisli ineklere ait toplam 83 adet süt örneğinin 75 (%90,7) adetinden kültür pozitif sonuç alındı. Örneklerin 3 adetinden iki farklı patojen türünün ürediği görüldü. Toplam 78 bakterinin 14 (%17,9)'ü *S. aureus*, 12 (%15,4)'si *Streptococcus uberis*, 36 (%46,2)'sı *Streptococcus* spp., 6 (%7,7)'sı *E. coli*, 5 (%6,4)'i *Enterococcus* spp., 3 (%3,8)'ü maya ve 2 (%2,6)'si ise koagulaz negatif stafilokok (CNS) olarak identifiye edildi (Tablo 1, Şekil 2-3).



Şekil 2a) Çiftlikte kültür sistemi olarak hazırlanan MacConkey ve Kanlı agarda üremiş *E. coli*. **b)** *Streptococcus spp*. için Edwards medium, Kanlı agar ve CNA da üremesinin gösterilmesi



Şekil 3. Kanlı agar ve CNA'da üremiş *S. aureus* kolonisi.

Araştırma kapsamında izole edilen *S. aureus*'un %100,0 (14 örnek)'ü kanlı agar ve CNA'da; *Streptococcus* türlerinin %100,0 (48 örnek)'ü kanlı agar ve CNA'da, %89,6 (43)'sı Edwards medium; *E. coli*'nin %100'ü kanlı agar ve MacConkey agarda; *Enterococcus* türlerinin %100,0 (5 örnek)'ü kanlı agar ve MacConkey agarda, %60,0 (3 örnek)'ı Edwards medium ve %60,0 (3 örnek)'ı CNA'da, maya türlerinin %100,0 (3 örnek)'ü kanlı agar, %33,3 (1 örnek)'ü MacConkey agar, %33,3'ü Edwards medium ve %100,0'ü CNA'da, KNS türlerinin %100,0 (2)'ü kanlı agar ve CNA besi yerlerinde üredi (Tablo 3).

Antibiyotik duyarlılık testin bulguları: Araştırmamızda identifiye edilen *S. aureus, Streptococcus* spp. ve *E. coli* suşlarının amoksisilin-klavulanik asit, tetrasiklin, kanamisin, penisilin, sefapirin, klindamisin, sefalotin ve neomisin/basitrasin/tetrasikline duyarlılıkları Tablo 4'de sunuldu. Çalışmada genel olarak *Enterococcus* suşlarının tetracycline, *E. coli* suşlarının amoxicillin-klavulonik asit, klindamisin ve penisiline, *Streptococcus* spp. ve *S. aureus* suşlarında ise test edilen antibiyotiklere farklı olranlarda dirençli oldukları saptandı.

Tartışma

Sağlıklı beslenme açısından temel gıda ürünlerinden biri olan sütün, söz konusu olumlu etkileri gösterebilmeleri için, süt ve süt ürünlerinin sağlıklı hayvanlardan elde edilmesi büyük önem taşımaktadır. Meme bölgesindeki bazı fiziksel, kimyasal ve biyolojik nedenlerin yanında, süt akışını engelleyen birtakım etkiler sonucunda mastitis oluşumu gözlenebilmektedir (Stuhr ve Aulrich, 2010). Gerek klinik gerekse subklinik mastitis vakalarında, süt örneklerinde çıplak gözle farkedilen veya fark edilemeyen önemli değişiklir gelişmektedir (Elizabeth ve ark., 2015; Stuhr ve Aulrich, 2010). *Staphylococcus* spp., *Streptococcus* spp. ve *E. coli* gibi patojenler, ineklerdeki mastitis vakalarının primer bakteriyel etkenleri arasında bulunmaktadır. Gerçekleştirilen bu çalışmada, 83 adet klinik olarak mastitis teşhisi konuşlan ineklere ait süt örneği test edildi.

Tablo 1. Mastitisli sığırlardan elde edilen sütlerden izole edilen bakteri türü ve sayısına ilişkin veriler.

Türler	n	%
Staphylococcus aureus	14	17,9
Streptococcus uberis	12	15,4
***Streptococcus* spp.**	36	46,2
Escherichia coli	6	7,7
***Enterococcus* spp.**	5	6,4
KNS	2	2,6
Maya	3	3,8
TOPLAM	78	100

Konuyla ilgili gerek klinik (Ferreira ve ark., 2011; Kaya ve ark., 1999; Özdemir, 2006; Tel ve ark., 2009) gerekse subklinik (Barrett ve ark., 2005; Macun ve ark., 2011; Tenhagen ve ark., 2006) mastitis vakalarında, bakteriyel patojenlerin rolü araştırılmış ve oldukça farklı veriler elde edilmiştir. Bu araştırmada mastitisli ineklerden alınan süt numuneleri mikrobiyolojik açıdan analiz edilmiş ve örneklerin %92,8'inde bakteriyel izolasyon gerçekleşirken, örneklerin %7,2'sinde ise herhangi bir üremenin olmadığı görülmüştür. Çokal ve Konuş (2012), Bandırma'da mastitisli süt sığırlarına ait örneklerin mikrobiyolojik analizinde, materyallerin %75'inde aerobik bakteri izole ettiklerini bildirmişlerdir. Benzer şekilde Fox (2009), mastitisli düvelere ait süt örneklerinde %43-87,7 arasında farklı oranlarda etken tespit ettiğini rapor etmiştir. Özdemir (2006), klinik mastitisli inek sütlerinden *Staphylococcus* cinsine ait bakteri izole etmeyi amaçladığı çalışmasında, toplam 448 örneğin %65,2'sinden pozitif sonuç aldığını bildirimiştir. Tel ve ark. (2009), Şanlıurfa yöresinde mastitis vakalarının 258'inden (%77,7) aerobik bakteriyel etkenleri izole ettiklerini bildirmişlerdir. Gerçekleştirilen bu çalışmaya ait bulguların, konuyla ilgili çalışmalara benzerlik gösterdiği görülmektedir. Örneklerin %7,2'sinden her hangi bir aerobik bakteriyel etkenin üretmemiş olması, hayvanların yakın tarihte antibiyotik alması veya *Mycoplasma* spp. gibi özel şartlarda üreyen bekterilerin etiyolojiden sorumlu olmasıyal açıklanabilir.

Tablo 2. Süt örneklerinin elektrik iletkenliğine ilişkin bulgular.

Elektrik İletkenliği Değeri	n	%
6	9	10,8
7	25	30,1
8	40	48,2
9	9	10,8

N: Örnek sayısı

Bu araştırmada kültür pozitif örneklerin %17,9'ünde *S. aureus*, %61,6'sinde *Streptococcus* spp., %7,7'sinde *E. coli*, %6,4'ünde *Enterococcus* spp., %2,6'sında KNS ve %3,8'inde de maya izolasyonu gerçekleştirilmiştir. Çelik ve ark. (2021), California Mastitis Testi (CMT) pozitif 226 adet ineğe ait süt örneklerinin 128'inde (%56,64) patojen mikroorganizmalar tespit etmişler. Üreyen 178 adet mikroorganizmanın 77'sinin (%43,75) kontagiyöz, 99'unun (%56,25) çevresel mastitis etkeni, 2'sinin (%1.12) ise maya olduğu bildirmişlerdir. Araştırıcılara, *Streptococcus* spp., *Stapylococcus* spp., *Corynebacterium* spp., *Bacillus* spp. ve koliform grubu bakterilerin en yüksek oranlarda identifiye edildiğine dikkat çekmişlerdir.

Tablo 3. Sütlerden izole edilen bakteri türü ve sayısı.

	CNA	**E.M**	**MCC**	KA
Stapylococcus aureus	14	-	-	14
Streptococcus	36	31	-	36
Streptococcus uberis	12	12	-	12
Escherichia coli	-	-	6	**6**
***Enteroccocus* spp.**	3	3	3	5
Maya	3	1	1	3
KNS	2	-	-	2

K.A: Kanlı agar, MCC: Macconkey Agar, E.M: Edwards medium, CNA: Columbia CNA agar.

Tablo 4. Araştırmada izole ve identifiye edilen bakteri türlerinin antibiyotik direncine ilişkin bulgular. S: Duyarlı, I: Orta duyarlı R: Dirençli

	Staphylococcus aureus (n: 12)			**Streptococcus spp. (n: 48)**			**E. coli (n: 7)**			**Enterococcus spp. (n: 5)**			**KNS (n: 2)**		
Antibiyotikler	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**	**S (%)**	**I (%)**	**R (%)**
Sefapirin	10 (83,4)	-	2 (16,6)	27 (56,3)	9 (18,8)	12 (24,9)	3 (42,8)	2 (28,6)	2 (28,6)	-	2 (40,0)	3 (60,0)	1 (50,0)	-	1 (50,0)
Kanamisin	9 (75,0)	2 (16,7)	1 (8,3)	15 (31,3)	2 (4,2)	31 (64,5)	3 (42,8)	-	4 (57,2)	1 (20,0)	1 (20,0)	3 (60,0)	1 (50,0)	-	1 (50,0)
Klindamisin	10 (83,4)	1 (8,3)	1 (8,3)	20 (41,7)	3 (6,3)	25 (52,0)	-	-	7 (100,0)	2 (40,0)	-	3 (60,0)	2 (100,0)	-	-
Amoksisilin/klavulonik asit	7 (58,3)	2 (16,7)	3 (25,0)	26 (54,2)	6 (12,5)	16 (33,3)	-	-	7 (100,0)	3 (60,0)	-	2 (40,0)	1 (50,0)	-	1 (50,0)
Sefalotin	6 (50,0)	2 (16,7)	4 (33,3)	13 (27,1)	3 (6,3)	32 (66,6)	2 (28,6)	2 (28,6)	3 (42,8)	-	2 (40,0)	3 (60,0)	-	-	2 (100,0)
Penisilin	8 (66,7)	-	4 (33,3)	30 (62,5)	-	18 (37,5)	-	-	7 (100,0)	2 (40,0)	-	3 (60,0)	1 (50,0)	-	1 (50,0)
Novobiyosin/basitrasin/terasiklin	12 (100,0)	-	-	44 (91,6)	1 (2,1)	3 (6,3)	3 (42,8)	2 (28,6)	2 (28,6)	5 (100,0)	-	-	1 (50,0)	-	1 (50,0)
Tetrasiklin	6 (50,0)	2 (16,7)	4 (33,3)	18 (37,5)	10 (20,8)	20 (41,7)	3 (42,8)	2 (28,6)	2 (28,6)	-	-	5 (100,0)	1 (50,0)	-	1 (50,0)

Türkiye'de yapılan başka bir çalışmada, test edilen örneklerin %41'inden *S. aureus*, %17'sinden KNS, %12'sinden *Enterobacter aerogenes*, %9,4'ünden *E. coli*, %8,5'inde *Streptococcus* spp., %6,8'inden *Klebsiella pneumonia*, %5,1'inde *Citrobacter* spp. ve %2,6'sından ise *Bacillus* spp. sorumlu tutulmuştur (Çokal ve Konuş, 2012). Tel ve ark. (2009), Şanlıurfa yöresinde CMT pozitif 181 (%72,4) inekten aldıkları 332 (%33,2) adet süt örneğinin mikrobiyolojik analizinde, örneklerin 84'ünden (%32,5) *S. aureus*, 71'inden (%27,5) KNS, 23'ünden (%8,9) *Streptococcus* spp., 16'sından (%6,2) *E. coli*, 15'inden (%5,8) *Arcanobacterium pyogenes*, 9'undan (%3,4) *Bacillus* spp., 8'inden (%3,1) *Corynebacterium bovis*, 7'sinden (%2,7) *Micrococcus* spp., 5'inden (%1,9) *Enterobacter aerogenes*, 5'inden (%1,9) *Candida* spp., 4'ünden (%1,5) *Pasteurella multocida*, 4'ünden (%1,5) *Klebsiella pneumoniae*, 4'ünden (%1,5) *Citrobacter diversus* ve 3'ünden (%1,1) ise *Pseudomonas auriginosa* olmak üzere toplam 258 aerobik bakteri izole ettiklerini bildirmişlerdir. Kaya ve ark. (1999) Aydın bölgesindeki 141 adet klinik mastitisli inekten aldıkları süt örneklerini, mikrobiyolojik açıdan inceleyerek, antibiyotiklere dirençlerini araştırmışlardır. Araştırmada bakteriyolojik üreme görülen vakaların %57'sinde *S. aureus,* %12'sinde *Streptococcus* spp., %5'inde *E. coli*, %5'inde *Lactobacillus* spp., %5'inde *Klebsiella pneumonia*, %4'ünde *Corynebacterium pyogenes* ve %13'ünde ise *Candida albicans* tespit edilmiştir. Bu bulgular, araştırmamıza ait verilerle yüksek oranda benzerlik göstermektedir.

Bu çalışmada izole edilen *Enterococcus* cinsine ait bakterilerin genel olarak tekrasikline, *E. coli* suşlarının amoksisilin/klavulonik ait, klindamisin ve penisiline; *Streptococcus* spp. ve *S. aureus* suşlarının ise tüm antibakteriyellere çeşitli oranlarda dirençli oldukları görüldü. Bakteriyel nedenli mastitis vakaların sağaltımında kullanılacak antibiyotiklerin belirlenmesine yöneli çeşitli çalışmalar yapılmıştır (Erskine ve ark., 2002; Macun ve ark., 2011; Sharun ve ark., 2021; Tel ve ark., 2009). Yapılan çalışmalarda etkenlerin antibiyotiklere dirençliliklerinde belirgin faklılkarın görülmesi, bilinçsiz ve rasgele antibiyotik kullanımı, tedavi süresinin gereğinden kısa tutlması ve gelişen genetik dirençlilikle açıklanabilir.

Bu çalışmada, çiftlikte kültür yöntemiyle standart mikrobiyolojik kültür yöntemi karşılaştırılmış ve izole edilen mikroorganizmalar yönünden benzer sonuçlar elde edilmiştir. *S. aureus* ve *E. coli* her iki yöntemde de aynı oranda tespit edilmiştir. Jones ve ark. (2009), Yeni Zelanda 292 süt örneğini çiftlikte kültür yöntemiyle incelemişler ve standart mikrobiyolojik kültür yöntemine benzer sonuçlar aldıklarını bildirmişlerdir. Ferreira ve ark. (2018), klinik mastitise neden olan bakteriyel patojenleri tanımlamak için farklı ticari çiftlikte kültür sistemlerini (Accumast, Minnesota, Easy System, SSGN ve SSGNC Quad plates) karşılaştırdıkları çalışmalarında, Accumast'ın en doğru sonuç veren sistem olduğunu belirtmişlerdir.

Sonuç olarak bu çalışmada, çiftlikte kültür yönteminin mastitisli inek sütlerindeki bazı aerobik bakteriyel patojenlerin saptanmasına yönelik, konvasiyonel kültür yöntemine benzer sonuçlar verdiği görüldü. Çiftlikte kültür yönteminin kullanımının, bakteriyel nedenli mastitis patojenlerinin tespiti ve oluşturulacak koruma/kontrol programlarında antimikrobiyal kullanımını azalatarak, etkili kontrol stratejilerinin oluşturmada yardımcı olacağı düşünüldü.

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Kaynaklar

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Turkish Migratory Beekeepers' Opinions Towards the Current State and Problems of Apiculture Sector: A Descriptive Study in Afyonkarahisar

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Abstract: This research was carried out to examine the opinions of the migratory beekeepers in Afyonkarahisar regarding the current situation and problems of the sector. A total of 37 items were assembled under four subgroups in the questionnaire to collect the research data. The questionnaire was applied through face-to-face interviews with beekeepers during the visits to the apiaries of the enterprises. The migratory beekeepers emphasized that marketing is the most crucial problem. They stressed that the marketing assembled with a small number of intermediary companies and the labeling not based on branding and quality standards reduce the retail price of bee products. Other significant problems were unannounced agricultural pesticide spraying, increasing decline in the flora of honey plants, high equipment costs, and the lack of specific legislation for migratory beekeeping. Also, it has been stated that there were challenges in obtaining quality queen bees, finding location areas and meeting the living needs on the migration route, and accessing training on topics such as bee diseases, organic beekeeping, and Apitherapy. As a result, it has been concluded that supporting migratory beekeepers with good input and marketing management, regulations that will facilitate migration and beekeeper accommodation, and policies to increase product quality and bee health can significantly contribute to improving their capacity to adapt to innovative and competitive national strategies to be developed for beekeeping in the future.
Keywords: *Afyonkarahisar, Beekeeping industry, Current state problems, Turkish migratory beekeepers' opinion.*

Türk Göçmen Arıcılarının Arıcılık Sektörünün Mevcut Durumu ve Sorunlarına İlişkin Görüşleri: Afyonkarahisar'da Tanımlayıcı Bir Araştırma

Özet: Bu araştırma Afyonkarahisar'daki göçmen arıcıların arıcılık sektörünün mevcut durumu ve sorunlarına ilişkin düşüncelerini incelemek amacıyla yapılmıştır. Araştırmanın verilerinin toplanması için geliştirilen ankette 37 adet madde dört bölüm altında toplanmıştır. Anket işletmelerin arılıklarına yapılan ziyaretler sırasında göçmen arıcılar ile yüz yüze görüşülerek uygulanmıştır. Göçmen arıcılar pazarlamayı en önemli problem olarak görmüşler, özellikle az sayıdaki aracı işletmeler ile yapılan pazarlama ile markalaşma ve kalite standartlarına dayalı olmayan etiketleme sorunlarının arı ürünlerinin perakende satış fiyatını düşürdüğünü vurgulamışlardır. Diğer önemli problemler ise habersiz yapılan tarımsal ilaçlama, ballı bitkiler florasında giderek artan daralma, yüksek ekipman maliyeti ve göçmen arıcılığa özel bir mevzuatın yokluğudur. Ayrıca kaliteli ana arı temini, göç rotası üzerinde konaklama yeri bulma ve barınma ihtiyaçların karşılanması ile arı hastalıkları, organik arıcılık ve Apiterapi gibi konularda eğitime ulaşmada zorluklar olduğu belirtilmiştir. Sonuç olarak, göçmen arıcıların iyi girdi ve pazarlama yönetimi, göç ve konaklamayı kolaylaştıracak düzenlemeler, ürün kalitesini ve arı sağlığını artırıcı politikalarla desteklenmesinin gelecekte arı yetiştiriciliği için geliştirilecek yenilikçi ve rekabetçi ulusal stratejilere uyum kapasitelerinin arttırılmasına önemli katkı yapabileceği kanaatine varılmıştır.
Anahtar Kelimeler: *Afyonkarahisar, Arıcılık sektörü, Mevcut durum ve sorunlar, Türk göçmen arıcıların düşünceleri.*

Introduction

According to 2019 data, 1.85 million tons of honey are produced in a total of 90.11 million hives worldwide. The part of global honey and beeswax produced in Turkey, which has 8.1 million hives, is 6.2% (114 thousand tons) and 7.2% (4.737 tons), respectively (Burucu, 2021). Most of the honey production in Turkey, which ranks second in world honey production, is carried out by enterprises engaged in migratory beekeeping (Günbey, 2007; Kösoglu et al., 2019; TKDK, 2016). Kekeçoğlu et al. (2014) also emphasized that migratory beekeeping should be performed for high production and profitability. According to 2020 data, before the forest fires on the Aegean and Mediterranean coasts in 2021, the first three regions with the most annual honey production in Turkey are the Eastern Black Sea Region (22.5%), Mediterranean Region (19.2%) and Aegean Region (13.4%) (Burucu, 2021). 95% of beekeeping enterprises in the Eastern Black Sea Region are migratory beekeepers (Kuvancı et al., 2017). Likewise, the Aegean region is the leader in hive prevalence (1.7 million) and the third-largest

honey producer, the rate of migratory beekeeping enterprises has been reported as 82% (Özbilgin et al., 1999; Korkmaz et al., 2018). There are differences between beekeeping enterprises engaged in migratory or stationary beekeeping activities in Turkey. These differences are more pronounced in terms of structural features (Takma et al., 2019), beekeeping practices (Çakmak et al., 2003), and input and output management features (Güneşdoğdu and Akyol, 2019). Compared to stationary beekeeping enterprises, migratory beekeeping enterprises have a high hive capacity (Özbilgin et al., 1999), and they use honey bee colonies with superior (Cengiz and Dülger, 2018). Migratory beekeepers can extend the honey season by moving hives on a planned route with timing to coincide with the flowering periods of honey plants (Korkmaz et al., 2018). It is reported that there are differences between the technical knowledge and skills or their approaches to the beekeeping sector of migratory and stationary beekeepers (Borum, 2017; Erkan and Aşkın, 2001). While stationary beekeepers market the honey they produce in their retail, migratory beekeepers use marketing channels with intermediary institutions in the sector (Tabur and Aziz, 2019). It has also been reported that migratory beekeepers can independently replace the queen bees of the colonies and recognize and distinguish more bee diseases and pests (Erkan and Aşkın, 2001). According to Tabur and Aziz (2019), it has been reported that migratory beekeepers keep operating and production records more regularly, and they produce with more staff, even though they are mostly family members (Takma et al., 2019). Migratory beekeepers have additional skills to continue their lives in rural areas (Sandal and Kan, 2013; Seven and Akkılıç, 2005), and most of them drive their transport vehicles to transport beehives (Akpınar and Bozkurt, 2021). Technical beekeeping processes, production capacity, differences in local and bureaucratic procedures, and difficulties experienced with other stakeholders related to finding apiaries and accommodation areas also affect the professional perspective and attitude of migratory beekeepers towards the beekeeping sector and their thoughts on problems and needs (Sandal and Kan, 2013, Seven and Akkılıç, 2005). For this reason, to increase the performance of bee product production in Turkey, it is crucial to analyze the sectoral and personal problems faced by migratory beekeepers well in terms of developing permanent solution strategies. This research was carried out to examine the opinions of the migratory beekeepers who carry out beekeeping activities in Afyonkarahisar on the current situation and problems in the beekeeping sector.

Material and Methods

The research data were collected with a four-part questionnaire, including the current and most important problems of the beekeeping sector in Turkey. In the first part of the questionnaire developed in this research, the structural problems of the beekeeping sector (9 items); in the second part, the particular problems of migratory beekeeping (11 items); in the third part, the problems related to queen bee production and vocational training (7 items), and in the fourth part, the problems related to the marketing of bee products (10 items) were included. During the development of the survey questions, besides the current problems and essential dilemmas of the beekeeping sector, national reports and scientific research on the beekeeping sector were also used (Cengiz and Dülger, 2018; Güneşdoğdu and Akyol, 2019; Korkmaz et al., 2018; TKDK, 2016). Each item in the questionnaire was subjected to a 5-point Likert-type rating as 1 = Strongly Disagree, 2 = Disagree, 3 = Neutral, 4 = Agree, and 5 = Strongly Agree. The participants of the research migratory beekeepers who the owners of the beekeeping enterprises the study of Akpınar and Bozkurt (2021) was conducted, sampling method and sample size for the migratory beekeeping enterprises were planned according to the reports of Sekaran (2003) and the Ural and Kılıç (2013). A total of 92 questionnaires were administered through face-to-face interviews, and 84 questionnaires with no missing and incorrect data were evaluated. This study was approved by Afyon Kocatepe University Local Animal Ethics Committee (AKUHADYEK -140-18, 14 January 2019).

Statistical Analysis: The thoughts of migratory beekeepers about the problems of the beekeeping sector were determined by the frequency and percentage distributions, as well as arithmetic, mean, and standard deviation values. Cronbach's Alpha coefficient was calculated to define the reliability of the developed questionnaire and its four sub-dimensions. SPSS 22.0 for the Windows package program and Microsoft Excel 2007 program were used for statistical analysis of the data collected (SPSS, Inc., Chicago).

Results

Cronbach's Alpha coefficients, arithmetic mean, and standard deviations of the questionnaire and its sub-dimensions are given in Table 1. The Cronbach's Alpha value, the internal consistency coefficient for the reliability of the questionnaire, and the general mean values were 3.99 and 0.878. The average

values and Cronbach's Alpha coefficients for the sub-dimensions of the beekeeping sector, including structural, migratory beekeepings' specific problems and current marketing problems, were 4.03, 3.54, 4.02, and 4.44

Table 1. Cronbach's Alpha coefficients, arithmetic mean and standard error values for the Questionnaire developed.

Features	n	Cronbach's Alpha	$\overline{X}$	SD
Questionary	84	0.878	3.99	0.50
Sub dimensions				
Structural problems	84	0.568	4.03	0.55
Migratory beekeeping's specific problems	84	0.677	3.54	0.65
Queen bee production and vocational training problems	84	0.756	4.02	0.72
Marketing problems	84	0.889	4.44	0.69

and were 0.568, 0.677, 0.756 and 0.889, respectively. The descriptive statistics of the migratory beekeepers' opinions on the structural problems of the beekeeping sector are given in Table 2. Migratory beekeepers stated that "local farmers spray pesticides without notice" ($\overline{X}$=4.46) and "Short flora and insufficient honey forests" ($\overline{X}$=4.39) items participated in the items at a high rate. The lowest participation is "Difficulties in accessing service from professionals and experts in bee diseases" ($\overline{X}$=3.25) and "a limited number of experts and institutions receive consultancy service in beekeeping "($\overline{X}$ =3.94). The descriptive statistics of participants' opinions on the specific problems of migratory beekeeping are presented in Table 3. Most of the interviewed migratory beekeepers participated in items such as "The lack of a beekeeping law and the narrow scope of the current beekeeping regulation" ($\overline{X}$=4.18) and "Insufficient beekeeping support payment" ($\overline{X}$=4.15). However, their level of participation in items like "Difficulties in official applications for finding apiary area and colony movements" ($\overline{X}$=2.50) and "Insufficiency of professional, experienced drivers for bee colony transport " was the least. The results of migratory beekeepers' opinions on problems related to queen bee production and vocational training are given in Table 4. In this study, the highest mean values were calculated for items "Difficulties in communicating and cooperating with universities" ($\overline{X}$=4.38) and "Short-quality training opportunities for beekeeping" ($\overline{X}$=4.23). Again, the mean value was the lowest for the item "Challenges in the supply of queen bees and poor-quality problems of the queens" ($\overline{X}$=3.54). The descriptive statistics of migratory beekeepers' opinions on the problems of bee product marketing are shown in Table 5. The most favorable views on the questionnaire were "Few large intermediary companies are in the market, so honey prices are low" ($\overline{X}$=4.55), whereas the most negative responses, were given to "Lack of label and price policies based on the honey quality classification" ($\overline{X}$=4.27).

Discussion and Conclusion

The migratory beekeepers paid great attention to unannounced agricultural pesticide spraying by local farmers, the narrowness of the honey plant flora and the low number of honey forests, and the expense of the hives and equipment. In Afyonkarahisar, migratory beekeepers locate close to agricultural flowering plants and orchards in spring and early summer. Güneşdoğdu and Akyol (2019) also reported similar results from Adana. Küçük et al. (2022) and Ergün and Altıntaş (2022) stated that bee colony losses are increasing gradually (up to 42%) in Turkey. In this study, participants were also worried about the insufficient plant flora. The beekeepers show great importance to global warming, climate change, and plant loss and even take this problem relatively more seriously than mass bee deaths. This finding indicates that migratory beekeepers are highly aware of the potential negative impact of global climate change on the ecosystem and the beekeeping sector. Topal et al. (2016) and Kösoğlu et al. (2021) also reported that climate change affects the phenology of plants and sudden temperature changes affect pollen and nectar sources, leading to weak colonies, spreading disease, and increasing the threat of bee extinction. Beekeepers noted that beekeeping equipment and materials are expensive. Tunca and Çimrin (2012) reported that the hive costs of beekeeping enterprises in Thrace and Kırşehir were high. The Apiculture Regulation does not include any special requirements on migratory beekeeping, except regarding allocating apiary lands for migratory beekeepers and identifying actual or legal entities with which beekeepers have to make agreements for these apiaries.

Table 2. The descriptive statistics of the migratory beekeepers' opinions on the structural problems of the beekeeping sector.

	Items	Agreement Level (%)						
		1	2	3	4	5	$\overline{X}$	SD
1	Difficulties in accessing and receiving service from professionals and experts in bee diseases	22.60	6.00	14.30	38.10	19.00	3.25	1.43
2	A limited number of experts and institutions receive consultancy services in beekeeping	8.30	2.40	14.30	36.90	38.10	3.94	1.17
3	Lack of standards for hives	8.30	0.00	8.30	31.00	52.40	4.19	1.15
4	Beekeeping equipment is expensive, and controls in this regard are insufficient	2.40	1.20	10.70	28.60	57.10	4.37	0.90
5	Climate change is due to global warming (rains, water overflows, aridness, etc.).	2.40	2.40	10.60	28.60	56.00	4.33	0.93
6	There are few honey plant varieties in flora and a shortage of honey forests	4.80	1.20	8.30	21.40	64.30	4.39	1.03
7	Bee and colony deaths.	9.50	7.10	16.70	25.00	41.70	3.82	1.31
8	Unannounced agricultural pesticide spraying by local farmers	1.20	1.20	13.10	19.00	65.50	4.46	0.85
9	Challenges in communication and cooperation with the public in the migrated region	21.40	4.80	13.10	27.40	33.30	3.46	1.52

Table 3: The descriptive statistics of migratory beekeepers' opinions on the specific problems of migratory beekeeping.

	Items	Agreement Level (%)						
		1	2	3	4	5	$\overline{X}$	SD
1	Apiary location fee requested from migratory beekeepers	13.10	2.40	19.00	28.60	36.90	3.74	1.33
2	Complications regarding the duties and responsibilities regulated by the Beekeeping Regulation	14.20	0.00	16.70	40.50	28.60	3.69	1.28
3	Accommodation problems in apiary locations such as shelter, water, electricity, communication, etc.	11.90	0.00	14.30	26.20	47.60	3.98	1.30
4	Security issues at the accommodation (robbery, predator wild animal attacks, etc.) in apiary locations.	16.70	3.60	21.40	22.60	35.70	3.57	1.43
5	Discussions among beekeepers when apiaries are located in high density in a region.	15.50	3.60	10.70	33.30	36.90	3.73	1.40
6	Difficulties in official applications for finding apiary area and colony movements.	44.00	4.80	19.00	21.40	10.80	2.50	1.49
7	Insufficiency of professional, experienced drivers for driving bee colony transport vehicles	30.10	6,00	21.70	33.70	8.50	2.84	1.39
8	Practice non-standard techniques and poor-quality problems in beeswax sheet production	28.60	2.40	20.20	25.00	23.80	3.13	1.54
9	Lack of forceful cooperation between NGOs to increase beekeeping support payments	17.90	4.80	19.00	28.50	29.80	3.48	1.42
10	Insufficient beekeeping support payment	6.00	1.20	16.70	23.80	52.30	4.15	1.12
11	The lack of a beekeeping law and the narrow scope of the current beekeeping regulation	4.80	1.20	9.50	40.50	44.00	4.18	0.99

Table 4: The descriptive statistics of migratory beekeepers' opinion on problems related to queen bee production and vocational training.

	Items	Agreement Level (%)						
		1	**2**	**3**	**4**	**5**	$\overline{X}$	**SD**
1	Inability to produce own queen bee and high queen bee fees on the market	15.50	1.20	10.70	44.00	28.60	3.69	1.32
2	Challenges in the supply of queen bees and poor-quality problems of the queens	16.70	3.60	15.50	38.10	26.10	3.54	1.36
3	The lack of Turkish resources and tools is suitable for beekeepers' training on bee breeding and diseases	7.10	1.20	13.20	33.30	45.20	4.08	1.13
4	Insufficient knowledge of beekeepers in organic beekeeping issues	8.30	2.40	21.40	23.80	44.10	3.93	1.23
5	Inadequate understanding of beekeepers on other current topics such as apitherapy	0.00	2.40	17.90	26.20	53.50	4.31	0.85
6	Short-quality training opportunities for beekeeping	3.60	2.40	11.90	32.10	50.00	4.23	0.99
7	Difficulties in communicating and cooperating with universities	2.40	0.00	10.60	31.00	56.00	4.38	0.86

Table 5: The descriptive statistics of migratory beekeepers' opinion on the problems of the bee product marketing.

	Items	Agreement Level (%)						
		1	**2**	**3**	**4**	**5**	$\overline{X}$	**SD**
1	Difficulties in establishing quality standards for honey and honey products in the market	3.60	0.00	7.10	29.80	59.50	4.42	0.91
2	Lack of label and price policies based on the honey quality classification	2.40	1.20	14.30	31.00	51.10	4.27	0.92
3	Inspections on honey quality are not widespread enough; the number of testing laboratories is small	3.60	0.00	4.80	22.60	69.00	4.54	0.88
4	The increasing market size of fake or cheap honey	6.00	0.00	3.60	25.00	65.40	4.44	1.02
5	Marketing problems due to undesirable residue problem	6.00	1.20	6.00	26.10	60.70	4.35	1.07
6	Deterioration of the natural structure of honey due to the addition of sugar or starch syrups (fraud in honey quality)	6.00	0.00	9.50	22.60	61.90	4.35	1.07
7	Veterinary drug residues in honey after treating the honeybee	6.00	0.00	3.60	19.00	71.40	4.50	1.02
8	Few large intermediary companies are in the market, so honey prices are low	3.60	0.00	6.00	19.00	71.40	4.55	0.89
9	Packaging and branding problems for honey	3.60	0.00	8.30	15.50	72.60	4.54	0.92
10	Incapacity of beekeepers to market their honey	3.60	3.60	6.00	11.80	75.00	4.51	1.01

A crucial stage of migratory beekeeping is bee colony transport. A Domestic Veterinary Report for Animal transport during the journey is mandatory, but no measure is envisaged to protect bee health and welfare. Since the transportation process is quite costly, beekeepers prefer vehicles that are not very suitable for bee transportation, which leads to the transfer of diseases and pests from one place to another along with the bees. In addition, most participants drove their bee transport vehicles (Akpınar and Bozkurt, 2021), but they stated that they were not professional drivers for these vehicles; they were concerned about the adverse effects of poor driving on bee health and welfare.

According to the participants, support payments are insufficient (10 TL support per hive). Participants said that the cost of mobile beekeeping activities is higher. Kösoğlu et al., (2021) emphasized that beekeeping policies and support payments should be updated by considering the field's realities. Problems related to apiary land (Sandal and Kan, 2013; Tunca and Çimrin, 2012) and beekeepers' accommodation in forest areas along the migration route have also been reported in many parts of the country (Korkmaz et al., 2018; Küçük et al., 2022).

The results show that both migratory beekeepers have sufficient awareness and skills about the official procedures, and Agriculture and Forestry Directorates provide beekeeper-friendly support. Nevertheless, migratory beekeepers stated that providing apiary land is challenging and complained about the problems such as high apiary land cost, insufficient security (theft, animal attacks, etc.), accommodation challenges (shelter, water, communications, electricity, etc.), and added the conflicts between the beekeepers that were located very close to each other in the same region. Similar results were reported by other researchers (Güneşdoğdu and Akyol 2019; Korkmaz et al., 2018; Küçük et al., 2022; Sandal and Kan 2013).

The beekeepers said they could not raise the queen they needed and had difficulty obtaining quality queen bees. So, it was thought that the queen bee enterprises could not meet the queen bee needs of the sector. Karaca and Karaman (2018) reported that although queen bee enterprises have low capacity, they raise queen bees with only half of the operating capacity due to high production costs and produce live bees and honey with the other half. Difficulties in obtaining queen bees were also detected in beekeeping enterprises in Trabzon (Küçük et al. 2022), Malatya (Kutlu and Kılıç 2020), and Diyarbakır (Demen, 2015).

In this study, migratory beekeepers said they need training on beekeeping and diseases. They reported difficulty accessing the required training (courses, Turkish books, online education tools, etc.). In general, it has been well-recognized that the educational status of beekeepers in Turkey is low (Küçük et al., 2022). However, studies on vocational training needs in beekeeping are scarce. In this study, migratory beekeepers reported their training needs for bee diseases, monitoring of honey quality, marketing, and some issues such as Apitherapy or organic beekeeping. It was thought that the migratory beekeepers were highly aware of their professional problems, so their training demands were high. Erkan and Aşkın (2001) also determined that migratory beekeepers feel more lack of knowledge and demand more training than stationary beekeepers.

For the participants, the most crucial problem of the beekeeping industry in Turkey is marketing. Beekeepers in Elazığ (Seven and Akkılıç, 2005), İzmir (Onuç et al., 2019), and Malatya (Kutlu and Kılıç, 2020) also emphasized the importance of marketing. They produce high-quality honey but have to market their products at a lower price than their quality, so they have difficulty competing with fake or low-quality honey in the same market. Onuç et al. (2019) reported that by producing residue-free and additive-free bee products beekeepers could market their products at a worthwhile price so businesses can gain a competitive and sustainable structure. The respondents said they do not know where and how to get these services and the cost of quality, residue, or fraud analysis. These results showed that migratory beekeepers demanded that the deficiencies in the beekeeping sector's production, quality, and organization axis be eliminated. Karacaoğlu et al. (2020) said that the herbal origin of honey (single-plant honey, multi-floral honey, etc.) or the labeling of it using geographical indications could reflect the quality of honey and increase the sales price. Küçük et al. (2022) reported that three-quarters of the beekeepers in Trabzon did not have residue analysis in honey, and those who did apply to institutions hundreds of kilometers away through the Rize Beekeepers Association. The migratory beekeepers in this research said they could not make enough progress in product packaging and branding. Bayramoğlu et al., (2016) also stated that the marketing of Bayburt honey by the producer increases informality. Migratory beekeepers argued that a few intermediary companies have an essential role in the lower wholesale price of honey, and they complain that honey quality inspections on honey quality are not widespread enough. There are not enough laboratories to test their honey's quality. Similar beekeeper complaints were reported from other provinces (Karahan et al., 2021; Küçük et al., 2022; Seven and Akkılıç, 2005). As a result, according to the migratory beekeepers in Afyonkarahisar, the most critical problems of the Turkish beekeeping

sector are that honey and other beekeeping products are not priced based on quality standards, unannounced agricultural pesticide spraying, the declining steadily of honey plant flora due to global climate change, apiary location, and accommodation problems, inability to obtain quality queen bees quickly, insufficient consultancy and training opportunities on bee diseases and beekeeping and high production costs. Increasing public economic support to improve the production performance and job satisfaction of migratory beekeepers who dominate honey production in Turkey are beneficial to increase the adaptability of migrating beekeeping to innovative breeding and competitive marketing strategies by supporting public regulations facilitating migration and beekeeper accommodation, determining quality standards in honey and other beekeeping products, and supporting the association of beekeepers.

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Conflict of Interest

We did not have any real, potential or perceived conflict of interest.

Ethical Approval

Permission was obtained for this study with the number AKUHADYEK-140-18. In addition, the authors declared that they comply with the Research and Publication Ethics.

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Investigation of Relationship among Dietary Fatty Acids, Milk Urea Nitrogen and Fertility Problems in Dairy Cattle Farms

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Abstract: The aim of this study was demonstrated the relationship between the nutritional variables of ration and the fertility parameters in the postpartum period in dairy cattle farms. All dairy cattle farms used in the present study had fertility problems (calving range ≥14 months and artificial insemination number ≥1.8). Ration and milk samples were taken from selected dairy cattle farms. Fertility records from herd registration systems were examined. In the study, milk urea nitrogen (MUN) levels of the milk samples were different between the farms; the lowest was 7.37 mg/dL, and the highest was 32.92 mg/dL (P<0.001). The artificial insemination number was negatively correlated with the monounsaturated fatty acid (MUFA) concentration of total mix ration (TMR) (r=-0.502; P<0.01). The rations at the beginning of lactation included average 31.09% of w-6 fatty acids, 1.99% of w-3 fatty acids, and 2.95% of w-9 fatty acids. The MUN concentration of milk was negatively correlated with long-chain fatty acids (LCFA) and linoleic acid concentrations of TMR (P<0.05). As a result, it can be said that the easy soluble carbohydrates, crude protein, oleic acid, w-3 and w-6 fatty acids and energy levels that may be related to fertility in dairy cattle should be well adjusted. It was concluded that targeted milk production and fertility could be achieved by feeding as many nutrients as genetic capacity allowed.
Keywords: *Dairy cattle, Energy, Fatty acids, Feed intake, Fertility, Milk urea nitrogen.*

Süt Sığırı Çiftliklerinde Rasyon Yağ Asitleri ile Süt Üre Azotu ve Fertilite Sorunları Arasındaki İlişkinin İncelenmesi

Özet: Bu çalışmanın amacı, süt sığırı işletmelerinde postpartum dönemde rasyonun besinsel değişkenleri ile döl verimi arasındaki ilişkiyi göstermekti. Çalışmada kullanılan süt sığırı işletmelerinin hepsinde infertilite sorunu (buzağılama aralığı ≥14 ay ve suni tohumlama sayısı ≥1.8) vardı. Seçilen süt sığırı işletmelerinde rasyon ve süt örnekleri alındı. Sürü kayıt sistemlerinden fertilite kayıtları incelendi. Çalışmada, süt örneklerinin süt üre nitrojeni (MUN) seviyeleri işletmeler arasında farklı olup, en düşük 7.37 ve en yüksek 32.92 mg/dL idi (P<0.001). Suni tohumlama sayısı, total miks rasyonun (TMR) tekli doymamış yağ asiti (MUFA) konsantrasyonu ile negatif korelasyon gösterdi (r=-0.502; P<0.01). Laktasyon başlangıcında infertilite sorunu rasyonların ortalama yağ asidi konsantrasyonları w-6 yağ asitlerinin %31.09'u, w-3 yağ asitlerinin %1.99'u ve w-9 yağ asitlerinin %2.95'i idi. Sütün MUN konsantrasyonu, uzun zincirli yağ asitleri (LCFA) ve TMR'nin linoleik asit konsantrasyonları ile negatif korelasyon gösterdi (P<0.05). Sonuç olarak, süt sığırlarında fertilite ile ilişkili olabilecek kolay çözünür karbonhidrat, ham protein, oleik asit, w-3 ve w-6 yağ asitleri ile enerji seviyesinin iyi ayarlanması gerektiği sonucuna varılabilir. Hedeflenen süt üretimi ve doğurganlığın, genetik kapasitenin izin verdiği ölçüde besinlerle beslenerek sağlanabileceği sonucuna varıldı.
Anahtar Kelimeler: *Enerji, Fertilite, Süt sığırı, Süt üre azotu, Yağ asitleri, Yem tüketimi.*

Introduction

The ration, such as crude protein (CP), rumen degradable protein (RDP), urea, and fatty acid profile, effects fertility efficiency in dairy cattle. It is desirable to have a certain level of ammonia formed by fermentation in the rumen (Elrod et al., 1993; Otto et al., 2014; Roy et al., 2011). Microorganisms that ferment non-structural carbohydrates (starch and sugar) in the rumen benefit from the protein (microbial protein) formed as a nitrogen source: microorganisms fermenting structural carbohydrates (cellulose, hemicellulose) require only ammonia as the nitrogen source for their metabolism (Russel et al., 1992). In addition to the need for ammonia for microbial growth in the rumen, it has been demonstrated that ammonia is needed for effective fiber digestion (Griswold et al., 1996). The most important factor that causes an increase in blood urea nitrogen (BUN) or milk urea nitrogen (MUN) values are the RDP, which is a ruminal ammonia source (NRC 2001). This excess urea may adversely affect fertility in dairy cattle in the postpartum period (McCormick et al., 1999; Roseler et al., 1993). Notably, high urea nitrogen values in the postpartum period delay the re-

initiation of ovarian activities of dairy cattle in the postpartum period, prolongs the postpartum first insemination period, and increase the interval between conception and calving (Elrod and Butler, 1993; Tamminga et al., 1997). The concentration of MUN in milk is used to determine how much of the CP taken by TMR is not used for microbial protein synthesis by the microorganism but is transferred to the general circulation. The dairy cattle TMR, rich in alpha-linolenic acid, can increase blood progesterone concentration. This hormone is necessary for the healthy continuation of pregnancy in dairy cattle. This hormone stimulates follicular and luteal cells, and progesterone synthesis increases (Lopez et al., 2005). The presented study hypothesizes that the difference in nutritional variables may cause infertility problems in dairy farms under field conditions. The present study aims to investigate, in terms of these nutrition criteria, the reproductive issues such as non-fertility and low pregnancy rates frequently encountered in the postpartum period in existing dairy farms. The data to be obtained will determine what measures the breeders can take against the problems arising from the ration.

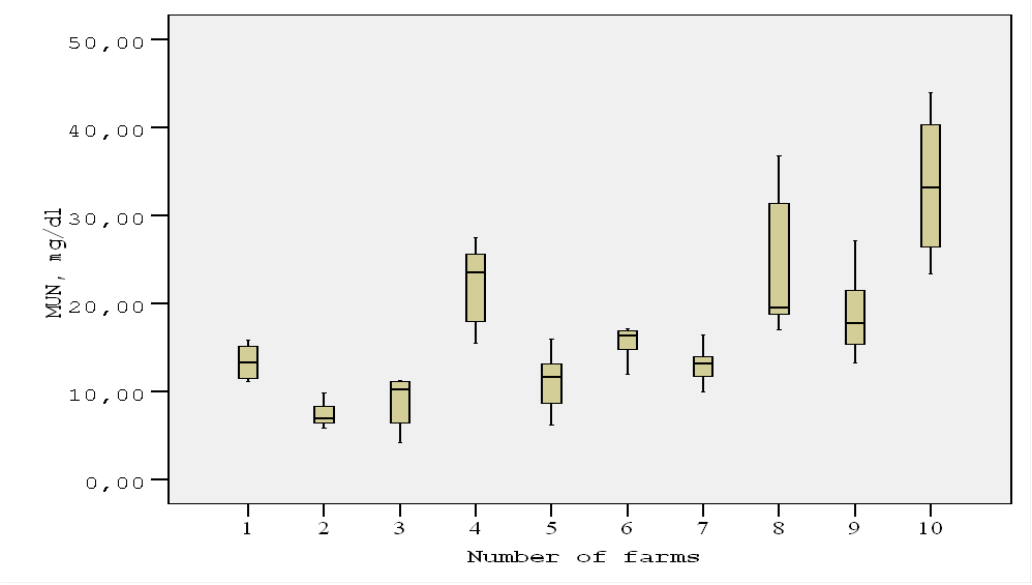

Figure 1. The MUN (milk urea nitrogen) concentrations of milk samples taken from dairy cattle.

Material and Methods

In the study, feed and milk samples were taken in dairy cattle enterprises, so there was no need for ethics committee approval or legal permission since no application was made on live animals.

We investigate the dairy cattle farms (≥25 cows) with infertility problems in the Nevşehir Province of Turkey. The farms' milk production and fertility data were obtained from the Breeding Cattle Breeders Association's e-Breeding Database (Table 1). The total mix ration (forage and concentrate feed) (Table 2) used by these dairy cattle farms were collected and analysed. The feed and milk samples were collected at two-week intervals.

The feedstuffs samples were taken in 2 kg of each ration component and airtight nylon bags (two weeks apart). The dried samples were analysed for crude protein (CP), ash, ether extract (EE), neutral detergent fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) contents (AOAC 1995; Van-Soest et al., 1991). The urea levels of concentrated feeds were determined in the UV-VIS spectrophotometer (SI Analytics, Germany) (R^2 = 0.9995) (Balthrop et al., 2011). All analyses were carried out in triplicate. The non-fibrous carbohydrate (NFC) values of TMR's were calculated according to NRC (2001). The total digestibility nutrient matter (TDN), digestibility energy (DE), metabolizable energy (ME), and net energy lactation (NEL) were calculated according to Donker (1989). Milk samples were taken from individual infertile dairy cattle, approximately 50 mL twice, two weeks apart. The samples were taken from 10 infertile dairy cattle in 10 different dairy farms. The milk samples were analysed for milk urea nitrogen concentration (MUN) using commercial kits in a MUN analyses device (cdR FoodLab Junior MUN, Italy). The EE of TMR's were methylated with the three-stage procedure of Wang et al. (2015). The free methylated fatty acids in n-hexane were detected in a gas chromatograph with a flame ionization detector (GC-FID, Thermo Scientific, USA) (Kara, 2020). The one-way variance analysis was conducted on the parameters tested in different dairy cattle

Table 1. Reproductive efficiency and other parameters of farms having fertility problems.

Number of farms	Breed of cattle	Number of lactation	Average milk yield of farms (L/day)	Number of insemination until conception	Ovarian cyst	Calving range, month	Approximate weight in the first 100 days, kg	Average milk yield, kg/day	DM intake, kg/day	Daily given feedstuffs, kg as DM							
										Commercial concentrate feed	Corn silage	Corn flake	Lucerne hay	Sugar beet pulp	Wheat straw	Oat hay	Meadow hay
1	*Holstein*	Multiparous	22	1.8	+	14.0	625	22	15.85	7.20	-	1.80	3.60	1.45	1.80	-	-
2	*Simmental*	Uniparous	26	1.8	+	14.5	557	26	18.76	5.40	6.16	-	4.50	-	2.70	-	-
3	*Holstein*	Multiparous	20	2.4	+	15.0	680	20	19.70	7.20	5.80	-	4.90	-	1.80	-	-
4	*Simmental*	Multiparous	18	2.5	+	14.5	680	18	16.70	7.20	-	-	3.6	2.32	3.60	-	-
5	*Simmental*	Multiparous	24	2.3	+	15.0	625	24	18.74	7.20	6.14	-	3.60	-	1.80	-	-
6	*Holstein*	Multiparous	24	2.6	+	15.0	680	24	19.45	7.20	4.15	1.80	3.60	-	2.70	-	-
7	*Holstein*	Multiparous	18	1.9	+	16.5	680	18	13.99	5.40	3.39	-	3.40	-	1.80	-	-
8	*Holstein*	Uniparous	26	2.1	+	13.5	557	26	17.33	8.10	-	-	3.60	1.13	2.70	-	1.80
9	*Holstein*	Uniparous	28	2.2	+	14.0	557	28	21.53	9.90	-	1.80	3.60	0.83	1.80	3.60	-
10	*Holstein*	Multiparous	25	2.0	+	14.5	680	25	19.09	7.20	4.69	-	4.50	-	2.70	-	-

Table 2. The chemical analysis values of dairy cattle TMR.

Farm No	CP	EE	Ash	NDF	ADF	HC	HemC	NFC	ADIN*	TDN	ME	NEL	CP**	Urea**
1	12.62^{c}	2.92^{b}	6.79^{c}	38.00^{c}	25.12^{c}	22.86^{ab}	12.88^{c}	39.66^{a}	3.89	61.01^{a}	2.34^{a}	1.39^{ab}	15.85^{ab}	0.13^{a}
2	11.64^{d}	2.79^{b}	8.18^{bc}	55.79^{a}	29.50^{a}	27.33^{a}	26.29^{a}	21.59^{c}	1.65	59.47^{b}	2.27^{b}	1.34^{b}	17.29^{ab}	0.12^{a}
3	11.84^{d}	5.17^{a}	8.95^{b}	46.44^{bc}	26.08^{c}	22.21^{ab}	20.36^{bc}	27.28^{bc}	2.11	60.67^{ab}	2.33^{a}	1.38^{ab}	15.93^{ab}	0.11^{ab}
4	12.15^{c}	3.42^{ab}	8.20^{bc}	49.00^{b}	30.36^{a}	$26.84a^{b}$	18.63^{c}	27.21^{bc}	3.86	59.17^{b}	2.26^{b}	1.34^{b}	15.28^{ab}	0.11^{ab}
5	12.16^{c}	2.83^{b}	9.57^{a}	46.15^{bc}	25.72^{c}	20.37^{b}	20.43^{bc}	30.07^{b}	2.00	60.79^{ab}	2.33^{a}	1.38^{ab}	16.47^{ab}	0.10^{b}
6	11.36^{d}	2.70^{b}	7.72^{c}	48.77^{bc}	23.48^{c}	25.34^{ab}	25.29^{a}	29.43^{bc}	2.47	61.58^{a}	2.37^{a}	1.40^{a}	15.07^{a}	0.11^{ab}
7	13.02^{b}	3.23^{b}	9.93^{a}	47.66^{bc}	25.09^{c}	21.58^{ab}	22.57^{b}	26.15^{bc}	3.50	61.01^{a}	2.34^{a}	1.39^{a}	18.66^{ab}	0.10^{b}
8	13.20^{b}	2.83^{b}	8.95^{b}	42.95^{c}	24.99^{c}	21.94^{ab}	17.96^{c}	32.05^{ab}	1.78	61.05^{a}	2.35^{a}	1.39^{a}	18.43^{ab}	0.10^{b}
9	13.61^{a}	2.98^{b}	8.60^{b}	41.12^{c}	24.42^{c}	21.69^{ab}	16.69^{c}	33.68^{ab}	2.04	61.25^{a}	2.36^{a}	1.40^{a}	19.50^{a}	0.09^{b}
10	12.45^{c}	3.01^{b}	8.25^{bc}	49.45^{bc}	28.68^{b}	27.25^{a}	20.77^{bc}	26.82^{bc}	1.84	59.76^{b}	2.29^{ab}	1.35^{b}	17.66^{ab}	0.11^{ab}
Means	12.40	3.19	8.51	46.53	26.34	23.74	20.19	29.39	2.51	60.57	2.32	1.37	17.04	0.11
Max.	13.66	7.07	10.06	56.65	30.41	28.41	27.19	40.77	4.32	61.60	2.37	1.41	19.80	0.14
Min.	11.26	2.34	6.70	34.55	23.41	12.57	9.56	20.66	1.44	59.15	2.26	1.34	14.44	0.10
SEM	0.12	0.16	0.16	0.95	0.41	0.58	0.80	0.96	0.21	1.43	0.006	0.004	0.35	0.002
P value	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	<0.001	<0.001	0.120	<0.001	<0.001	0.004	0.01	0.001

CP: crude protein, EE: diethyl ether extract, NDF: neutral detergent fiber, ADF: acid detergent fiber, HemC: hemicellulose, NFC: non-fibrous carbohydrate, ADIN: acid detergent nitrogen, TDN: total digestible nutrients, ME: metabolic energy (as Mcal/kg DM), NEL: net energy lactation (as Mcal/kg DM). $^{a-c}$: The difference between the average values indicated by different letters in the same column is important.
* The ADIN value is given as % CP in the ADF residue. **: these are as % in concentrate mix feeds, SEM: Standard error of means

Table 3. The compositions of fatty acid (g/100g fat) in dairy cattle TMR's.

Number of farm	w-3	w-6	w-9	w-3/w-6	LA	AA	ALA	EPA	SFA	UFA	MUFA	PUFA	MCFA	LCFA	VLCFA
1	1.17^{d}	31.23^{ab}	5.50	0.037^{cd}	26.15^{a}	44.67^{abc}	0.41^{ab}	0.25^{b}	61.73^{abc}	38.18^{bc}	5.78	32.40^{bcd}	0.06^{b}	97.08^{a}	2.76^{b}
2	1.44^{bcd}	26.26^{b}	5.75	0.055^{bcd}	21.66^{b}	44.94^{ab}	0.29^{b}	0.25^{b}	66.33^{ab}	33.65^{bcd}	5.94	27.71^{cd}	0.07^{b}	94.91^{ab}	4.99^{ab}
3	1.17^{d}	40.25^{a}	2.74	0.029^{d}	26.59^{a}	33.56^{bcde}	0.42^{ab}	0.25^{b}	55.01^{c}	44.99^{a}	3.57	41.42^{a}	0.19^{b}	95.71^{ab}	4.08^{ab}
4	1.26^{cd}	32.55^{ab}	3.34	0.038^{cd}	24.16^{b}	40.66^{abcd}	0.37^{ab}	0.24^{b}	62.62^{abc}	37.40^{bc}	3.58	33.82^{bc}	0.18^{b}	92.72^{ab}	7.11^{ab}
5	2.14^{abcd}	31.71^{ab}	0.29	0.067^{abcd}	24.83^{b}	40.27^{abcd}	0.69^{ab}	0.65^{ab}	65.59^{ab}	34.47^{bcd}	0.61	33.86^{bc}	0.20^{b}	96.08^{a}	3.70^{ab}
6	1.72^{bcd}	28.06^{b}	0.21	0.062^{bcd}	24.51^{b}	47.46^{a}	0.60^{ab}	0.53^{ab}	69.75^{a}	30.25^{d}	0.46	29.79^{bcd}	0.08^{b}	98.12^{a}	1.80^{b}
7	3.19^{a}	32.23^{ab}	1.89	0.099^{a}	22.27^{b}	31.87^{de}	0.88^{a}	0.77^{a}	62.82^{abc}	37.76^{bc}	2.33	35.42^{b}	0.41^{ab}	91.95^{ab}	7.93^{ab}
8	2.38^{ab}	26.34^{b}	3.13	0.091^{ab}	18.07^{b}	28.44^{de}	0.65^{ab}	0.65^{ab}	67.31^{a}	32.12^{cd}	3.39	28.72^{cd}	0.86^{a}	90.19^{ab}	8.15^{ab}
9	2.29^{abc}	31.22^{ab}	2.63	0.073^{abc}	21.66^{b}	32.44^{cde}	0.69^{ab}	0.56^{ab}	63.77^{abc}	36.40^{bcd}	2.88	33.51^{bc}	0.34^{ab}	91.06^{ab}	8.50^{ab}
10	3.13^{a}	31.05^{ab}	4.05	0.102^{a}	19.00^{b}	25.14^{e}	0.66^{ab}	0.57^{ab}	56.70^{bc}	38.63^{b}	4.45	34.18^{bc}	0.94^{a}	83.32^{b}	10.52^{a}
Means	1.99	31.09	2.95	0.066	22.89	36.94	0.56	0.47	63.16	36.38	3.30	33.08	0.33	93.11	5.95
Max.	3.45	42.98	9.44	0.12	32.28	49.22	0.95	0.83	70.59	48.54	9.88	43.73	1.63	99.34	11.65
Min.	0.75	24.90	0.18	0.02	13.42	21.79	0.20	0.06	46.92	29.43	0.38	26.60	0.03	71.16	0.61
SEM	0.79	4.16	2.60	0.02	0.68	1.49	0.04	0.04	1.65	0.79	0.48	0.74	0.35	5.52	3.53
P value	<0.001	<0.001	0.085	<0.001	0.033	<0.001	0.016	0.001	0.001	<0.001	0.109	<0.001	<0.001	0.019	0.008

ALA: α-Linolenic acid (C18:3n3), LA: linoleic acid, AA: Arachidic acid, EPA: cis-5,8, 11,14,17-Eicosapentaenoic Acid (C20:5n3), DHA: cis-4,7,10,13,16,19-Docosahexaenoic Acid (C22:6n3), LCFA: Long chain fatty acids, MCFA: Medium chain fatty acids, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids, SFA: Saturated fatty acids, UFA: Unsaturated fatty acids, VLCFA: Very long chain fatty acids, SEM: Standard error of means, $^{a-e}$: The difference between the average values indicated by different letters in the same column is important.

Table 4. Comparison of the ration and milk variables analysed with Pearson correlation(*r*).

	ME	NEL	CP	NDF	ADF	HemC	NFC	ADIN	MUN	AIN	MUFA	PUFA	LCFA	LA	ALA
TDN	0.996**	0.990**	0.297	-0.562**	-1.000**	-0.159	0.520**	-0.033	-0.183	0.610**	0.362*	0.021	0.257	0.111	0.419*
ME	1	0.986**	0.336	-0.588**	-0.996**	-0.191	0.538**	-0.027	-0.154	0.599**	-0.362*	0.045	0.224	0.095	0.441*
NEL		1	0.333	-0.581**	-0.990**	-0.187	0.532**	-0.008	-0.209	0.571**	-0.355	0.033	0.272	0.127	0.403*
CP			1	-0.586**	-0.297	-0.547**	0.431*	0.066	0.249*	-0.278	0.078	0.018	-0.384*	-0.378*	0.415*
NDF				1	0.562**	0.906**	-0.953**	-0.187	-0.060	-0.073	0.002	-0.126	-0.055	-0.076	-0.127
ADF					1	0.159	-0.520**	0.033	0.183	-0.611**	0.362*	-0.021	-0.257	-0.111	-0.418*
HemC						1	-0.871**	-0.236	-0.166	0.226	-0.183	-0.140	0.066	-0.034	0.063
NFC							1	0.192	0.067	0.056	-0.012	-0.050	0.143	0.104	0.059
ADIN								1	-0.071	-0.393	0.187	0.136	0.185	0.355	-0.034
MUN									1	-0.065	0.008	0.000	-0.597**	-0.376*	0.189
AIN										1	-0.502**	0.193	0.226	0.174	0.204
MUFA											1	-0.217	-0.327	-0.353	-0.548**
PUFA												1	0.081	0.500**	0.251
LCFA													1	0.781**	0.086
LA														1	0.027

NDF: neutral detergent fiber, ADF: acid detergent fiber, HemS: hemicellulose, NFC: fiber non-carbohydrate, ADIN: acid detergent nitrogen, CP: crude protein, MUN: Milk urea nitrogen, TDN: total digestible nutrients, ME: metabolic energy, NEL: net energy lactation, LCFA: long chain fatty acids, MUFA: monounsaturated fatty acid, PUFA: polyunsaturated fatty acid, LA: linoleic acid, ALA; Alpha linolenic acid, AIN: artificial insemination number. **: P< 0.01, *: P<0.05.

farms. Significance was defined at P values of <0.05. The relationship between the ration and milk variables was determined by the Pearson correlation and SPSS 15.0 package program.

Results

The amount and energy-protein content of rations at the beginning of lactation in dairy cattle farms in the Cappadocia region are given in Table 1. The nutrient matter and energy values of TMR's are given in Table 2. The MUN concentrations of milk samples taken from dairy cattle showed significant differences among farms (P<0.001); the lowest value was 7.37 mg/dL (in number 2 from farms), and the highest value was 32.92 mg/dL (in number 10 from farms) (Figure 1). The ratio of PUFA in the fatty acid profile of the TMR's ranged from 27% to 41%. Linoleic acid levels were between 18.07 and 26.59%. The alpha-linolenic acid level was between 0.29 and 0.88% (Table 3). The ME and NEL values of dairy cattle TMR were positively correlated with ALA and NFC levels of dairy cattle TMR and artificial insemination number and negatively correlated with NDF and ADF values of TMR (P<0.01). The CP value of TMR was positively correlated with MUN concentration (P<0.05). The MUN was negatively correlated with LCFA and linoleic acid of TMR (P<0.05). The artificial insemination number was negatively correlated with the MUFA of TMR (P<0.01) (Table 4).

Discussion

The DM consumption of the dairy cattle in some dairy cattle farms differed according to the amount of milk yield. In some farms, the animals decreased the feed consumption milk yield and the postpartum physiological needs of dairy cattle (NRC 2001). Previous researchers associated the low DM consumption of dairy cattle in early lactation with plasma cholecystokinin increase linearly during postpartum periods (Choi and Palmquist, 1996; Opara et al., 1994). Uterine involution at the beginning of lactation to 30-35 days to continue to reach maximum feed intake by the rumen is another reason (NRC, 2001). The CP levels were slightly lower than the CP requirement calculated by the formula, but these values were lower than the genetic capacity of the herd (NRC, 2001). Likewise, it was found that the ME and NEL values of TMR's were enough according to the current milk yield of the animals, but they were far from the high milk production targeted by the genetic capacity (Alderman et al., 2001). In general, it is understood that dairy cattle in the farms are undernourished in terms of milk yield and have a low milk yield average. For ideal rumen fermentation in dairy cattle diets, 25-33% NDF, 17-21% ADF, and 44-36% NFC in the TMR are required (NRC, 2001). The mean TDN values (60.5%) of dairy cattle TMR were lower than the recommended TDN values (68 or 78%) of TMR's at early lactation for large dairy cattle breeds by NRC (2001). The low energy and TDN of the TMR consumed by dairy cattle may be related to the problem of fertility. Ovarian follicles in dairy cattle contain insulin receptors (Bossaert et al., 2010). Postpartum ovarian activity resumption and average oestrus cycle retention in cows can delay due to insufficient peripheral insulin levels in the immediate postpartum period with low NFC levels of TMR (Van holder et al., 2005). Therefore, the glycogenic TMR's in the postpartum period are recommended to increase peripheral insulin concentrations and for normal ovarian activities of dairy cattle (Gong et al., 2002). The dairy cattle organism directed to gluconeogenesis due to the high energy is needed for milk yield after calving. When plasma glucose levels decrease, the fat mobilizes from the fat stores in the organism and tries to provide the necessary energy until the energy balance shifts to positive (Gong et al., 2002; Adewuyi et al., 2005). In the present study, it is thought that dairy cattle farms experience fertility problems at the beginning of lactation due to low NFC, fat and energy levels, and possible ration digestibility.

The CP contents of dairy cattle TMR's in the investigated farms had a positive correlation with MUN values that were parallel with the results of Elrod et al. (1993). The effect of dietary CP or nitrogenous compounds on milk MUN value can make occur the urea formation in the liver with a breakdown of RDP in the rumen and the absorbed amino acids by the degrading of RUP in the intestine. Under normal conditions, microbial protein is produced by microorganisms in the presence of alpha-keto acids by microbial fermentation from ammonia in the rumen. However, excess ammonia is absorbed from the rumen and mixed with the liver to the general circulation, causing blood and MUN levels to rise (Roy et al. 2011). Consistent with our results, Roy et al. (2011) stated that the high NDF value in dairy cattle TMR's and the increase in rumen pH value could increase $NH4^+$ absorption and blood transfer from the rumen wall and increase the MUN value. The 10-14 mg/dL MUN concentration is considered normal for the MUN value in milk, whereas <10 mg/dL can be association with low CP or RDP in TMR or effective conversion of ruminal ammonia to microbial protein, and >14 mg/dL MUN value may be effective NFC insufficiency in TMR (Aydın and Güler, 2004; Aydın, 2007). In the present study, the milk MUN values in the two farms were

less than 10 mg/dL, although the CP value of TMR's did not lower significantly may be ruminal low NFC concentration. The MUN value was found to be higher than 14 mg/dL in half of the investigated farms. The difference in MUN values despite similar CP consumption in the farms examined suggests that other TMR contents and environmental conditions may also be helpful. Elrod et al. (1993) reported that the protein in the TMR is effective in the uterine environment and that high pH in the ration may be associated with decreased fertility in the uterus. In the present study, it is thought that increasing MUN value may decrease uterine pH and may adversely affect embryo implantation.

Most of the w-3's found in dairy cattle TMR's are obtained from forage. Significant levels of LA, oleic acid and ALA are feedstuffs of sunflower, rapeseed, flaxseed, soybean, corn, safflower, flaxseed, soybean, peanut and canola (Chong et al., 2006). Infertility is considered a grave problem in the dairy industry due to the increased number of artificial inseminations per conception and the irregular shaping of the oestrus (Lopez et al., 2005). A negative correlation between the number of seeding and ration MUFA concentration in the present study seems to be related to oleic acid concentration. Mobilization of fatty acids from adipose tissue during metabolic stress at the onset of lactation will increase the amount of free fatty acids in the blood and follicular fluid and thus affect oocyte quality. In a previous study, the effect of three fatty acids (saturated palmitic and stearic acid and unsaturated oleic acid) on lipid storage and development of the oocyte was investigated and it was found that palmitic and stearic acid had an inhibitory effect on oocyte development, but MUFA oleic acid eliminated this adverse effect and has a positive impact (Aaderma et al., 2011). In the present study, a negative correlation between milk MUN concentration and ration LCFA and linoleic acid levels, and the use of ammonia for microbial protein production by rumen microorganisms may be due to LCFA and linoleic acid reduction.

In conclusion, this study demonstrated that possible problems related to TMR in dairy cattle farms having fertility problems can be listed as follows: It is thought that dairy farms experience fertility problems in the postpartum period due to low energy levels and possible low digestibility (approximately 60% TDN) in TMR's. The low levels of oleic acid and w-3 and w-6 fatty acids are thought to be the cause of fertility problems in dairy cattle. High NDF values in dairy cattle TMR's increase in rumen pH may increase NH_4^+ absorption and increase MUN value in the milk. Not enough ammonia is used in microbial protein production, which may result from low NFC levels in diets, and the urea in general circulation may decrease the pH of the uterus. Due to the high level of NDF and ADF, dairy cattle may enter a negative energy balance.

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Antimicrobial Resistance and Virulence Genes of Enterococci Isolated from Water Buffalo's Subclinical Mastitis

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Abstract: This study aimed to investigate the antimicrobial resistance and virulence genes of enterococci isolated from water buffalo's subclinical mastitis cases. The antimicrobial susceptibilities of the isolates were determined by the disc diffusion method. Identification at the species level of enterococci, virulence [aggregation substance (*asa*1), gelatinase (*gel*E), cytolysin (*cyl*A), enterococcal surface protein (*esp*), and hyaluronidase (*hyl*)] and resistance genes [macrolide (*erm*A, *erm*B, *mef*A/E) and tetracycline (*tet*K, *tet*L, *tet*M, *tet*O, and *tet*S)] were investigated by polymerase chain reaction (PCR). Overall, *Enterococcus* spp. was recovered from 65 of 200 (32.5%) mastitic milk samples, comprising *E. faecium* (n=26), *E. durans* (n=22), *E. faecalis* (n=12), and *E. hirae* (n=5). Most isolates (56.9%) were susceptible to all tested antibiotics. The rest of the isolates showed various rate of resistance against rifampicin (23.1%), tetracycline (21.5%), quinupristin-dalfopristin (10.8%), ciprofloxacin (7.7%), erythromycin (6.2%), and chloramphenicol (3.1%). Out of 65 enterococci, only 16 (24.6%) were detected to have virulence genes, of which 12 were positive for *gel*E, seven were positive for *esp*, two were positive for *asa*1, and one was positive for *hly*A. The gene *cyl*A was not detected in any isolate tested. Resistance to tetracycline was mainly associated with *tet*M. Two erythromycin-resistant isolates were positive for *erm*B, and one was positive for *mef*A/E. This study was the first to report species distribution, antimicrobial susceptibility, and virulence traits of enterococci isolated from subclinical mastitis of water buffaloes in Çorum Province, Türkiye.
Keywords: *Antimicrobial resistance*, *Enterococci*, *Subclinical mastitis*, *Virulence*, *Water buffalo.*

Subklinik Manda Mastitislerinden İzole Edilen Enterokok Türlerinde Antimikrobiyal Direnç ve Virulans Genleri

Özet: Bu çalışmada subklinik manda mastitis vakalarından izole edilen enterokokların antimikrobiyal direnç ve virülans genlerinin araştırılması amaçlanmıştır. İzolatların antimikrobiyal duyarlılıkları disk difüzyon yöntemi ile belirlendi. Enterokokların, tür düzeyinde identifikasyonu, virülans (*asa1*, *gelE*, *cylA*, *esp* ve *hyl*) ve direnç genleri [makrolid (*ermA*, *ermB*, *mefA/E*) ve tetrasiklin (*tetK*, *tetL*, *tetM*, *tetO* ve *tetS*)] polimeraz zincir reaksiyonu (PZR) ile araştırıldı. İncelenen 200 mastitisli süt örneğinin 65'inden (%32,5) *Enterococcus* spp. izole edildi ve izole edilen türler *E. faecium* (n=26), *E. durans* (n=22), *E. faecalis* (n=12) ve *E. hirae* (n=5) olarak identifiye edildi. İzolatların %56,9'u test edilen tüm antibiyotiklere duyarlı bulundu. İzolatların geri kalanı rifampisin (%23,1), tetrasiklin (%21.5), kuinupristin-dalfopristin (%10,8), siprofloksasin (%7,7), eritromisin (%6,2) ve kloramfenikol (%3,1)'e karşı çeşitli direnç oranları gösterdi. İzole edilen 65 *Enterococcus* spp.'nin sadece 16'sının (%24,6) virülans genlerine sahip olduğu tespit edildi. Virulens genlerine sahip izolatların 12'si *gelE*, yedisi *esp*, ikisi *asa1* ve biri de *hlyA* yönünden pozitif bulundu. *cylA* geni incelenen hiçbir izolatta saptanmadı. Tetrasikline direncin esas olarak *tetM* ile ilişkili olduğu saptanırken; eritromisine dirençli iki izolat *ermB* ve bir izolat ise *mefA/E* geni yönünden pozitif bulundu. Bu çalışma, Türkiye'nin Çorum ilinde yetiştirilen mandalarda saptanan subklinik mastitisli süt örneklerinden izole edilen enterokokların tür dağılımı, antimikrobiyal duyarlılık ve virülans özelliklerini bildiren ilk çalışmadır.
Anahtar kelimeler: *Antimikrobiyal direnç*, *Enterococcus* spp., *Manda, Subklinik mastitis*, *Virulens.*

Introduction

Based on the data received from Turkish Statistical Institute (TSI), 63 643 tons of milk were obtained from the registered 185 574 water buffalos in Türkiye in 2021 (TSI, 2021). Water buffalo milk is about 5% of the total world milk production (Atasever and Erdem, 2008), and 0.3% of the milk production in Türkiye (TSI, 2021). Even though milk yield per animal is low in comparison with cows, the quantity and quality of the water buffalo milk are of great importance both for producers and consumers (Şahin and Yıldırım, 2015). Therefore, water buffalo breeding started to be supported by the Ministry of Agriculture and Forestry in recent years (Sarıözkan, 2011).

As in other animal species, there are many factors affecting milk yield in water buffalos, such as the number of lactations, calving season, and diseases. Of these diseases, mastitis is considered a global problem that affects milk yield and quality, thus causing serious economic losses (Singha et al., 2021). Mastitis pathogens are divided into two groups depending on the infection source: contagious or environmental pathogens. Although mastitis control programs are effective against contagious mastitis pathogens, these mastitis control programs are less effective against environmental pathogens, such as enterococci (Yang et al., 2019).

Antimicrobials are widely used for the control and prevention of mastitis cases. However, treatment success is mainly limited due to the antimicrobial resistance against these pathogens (Saini et al., 2019). Another factor that affects the frequency of mastitis cases is the virulence traits of pathogens (Yang et al., 2019). Several virulence factors have been described in enterococci including cytolysin (*cyl*A), hyaluronidase (*hyl*), aggregation substance (*asa*), enterococcal surface protein (*esp*), and gelatinase (*gel*E) (Vankerckhoven et al., 2004). Of these virulence genes, cytolysin is a bacteriocin-type exotoxin, exerts its effects on erythrocytes, leukocytes, and macrophages. Geletinase is zinc-dependent metalloendopeptidase, is capable of hydrolysing gelatine, elastin, collagen, haemoglobin. Aggregation substance is enterococcal surface protein that contributes the formation of mating aggregates facilitating bacterial conjugation. Enterococcal surface protein is known to be involved in biofilm formation. Biofilm production has been shown to play an important role in the exchange of antibiotic resistance genes between cells and to increase their resistance to antibiotics. Hyalurinidase plays a role in destroying mucopolysaccharides of the connective tissue and cartilage and, consequently, in spreading bacteria (Chajęcka-Wierzchowska et al. 2017).

Little information exists about enterococci from milk samples of water buffaloes both in Türkiye and in the world. Therefore, in this study, it was aimed to investigate the antimicrobial resistance and virulence genes of enterococci isolated from subclinical mastitic milk samples of water buffaloes in Türkiye.

Materials and Methods

Ethical statements: This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Milk Samples: A total of 200 mastitic milk samples of water buffaloes were collected from family-sized farms located in Çorum, Türkiye, between June 2018 and July 2018. CMT test was applied to buffaloes that did not show clinical signs in mammary tissue and milk, and subclinical mastitis was evaluated by CMT test results.

Bacterial isolation and identification: The milk samples (100 µl) were inoculated into Enterococcosel broth (BD, UK) and incubated at 37 ^{o}C for 24-48 h. When the color change occurred in the Enterococcosel broth tubes, a loopful of culture was inoculated on Vancomycin Resistant Enterococci (VRE) agar plates and incubated for 48 h at 37 ^{o}C. Subsequently, one presumptive colony from each plate was randomly selected and streaked onto Blood agar (Merck, Germany) supplemented with 5% defibrinated sheep blood to obtain pure culture. Following Gram staining and biochemical tests, the isolates were confirmed and identified by the PCR method (Layton et al., 2010).

Antimicrobial susceptibility testing: Antibiotic susceptibilities of the isolates were determined by disc diffusion methods using Mueller Hinton Agar (Merck, Germany), following Clinical and Laboratory Standards Institute (CLSI) criteria (CLSI, 2021). The antimicrobial agents (Bioanalyse, Türkiye) were as follow: ampicillin (10 µg), quinupristin/dalfopristin (15 µg), chloramphenicol (30 µg), tetracycline (30 µg), ciprofloxacin (5 µg), erythromycin (15 µg), vancomycin (30 µg), rifampicin (5 µg), and gentamicin (120 µg). *Staphylococcus aureus* ATCC 25923 was used as a quality control strain. Multidrug resistance (MDR) was defined as resistance against at least 3 antimicrobial agents belonging to different antimicrobial classes (Magiorakos et al., 2012).

Detection of macrolide and tetracycline resistance genes: The presence of macrolide (*erm*A, *erm*B, *mef*A/E) and tetracycline resistance genes (*tet*K, *tet*L, *tet*M, *tet*O, and *tet*S) were investigated using PCR as previously reported by Malhotra-Kumar et al. (2005).

Detection of virulence genes: Virulence genes (*asa1*, *cylA*, *esp*, *gelE*, and *hyl*) were investigated as previously reported by Vankerckhoven et al. (2004).

Results

Isolation and identification: Of the 200 mastitic milk samples, 65 (32.5%) *Enterococcus* spp. were isolated and the distribution of enterococci was detected as follows: 26 *E. faecium* (40%), 22 *E. durans* (33.8%), 12 *E. faecalis* (18.5%), and 5 *E. hirae* (7.7%) (Figure 1-2).

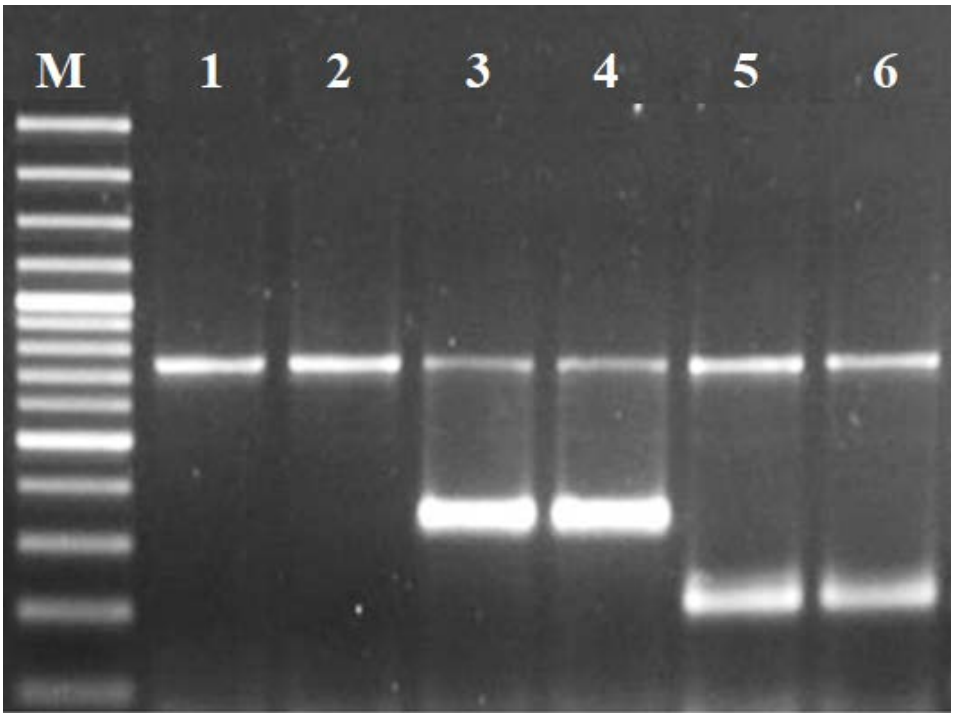

Figure 1. Agarose gel electrophoresis of *Enterococcus* species. Lane M: 100 bp plus molecular marker, Lane 1-2: *Enterococcus* spp. (733 bp), Lane 3-4: *E. faecalis* (360 bp), Lane 5-6: *E. faecium* (214 bp).

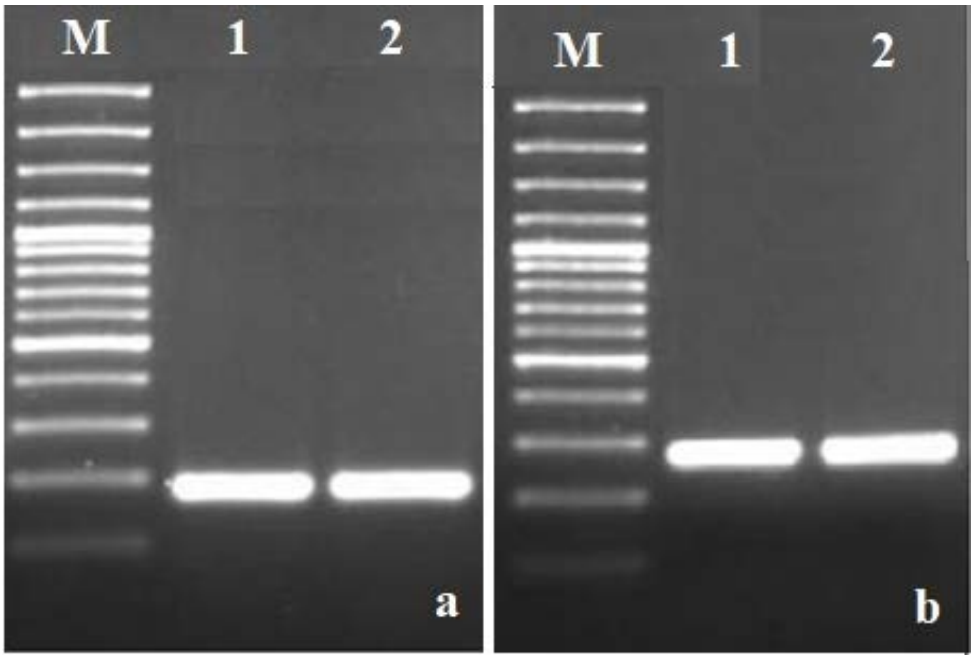

Figure 2. Agarose gel electrophoresis of *E. durans* and *E. hirae* isolates. Lane M: 100 bp plus molecular marker. **Figure 2a.** *E. hirae* (186 bp), F**igure 2b:** *E. durans* (286 bp)

While 37 (56.9%) of the isolates were susceptible to all antibiotics tested, 28 (43.1%) isolates showed various rate of resistance to tetracycline (21.5%, 14/65), rifampicin (23.1%, 15/65), ciprofloxacin (7.7%, 5/65), erythromycin (6.2%, 4/65), quinopristin-dalfopristin (10.8%, 7/65), and chloramphenicol (3.1%, 2/65).

Determinants of erythromycin and tetracycline resistance: In tetracycline-resistant isolates, 12 isolates carried *tet*M and one isolate carried *tet*L. Among erythromycin-resistant isolates,

three isolates were positive for *erm*B and one isolate was positive for *mef*A/E.

Virulence gene profiles of the isolates: In 21.5% (14/65) of the isolates virulence genes were detected. These isolates were found to carry one or more virulence genes. Twelve isolates were positive for *gel*E, seven were positive for *esp*, two were positive for *asa*1 and one was positive for *hly*A. The gene *cyl*A was not detected in any isolate tested (Figure 3). Resistance phenotypes, virulence, and resistance genes determined in *Enterococcus* spp. are given in Table 1.

Table 1. Resistance phenotypes, virulence and resistance genes determined in *Enterococcus* spp.

Species	**Resistance Phenotype***	**Resistance Genotype**	**Virulence Gene(s)**
E. faecium (n=2)	CIP, RA	-	-
E. faecium	E	*mef*A/E, *tet*K	-
E. faecium	E	-	-
E. faecium	RA	-	*gel*E, *esp*
E. faecium	TE, SYN	-	-
E. faecium (n=2)	RA	-	-
E. faecium (n=3)	CIP	-	-
E. faecalis	TE, RA, SYN	*tet*M	-
E. faecalis	TE, RA, SYN	*tet*M	*gel*E, *esp*
E. faecalis	TE	*tet*M	*gel*E, *esp*
E. faecalis	SYN	-	*asa*1, *gel*E
E. faecalis	SYN	-	*gel*E
E. faecalis (n=2)	TE, RA	*tet*M	*gel*E, *esp*
E. faecalis	TE, RA	*tet*M	*gel*E
E. faecalis	TE, RA	*tet*M	*gel*E
E. faecalis	E, TE, RA, SYN, C	*erm*B, *tet*M	*esp*
E. faecalis	E, TE, RA, SYN, C	*erm*B, *tet*M	*esp*
E. durans (n=3)	TE	*tet*M	-
E. durans	TE	*tet*M, *tet*L	*asa*1, *gel*E
E. durans (n=2)	RA	-	-
E. hirae (n=1)	Sensitive	-	*hlyA*
E. durans (n=1)	Sensitive	*erm*B	-
E. durans (n=15)	Sensitive	-	-
E. hirae (n=4)	Sensitive	-	-
E. faecium	Sensitive	-	*gel*E, *esp*
E. faecalis	Sensitive	-	*gel*E
E. faecium	Sensitive	-	*gel*E
E. faecium (n=13)	Sensitive	-	-

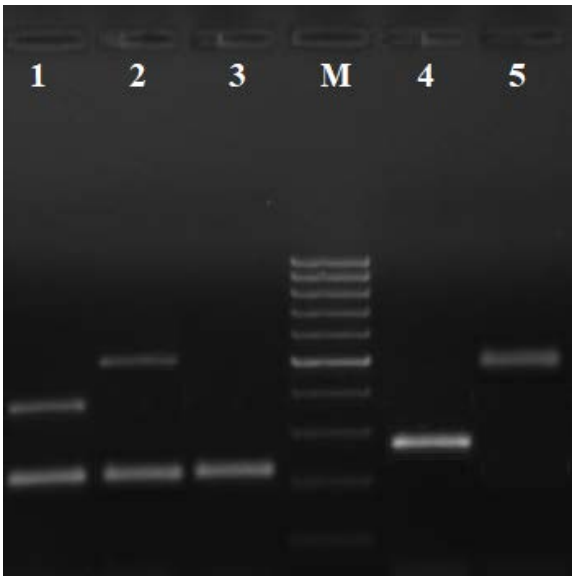

Figure 3. Agarose gel electrophoresis of virulence genes. Lane M: 100 bp molecular marker. Lane 1: *asa1* (375 bp)+*gelE* (213 bp), Lane 2: *esp* (510 bp)+*gelE* (213 bp), Lane 3: *gelE*, Lane 4: *hlyA* (276 bp), Lane 5: *esp*

Discussion

Although enterococci are considered as commensal inhabitants of gastrointestinal microbiota both in humans and animals, these agents have increasingly been reported in mastitic milk samples (Yang et al., 2019). In this study, the prevalence of enterococci was determined as 32.5%. In different studies carried out in Türkiye, the prevalence of enterococci in milk samples of bovine subclinical mastitis cases was reported to be 0.7% (3/421) in Samsun (Gürler et al., 2015), 10.9% (43/392) in Afyon (Kuyucuoğlu, 2011), and 16% (96/600) in Aydın (Herkmen and Türkyılmaz, 2016). On the other hand, in the studies conducted abroad, the prevelance of enterococci were reported as 4.8% (105/2185) in Korea (Nam et al., 2010), 15.23% (27/177) in Canada (Cameron et al., 2016), 16.7% (112/669) in the Czech Republic (Cervinkova et al., 2013), and 21.3% (426/2000) in Poland (Różańska et al., 2019). In the above-mentioned studies, the presence of different Enterococcus species has also been reported. In the majority of these studies, *E. faecalis* was identified as the predominant species (Kuyucuoğlu, 2011; Nam et al., 2010; Cameron et al., 2016; Różańska et al., 2019). In contrast, *E. faecium* and *E. durans* were found to be the predominant species in this study. While similar observation was reported by Kateete et al. (2013) in Uganda, Klimiene et al. (2011) found *E. durans* as the predominant species in Lithuania. Variations in prevalence rates and species distribution could be attributed to the isolation methods, geographical origins of the samples, and differences in rearing conditions.

Despite enterococci having intrinsic resistance to antimicrobials such as beta-lactams, lincosamides, cephalosporins, trimethoprim, and aminoglycosides (low level), emergence and dissemination of acquired resistance is mainly related to over-and misuse of antimicrobial agents e.g. tetracyclines, ciprofloxacin, daptomycin, erythromycin, linezolid, quinupristin-dalfopristin, and vancomycin. While most of the isolates were susceptible to antimicrobials tested, the rest showed moderate levels of resistance to rifampicin (23.1%) and tetracycline (21.5%) in this study. In previous studies, high resistance rates to tetracycline and erythromycin has been reported in enterococci isolated from subclinical bovine mastitis cases (Yang et al., 2019; Kuyucuoğlu, 2011; Nam et al., 2010). The high resistance observed for these agents could be explained by the long-term and widespread use of these antimicrobials in food-producing animals (Yang et al., 2019).

Horizantal transfer of antimicrobial resistance genes between bacteria is of important concern for both human and veterinary medicine (Aslam et al., 2012). In this study, the *tet*M was the most frequent resistance gene detected among the tetracycline-resistant isolates. Similarly, the dominance of *tet*M in tetracycline-resistant enterococci from different sources such as meat (Yılmaz et al., 2016), cheese (Kürekci et al., 2016), and dogs (Boyar et al., 2017) have been reported in previous studies conducted in Türkiye. Kim et al. (2019) explained the widespread occurrence of *tet*M (providing ribosomal protection) in tetracycline-resistant enterococci by localization of this gene on conjugative transposons such as Tn916, Tn1545, and Tn5385 leading to the easy spread of this gene among enterococci. Moreover, among four erythromycin-resistant enterococci, *erm*B in three isolates, and *mef*A/B in one isolate was detected. But, one isolate did not carry any gene studied. Similarly, previous studies revealed the *erm*B gene was the most dominant gene found in enterococci from animals (Boyar et al., 2017; Aslantaş, 2019) and food of animal origin (Yılmaz et al., 2016).

Enterococci are capable of producing various virulence factors that play an important role in the pathogenesis of the infections they cause (Mundy, 2000). The five virulence genes examined in this study were reported to contribute to the virulence of enterococci (Vankerckhoven et al., 2004). Of these virulence genes, the *gel*E is a metalloproteinase, capable of hydrolyzing casein, hemoglobin, collagen, gelatine, elastin as well as various peptides and proteins (Chajęcka-Wierzchowska et al., 2017). In this study, the *gel*E was found in 18.5% of the

isolates. In contrast, Yang et al. (2019) reported a higher prevalence rate (70.4%) in *E. faecalis* isolates. Herkmen and Türkyılmaz (2016) found *gel*E gene in 30.7% of *E. faecium* isolates. Another virulence factor, the aggregation substance encoded by the *asa*1 gene is an enterococcal surface protein contributing to the formation of mating aggregates that facilitates the conjugation of bacteria (Sava et al., 2010). In the current study, 3.1% of the isolates carried the *asa*1 gene. In contrast, a higher rate of prevalence (24.7%) of this gene was reported by Yang et al. (2019) in China. The *esp* gene encoding enterococcal surface protein has been reported to be related to biofilm formation in enterococci (Sava et al., 2010). This gene was found in seven (10.8%) isolates in this study. The prevalence of this gene was reported as 85.2% by Yang et al. (2019) and 30.7% by Herkmen and Türkyılmaz (2016). Hyaluronidase is an enzyme with a molecular weight of approximately 45 kDa and encoded by the *hyl* gene. The enzyme plays important role in degrading the connective and cartilage tissue glycosaminoglycans (mucopolysaccharides), consequently leading to the spread of the bacteria (Chajęcka-Wierzchowska et al., 2017). This gene was detected in only one (1.5%) *E. hirae* isolate in the study. Similarly, in China, Yang et al. (2019) reported that they found the *hyl* gene only in a few *E. faecalis* isolates (2.5%, 2/81) from subclinical bovine mastitis.

In conclusion, the results of the study showed that enterococci isolated from subclinical buffalo mastitis had low levels of resistance to antimicrobials tested and revealed a low carriage rate of virulence genes. This might be explained by no or low transmission of virulent and antimicrobial-resistant enterococci via human intervention.

Conflict of Interest

The authors stated that they did not have any real, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Similarity Rate

We declare that the similarity rate of the article is 14% as stated in the report uploaded to the system.

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Author Contributions

Motivation / Concept: ÖA, EKÜ, MAY
Design: ÖA, EKÜ, YE
Control/Supervision: ÖA
Data Collection and Processing: ET, ÖA, MAY
Analysis and Interpretation: ET, ÖA, EKÜ
Literature Review: ÖA
Writing the Article: ÖA, EKÜ
Critical Review: ÖA

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Fundus Imaging via Infrared Camera without Mydriatics in Holstein Calves

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Abstract: This study aimed to record the ocular fundus images of healthy Holstein calves under field conditions. The fundus of 34 eyes of healthy 17 Holstein calves was examined with a fundus camera, which does not require mydriatics, as it has been designed especially for animals and provides imaging with infrared light. The findings showed that the green-yellow tapetal zone was dominant in all calves, the optic disc was oval, and the number of primary arteries and veins originating from the center varied between 4 and 5. The vascular pattern was holangiotic. A remnant of the hyaloid artery (Bergmeister's papilla) was detected as a gray dot in the middle of the disc. It was noted that the non-tapetal area was homogeneous, brown-black, and rich in choroidal vascular structure. Imaging the ocular fundus is essential in diagnosing some systemic and hereditary diseases in farm animals. However, herd-based ophthalmoscopic screening in farm animals is difficult under field conditions. By using this portable fundus camera, fundus examination can be performed easily under field conditions without taking the animals to the hospital. The standard ophthalmoscopic fundus images of healthy Holstein calves presented in this study will contribute to the literature.
Keywords: *Calf, Fundus photography, Ocular fundus, Retina*.

Holstein Buzağılarda Midriyatik Ajan Kullanmadan İnfrared Kamerayla Fundus Görüntülenmesi

Özet: Çalışmanın amacı sağlıklı Holstein buzağıların oküler fundusunun saha şartlarında görüntülenmesidir. Sağlıklı 17 Holstein buzağının 34 fundusu midriatiklerin kullanımını gerektirmeyen, hayvanlar için özel olarak üretilmiş bir fundus kamerası ile incelendi. Tüm buzağılarda yeşil sarı tapetal bölge baskın, optik disk ovaldi. Merkezden çıkan primer arter ve toplardamar sayısı 4-5 arasında değişmekteydi. Vasküler desen holanjiyotik olarak kaydedildi. Tüm buzağılarda diskin ortasında gri nokta şeklinde hyaloid arter kalıntısı (Bergmeister's papilla) tespit edildi. Tapetal olmayan bölgenin homojen, kahverengi-siyah renkte ve koroid damar yapısından zengin olduğu kaydedildi. Çiftlik hayvanlarında bazı sistemik ve kalıtsal hastalıkların tanısında oküler fundusun görüntülenmesi çok önemlidir. Ancak çiftlik hayvanlarında sürü bazlı oftalmoskopik tarama saha koşullarında oldukça zordur. Portatif fundus kamerası ile hastaların saha şartlarında hastaneye getirilmeden kolaylıkla fundus muayenesi yapılabilmiştir. Bu çalışmada sağlıklı Holstein buzağıların normal oftalmoskopik fundus görüntüleri sunulmuştur.
Anahtar Kelimeler: *Buzağı, Fundus görüntüleme, Oküler fundus, Retina*.

Introduction

The retina is the eye layer containing light-sensitive cells enabling vision. Defects and disorders formed in the retina, which is associated with the brain, affect the sense of sight. In order to detect visual impairments, we need to know the normal function of the retina and the diseases affecting the retina. Early identification of conditions affecting the retina without causing irreversible damage is vital in terms of early treatment and prognosis. Diagnosis of retinal disorders can be made with direct ophthalmoscopy, indirect ophthalmoscopy, smartphone-based ophthalmoscopy, scanning laser ophthalmoscope, optical coherence tomography, fluorescein angiography, ultrasonography, and electroretinography methods (Pearce and Moore, 2013; Şirin, 2020).

Diseases such as bovine virus diarrhea (BVD), rabies, malignant catarrhal fever (MCF), infectious meningoencephalitis, listeriosis, septicemia, particularly in calves with scours and umbilical infections, tuberculosis, and toxoplasmosis can cause retinal damage. Findings of tapetal color changes and loss of texture, attenuation of retinal vessels, focal depigmentation, varying degrees of retinitis, and choroiditis in the non-tapetal region can be observed (Martin et al., 2019). Thorough knowledge of the normal ocular fundus image is essential to diagnose diseases.

Considering how common eye diseases are in bovine (Gökçe and Gençelep, 2021; Han et al., 2019; İşler et al., 2014; Sarierler and Kilic, 2003), it is necessary to have a good knowledge of the healthy fundus image to perform a detailed eye examination. The normal fundus of animals consists of the neurosensory retina, retinal pigment epithelium, choroid, sclera, optic nerve head, and tapetum. Each

species has a typical retinal fundus (Rajathi and Muthukrishnan, 2020). There are some detailed studies on the presence of retinal tapetum, visibility of choroidal vessels, retinal vascular pattern, optic disc position and shape, nerve fiber location, and gross anatomic findings in cattle (Çatalkaya and Özaydin, 2019).

Due to the large number of animals in dairy cattle farms, veterinarians have limited time. Fundoscopy with fundus cameras requires the use of mydriatic agents. Fundus camera needs a wired computer connection. There are many determining factors such as examination area, the need for more staff for restrain, waiting time for mydriasis, and drug cost. In dairy cattle farms, a fundoscopic examination should be fast, easy to apply, and inexpensive. This study aimed to obtain detailed standard fundus imaging of Holstein's calves under farm conditions with a portable non-mydriatic fundus camera.

Material and Methods

Animals: The study was conducted on the Arif Gürdal Dairy Farm. The Evaluation was made of 17 healthy male and female Holstein calves aged 3.5-4.5 months.

Ethical approval was granted by The Animal Research Ethics Committee ADÜ HADYEK (64583101/2021/169).

Fundus Examinations: All the calves included in the study underwent ophthalmological examination. All the animals were healthy, with no ophthalmological anomalies. The fundus examination of 34 eyes was performed with no drug administration to the animals using Ocellus-Vet (Vivente, Türkiye) fundus camera (Figure 1).

Animals were examined without anaesthesia, with proper restraining by one farm staff. While the eyelids were opened with the left hand, the camera was held with the right hand. Starting at a distance

Figure 1. Fundoscopy with a non-mydriatic portable fundus camera.

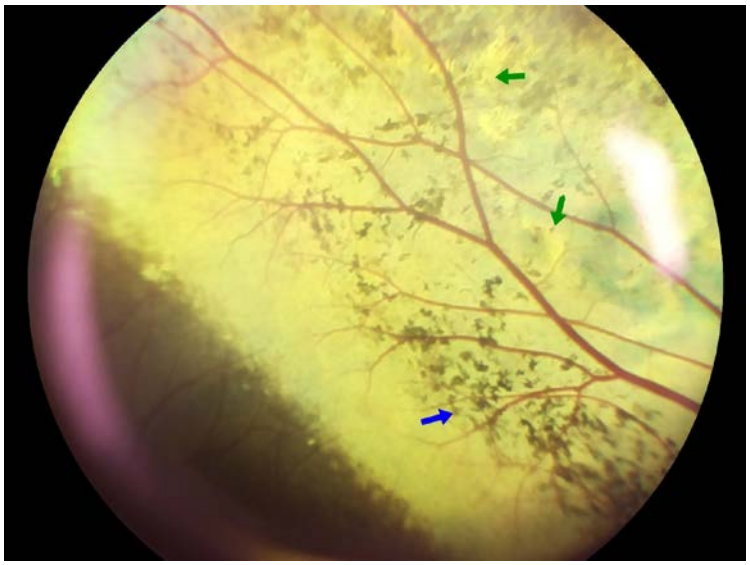

Figure 2. The Tapetal zone appears green-yellow. A rich vascular pattern in tapetal and non-tapetal areas is also seen. There are multifocal areas of pigmentation of the retinal pigment epithelium (blue arrow). Dark spots/Winslow stars are observed in the tapetal area (green arrows).

of 10 cm, we approached the eye until we had a clear fundus image. The device consists of an infrared LED (white), an optical lens, and a camera with infrared and daylight features to illuminate the eye. The device features include a sensor resolution of 8 megapixels, a field of view of 40°, and the required minimum pupil size of 3,5 mm. The obtained images were recorded on the device and transferred to the computer in jpeg format with a USB data cable.

In fundus images, tapetal colour, shape of the tapetal area, homogenicity of the tapetum, junction of tapetal and non-tapetal border, vascularization (artery, vein, small vessels), structure and color of the optic disc, and position of the optic disc were evaluated.

Results

Fundoscopy was performed on 34 eyes of 17 Holstein calves, eight males and nine females aged between 113-137 days.

The tapetal region, non-tapetal region, optic disc, and retinal vessels were examined. The green-yellow tapetal zone was dominant in all calves (Figure 2). The optic disc was dark and oval (Figure 3) and located in the non-tapetal area juxtaposed to the tapetal area (Figure 4) in all calves. The disc was seen to be cupped, with veins emerging near the center. The number of primary arteries and veins originating from the center varied between 4 and 5 (Figures 2-11). In some calves, artery-vein pairs traversing in the tapetal area were intertwined (Figure 6). The periphery of the main artery and vein pairs in the non-tapetal region appeared paler than their central parts (Figure 7). The small vessels originating from the disc largely disappeared about 1-2 disc diameters away (Figure 8). The vascular pattern was holangiotic. Hyaloid artery remnant (Bergmeister's papilla) was detected as a gray dot in the middle of the disc (Figure 9) in all 17 calves. The non-tapetal area was homogeneous with brown-black in color and rich in choroidal vascular structure (Figure 10).

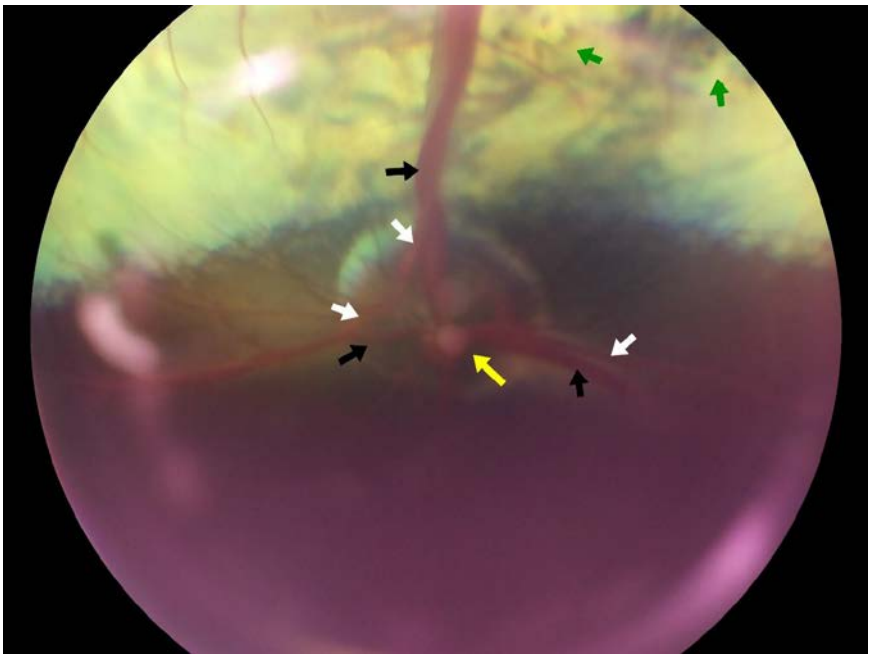

Figure 3. Oval and dark optic disc. The Tapetal zone appears green-yellow. Winslow stars are visualized in different sizes and morphology (green arrows). Bergmeister's papilla is also seen (yellow arrow). Large primary retinal vessels emerge from the optic disc's center. The periphery of the artery (white arrows) and vein (black arrows) pairs originating from the optic disc is pale in the non-tapetal region.

Discussion

Fundus examination is one of the most challenging but essential methods of the ophthalmic examination. It is necessary to know the normal fundus appearance to recognize any abnormality. Factors such as species, breed, age, sex, and coat color affect the normal fundus appearance (Martin et al., 2019). The animals were housed according to specific age groups in the establishment where the study was carried out. We preferred weaned calves regardless of gender. The age range was kept narrow to reduce the effect of the age factor.

In this study, the fundus examination was performed using an Ocellus-Vet fundus camera which can operate with infrared light. Infrared

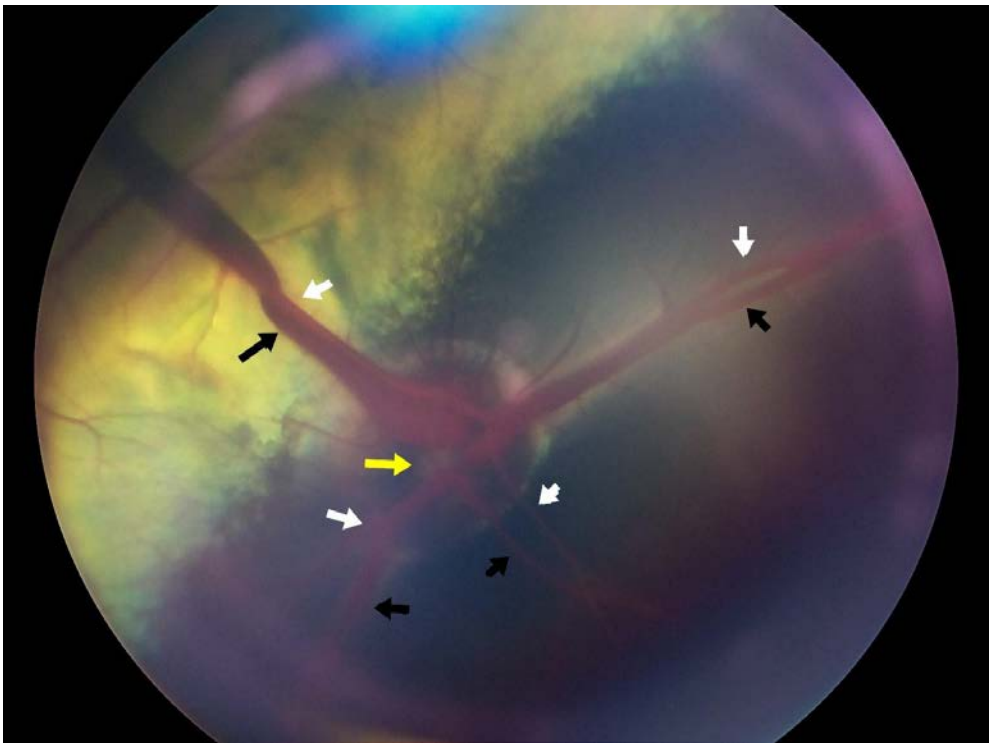

Figure 4. The optic disc is located in the non-tapetal zone, slightly below the junction of the tapetal and non-tapetal fundi. There are four primary arteries (white arrows) and four primary veins (black arrows). In the center of the optic disc is a remnant of the posterior hyaloid vessels/Bergmeister's papilla (yellow arrow).

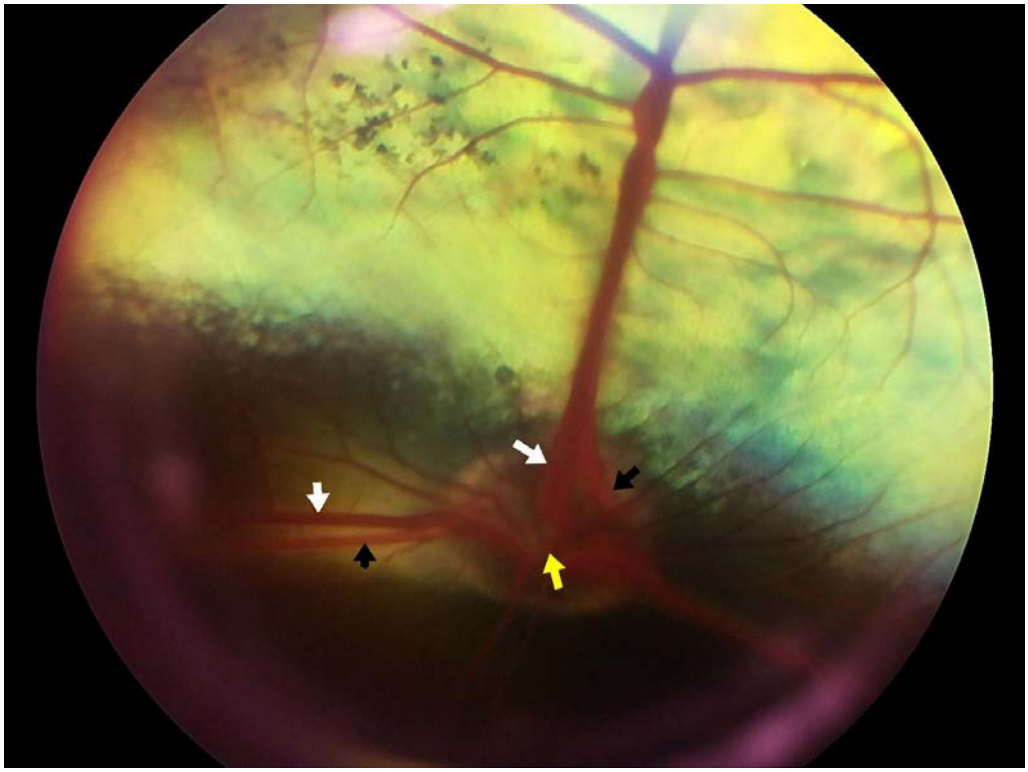

Figure 5. White arrows show arteries, and black arrows show veins. There are seven primary vessels, one artery, one vein in the tapetal zone, and three arteries and four veins in the non-tapetal zone. The yellow arrow shows Bergmeister's papilla.

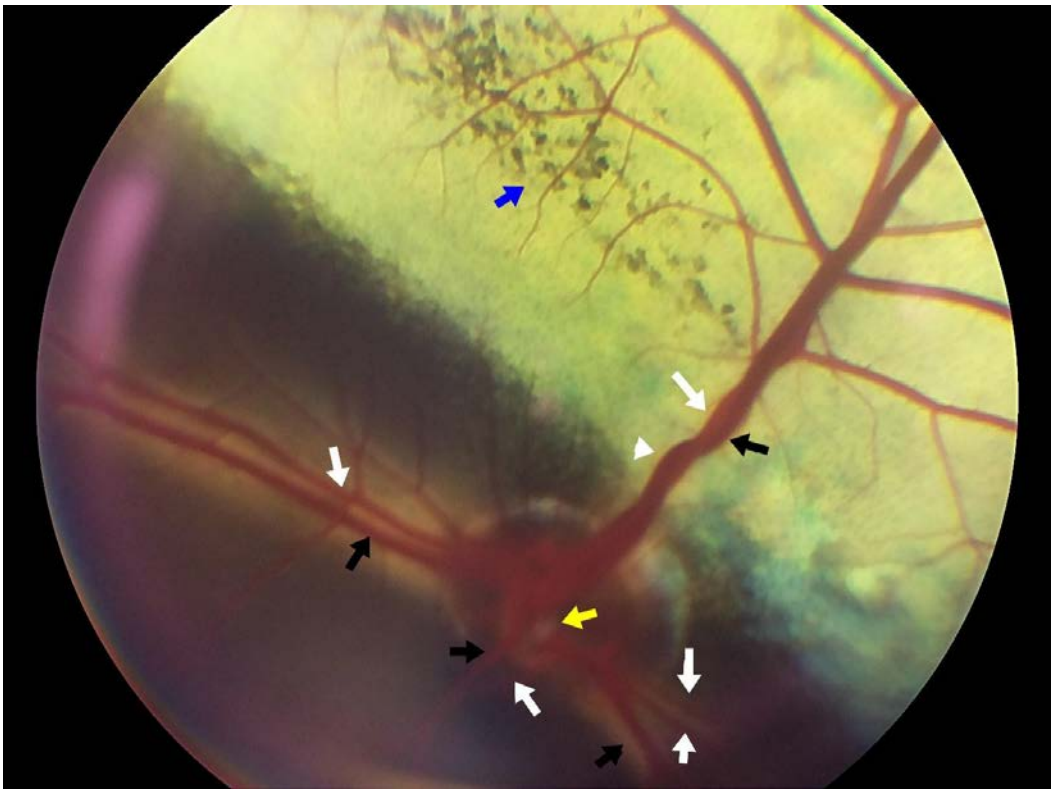

Figure 6. White arrowhead shows intertwined artery and vein. Multifocal pigmented areas are found (blue arrow) in the tapetal zone. There are five primary arteries (white arrows) and four veins (black arrows) originating from the optic disc. The yellow arrow shows Bergmeister's papilla.

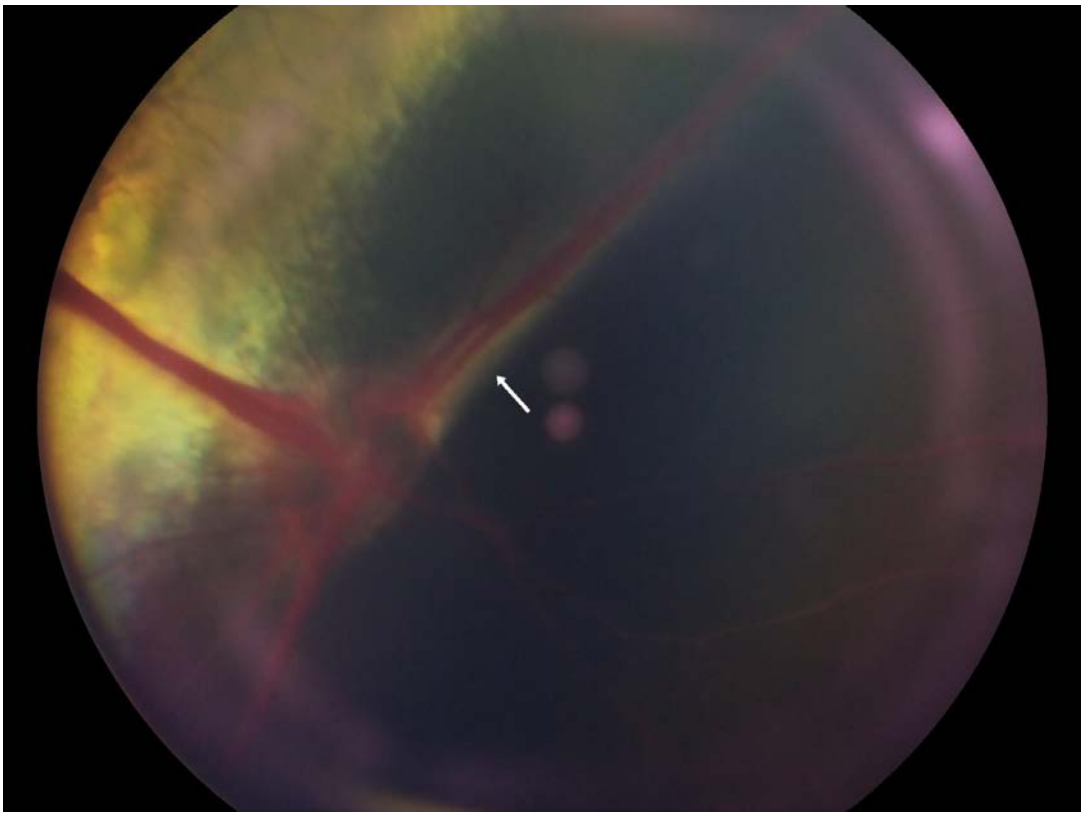

Figure 7. The arrow shows the periphery of an artery-vein pair. There are four primary arteries and five veins originating from the optic disc.

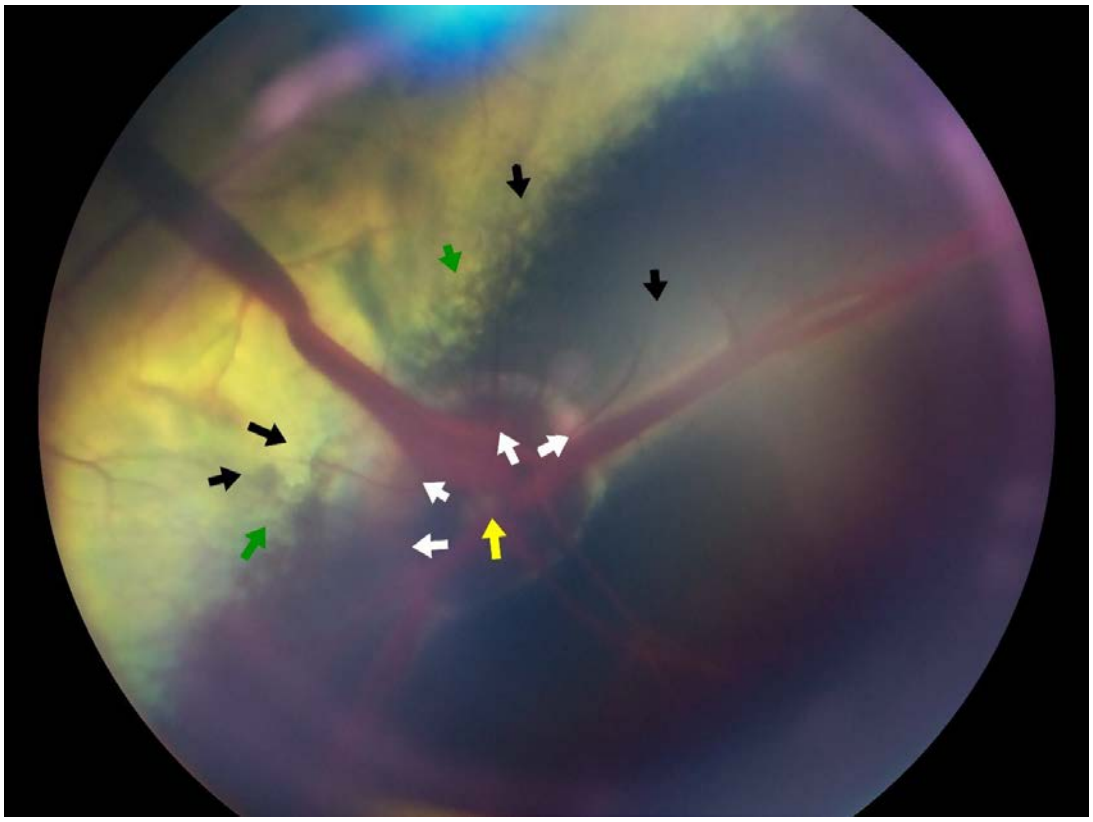

Figure 8. White arrows show the origin of small veins, and black arrows indicate where they disappear. The yellow arrow shows Bergmeister's papilla. Winslow stars appear in different sizes (green arrows).

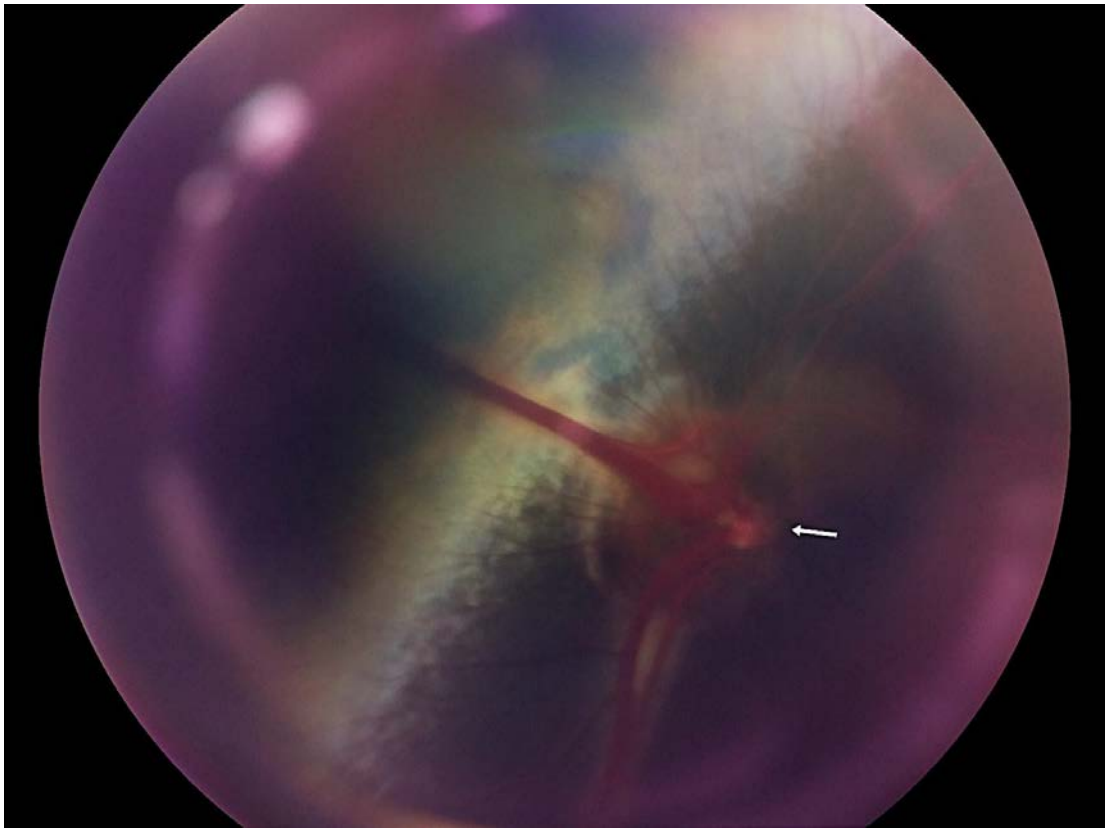

Figure 9. Bergmeister's papilla.

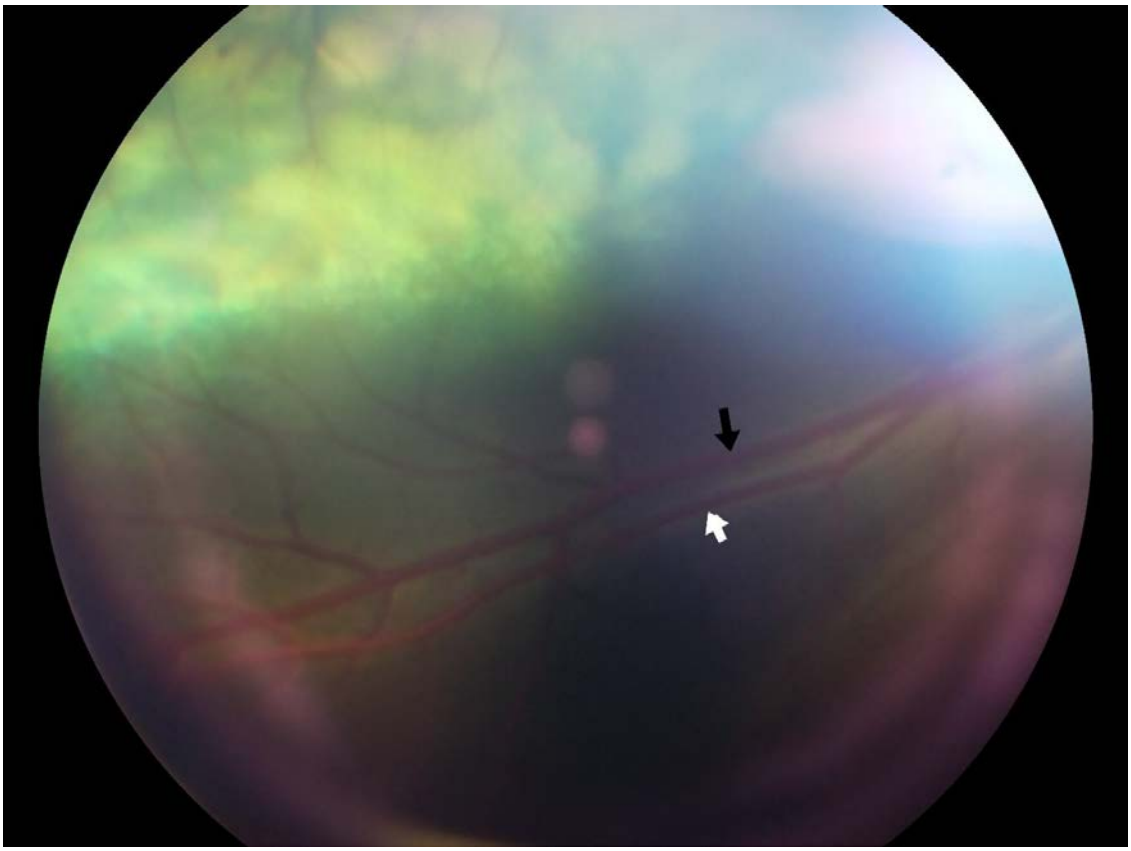

Figure 10. Non-tapetal zone. The white arrow shows the artery, and the black one shows the vein.

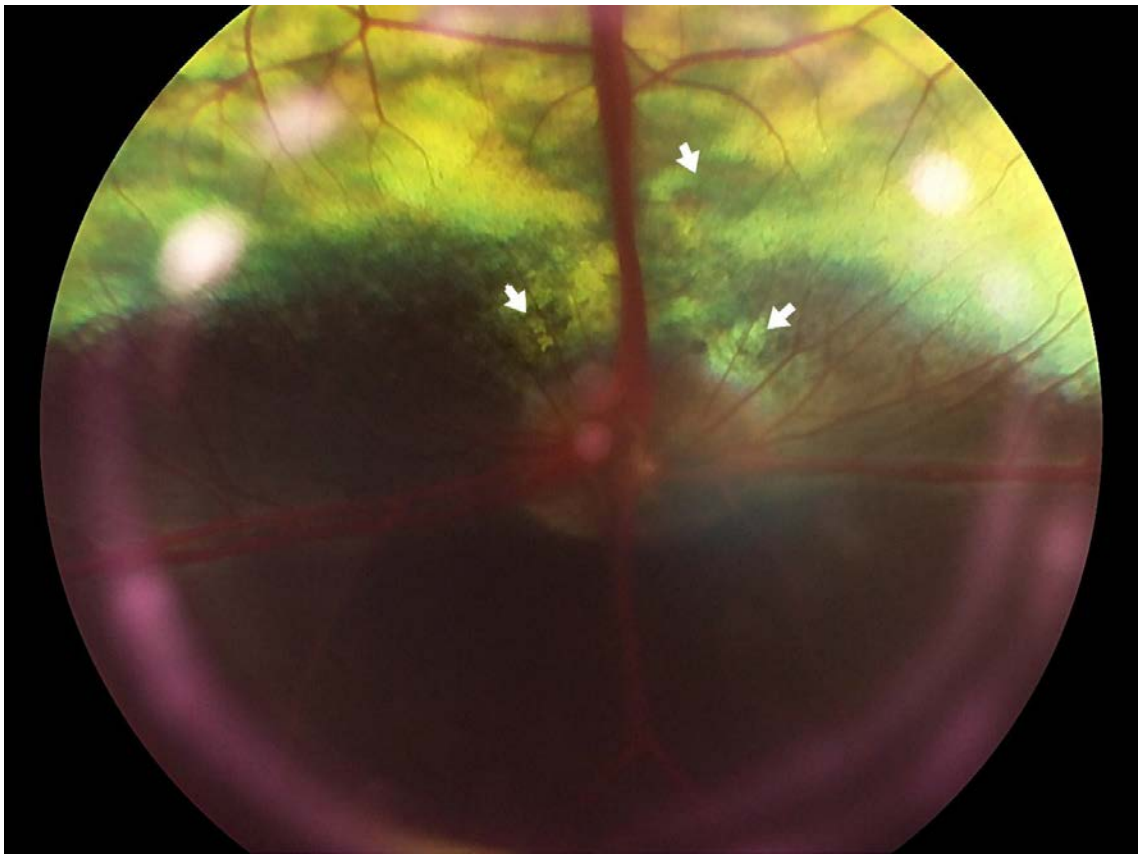

Figure 11. White arrows show Winslow stars surrounded by a yellowish area.

illumination does not cause pupil constriction, eliminating the need for using mydriatics. It also provides a tremendous advantage for animals because of its handheld portability, especially in field conditions. Although there are various examination methods in the hospital environment, a fundus camera that offers ease of use in field conditions will significantly facilitate the work of veterinarians and farm owners.

The bovine optic disc is oval with a long horizontal axis. Çatalkaya and Özaydın (Çatalkaya and Özaydin, 2019) reported that the optic disc could be observed in the tapetal region, in the non-tapetal region, or in between. In the present study, the optic disc was seen to be located in the non-tapetal region, with its dorsal aspect touching the tapetum. The optic disc appears dark because it is unmyelinated (Martin et al., 2019). In this study, the optic disc of the calves was oval and dark.

The retinal vascular pattern is holangiotic in dogs, cats, sheep, and primates, where the entire retinal surface receives a direct blood supply (Maggs et al., 2008; Şirin, 2020). In line with the literature, the vascular pattern was holangiotic. The dorsal arteriole and vein pair are intertwined in small ruminants (Çatalkaya and Özaydin, 2019; Galán et al., 2006; He et al., 2012; Kang et al., 2017; Şirin, 2020), the structure of the vessels of the calves was similar in this study. Arterioles originate more peripherally on the disc. There can be 15 to 20 smaller vessels originating from the disc and then disappearing 1-2 disc diameters away from the disc and these results are similar to the literature (Maggs et al., 2008).

Mild glial proliferation on the disc surface, known as Bergmeister's papilla, represents the remnant of a persistent hyaloid artery. The Bergmeister's papilla is routinely seen as a gray, translucent linear structure protruding from the center of the optic disc into the vitreous cavity in ruminants such as cows and sheep (Galán et al., 2006; Maggs et al., 2008; Martin et al., 2019; Shunmugam et al., 2020). In the current study, the hyaloid artery was detected in all calves.

The larger vessels disperse epiretinally rather than running through the nerve fiber layer. In calves, blood is usually present in the hyaloid vein for several months (Martin et al., 2019). However, we detected no blood in any calf in this study. In small ruminants, it has been reported that the area around the artery and vein pair in the non-tapetal region appears pale (Galán et al., 2006). In this study, the part of the pairs of major arteries and veins advancing to the non-tapetal region was paler than the rest because the optic nerve fibers appear to radiate outward from the temporal portion of the myelin disc.

Tapetal colors were seen to vary but were usually dark green with scattered dark spots or Winslow stars (Figure 11). Similar to the results of Martin et al. (Martin et al., 2019), there were often prominent pigment islets in the nasal tapetal region bilaterally. The non-tapetal region was also detected as dark brown. This result is similar to that reported in goats (Şirin, 2020). Green-yellow colors were dominant in the tapetal region of all the calves. In another study (Çatalkaya and Özaydin, 2019), a similar tapetal color was mentioned in some calves. It has been previously reported that age is associated with the tapetal area (Aksoy et al., 2011). Calves of similar ages were preferred in the present study. The observation of similar colors in the tapetal region of all calves examined in the current study is thought to be related to the evaluation of only one breed (Holstein) of similar age.

Conclusion

Ophthalmological disease on dairy farms causes significant economic losses along with individual pain and suffering in animal welfare (Irby and Angelos, 2018). Retinal fundus examination provides information about the presence and severity of many systemic and hereditary diseases, which will form the basis for disease diagnosis. Knowledge of healthy fundus images is of great value for identifying abnormal conditions. The fundus images of healthy Holstein calves presented in this study may guide for future studies and contribute to diagnosing ophthalmological diseases.

A limitation of the study is that only calves were examined, so future studies should include adult cattle.

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Conflict of Interests

The authors stated that they did not have any real, potential or perceived conflict of interest.

Ethical Approval

The current research was carried out at the Arif Gürdal Dairy Farm. ADÜ–HADYEK (64583101/2021/169).

Similarity Rate

We declare that the similarity rate of the article is 11% as stated in the report uploaded to the system.

Author Contributions

Idea/Concept: BKK
Design: BKK
Supervision-/Consultancy: BKK, AB, MS
Data Collection and/or Processing: BKK
Analysis and/or Interpretation: BKK
References Scanning: BKK, AB, MS
Writing of the Article: BKK
Critical Review: BKK, AB, MS

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Fötal Gelişim Süresince Sığır Karaciğerindeki Sitokeratin 8 ve Sitokeratin 18 Proteinlerinin Dağılımı

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Özet: Embriyonik gelişimin kontrolünde kritik rol alan sitokeratinler (CK), embriyogenezis esnasında epitel hücre gelişiminin değişen aşamalarında farklı keratinler şeklinde ekspresse edilir. CK8 ve CK18 proteinleri; çeşitli parankimatöz epitel başta olmak üzere basit epitel hücrelerinin primer keratin çifti olarak bilinmektedirler. Karaciğer; embriyonal dönemde kan yapımı ve kan hacmi regülasyonu, protein sentezi, bağışıklık sistemine katkı, büyüme sinyal yollarının endokrin kontrolü, metabolitleri depolama, safra salgısı ve detoksifikasyon gibi çok sayıda fizyolojik rollere sahiptir. Bu çalışma; CK8 ve CK18 proteinlerinin sığır fötal karaciğer hücrelerindeki dağılım ve lokalizasyonlarının immunohistokimyasal yolla belirlenmesi amacıyla yapılmıştır. Çalışmada özel kesimhanelerden temin edilen 27 adet sığır fetüsü kullanıldı. Yaşları belirlenen fetüsler; gebeliğin birinci (69-89 günlük / 9 fetüs), ikinci (99-178 / 9 fetüs) ve üçüncü (190-269 / 9 fetüs) dönemlerine ait olacak şekilde gruplandırıldı. Fetüslerden alınan karaciğer örnekleri 18 saat boyunca %10'luk formol-alkolde tespit edildi. Rutin histolojik prosedürlerden sonra elde edilen kesitlere immunohistokimyasal boyamalar yapıldı. Boyama sonucunda; CK8 ve CK18'in gebelik süresince safra kanalı epitel hücrelerinde çok güçlü seviyede ekspresse olduğu görüldü. Bununla birlikte hepatositlerde ise CK8 ve CK18'in gebelik dönemlerine göre değişen yoğunluklarda ekspresyon gösterdikleri belirlendi. Böylece CK8 ve CK18'in; sığır karaciğer gelişiminin kontrolü, hepatositlerin ve safra kanal epitel hücrelerinin bölünmesi, çoğalması ve farklılaşmaları gibi birçok role sahip olabilecekleri düşünüldü.
Anahtar Kelimeler: *CK8, CK18, Fötal karaciğer, İmmunohistokimya, Sığır, Sitokeratinler.*

Distribution of Cytokeratin 8 and Cytokeratin 18 Proteins in Bovine Liver During Fetal Development

Abstract: Cytokeratins (CK), which play a critical role in the control of embryonic development, are expressed as different keratins at varying stages of epithelial cell development during embryogenesis. The CK8 and CK18 proteins are the primary keratin pair of simple epithelial cells, especially of various parenchymatous epithelium. The liver has many physiological roles, such as blood production and blood volume regulation, protein synthesis, contribution to the immune system, endocrine control of growth signaling pathways, metabolite storage, bile secretion, and detoxification during the embryonal period. This study was conducted to determine the distribution and localization of CK8 and CK18 proteins in bovine fetal liver cells by immunohistochemical method. In the study, 27 bovine fetuses obtained from private slaughterhouses were used. The fetuses whose ages were determined; were grouped as belonging to the first (69-89 days / 9 fetuses), second (99-178 days / 9 fetuses) and third (190-269 days/ 9 fetuses) periods of pregnancy. Then, liver tissue samples were taken from the fetuses and fixed in 10% formol-alcohol for 18 hours. The tissue samples were then subjected to routine histological procedures and immunohistochemistry staining. As a result of staining, it was observed that CK8 and CK18 were strongly expressed in bile duct epithelial cells during pregnancy. However, it has been determined that CK8 and CK18 are expressed in hepatocytes with varying densities according to the pregnancy periods. Thus, it was thought that CK8 and CK18 might have many roles, such as controlling the development of the bovine liver, division, proliferation, and differentiation of hepatocytes and bile duct epithelial cells.
Keywords: *Bovine, CK8, CK18, Cytokeratins, Fetal liver, Immunohistochemistry.*

Giriş

Sitokeratinler (CK), keratin içeren bir ara filament olup genellikle epitel dokusunun intrasitoplazmik hücre iskeletinde bulunmaktadırlar. Ara filament proteinlerinin tanımlandığı 1970'lerde sitokeratin (CK) olarak kullanılan terim, 2006'daki yeni sistemik terminolojiye göre sitokeratinler ile birlikte keratinler (k) olarak da kullanılmaya başlanmıştır (Kumar ve Jagannatha, 2018). Sitokeratinler kendi içerisinde; yüksek moleküler ağırlıklı veya bazik sitokeratinler (CK/K 1-9) ve düşük moleküler ağırlıklı veya asidik sitokeratinler (CK/K 10-20) şeklinde gruplandırılmıştır (Moll ve ark., 1982). Sitokeratinler her organa özel olacak şekilde ekspresse edilirken, bir epitel hücresinde ekspresse olan sitokeratinlerin alt üyeleri epitel tipine ve farklılaşma modeline göre değişkenlik gösterebilir. Bununla birlikte sitokeratinlerin en dikkat çekici tarafı ise herhangi bir epitel dokusunda görülen sitokeratinin aynı gruptakilerden en az biri ile her zaman birlikte ekspresse olmasıdır (Kumar ve Jagannatha, 2018).

Embriyonik gelişimin kontrolünde rol alan sitokeratinler, embriyogenezis esnasında epitel hücre gelişiminin değişen aşamalarında farklı keratinler şeklinde ekspresse edilir (Kumar ve Jagannatha, 2018; Steinert ve ark., 1993).

Genelde birlikte ekspresse olduğu ifade edilen CK8 ve CK18 proteinleri; çeşitli parankimatöz epitel başta olmak üzere basit epitel hücrelerinin primer keratin çifti olarak bilinmektedirler. Bununla birlikte bu proteinlerin; embriyogenezisde, embriyonun ilk oluşum anında ortaya çıktıkları ve filogenez sırasında en eski keratinler olarak görüldükleri de ifade edilmiştir. CK8 ve CK18; kanal kaplayan hücreler, bağırsak ve mezotel hücreleri gibi basit, tek katmanlı epitel hücrelerinin birincil hücre iskeleti proteinleridir (Kumar ve Jagannatha, 2018; Liao ve ark., 1995). Yine keratin 8 (K8) ve keratin 18'in (K18), spesifik olarak hepatositlerde eksprese edilen başlıca ara filament proteinleri olduğu ve bu proteinlerin hücrelerde sitozol boyunca yayılarak mekanik olarak destek sağladığı belirtilmiştir (Pan ve ark., 2013; Strnad ve ark., 2012; Toivola ve ark., 2015). Ayrıca CK8 / CK18, sinyallemenin yapısal desteği ve düzenlenmesi yoluyla hepatositleri koruduğu da ifade edilmiştir (Lim ve Ku, 2021).

Vücudun en büyük bezi olarak tanımlanan karaciğer hem ekzokrin hem de endokrin kısımlardan meydana gelir. Karaciğerin ekzokrin olarak ifade edilen kısmı safrayı, endokrin kısmı ise glikojenden ve bazı plazma proteinlerinden glikoz gibi kimyasal maddeler salgılar (Michalopoulos, 2007; Naik ve ark., 2020; Subhaana ve ark., 2020). Bu metabolik görevlerinin yanı sıra karaciğerin yetişkinlerde sindirim ve bağışıklık başta olmak üzere birçok fizyolojik sürece pozitif anlamda etki ettiği bildirilmiştir. Ayrıca karaciğerin canlılarda özellikle metabolizma ile ilgili olarak embriyonal dönemde kan yapımı ve kan hacmi regülasyonu, protein sentezi, bağışıklık sistemine katkı, büyüme sinyal yollarının endokrin kontrolü, metabolitleri depolama, safra salgısı ve detoksifikasyon gibi çok sayıda fizyolojik rollere sahip olduğuda ifade edilmiştir (Akiyoshi ve Inoue, 2012; Trefts ve ark., 2017). Karaciğer farklı embriyolojik hücre tiplerinden (hepatositler, biliyer epitel hücreleri (kolanjiyositler), stellate hücreleri, Kupffer hücreleri ve karaciğer sinüzoidal endotel hücreleri) oluşur. Bu hücre tiplerinin her biri, karaciğer fonksiyonunu iş birliği içinde kontrol eden eşsiz görevlere sahiptir. Karaciğerin birincil epitel hücre popülasyonu olan hepatositler, karaciğer hacminin çoğunu (%60'ı) oluşturur ve birçok işlevi yerine getirirler. Hepatositlerin, besinlerin taşınması, fötal büyüme ve gelişimdeki kritik düzenleyici hücreler olduğu gösterilmiştir. (Ham ve ark., 1979; Naik ve ark., 2020; Trefts ve ark., 2017).

Sunulan çalışma; epitel hücrelerin ve hepatositlerin yapısına katıldığı belirtilen CK8 ve CK18 proteinlerinin sığır fötal karaciğer hücrelerindeki dağılım ve lokalizasyonlarını immunohistokimyasal yöntemle belirlemek amacı ile yapılmıştır.

Materyal ve Metot

Materyal Temini ve Doku Hazırlama: Sunulan araştırmada özel kesimhanelerden temin edilen gebeliğin farklı dönemlerine ait ve klinik olarak sağlıklı 27 adet fetüs kullanıldı. Alınan fetüslerin yaş tayinleri; alın-sağrı uzunluğu (Crown-Rump Lenght; CRL) ölçümüne dayalı olan,''y:54,6 cm+2,46(x) cm'' eşitliği kullanılarak belirlendi. Uygulanan bu formülasyona göre ''x'' alın-sağrı uzunluğunu, ''y'' ise fötal yaşı gün cinsinden göstermektedir (Harris ve ark. 1983). Yaşları belirlenen fetüsler; gebeliğin birinci (69-89 günlük / 9 fetüs), ikinci (99-178 / 9 fetüs) ve üçüncü (190-269 / 9 fetüs) dönemlerine ait olacak şekilde gruplandırıldı. Bu gruplandırma dikkate alınarak tüm fetüslerden karaciğer doku örnekleri alındı. Alınan doku örnekleri tespit edilmek amacı ile 18 saat boyunca %10'luk formol-alkole tabi tutuldu. Ardından dereceli alkol serilerinde dehidre edildi. Dehidre edilen doku örnekleri daha sonra metilbenzoat, benzol ve benzol-paraplast serilerinden geçirilerek parafin blokları haline getirildi. Ardından parafine gömülmüş doku örneklerinden rotary mikrotomunda (Leica RM-2125, Germany) 5 mikrometre kalınlığında seri kesitler alındı. Alınan kesitler immunuhistokimya boyaması için önceden APES (3 amino propyl triethoxysilan; Sigma–Aldrich Chemicals, St.Louis, MO, USA) ile kaplı lamlar üzerine alındılar.

Hayvan Deneyleri Etik kurullarının çalışma usul ve esaslarına dair yönetmeliğin (15.02.2014) 8. maddesinin k bendi 1. fıkrasında belirtildiği üzere çalışmamızda kullandığımız materyal; mezbaha materyali olduğundan HADYEK iznine tabi değildir.

İmmunohistokimyasal Prosedür: APES ile kaplı lamlara alınan seri kesitler, streptavidin-peroksidaz prosedürü ve Zymed Histostain Plus Bulk Kit (South San Francisco, CA, ABD, cat no: 85-9043) tekniğine uygun olarak immunohistokimya (IHC) boyamasına tabi tutuldu. Öncelikle alınan kesitler kuruması için bekletildi. Bu kurutma işleminden sonra kesitler deparafinizasyon (ksilol ile 5'er dakikadan iki kez) ve rehidrasyon (dereceli alkoller ile 3'er dakika) süreçlerden geçirilerek distile suda yıkanmaya alındı. Daha sonra doku örneklerindeki endojen peroksidaz aktivitesini ortadan kaldırmak amacı ile kesitler metanolde hazırlanmış %3'lük H_2O_2 solüsyonu içerisinde 20 dk bekletildi ve sonrasında 5'er dk (üç kez) PBS (Phosphate Buffer Saline, pH: 7.4, 0.01 M) çözeltisinde yıkandı. Preperatlar daha sonra

antikorun bağlanacağı antijenik bölgelerin açığa çıkarılması amacı ile sitrat buffer çözeltisinde (pH: 6) 95ºC'de 30 dakika boyunca bekletildi ve süre sonunda oda ısısına gelinceye kadar solüsyon içinde bırakıldı. Ardından kesitlerde, primer antikorun spesifik olmayan bağlanmalarını önlemek amacı ile bloklayıcı solüsyonda (Ultra V Blok, catalog: TA-125-UB, Thermo Scientific) 15 dk tabi tutuldu ve ardından solüsyonun uzaklaşması sağlanarak kesitler, 1:200 oranında sulandırılmış CK8 (Mouse Monoclonal Antibody Cytokeratin 8, katalog no: ab2530 Abcam) ve CK18 (Mouse Monoclonal Antibody Cytokeratin 18, katalog no: ab668 Abcam) antikorları ile +4 ^{o}C de bir gece reaksiyona tabi tutuldu. Bu işlemin ardından kesitler 5'er dk (üç kez) PBS içerisinde yıkandıktan sonra oda ısısında 20 dk biyotin işaretli sekonder antikor (Biotinylated Goat Anti-Polyvalent, catalog:TP-125-BN, Thermo Scientific) ile inkube edildi. Sonrasında 5'er dk (3 kez) PBS içerisinde yıkamayı takiben kesitlere oda ısısında 20 dk süreyle streptavidin peroksidaz (Thermo Fisher Scientific, catalog: TA-125-HR) uygulandı. Son olarak kesitler üzerine 3.3 diaminobenzidin (DAB Substrate, Thermo Scientific, katalog no: TA-125-HD) damlatılarak reaksiyonun şekillenme süresine göre ortalama 5-15 dk bekletildi. Sonrasında distile suda yıkanan kesitler Mayers'in Hematoksilen solüsyonu damlatılarak 2 dk boyunca çekirdeklerin boyanması sağlandı. Ardından akan çeşme suyu altında 5 dk boyunca yıkanan kesitler artan dereceli alkol serilerinden geçirilerek suyun uzaklaştırılması sağlandı ve ksilol'de parlatıldıktan sonra dokular entellan damlatılarak lamel ile kapatıldı. Boyanan preparatlar DS-RI1 video kamera (DS-U3, Nikon, Tokyo, Japan) ataçmanlı Nikon Eclipse E400 araştırma mikroskobunda (Nikon, Tokyo, Japonya) değerlendirildi ve fotoğrafları çekildi.

Yapılan immunohistokimya metodunun doğruluğunu göstermek amacı ile negatif kontroller kullanıldı. Bunun için de alınan karaciğer kesitleri birincil antikor yerine PBS tamponu ile inkübasyona tabi tutuldu.

Yarı Kantitatif Değerlendirme: İmmunohistokimyasal boyanmaların değerlendirilmesi yoğunluk skoru (intensity score) üzerinden yapıldı. CK8 ve CK18 ekspresyonları için pozitif reaksiyon gösteren hücreler kalitatif olarak değerlendirildi. Pozitif hücreler boyanma yoğunluklarına göre (-) negatif; (+) zayıf; (++) orta veya (+++) güçlü olmak üzere dört seviyede skorlandı (Devkota ve ark., 2006). Pozitif boyanan hücrelerin değerlendirmelerini birbirinden bağımsız uzman araştırmacılar (U.T ve M.E.A) tarafından yapıldı ve ortalama skorlar hesaplandı. Karaciğer kesitlerinde CK8 ve CK18'in ekspresyonları X40, X100 ve X400 büyütmede incelendi. Karaciğerin her bir bölgesinde, her bir kesit için üç rastgele seçilmiş alan değerlendirildi. Sonuçlar; hepatositler, portal ven, arteria hepatika ve safra kanalı epitel hücreleri olmak üzere ayrı ayrı değerlendirildi (Tablo 1).

Tablo 1: CK8 ve CK18'in immunohistokimyasal boyanma yoğunluklarına ait semikantitatif değerlendirmeler.

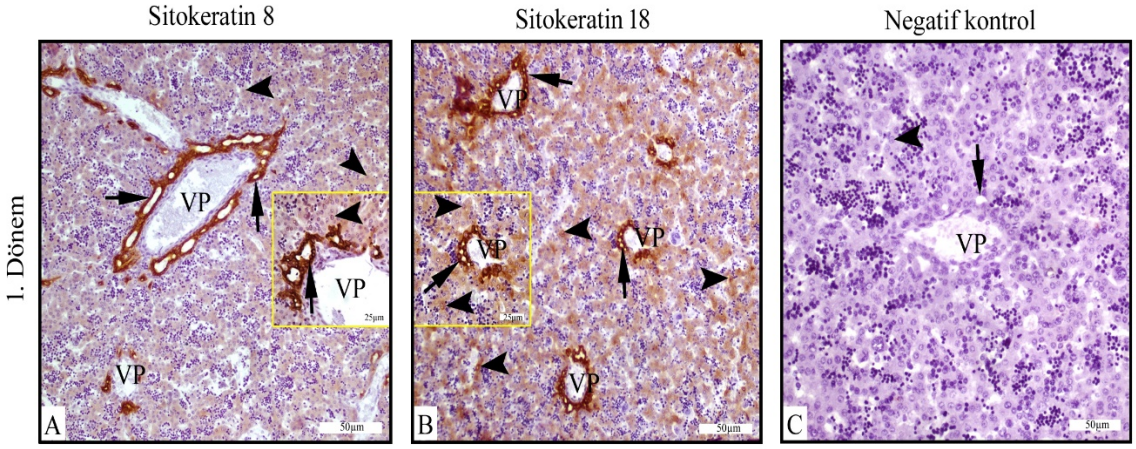
Antikorlar	Karaciğer Hücreleri	1.Dönem	2. Dönem	3. Dönem
CK8	Hepatositler	+/++	-	+
	Vena Portalar	-	-	-
	Hepatik Arterler	-	-	-
	Safra kanal epiteli	+++	+++	+++
CK18	Hepatositler	++/+++	++/+++	-
	Vena Portalar	-	-	-
	Hepatik Arterler	-	-	-
	Safra kanal epiteli	+++	+++	+++

Bulgular

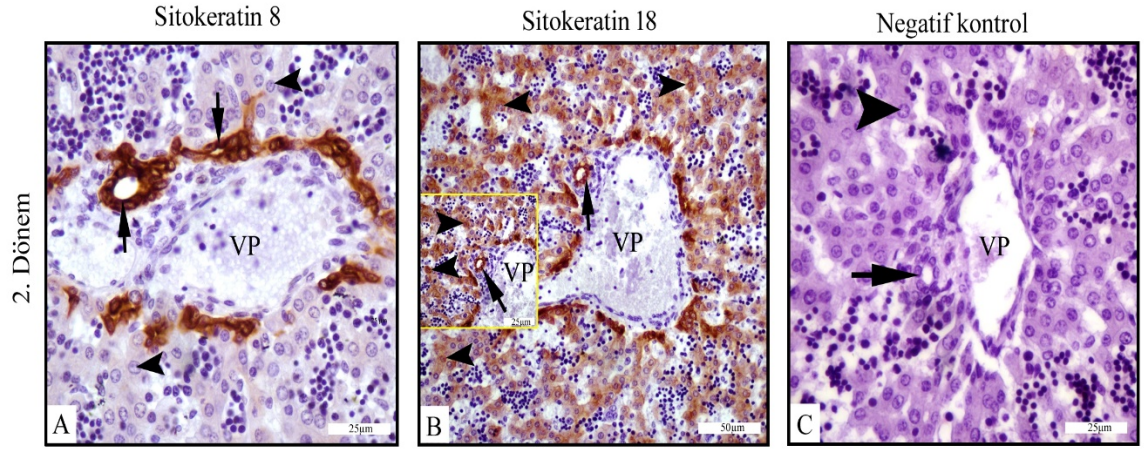
İmmunohistokimya boyama sonucunda elde edilen bulgularda, CK8'in hepatositlerde gebeliğin birinci döneminde zayıftan ortaya yakın yoğunlukta olduğu (Şekil 1A), gebeliğin ikinci ve üçüncü dönemlerinde ise immunreaksiyonun negatif olduğu belirlendi (Şekil 2A, 3A). Bununla birlikte gebelik süresince vena porta ve hepatik arterlerde de immunreaksiyonun meydana gelmediği gözlendi. Ancak safra kanalı epitel hücrelerinde gebeliğin her üç döneminde güçlü düzeyde bir immunreaksiyon şekillendiği tespit edildi (Şekil 1A, 2A, 3A).

CK18 proteini için yapılan immunohistokimya sonucunda da bu proteinin hepatositlerde gebeliğin birinci ve ikinci dönemlerinde orta ve ortadan güçlü yoğunluğa doğru bir immunreaksiyon şekillendirdiği ancak üçüncü dönemde ise negatif olduğu görüldü (Şekil 1B, 2B, 3B). Ayrıca CK8 de olduğu gibi gebelik süresince vena portalarda ve hepatik arterlerde immunreaksiyonun negatif olduğu saptandı. Ancak gebelik süresince safra kanalı epitel hücrelerinde güçlü düzeyde bir immunreaksiyon meydana geldiği tespit edildi (Şekil 1B, 2B, 3B).

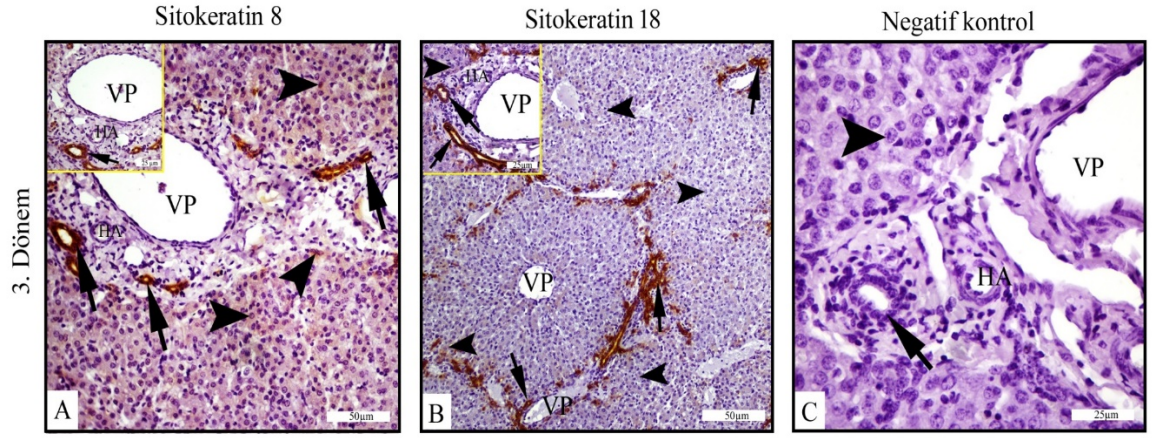
Boyanmanın özgünlüğünü ortaya koymak amacı ile negatif kontroller kullanıldı (Şekil 1C, 2C, 3C).



Şekil 1: Gebeliğin birinci dönemindeki fötal sığır karaciğerinde sitokeratin 8 (CK8) ve sitokeratin 18 (CK18) proteinlerinin immunohistokimyasal dağılımı. VP: Vena Porta, Ok: Safra kanal epitel hücreleri, Ok başı: Hepatositler.



Şekil 2: Gebeliğin ikinci dönemindeki fötal sığır karaciğerinde sitokeratin 8 (CK8) ve sitokeratin 18 (CK18) proteinlerinin immunohistokimyasal dağılımı. VP: Vena Porta, Ok: Safra kanal epitel hücreleri, Ok başı: Hepatositler.



Şekil 3: Gebeliğin üçüncü dönemindeki fötal sığır karaciğerinde sitokeratin 8 (CK8) ve sitokeratin 18 (CK18) proteinlerinin immunohistokimyasal dağılımı. VP: Vena Porta, HA: Hepatik Arter, Ok: Safra kanal epitel hücreleri, Ok başı: Hepatositler.

Tartışma ve Sonuç

Karaciğer gelişim sürecinin, epigenetik modülasyon ile belirlenen karmaşık bir gen ekspresyonunun dinamik olarak değişimine bağlı olduğu bilinmektedir. Buna paralel olarak karaciğer gelişimi için sitokeratinler, fetal proteinler ve adezyon molekülleri başta olmak üzere tüm gen ekspresyon ürünleri koordine edilmelidir (Feng ve ark., 2021). Sitokeratinler; hücre bölünmesi, hareketlilik, hücresel mekanik bütünlüğünün korunması ve vezikül taşınması gibi önemli hücresel işlevleri yerine getiren hücre iskelet proteinlerinin ara filamentleri ağında yer alırlar (Gonsebatt ve ark., 2007). Tipik olarak epitel hücrelerinde ekspresse olduğu bilinen sitokeratinlerden CK8 ve CK18'in; karaciğer, ekzokrin pankreas ve bağırsak gibi basit epitellerde değişken ekspresyon oranlarında görüldüğü bildirilmiştir (Gonsebatt ve ark., 2007; Schöniger-Hekele ve ark., 2006).

CK8 ve CK18 proteinlerinin normal karaciğerdeki hepatositler ve safra kanal hücreleri için belirteç proteinler olarak görüldüğü ifade edilmiştir (Korver ve ark., 2021). CK8, 18 ve 19'un embriyonal hepatositlerde ekspresse olduğu, ancak erişkin karaciğerin ise sadece CK8 ve CK18 proteinlerini içerdiği bildirilmiştir (Basturk ve Adsay, 2011). Yine başka bir araştırmada insan karaciğer gelişiminin 3.ve 4. haftalarında hepatosit öncü hücreleri olan hepatoblastlar ile birlikte daha sonra hepatositlerde ve safra kanal epitel hücrelerinde CK8 ve CK18'in ekspresse olduğu gösterilmiştir (Feng ve ark., 2021; Terada, 2017). Diğer araştırmalarda da aynı şekilde embriyonal insan karaciğerinde CK8 ve CK18 proteinlerinin hepatositlerde ve safra kanalı hücrelerinde lokalize oldukları belirtilmiştir (Bateman ve Hübscher, 2010; Stosiek ve ark., 1990). Yetişkin insan karaciğerinde ise sitoplazmik intermedier filament çifti olarak CK8 ve CK18'in hepatositlerde ve safra kanalı hücrelerinde ekspresse olduğu ve bu proteinlerin eksikliğine bağlı olarakta başta karaciğer olmak üzere birçok hastalığın gelişimine neden olduğu ifade edilmiştir (Eyken ve ark., 1988; Strnad ve ark., 2008). Genel olarak normal doku ve organlarda gözlenen keratinlerin canlılarda meydana gelebilecek hastalıklarda ekspresyon düzeylerinde dinamik farklılıklar gözlenir. Örneğin çeşitli insan karaciğer rahatsızlıklarında aşırı ekspresyon yoğunluğunun olduğu ve keratin 8 ile 18'in karaciğerde bulunma yoğunluğunun bozulduğu gözlenmiştir (Guldiken ve ark., 2014; Kucukoglu ve ark., 2014). Böylece bu bilgilere dayanarak karaciğer hasarının keratin 8 ve 18 boyaması ile tespit edilebileceği de belirtilmiştir (Toivola ve ark., 2015).

Sıçanlarda CK8 / 18'in karaciğerdeki aşırı ekspresyon artışı ile bozulan regülasyonun sıçan hepatokarsinogenezise ve hepatoselüler karsinom oluşumuna pozitif etki ettiği ifade edilmiştir. Buna bağlı olarak da bu proteinlerin karaciğer kanserinde erken teşhis aracı olarak kullanılabileceği belirtilmiştir (Kakehashi ve ark., 2010). Keratin 8, 18 19 proteinlerinin inaktif edildiği farelerde karaciğer gelişiminin devam ettiği ve böylece düzenli karaciğer gelişiminin bu proteinlere bağlı olmadığı bildirilmiştir (Ku ve ark., 2007). Ancak bunun yanında bu proteinlerin eksikliğine bağlı olarak bu farelerde hafif kronik hepatit, hepatosit hücre yapısının bozulması ve çeşitli stres koşullarına karşı hassasiyet artışı olduğu bildirilmiştir (Ku ve ark., 2007; Omary ve ark., 2002; Zatloukal ve ark., 2004). Embriyonal dönemdeki farelerde yapılan çalışmada gebeliğin ilk haftasında CK8 ve CK 18'in hepatositlerde zayıf bir immunreaksiyon şekillendirdiği ve gebeliğin ilerlemesine bağlı olarakta bu immunreaksiyonun normal ve hasarlı hepatositlerin çekirdek ve sitoplazmasında daha yoğun olduğu bildirilmiştir (Jeong ve ark., 2005). K8/K18'in nakavt veya mutasyon kaynaklı kusurları karaciğer hasarını şiddetlendirir ve transgenik farelerdeki karaciğeri belirli hastalıklara yatkın hale getirir. Kronik karaciğer hasarı hepatosellüler karsinom (HCC) insidansını yükselttiğinden, CK8/CK18 defektleri HCC insidansını artırabilecek faktörlerden biri olabilir. Ayrıca, bol miktarda sitoplazmik CK8/CK18, sinyal faktörleriyle etkileşime girerek ve bunların mevcudiyetini ve konumunu değiştirerek sinyal yollarını düzenlediği de bildirilmiştir (Strnad ve ark., 2008). Tavşan karaciğerinde CK18'in hepatositlerde ekspresse olduğu ancak portal damarlarda gözlenmediği gösterilmiştir (Wells ve ark., 1997). Köpeklerin normal karaciğer hepatositlerinde ve safra kanal hücrelerinde sitokeratin ekspresyonunun negatif, tümoral karaciğerde ise pozitif olduğu belirtilmiştir (Shiga ve Shirota, 2000). Bir başka araştırmada CK18'in sağlıklı köpek karaciğerinde gözlendiği ve kronik hepatitis ile ekspresyon yoğunluğunun arttığı saptanmıştır (Lawrence ve ark., 2018).

İnsan (Basturk ve Adsay, 2011; Bateman ve Hübscher, 2010; Feng ve ark., 2021; Stosiek ve ark., 1990; Tereda, 2017), fare (Jeong ve ark., 2005; Ku ve ark., 2007; Omary ve ark., 2002; Zatloukal ve ark., 2004), tavşan (Wells ve ark., 1997) ve köpeklerde (Lawrence ve ark., 2018) yapılan çalışmalara çoğunlukla paralel olacak şekilde bizim çalışmamızda da CK8 ve CK18'in sığır fötal karaciğerinde ekspresse oldukları gözlenmiştir. Gebelik süresince her iki proteinin; safra kanal epitel hücrelerinde güçlü seviyede ekspresse olması bu hücrelerin gelişimi, çoğalması ve farklılaşmasında önemli rollere sahip olabileceklerini düşündürmüştür. Ayrıca insan, fare, tavşan ve bazı köpeklerde olduğu gibi sunulan çalışmada da CK8 ve CK18'in hepatositlerde lokalize olduğu görülmüştür. Fakat gebeliğin ikinci

döneminde CK8'in, gebeliğin üçüncü döneminde ise CK18 proteinin negatif olduğu belirlenmiştir. Bununda bu dönemlerdeki hücrelerin farklılaşma durumuna ve buradaki hücrelerin fizyolojik aktivitelerine göre değişkenlik gösterebildiğini akla getirmiştir. Ayrıca insan ve farelerin aksine CK18'in gebeliğin son döneminde negatif olması türler arasındaki farklılıktan da kaynaklanabileceğini düşündürmüştür.

Sonuç olarak CK8 ve CK18 proteinlerinin sığır karaciğerindeki hepatositler ve safra kanal epitel hücreleri içinde ayrıt edici belirteçler olarak kabul edilebilecekleri düşünülmüştür. Ayrıca bu proteinlerin immunohistokimyasal lokalizasyonlarına göre; sığır karaciğer gelişiminin kontrolü, hepatositlerin ve safra kanal epitel hücrelerinin bölünmesi, çoğalması ve farklılaşmalarının yanı sıra birçok metabolik ve fizyolojik süreçlerde kritik rollere sahip olabilecekleri akla getirilmiştir. Bunlar doğrultusunda da CK8 ve CK18'in sığırlarda fötal yaşamın sürdürülmesine katkı sağlayarak tür devamlılığına pozitif anlamda etki edebilecekleri kanaatine varılmıştır.

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Yazar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmiştir.

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Yazar Katkıları

Fikir/Kavram: UT
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Evaluation of the treatment of traumatic coxofemoral luxations in dogs using toggle pin technique

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Abstract: This study was performed on 11 dogs diagnosed with traumatic coxofemoral luxation. The direction of the luxations, the operation details, postoperative complications, and radiographic evaluation were recorded during the study. The study utilized the toggle pin technique, which provided open-reduction techniques for the treatment of coxofemoral luxations. Polyester multifilament braided sutures with commercial toggle pins were employed in three cases, whereas toggle pins obtained from Kirschner wire and monofilament nylon suture material were performed in eight patients. Clinical and radiographic monitoring of postoperative lameness, complications, pain, and infection were assessed and recorded for comparison. Postoperative reluxation was observed in two cases in which multifilament braided polyester suture material was performed without other complications. In the cases without reluxation, it was noted that the animals used their limbs comfortably on the 1st postoperative day, and their lameness scores were 0 on the 7th postoperative day. As a result of the findings, the toggle pin technique using monofilament nylon suture could effectively treat coxofemoral luxations in dogs.
Keywords: *Dog, Luxation, Suture material, Toggle pin.*

Köpeklerin travmatik koksofemoral luksasyonlarının toggle pin tekniği ile tedavisinin değerlendirilmesi

Özet: Bu çalışma, travmatik koksofemoral luksasyon tanısı konulan 11 köpekte gerçekleştirilmiştir. Olgulardaki luksasyonların yönü, operasyon detayları, operasyon sonrası oluşan komplikasyonlar ve radyografik görüntüleri kaydedildi. Çalışmada açık redüksiyon tekniklerinden birisi olan toggle pin tekniği kullanıldı. Olgulardan 3 tanesinde kullanıma hazır halde satılan toggle pin ve multifilament örgülü polyester, 8 hayvanda ise kirschner telinden yapılmış toggle pinler ile monofilament nylon sutur materyali kullanıldı. Olguların postoperatif topallık skorlaması, komplikasyonlar, ağrı ve enfeksiyon yönünden klinik ve radyografik takibi yapıldı. Monofilament örgülü polyester sutur materyali kullanılan iki olguda postoperatif reluksasyon görülürken, diğer olgularda herhangi bir komplikasyona rastlanmadı. Reluksasyon görülmeyen olgularda hayvanların postoperatif 1. günde ekstremitesini rahatlıkla kullandığı dikkat çekti ve postoperatif 7. gündeki topallık skorları 0 olarak belirlendi. Elde edilen bulgular sonucunda köpeklerin travmatik koksofemoral luksasyonlarında monofilament nylon sutur kullanılarak uygulanan toggle pin tekniğinin oldukça etkili olduğu görülmüştür.
Anahtar Kelimeler*: Köpek, Luksasyon, Sütur materyal, Toggle pin.*

Introduction

The hip joint is a diarthrodial joint composed of the acetabulum and femoral head. The ball-and-socket design provides the joint with a broad range of motion and stability. The ligamentum capitis ossis femoris, joint capsule, and dorsal acetabular rim serve as the primary stabilizers of the hip joint (Evans, 1993). Luxation occurs when at least two primary stabilizers lose their functional capacity (Holsworth and DeCamp, 2003; Smith et al., 1963). Coxofemoral luxation, which accounts for 90% of all luxation cases in dogs, is a common problem. Luxations are typically traumatic, and 60% of these traumas are caused by motor vehicle accidents (Demko et al., 2006; Trostel and Fox, 2020). As a result of trauma-induced forces and the gravitational pull of the gluteal and iliopsoas muscles, craniodorsal luxations occur most frequently (Basher et al., 1986). Cranioventral, caudodorsal, and caudoventral luxations are less common and may be related to an avulsion fracture of the trochanter major coincidingwith the luxation (Harari et al., 1984; Schrader, 1994).

Closed reduction is the first treatment technique for coxofemoral luxations, but the success rate for craniodorsal luxations ranged from 50 to 78%. Open reduction and stabilization may be considered if closed reduction cannot be performed, reluxation occurs after reduction, or if the dog has multiple injuries and requires urgent hip stabilization. Numerous surgical techniques have been described to treat coxofemoral luxations (Martini et al., 2001). Because of the coxofemoral joint's anatomical structure, applying a toggle pin is frequently preferred. This technique is effective

because it replaces the ligamentum capitis ossis femoris, which contributes to passive stability. It has been reported that patients undergoing toggle pin stabilization have a low relapse rate, and it is a beneficialtechnique, particularly for patients with polytrauma (Bone et al., 1984; Demko et al., 2006; Scott and McLaughlin, 2007).

This study evaluated the effectiveness of the materials used in toggle pin stabilization, relapse rates, and postoperative process.

Materials and Methods

Permission was obtained from the "Kırıkkale University Clinical Practices Ethics Committee," and the animal owners were informed about the study, and a consent form was obtained for this study. The study material consisted of 11 dogs presented to Kırıkkale University Veterinary Faculty Research and Application Hospital between June 2020-2022 with a complaint of lameness in the hind limbs, diagnosed with coxofemoral luxation and treated with toggle pins. In the clinical examinations, all of the dogs had unilateral coxofemoral luxation. There was no concurrent injury.

Anesthesia and analgesia: The dogs were premedicated with medetomidine hydrochloride (80 mcg/kg) (Domitor; Zoetis, Finland) and induced with ketamine HCl (5 mg/kg) (Ketasol 10%; Richterpharma, Austria) via intravenous (IV) route. Butorphanol (0.4 mg/kg) (Butomidor; Richterpharma, Austria) was administered as an analgesic via IV route. Afterward, the animals were intubated, and anesthesia was maintained with isofluorane (Adeka, Turkey). Intravenous Cefazolin sodium (22 mg/kg) (Eqizolin, Tüm Ekip Ilac AS, Turkey) was administered 30 min before the operation and in the perioperative period.

Operative Approach: According to Çetinkaya and Olcay (2011), some toggle pins used in operation were formed from Kirschner wires, while others were purchased ready-made and sterilized. The ligament capitis ossis femoris was replaced with a monofilament nylon or a polyester multifilament braided yarn.

Then a hole was drilled in the acetabular fossa with a 2.7 mm or 3.5 mm diameter drill depending on the size of the animals, and the toggle pin was pushed through the created hole into the canalis pelvis (Figure 1A and 1B). Thus, when the thread was pulled back, the toggle pin rested against the medial wall of the acetabulum (Figure 1C). Guidewire was used to tunnel from the fovea capitis to the third trochanter, and a 2.0 mm or 2.7 mm diameter drill was used depending on the animal's size (Figure 1D). Under the guidance of a 0.80 mm Kirschner wire, the suture material attached to the toggle pin was fed through the tunnel (Figure 1E). After joint reduction, a button made of Kirschner wire was used to secure the end of the monofilament nylon to the proximal lateral surface of the femur at the level of the third trochanter (Figure 1F). As stated in the literature (Wardlaw and Mclaughlin, 2018), the objective was to preserve joint reduction until the formation of periarticular fibrous connective tissue. Monofilament nylon was used as suture material, and polyester multifilament braided thread in 8 and 3 dogs, respectively.

Postoperative Care and Control: All animals received subcutaneously 0.2 mg/kg meloxicam (Bavet; meloxicam; Turkey) for five days, to avoid postoperative pain and administered orally 12.5 mg/kg amoxicillin clavulanic acid (Synulox; Zoetis, Finland) for one week. No support material was applied to the extremity, and patients were advised to restrict movement for three weeks. Postoperatively, the Elizabethan collar was used for ten days (until the sutures were removed) to prevent the risk of suture damage. Clinical examinations and control radiographs were taken at 1, 2, 4 weeks, and two months postoperatively to evaluate the joint status. Pain, infection, crepitation, and degree of lameness were also checked. The differences between the animals were evaluated with the findings obtained. Postoperative lameness scoring was numbered from 0 to 5 (0: no lameness, 5: no weight bearing on the limb) according to a previous study (Ramirez et al., 2015).

Results

The average age of the different breeds and sexes of operated animals was 19.2 months (min:5-max:38), and their average body weight was 20.5 kg (min:11-max:28). On average, surgical intervention was performed 2.55 days (min:1-max:4) after trauma. Seven operated animals were male, and four were female. Breeds of dogs were Golden Retriever (n=2), Crossbreed (n=5), Pointer (n=1), Chow Chow (n=1), Staffordshire (n=1), and Cane Corso (n=1). Craniodorsal luxation was detected in all patients. Congenital hip dysplasia was observed in onepatient (case 11). No orthopedic abnormality except for luxation was observed in the other animals. In three of the animals (cases 4, 6, and 7), toggle pins made of commercially available polyester multifilament braided yarn were used (Table 1). In two of the animals (cases 4 and 6, in which polyester multifilament braided yarn was used), lameness was observed four and five days after the operation, and reluxation was detected in the radiographic examination (Figure 2). Caput femoris degenerations were discovered during the reoperation on these

two patients, and it was decided to perform excision arthroplasty. Patients who did not experience reluxation began using their extremities on the 1st postoperative day, and no abnormality was postoperatively observed in the radiographic and clinical examinations in the following weeks (Figures 3 and 4). Median scores of lameness was 1 (range 0-5) on the first day and was 0 on the seventh day postoperatively (P=0.008) for nine patients without reluxation. (Table 2).

Figure 1. A) A hole was drilled in the acetabular fossa. **B)** Toogle button advanced into the acetabular hole. **C)** Toggle button seat on the medial wall of acetabulum. **D)** A guidewire placed from fovae capitis to third trochanter and bone tunnel was created. **E)** Passing the toggle suture material through the femoral bone tunnel. **F)** Knotting of the suture material at the level of the third trochanter after reduction.

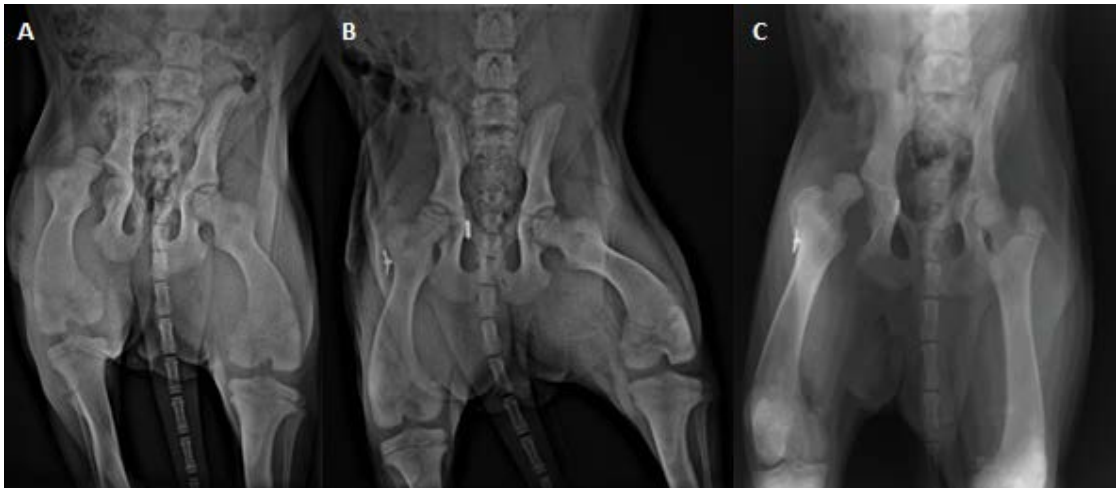

Figure 2: Ventrodorsal radiographic images of case 4, A) preoperative, B) immediately after surgery, C) 5th day after surgery.

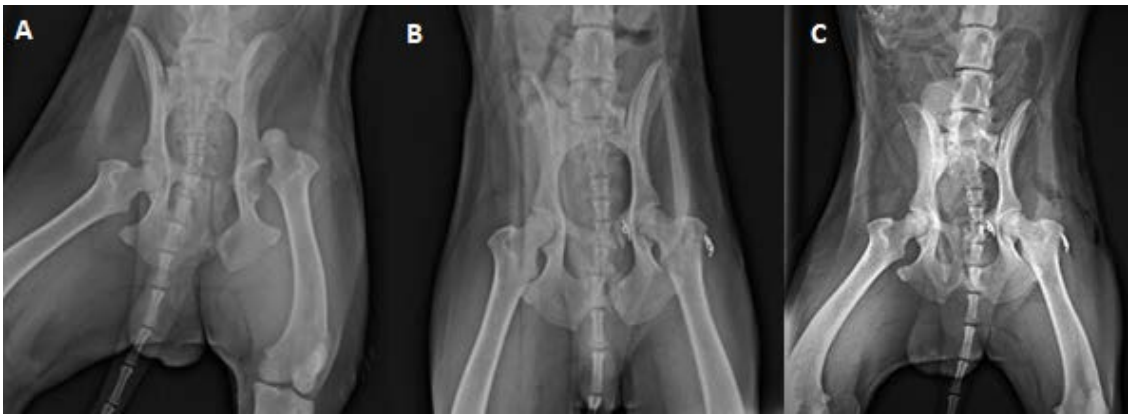

Figure 3: Ventrodorsal radiographic images of case 9, A) preoperative, B) immediately after surgery, C) 2 months after surgery.

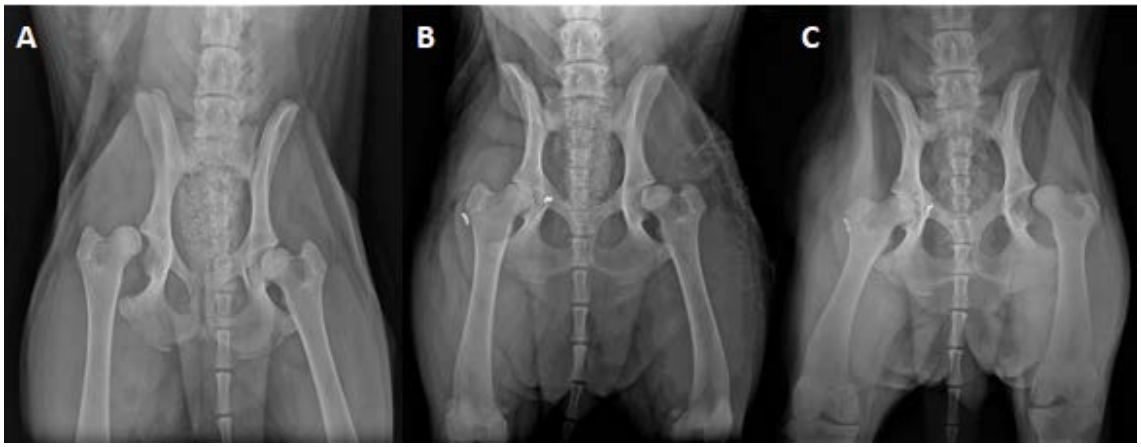

Figure 4: Ventrodorsal radiographic images of case 11, A) preoperative, B) immediately after surgery, C) 14th day after surgery.

The animal was placed in the supine position with the injured limb on top. The operation area was shaved, decontaminated, and prepared for aseptic surgery. A craniolateral approach involved external rotation and adduction of the extremity in reaching the hip joint. The acetabulum was cleared of debris, hematoma, fibrin, granulation tissues, and the remnants of the detached ligament capitis ossis femoris.

Discussion

Different reduction techniques, including intra- and extra-capsular, have been reported in coxofemoral luxations (Ash et al., 2011; Demko et al., 2006; Rocherau and Bernard, 2012; Venzin and Montazon, 2007) and some studies are recommend using different suture materials in toggle pin fixation one of these techniques (Bone et al., 1984; Çetinkaya and Olcay, 2011; Hoim et al., 2005; Martini et al., 2001). In the study by Spranklin et al. (2006), the weight-bearing capacities of various materials were examined in the toggle pin technique applied to cadavers and They reported that monofilament suture material could not support a weight of 40 kg, causing damage to the line on the caput femoris and causing it to rupture. Demko et al. (2006) used monofilament nylon sutures in large breed dogs and reported very low (11%) reluxation problems. In all studies, toggle pin or rod fractures, ruptures in the prosthetic ligament, fractures of the caput femoris or colum femoris, and related reluxations were reported as primary complications. Infection and sinus formation are said to be related to the artificial ligament material used. Reluxation cases are reported to occur within two weeks postoperatively (Kieves et al., 2014; Martini et al., 2001; Trostel and Fox, 2020). Previous studies have reported that polyester multifilament braided rope is more elastic and resistant to stretching thanks to its knitting structure (Martini et al., 2001). A previous study conducted by Çetinkaya et al. (2011) reported that monofilament nylon was more durable than braided ropes in coxofemoral luxations in their study. Another study stated that there was no difference

Table1: Details and clinical outcomes of 11 dogs postoperatively.

Case	Breed	Sex	Age (month)	Body Weight (kg)	Luxation direction	Duration of luxation (day)	Toggle suture material	Evaluation of outcomes postoperatively
1	Mix Breed	M	17	18	CD/R	3	Monofilament nylon	Excellent
2	Golden Retriever	F	13	25	CD/L	3	Monofilament nylon	Excellent
3	Mix Breed	M	11	22	CD/L	4	Monofilament nylon	Excellent
4	Cane corso	M	5	11	CD/R	2	Polyester multifilament braided	Reluxated (5th day)
5	Stafford shire terrier	M	12	28	CD/L	2	Monofilament nylon	Excellent
6	Mix Breed	F	26	20	CD/L	1	Polyester multifilament braided	Reluxated (4th day)
7	Mix Breed	M	38	21	CD/R	3	Polyester multifilament braided	Excellent
8	Golden retriever	M	18	23	CD/L	2	Monofilament nylon	Excellent
9	Mix Breed	F	24	19	CD/L	2	Monofilament nylon	Excellent
10	Pointer	F	23	18	CD/L	3	Monofilament nylon	Excellent
11	Chow Chow	M	24	20	CD/R	3	Monofilament nylon	Excellent

Table2: Lameness scores at the 1st and 7th day postoperatively**.**

	Case1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7	Case 8	Case 9	Case 10	Case 11
Post-op 1st day	2	1	2	1	1	1	2	1	2	2	1
Post-op 7th day	0	0	0	5	0	5	0	0	0	0	0

between braided polyester ropes and monofilament nylon (Flynn et al., 1994). We used monofilament nylons and polyester multifilament braided ropes as artificial ligament materials in toggle pin applications, frequently used in coxofemoral luxation cases. We observed that the monofilament nylon was more durable than polyester multifilament braided rope and could be successfully applied in animals under 30 kg. This study determined that the monofilament nylon did not break in the postoperative period, and the animals still used the relevant leg very comfortably in the second month of control. The findings are in good agreement with the study of Çetinkaya et al. (2011). We suggested that using monofilament nylons in intra-articular stabilization is more satisfactory than polyester multifilament braided yarn. No radiographic or clinical evidence of osteoarthritis was found in the second month of postoperative follow-up. It should be noted, however, that dogs weighing no more than 30 kg were used in this research. In accordance with previous studies, postoperative reluxation occurred within the first week after surgery in cases where polyester multifilament braided yarn was utilized. This is believed to be because polyester multifilament braided threads are less flexible than monofilament nylons and may deteriorate more rapidly when in contact with bone.

In coxofemoral luxations, the duration of the operation is an essential factor in preventing caput femoris damage, achieving reduction, and reducing the risk of reluxation. It has been reported that the longer the reduction time, the greater the risk of caput femoris damage and abnormal formations in the acetabular fossa, such as fibrin and granulation tissue (Matthews ve Bernhart, 2021). In the present study, animals were operated on within the fourth post-traumatic day. No damage to the caput femoris was found at the time of the operation. Of the two cases with reluxation, one was operated on the second day and the other on the first day. However, the reason for recurrence is thought to be the low durability of the suture material because no recurrence was observed in the repair performed with the monofilament nylon.

In the review by Trostel and Fox (2020), it is emphasized that the toggle pin technique has been the most common treatment for coxofemoral luxations in recent years, but the relapse rate has not decreased below 10% despite the use of different materials. In another study involving 128 dogs with coxofemoral luxation, the reluxation rate for toggle pin stabilization with monofilament nylon suture material was determined to be 24.2%. (Matthews and Bernhart, 2021). In this study, material-induced luxation was observed in animals with polyester multifilament braided yarn. No luxation was observed in animals with monofilament nylon suture material, and the rate of luxation was similar to Matthews and Bernhart (2021).

It has been reported that toggle pin stabilization in coccafemoral luxations gives positive results as long as no reluxation occurs. The most recently reported reluxation rate following toggle pin stabilization in large breed dogs (over 60 kg) was reported to be 11%, and monofilament nylon suture was used in 96% of cases (Trostel and Fox, 2020). In the study, it was observed that the animals weighed an average of 20 kg, and it was noteworthy that the animals with reluxation were animals weighing less than 20 kg. In addition to weight, it is believed that the flexibility and tensile strength of the material used are much more significant in the formation of reluxation.

Coxofemoral joint luxations have historically been treated with the Toggle pin technique. Although different materials have been tried in the studies, the weight and skeletal structure of the animal are among the factors that influence success. In this study, the lower recurrence rate of the line used for Toggle pin application compared to the polyester multifilament braided yarn mentioned in the literature is deemed to be a significant finding. It is believed, however, that stronger results will be obtained when supported by studies involving a greater number of animals and longer postoperative monitoring.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

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Evaluation of The Efficiency of Glutaraldehyde Coagulation Test in Some Cattle Diseases: 2021-2022 Retrospective Study

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Abstract: Metabolic disorders occurring in negative energy balance (NEB) in cows cause diseases related to immunosuppression and economic losses. Although treatment, animal welfare, and herd immunity are possible with comprehensive clinical diagnostics combined with laboratory evaluation, the applicability and availability of these analytes in the farm setting are often limited. Therefore, the glutaraldehyde coagulation (GC) test, a fast, practical, and inexpensive test, help diagnose inflammatory diseases in cattle. This study aims to evaluate the effectiveness of the GC test in certain bovine diseases whose etiology is classified as infectious or non-infectious due to clinical and laboratory examinations. The animal material was 40 Holstein cows with various clinical findings brought to Harran University, Veterinary Faculty Animal Hospital for diagnosis and treatment. Following the anamnesis, physical examinations of all the cows were performed. As a result of the hemogram analysis of the venous blood samples (8-10 mL) along with the physical examination findings, the cows were divided into two subgroups, Infectious (n:14) and Non-Infectious (n:26); GC test was performed from the venous blood samples. In the physical examination, the respiratory rate, heart rate, and body temperature of the cows in the Infectious Group were higher than those of the Non-Infectious Group ($p<0.008$). WBC and granulocyte levels of the Infectious Group were higher than the Non-Infectious Group ($p<0.000$). The GC test result was 70 seconds in the Infectious Group and 165 seconds in the Non-Infectious Group ($p<0.001$). The optimal cut-off value was 100 seconds (sensitivity: 87%, specificity: 72.7%) for detecting the presence of an inflammatory process in the ROC analysis. As a result, it was concluded that the GC test might give results ranging from mild to severe even in some non-infectious diseases such as rumen alkalosis, left displacement of the abomasum and ulcer, and when evaluated along with clinical findings in a farm setting, it can provide helpful information for the diagnosis of the diseases.
Keywords: *Cattle, Diagnosis, Glutaraldehyde coagulation test, Infectious, Inflammatory.*

Bazı Sığır Hastalıklarında Gluteraldehit Koagulasyon Testinin Etkinliğinin Değerlendirilmesi: 2021-2022 Retrospektif Çalışma

Özet: İneklerde negatif enerji dengesinde (NED) oluşan metabolik hastalıklar, immunsupresyona bağlı hastalıklara sebep olup ekonomik kayıplara yol açmaktadır. Hastalıkların tedavisi, hayvan refahı ve sürünün korunması, kapsamlı klinik diagnostikler ile birlikte laboratuvar değerlendirilmesi ile mümkün olsa da çiftlik ortamında bu analitlerin uygulanabilirliği ve bulunabilirliği genellikle kısıtlıdır. Bu sebeple hızlı, pratik ve ucuz bir test olan gluteraldehit koagulasyon (GK) testi, sığırların yangısal hastalıklarının tanısında faydalıdır. Bu çalışmanın amacı, klinik ve laboratuvar muayeneleri sonucu hastalık etiyolojisi enfeksiyöz veya non-enfeksiyöz olarak sınıflandırılmış bazı sığır hastalıklarında GK testinin etkinliğini değerlendirmektir. Hayvan materyali Harran Üniversitesi Veteriner Fakültesi Hayvan Hastanesine çeşitli klinik bulgularla, tanı ve tedavi amacı ile getirilmiş toplam 40 adet Holstein inekti. Anamnez bilgisini takiben tüm ineklerin fiziksel muayeneleri gerçekleştirildi. Fiziksel muayene bulguları ile birlikte alınan venöz kan örneklerinin (8-10 mL) hemogram analizi sonucu, çalışmaya dahil edilen inekler Enfeksiyöz (n:14) ve non-Enfeksiyöz (n:26) olarak iki alt gruba ayrıldı, tüm ineklerin venöz kan örneklerinden GK testi gerçekleştirildi. Fiziksel muayenede Enfeksiyöz Grubu ineklerinin solunum sayısı, kalp ritmi ve vücut sıcaklığı Non-Enfeksiyöz Gruba göre daha yüksekti ($p<0.008$). Enfeksiyöz Grubun WBC ve granülosit düzeyleri Non-Enfeksiyöz Gruba göre daha yüksekti ($p<0.000$). GK testi sonucu Enfeksiyöz Grubunda 70 saniye iken Non-Enfeksiyöz Grubunda 165 saniyeydi ($p<0.001$). ROC analizinde yangısal süreç varlığının tespiti için optimal cut-off değeri 100 saniyeydi (sensitivite: %87, spesifite: %72.7). Sonuç olarak, GK testinin enfeksiyöz etiyolojide olmayan rumen alkalozu, abomazumun sola deplasmanı ve ülseri gibi bazı hastalıklarda bile hafif şiddetliden şiddetliye değişen sonuç verebileceği ve çiftlik ortamında klinik bulgularla birlikte değerlendirildiğinde hastalık tanısına yardımcı bilgi sağlayabileceği kanısına varıldı.
Anahtar Kelimeler: *Enfeksiyöz, Gluteraldehit koagulasyon testi, Sığır, Tanı, Yangısal.*

Introduction

It is a well-known fact that negative energy balance (NEB) and immunosuppression are the endemic insidious underlying causes of cattle diseases in herds and cause significant economic losses

(Abramowicz et al., 2019; Lei and Simoes, 2021; Mostert et al., 2018). Metabolic diseases such as fatty liver and ketosis occur in cows due to the development of NEB (Mostert et al., 2018). There is an increase in the amount of non-esterified fatty acids (NEFA) due to the formation of fatty liver, and these fatty acids are esterified to triglycerides as a result of the oxidation of hepatic fatty acids. It has been reported that ketosis, hypocalcemia, reticulo pericarditis traumatica (RPT), retention, metritis, mastitis, and increased risk of immunosuppression and decreased reproductive performance can be seen as a result of fatty liver (Lei and Simoes, 2021).

Immunosuppression, which develops with metabolic diseases, especially in the NEB period, causes many diseases and economic burdens (Liang et al., 2017; Zhang and Ametaj, 2020). Immunity should be boosted to prevent the occurrence of diseases in this period. It is well-known that the modern cattle industry is based on balanced nutrition, appropriate zoo-hygienic care, animal welfare, and herd protection (Abramowicz et al., 2019; Roche et al., 2013). The persistence of this is possible with comprehensive clinical diagnostics and laboratory evaluation. Applicability and availability of clinical assessment methods, particularly in the farm setting, are often limited, and in addition to this, economic concerns come forth. Thus, the diagnostic effectiveness and practicality of these methods used diagnosing of various diseases have been discussed recently (Ok et al., 2009; Overton et al., 2017). The glutaraldehyde coagulation (GC) test is a fast, practical, and inexpensive test used to diagnose of inflammatory diseases in cattle for years (Aslan and Ok, 1991; Metzner et al., 2007). The GC test primarily indicates increased serum fibrinogen and globulin concentrations. Glutaraldehyde reacts first-degree chemical reaction with the free amino groups in fibrinogen and immunoglobulin to form a clot. Thus, the GC test's clotting time provides an estimated amount of the protein content produced in response to the inflammatory process (Akyüz ve Aydın, 2022; Tschone et al., 2021, Tschone et al., 2022).

This study aims to evaluate the diagnostic efficacy of GC tests in some bovine diseases whose etiology is classified as infectious or non-infectious due toclinical examinations along with hemogram analysis.

Materials and Methods

Ethical approval: This study was conducted with the approval of the Harran University Animal Experiments Local Ethics Committee dated 17/10/2022, number 2022/008.

Animal Material: The animal material of this study consisted of 40 Holstein cows with various clinical findings; all were admitted to Harran University Veterinary Faculty Animal Hospital for diagnostic and/or treatment purposes between the dates 2021 and 2022. Following the anamnesis, physical examinations including measurement of respiratory rate, heart rate, rectal body temperature, and evaluation of palpable lymph nodes along with lung, heart, rumen, and intestinal auscultation were performed. As a result of the detailed clinical assessment, further diagnostic examinations were carried out to determine whether the existing disease was infectious or non-infectious.

Diagnosis of Health Issues: Diseases detected in cows of the Infectious Group were pneumonia (n:7) and enteritis (n:7). Diagnosis of pneumonia was made based on the presence of fever, nasal discharge, cough, and changed respiratory character (Demir and Bozukluhan, 2012). For the diagnosis of enteritis, clinical findings such as diarrhea and dehydration were considered (Bonadiman et al., 2018).

Diseases detected in cows of the Non-Infectious Group were abomasum ulcer (n:2), presence of bezoars (n:1), downer cow (n:1), Hoflund syndrome (n:1), simple indigestion (n:2), hepatic lipidosis (n:6), reticulo pericarditis traumatic (RPT) (n:7), rumen alkalosis (n:4), subcutaneous emphysema (n:1) and left displacement of the abomasum (LDA) (n:1). For the diagnosis of abomasum ulcer, the presence of clinical findings such as abdominal pain, melena, pale mucous membranes, indigestion, and low milk yield were considered (Radostitis et al., 2000). For the presence of bezoars, ultrasonographic imaging with cessation of defecation, loss of appetite, abdominal pain, decreased rumen motility, and succussion splash sound in the abdomen was considered (Abutarbush and Naylor, 2006). For the diagnosis of Downer cow syndrome, the inability to stand up within 24 hours and/or lie in the sternal position for more than 24 hours without showing signs of systemic disease, despite hypocalcemia treatment, were taken as the criterion (Dahlberg, 2012). For Hoflund syndrome, posterior functional stenosis with rumen dilatation and nutritional fullness, recurrent tympani, gradual weakness, and bradycardic symptoms were considered (Radostitis et al., 2007). For the diagnosis of simple indigestion, the presence of abrupt changes in the structure, amount, or frequency of diet in the anamnesis along with anorexia and decrease in milk yield, and abnormal rumen content findings such as a decrease in the number, size, and activity of protozoa and change in ruminal pH were considered (Constable, 2010). The criteria for the diagnosis of hepatic

lipidosis were increased liver size, rounding of liver borders, hyperechoid structure of the liver parenchyma towards the abdominal wall, and inadequate visualization of hepatic vessels as a result of ultrasonographic examination performed through the 10th intercostal space on the left side (Ok et al., 2013). For the diagnosis of RPT, fever, anorexia, increased heart and respiratory rate, ruminal stasis, frequently recurring tympani, abdominal tension, abdominal pain, groaning, dehydration and weakening along with tachycardia, venous engorgement, positive venous pulse, heart churning or rubbing sound, and the presence of edema in the submandibular region were considered (Roth and King, 1991). For the existence of rumen alkalosis, the presence of pH >7.2 with a decrease in the movement and number of rumen infusoria was accepted (Guzelbekteş and Şen, 2014). For the diagnosis of subacute emphysema, painless subcutaneous crepitation on palpation, dyspnea, and loss of appetite were considered (Muhammad et al., 2017). For LDA, clinical findings such as sudden onset anorexia, presence of ping sound on auscultation, low milk yield, moderate abdominal pain, and costal posterior enlargement of the left fossa paralumbalis were evaluated (Fouda et al., 2004). In addition, the suspicion of LDA was confirmed by visualization of the abomasum on ultrasonographic examination.

Hemogram Analysis: Hemogram analysis was carried out from venous blood samples (8-10 mL, in K_3EDTA tubes) taken by vena jugularis venepuncture from all cows included in the study, and the analysis was performed using an autoanalyzer (Sysmex Hematology Analyser, poch-100i, Japan) within 5-10 minutes after sampling. Within the scope of hemogram analysis, leukocyte (WBC), granulocyte, lymphocyte, and monocyte levels were evaluated.

Forming Subgroups: The results of physical examination and hemogram analysis were used to determine whether the present health issues were infectious or non-infectious and to establish subgroups. Thus, the cows included in the study were divided into two subgroups Infectious (n:14) and Non-Infectious (n:26) groups.

Performing Glutaraldehyde Coagulation Test: A glutaraldehyde coagulation (GC) test was performed on all the cows' venous blood samples, divided into subgroups. For this reason, we used a solution prepared from 50 mL of 25% glutaraldehyde, 1 g of Na_2EDTA, and 1 liter of 0.9% NaCl solution (saline). The components were mixed homogeneously and maintained in dark glass receptacles for optimal storage. For the test, 2 mL of blood was collected and mixed with 2 mL of the aforementioned glutaraldehyde solution in an anticoagulant collection tube (K_3-EDTA) (Doll et al., 1985). The GC was measured for up to 15 minutes or until coagulation (Doll et al., 1985). We used a digital chronometer to keep time. We interpreted the results as 0 – ≤ 3 minutes for severe, >3 – ≤ 6 minutes for moderate, >6 – ≤15 minutes for mild, and > 15 minutes for a non-detectable inflammatory process (Aslan ve Ok, 1991; Doll et al., 1985; Turgut, 2000). GC test results of all the cows included in the study are presented in Table 3.

Statistical Analysis: All data were evaluated using SPSS 25.00 (SPSS for Windows) statistical software. A one-sample Kolmogorov–Smirnov test was used to determine whether all data were parametric or nonparametric. Nonparametric data were presented as median (min-max) using the Mann–Whitney U, Kruskal-Wallis test. To investigate how accurately the present diagnostic glutaraldehyde coagulation test discriminates the presence of an inflammatory state and to determine optimal sensitivity, specificity, and cut-off values, receiver operating characteristic curve (ROC) analyses were performed. Statistical significance was considered at a $p<0.05$ for all data.

Results

In the physical examination, the respiratory rate, heart rate, and body temperature of the cows in the Infectious Group were higher than that of the Non-Infectious Group ($p<0.008$). Physical examination findings are presented in Table 1.

As a result of hemogram analysis, it was determined that WBC and granulocyte levels of the Infectious Group were higher than the Non-Infectious Group ($p<0.000$). There was no statistical difference between the groups regarding lymphocyte and monocyte levels. Hemogram analysis results are presented in Table 2.

As a result of the GC test, the coagulation time of the Infectious Group was determined as 70 seconds, while the duration of the Non-Infectious Group was 165 seconds ($p<0.001$) (Table 3). In addition, as a result of the receiver operating characteristic (ROC) analysis, it was determined that the optimal cut-off value was 100 seconds (sensitivity: 87%, specificity: 72.7%) for the determination of the presence of an inflammatory process. The ROC curve is presented in Figure ,1 and ROC analysis results are shown in Table 4.

Discussion and Conclusion

Studies have shown that the GC test is a helpful test that maintains its importance among diagnostic tools for a long time without losing its currency (Akyüz and Aydın, 2022; Aslan and Ok, 1991;

Metzner et al., 2007; Tschone et al., 2021, Tschone et al., 2022). The easily accessible, cheap, and fast results make the test frequently preferred in determining the presence of inflammation,

Table 1. Physical examination findings.

Parameters	Infectious Group (n: 14) median (min-max)	Non-Infectious Group (n:26) median (min-max)	p value
Respiratory rate (breath/min)	64 (35-80)	37 (22-72)	0.000
Heart rate (beats/min)	80 (64-108)	64 (48-84)	0.000
Body temperature (°C)	39.1 (37.9-40.2)	38.4 (37.1-40)	0.008

Table 2. Hemogram analysis results.

Parameters	Infectious Group (n: 14) median (min-max)	Non-Infectious Group (n:26) median (min-max)	p value
WBC ($x10^9$ cells per liter)	**15.65 (14.1-29.5)**	**9.9 (4.9-18.5)**	**0.000**
Granulocyte ($x10^9$ cells per liter)	**11.95 (11.3-24.9)**	**6.1 (2.2-12.4)**	**0.000**
Lymphocyte ($x10^9$ cells per liter)	2.6 (2.1-5)	2.3 (0.8-8.1)	0.745
Monocyte ($x10^9$ cells per liter)	0.65 (0.2-1.1)	0.5 (0.2-1.7)	0.582

Table 3. Intergroup glutaraldehyde coagulation test results.

Parameters	Infectious Group (n: 14) median (min-max)	Non-Infectious Group (n:26) median (min-max)	p value
Glutaraldehyde coagulation time (seconds)	70 (30-280)	165 (60-1020)	0.001

Table 4. Diagnostic ROC analysis result of the glutaraldehyde coagulation test.

Area Under Curve	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval		Cut-off	Sensitivity	Specificity
			Lower Bound	Upper Bound			
0.866	0.068	0.001	0.733	0.998	100	87%	72.7%

The test result variable(s): Glutaraldehyde coagulation test has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

[a]Under the nonparametric assumption

[b]Null hypothesis: true area = 0.5

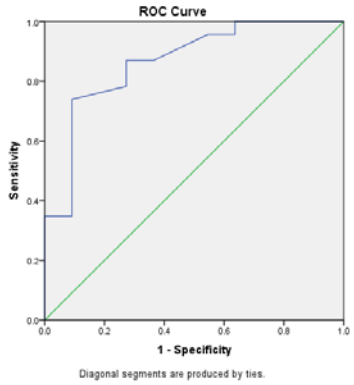

Figure 1. Diagnostic ROC curve of the glutaraldehyde coagulation test. Diagonal segments are produced by ties.

especially in a farm setting (Aslan and Ok, 1991; Turgut, 2000). In the present study, a significant difference was observed in GC test results between the Infectious and Non-infectious groups ($p<0.001$). Also, it was observed that the GC test is useful in understanding whether the diseases are of infectious etiology. According to the diagnostic ROC analysis, the cut-off value of the GC test in the differentiation of infectious and non-infectious diseases was determined as 100 seconds, considering the optimal sensitivity (87%) and specificity (72.7%) values (Table 4).

Respiratory system diseases of cattle are the most common diseases that harm the herd health and livestock industry (Peel, 2020). It is reported that the mortality of bovine respiratory system diseases in the United States causes an annual loss of 907 million United States dollars (USDA, 2017). The fact that respiratory system diseases cause high economic losses and are observed most frequently indicates that there is a need for up-to-date studies on the subject. A previous study reported that the GC test result was 2 minutes in cattle with pneumonia (Metzner et al., 2007). In a study of 30 calves in which the GC test was evaluated, it was reported that the coagulation time was less than 3 minutes (severe) in 18, between 3-6 minutes in 9 (moderate), and 6-15 minutes in 3 (mildly severe) out of 30 calves (Akyüz et al., 2022). The GC test time of the present study was 70 (35-90) seconds in cows of the Infectious Group was classified as severe, and the results were consistent with previous studies.

In cattle, especially calves, enteritis and diarrhea are important herd health problems, with the highest mortality causing significant economic loss following respiratory system diseases (Aygün and Yıldız, 2018;). In the literature review, we could not find a study in which the GC test was evaluated in cases of enteritis with an infectious etiology. However, it was reported that the GC test result ranged between 21-36 minutes (negative) in cases of enteritis due to alimentary simple indigestion etiology (Uhde et al., 2008). In the present study, the GC test result of cows in the Non-Infectious Group with simple indigestion was determined as 165 (70-200) seconds (severe). Compared with previous studies, the shortened GC time of the present study may be related to the amount and composition of feed consumed, and the time to hospital admission, which may affect the severity of the inflammation. In addition, due to the working principle of the GC test, a negative test result is a typical situation where the fibrinogen and globulin levels do not increase and/or slightly increase (Turgut, 2000). In the present study, the 70 (30-280) seconds (severe) GC time of the cattle with infectious enteritis was associated with increased amounts of globulin and fibrinogen during the infectious process compared with the alimentary enteritis cases. Investigation of the GC test in more cattle with enteritis of infectious etiology is recommended to demonstrate the diagnostic efficiency of the test.

Abomasum ulcer in cattle is an important condition that causes various clinical findings according to its grade, and diffuse peritonitis is a common finding. In cases of type-4 ulcers, the prognosis is unfavourable, and death usually occurs within 24-48 hours (Braun et al., 2019a, Braun et al., 2019b). In a study conducted in cattle with abdominal ulcers, GC test results were severe in 14% (<3 min), moderate in 17% (3-6 min), mild (6-15 min) in 67%, and negative in 2% (>15 min) (Braun et al., 2019 b). The GC time of the cattle with abomasum ulcers in the Non-Infectious Group of the present study was determined as 165 (104-230) seconds (severe). The higher values in the present study may be related to the grade of abomasal ulcer. It was reported that the severe GC test results in the previous study were observed in cows with third-grade abomasal ulcers (Braun et al., 2019a). Abomasal perforation was detected in two of the present cases, characterized by antemortem findings such as severe melena and pale mucous membranes and were referred to slaughter; thus, they were evaluated as grade 4. For this reason, more severe and widespread inflammation in grade 4 ulcer types causes a severe GC test result. Although the GC test results in cows with abomasum ulcer was defined as severe in the present study, it is recommended to investigate the diagnostic efficacy of the GC test in a study in which abomasum ulcers are classified according to their grade.

Reticuloperitonitis traumatica (RPT) is an important condition that causes different degrees of inflammation and complications in cattle due to sharp foreign bodies ingested with feed, penetrating the reticulum and damaging various tissues and organs (Braun et al., 2020). In a study, GC test results

were found to be severe in 80%, moderate in 15%, and mild in 5% of the group with adhesive RPT. In the same study, it was reported that in cows with non-adhesive RPT, 30% were severe, 45% were moderate, and 25% were mild (Akyüz and Aydın, 2022). In a study conducted in cases with acute and chronic RPT, it was reported that the GC time was 191 seconds in acute and 131 seconds in chronic cases (Ozba, 1996). In the present study, it was determined that the GC test result was 145 (33-360) seconds (severe) in cattle with RPT of the Non-Infectious Group (6 severe, 1 moderate). These findings may be related to the severe, acute, adhesive nature of RPT and the delayed admission time to the hospital.

Functional gastric stenosis (Hoflund syndrome) is a transitional disorder characterized by inhibition of gastric passage. Compression and/or damage of the various branches of the vagus nerve innervating the stomach prevent passage in the pylorus, ostium reticulomasi and/or cardia parts of the stomach and as a result, the disease occurs (Bilal, 2004; Gul and Issi, 2009). The results of the present study were in agreement with the previously reported results (Gül ve Issi, 2009). These findings may be related to the fact that denervation in the present case was associated with the presence of RPT (Bilal, 2004).

Although the presence of bezoar varies according to its localization, it can cause functional stenosis similar to Hoflund syndrome. In addition to stenosis, it can cause compression ischemia, and consequently necrosis, and gangrene in the affected region. Thus, the onset of an inflammatory process due to compression is expected (Cortés-Beltrán et al., 2022; Tschuor et al., 2010). Also, the results of the GC test may vary according to the severity of the inflammation in the region where the bezoars are located. The cows affected by the presence of bezoars in the present study had a GC time of 15 minutes (mild). The mild GC test result detected in the present study may be associated with ischemia and necrosis caused by the bezoar that initiates the inflammatory process (Tschuor et al., 2010).

Left displacement of the abomasum (LDA) is an important postpartum disease, especially in high-yielding dairy cows (LeBlanc et al., 2005). In a study conducted on 52 cows diagnosed with LDA, the GC test results were determined as moderately severe (6.5 (0.5-16) minutes) (Tschoner et al., 2021). In the present study, there was only 1 case with LDA, and the test was described as mild (10.5 minutes). The very different GC values in the previous study may indicate that the inflammatory process is related to the displacement's time of occurrence and severity. Since it was the first day of the cow's complaints in the present study, a mild GC test was considered as an expected result.

Although subcutaneous emphysema is mainly related to respiratory distress, it can also develop due to trauma, operative interventions, and gaseous gangrene (Rashid and Saqi, 2017). In the literature review, no study was found in which the GC test was evaluated in cows with subcutaneous emphysema. However, it was reported that the severity of inflammation and clinical findings that may occur in the cattle are highly variable depending on the etiology (Rashid and Saqi, 2017). GC test result was negative (17 min) in the cow with subcutaneous emphysema of the present study. Anamnestic data revealed that this case occurred due to traumatic reasons. During the physical examination, it was observed that the affected area was scanted and could not contribute significantly to generalized inflammation. Therefore, it was considered normal that the GC test did not result in a positive.

Causes such as sudden consumption of protein-rich feed in a short time, feeding with excessive amounts of urea, sudden ration changes, and providing large amounts of straw for a long time raise the rumen pH to 7.5 and above. This condition is called rumen alkalosis. (Vijayakumar et al., 2010). In the literature review, no study was found in which the GC test was evaluated in cows with ruminal alkalosis. Since the increase in rumen pH causes a decrease in the number of saccharolytic and amylolytic microorganisms and an increase in E. coli, Pseudomonas and Proteus group bacteria, the severe and moderate cases of the present study was associated with the inflammatory process due to overgrowth of these aforementioned bacteria (Guzelbekteş and Şen, 2014).

Especially in the first period of the postpartum period, the occurrence and prolongation of NEB state in high-yielding dairy cows increase the risk of hepatic lipidosis (Collins and Reid, 1980). Ketone bodies, which can reach pathological levels in the blood circulation as a result of NEB, cause generalized inflammation with metabolic acidosis (Ingvartsen, 2006; Lei and Simoes, 2021). Therefore, a positive GC test result is expected. In the Non-Infectious Group of the present study, 4 severe (128 (60-160) seconds) and 2 moderate (270 (180-360) seconds) cases were observed. These findings may be related to the presence of endotoxin, which enters the blood circulation from the rumen and contributes to the development of hepatic lipidosis and the activation of the immune system and acute phase response in cases of hepatic lipidosis (Ametaj, 2005).

This study has some limitations. Although the small sample size scenario is common in medical tests, a comprehensive study of small sample size properties of various methods for constructing the confidence/credible interval (CI) for the AUC has yet

to be included in the literature. As previously reported (Feng et al., 2017), it was observed that the larger the true AUC value and the smaller the sample size, the larger the discrepancy among the results of different approaches. Therefore, the major limitation of this study is the limited number of animals which may influence the results of the ROC-based diagnostic performance analyses. Hence, the authors recommend evaluating the promising results of this study with a larger number of animals with various diseases.

In the present study, the diagnostic efficacy of the GC test was evaluated along with clinical examinations and hemogram analysis results in infectious diseases such as pneumonia and enteritis and non-infectious diseases such as ulcer of the abomasum, presence of bezoars, downer cow, Hoflund syndrome, hepatic lipidosis, rumen alkalosis, and LDA. Conducting future studies on diseases that were evaluated in the present study, but for which the GC test has not been evaluated yet, may increase the effectiveness and prevalence of the GC test. Although the current study results are promising, there is a need for studies investigating the diagnostic and prognostic efficacy of GC tests with more animal material. As a result, it was observed that the GC test might give results ranging from mild to severe even in some non-infectious diseases such as rumen alkalosis, left displacement and, ulcer of the abomasum. It was concluded that, especially in the farm environment where comprehensive laboratory analyzes are difficult to perform, when evaluated along with the clinical findings, the GC test could provide helpful information for the diagnosis and prognosis of various diseases.

Conflict of Interest

The authors stated that they did not have any real, potential or perceived conflict of interest.

Ethical Approval

Permission was obtained for this study with the approval of the ethics committee of Harran University HADYEK 01-05, session number 2022/008 and dated 17.10.2022. In addition, the authors declared that they comply with the Research and Publication Ethics.

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Erzincan İli Süt Sığırcılığı İşletmelerinde Postpartum Dönem Klinik Mastitis İnsidensi ve İnsidense Etkili Bazı Faktörler

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Özet: Bu araştırma; Erzincan ilindeki süt sığırcılığı işletmelerinde postpartum dönemin önemli endemik hastalıklarından biri olan klinik mastitis vakalarının insidensi ve bu insidensin bazı yetiştirici ve işletme özellikleri ile koruyucu hekimlik tercihlerine göre nasıl bir değişim gösterdiğini tespit etmek amacıyla gerçekleştirilmiştir. Araştırmanın materyalini Erzincan il merkezine bağlı Damızlık Sığır Yetiştiricileri Birliğine üye 910 işletmeden rastgele seçilen 63 işletmenin 683 adet postpartum dönem verisi oluşturmaktadır. Postpartum dönem klinik mastitis insidensinin ağırlıklı ortalaması %10,68 olarak hesaplanmıştır. Bu insidensin ırklara göre dağılımı; Montofon ve melezi ırkta %6,20; Simental ve melezi %10,71; Holştayn ırkında %15,65 olarak hesaplanmıştır. Küçük ölçekli, kapalı tip barınak ve bağlı sisteme sahip işletmelerdeki ineklerde insidens oranları sırasıyla %13,29; %11,44; ve %12,12 olarak ortalamadan daha yüksek oranda olduğu ve farklılığın p<0,05 düzeyinde (anlamlı) olduğu tespit edilmiştir. Postpartum klinik mastitis insidensinin; düzenli CMT uygulamasının yapıldığı (%3,5), ücret karşılığında danışmanlık hizmetinin alındığı (%4,31), süt sığırcılığı eğitiminin alındığı (%7,39), sağım öncesi daldırma kabı uygulandığı (%8,77) işletmelerdeki ineklerde daha düşük seviyede olduğu belirlenmiştir (p<0,05). Postpartum klinik mastitis insidensinin hedef insidens değeri %5 olarak hesaplanmış olup, mastitis kontrol yönteminde ihmal edilen hususların olduğu ve sakınılabilir kayıpların oluştuğu işletmelerdeki ineklerde insidensin ortalama %15,85 olduğu tespit edilmiştir.
Anahtar Kelimeler: *Endemik, İnsidens, Klinik mastitis, Mastitis kontrol, Postpartum, Süt sığırcılığı.*

Incidence of Postpartum Clinical Mastitis and Some Factors That Influence This Incidence in Dairy Herds in Erzincan Provinces

Abstract: This study was conducted to determine the incidence of clinical mastitis cases, one of the endemic diseases of the postpartum period, in dairy cattle farms in Erzincan province and how this incidence varies according to some breeder and farm characteristics and preventive medicine preferences. A total of 683 postpartum period data from 63 enterprises were randomly selected from 910 animal enterprises members of the Erzincan Cattle Breeders Association. The weighted average of the incidence of postpartum clinic mastitis was calculated as 10.68%. The distribution of the mastitis incidence was calculated as percentage following 6.20% in the Montafon breed, 10.71% in the Simmental breed, and 15.65% in the Holstein breed. The incidences of mastitis in small-scale of herd sizes, closed barn type, and tie-stalls barn were 13.29%, 11.44% and 12.12%, respectively. Those percentages were higher than the general average incidence of mastitis (P<0.05). On the other hand, the incidences of postpartum clinic mastitis were also calculated according to the producer's criteria, such as regularly taking California Mastitis Test (3.50%), routinely taking counseling service (4.31%), being educated for dairy herds (7.39%), applied teat dipping (8.77%). The dairy herds with the producer criteria mentioned above had a lower incidence of postpartum clinic mastitis than the general average (P<0.05). The target incidence value of postpartum clinical mastitis incidence was 5%. It has been determined that the incidence in cows is 15.85% on average in enterprises where there are neglected issues in the mastitis control method, and avoidable losses occur.
Keywords: *Clinical mastitis, Dairy, Endemic, Incidence, Mastitis control, Postpartum.*

Giriş

Türkiye'nin Doğu Anadolu Bölgesinde yer alan Erzincan ili; büyükbaş hayvan varlığı, arazi dağılımı ve tarımsal örgütlenme durumu göz önüne alındığında hayvancılık açısından önemli bir potansiyele sahiptir. Türkiye büyükbaş hayvan varlığının %0,71'i Erzincan'da bulunup ilin tarımsal hasılasının %57,38'ini hayvansal üretim değerleri oluşturmaktadır. Hayvancılık için önemli olan çayır-mera alanları il arazi yüzölçümünün %36'sını oluşturmaktadır. İl genelinde 8.170 adet büyükbaş hayvan işletmesi bulunmakta olup, bu işletmelerin %27,93'ü 1-5 baş, %20,13'ü 6-10 baş, %24,60'ı 11-20 baş ve %27,33'ü 21 baş ve üzeri hayvan varlığına sahiptir. Bu hayvanların sadece %3,6'sı yerli ırk olup geri kalanı kültür ve melezi ırklardan oluşmaktadır (Anonim (1), 2022; Anonim (2), 2022).

Süt üretiminin ilk basamağı olan süt sığırcılığı işletmelerinden temin edilen sütün içeriğini ve

üretim miktarını etkileyen faktörlerden birisi de işletmelerde yaygın olarak görülen mastitistir. Mastitis, enfeksiyöz (bakteri, virüs, mantar ve algler) ve enfeksiyöz olmayan (fiziksel ve kimyasal) etkenlere karşı memenin göstermiş olduğu anatomik, hücresel ve humoral yanıt neticesinde meme bezinin yangısı olarak tanımlanmaktadır (Biggs, 2009; Vural ve ark., 2016). Temel olarak klinik ve subklinik olmak üzere 2 ayrı formu bulunmaktadır. Klinik mastitis; meme bezinde kızarıklık, şişkinlik, ağrı, genel durum bozukluğu ve sütün yapısının değişmesiyle gözle teşhis edilebilirken, subklinik mastitis ise sütteki somatik hücre sayısına bağlı olarak gelişir ve herhangi bir klinik bulgu oluşturmaz (Contreras ve Rodriguez, 2011).

Süt sığırcılığında genetik seleksiyon ve bakım-beslemedeki gelişmeler neticesinde süt verimlerinde önemli artışlar sağlanmıştır. Ancak verimdeki bu iyileşmeye karşın sağlık ve fertilite problemlerinde de önemli ölçüde artış meydana gelmiştir (Hailemariam ve ark., 2014; Ingvartsen ve ark., 2003). Bu artışlarda süt sığırcılığı işletmelerinde endemik seyirli sağlık problemlerinin (eradikasyonun mümkün olmadığı ve işletmelerde farklı düzeylerde görülen hastalıklar) ayrı bir önemi bulunmaktadır (Yıldız ve Yalçın, 2014). Süt sığırcılığı işletmelerinde sorun oluşturan endemik hastalıklar, özellikle peripartiruent sürecin (Drackley, 1999) (gebeliğin son 4 haftası ile laktasyonun ilk 4 haftasını) ikinci bölümü olan postpartum döneminde önemli ölçüde verim ve finansal kayıplara neden olmaktadır (Martins ve ark., 2013). Postpartum dönemde maternal rezervlerin hızla kullanılması ile birlikte besleme ve bakım şartlarının yetersiz olduğu ineklerde immun sistem baskılanmaktadır. Bu durumda inekler metabolik, reprodüktif ve meme hastalıklarına yatkın hale gelebilmektedir.

Postpartum dönemde, ineklerin %30-50'sinin yetiştirme hastalıklarından en az birinden etkilendiği (Leblanc, 2010), klinik mastitis vakalarının %23,4-65'i (Svensson ve ark., 2006; Waller ve ark., 2009; Zwald ve ark., 2004) bu dönemde meydana geldiği, klinik mastitise yakalanma olasılığının erken ve orta laktasyon döneminde geç laktasyon dönemine göre 1,5 kat daha fazla olduğu bildirilmektedir (Suriyasathaporn ve ark., 2000).

Endemik hastalıklar içerisinde de mastitis; hayvan refahı ve gıda güvenliği problemlerine yol açması, zoonotik karakterli bir hastalık olması gibi etkilerine ek olarak ciddi ekonomik kayıpların olduğu bir sorun olarak değerlendirilebilmektedir (Azooz ve ark., 2020; Seegers ve ark., 2003; Vural ve ark., 2016). Mastitis, hayvancılık işletmelerinde teşhis, ilaç ve tedavi, süt veriminin ve kalitesinin düşmesine bağlı kayıplar, işçilik gibi farklı düzeylerde direkt ve süt üretim kayıpları, gelecekteki üreme kaybı ve sürüden erken ayırma ve değiştirmeye bağlı kayıplar gibi indirekt kayıplara sebep olarak önemli düzeyde ekonomik etkilere yol açabilmektedir (Aghamohammadi ve ark., 2018; Rollin ve ark., 2015; Seegers ve ark., 2003). Holştayn ineklerde klinik mastitisin üreme ve süt verimine olumsuz etkilerinin olduğu, hastalığı geçirmeyen ineklere göre buzağılama aralığını 6,1 gün uzattığı ve 305 günlük laktasyon sürecinde 549,6 kg süt verim kaybın olduğu, pik süt verimine ulaşmadan önce hastalığa yakalanan ineklerin bu dönemden sonra hastalığa yakalananlara göre 506 kg verim kaybı olduğu hesaplanmıştır (Boujenane ve ark., 2015).

Klinik mastitis vakalarının finansal boyutuna bakıldığında, Türkiye'de Yıldız ve Yalçın (2014) tarafından yapılan araştırmada her klinik mastitis vakası sonucu oluşan ekonomik kayıpların (nüks dâhil) hastalığın hafif, orta ve şiddetli formlarında sırasıyla 158 lt, 436 lt, 1204 lt çiğ süt eşdeğeri olarak hesaplanmıştır. Sarıözkan (2019), 2019 yılı cari fiyatları ile hastalığın maliyetinin hafif/orta şiddetli vakalar için laktasyon süt veriminin %9,9'una, şiddetli vakalar için ise %22,6'sına karşılık geldiğini hesaplamıştır.

Postpartum döneme özgü PKM (postpartum klinik mastitis) vakasında ortalama 444$ (128$ direkt+316$ indirekt) maliyet hesaplanmıştır (Rollin ve ark., 2015). Yildiz (2018)'de PKM'de vaka başına ortalama finansal kaybın literatürde 73$ ile 621$ arasında değiştiğini ve bu kayıpların ortanca değerinin 212$'a karşılık geldiğini bildirmektedir.

Süt sığırcılığı işletmelerinde klinik mastitis kaynaklı insidens oranın artması ve dolayısı ile oluşan finansal kayıplarda, yetiştirici profilinin ve yetiştiricilerin işletmecilik prensiplerinde göz ardı ettikleri unsurların önemli etkisi bulunmaktadır (Suresh ve ark., 2017). Öyle ki, yetiştiriciler genel olarak kârlılıklarını devam ettirip süt üretim miktarlarını yükseltmek için uygun rasyon ile ineklerini beslemeye çalışırken, çok az bir kısmı yaşadıkları endemik hastalıklardan kaynaklanan finansal kayıpları göz önünde bulundurmaktadırlar (Ingvartsen, 2006; Yıldız ve Yalçın, 2014).

Ulusal Mastitis Konseyi, meme sağlığı kontrol uygulamalarını temelde 10 madde olarak belirlemiştir. Bu programda; hayvan refahına uygun, temiz ve kuru bir çevre, doğru sağım prosedürü, sağım ekipmanlarının uygun olması ve uygulanması, işletme kayıtlarının iyi tutulması, laktasyon döneminde tedavi, biyogüvenlik önlemleri, işletme kayıtlarının düzenli tutulması ile sürü bazında mastitis için gerçekçi hedeflerin belirlenerek programın periyodik olarak gözden geçirilmesi yer almaktadır (Küplülü ve Vural, 2016).

Mastitis kontrol yönteminde ihmal edilen hususlar hastalığın insidensini artırmakta ve ciddi ekonomik kayıplara neden olmaktadır (Küplülü ve Vural, 2016; Yıldız ve Yalçın, 2014). Bu araştırmada;

PKM insidensi ile insidensin yetiştirici, işletme özellikleri ve mastitis kontrol yöntemlerine göre nasıl bir değişim gösterdiği belirlenmeye çalışılmıştır.

Materyal ve Metot

Araştırmada yer alacak işletmelerin seçiminde; üyelik için en az 5 baş ineğe sahip, kısmen de olsa pazara dönük üretim yapan, soy kütüğü verilerini düzenli olarak tutan Damızlık Sığır Yetiştiricileri Birliğine üye işletmeler tercih edilmiştir.

910 Birlik üyesi işletmeden örneklem büyüklüğünün (n) hesaplanmasında aşağıdaki formül kullanılmıştır (Dean ve ark., 2013):

n = [DEFF*Np(1-p)]/ [($d^2/Z^2_{1-\alpha/2}$*(N-1)+p*(1-p)]

Formülasyonda; N=popülasyon büyüklüğü (910), p= yapılan araştırmalarda bildirilen hastalığın görülme sıklığı (%5), d=hata payı (%5), Z1-α/2=1,96 ve DEFF=1 (rastgele örnek etkisi) olarak yapılan hesaplamada %95 güven aralığında örneklem büyüklüğü 68 işletme (%94 güven aralığında 49 işletme) olarak tespit edilmiştir. Proje başlangıcında 90 işletme basit rastgele örnekleme yöntemiyle seçilerek veri temin formu ile işletme ziyaretlerine başlanılmıştır. İşletmeler 4 kere ziyaret edilebilmiş, ilk ziyaret bilgilendirme amaçlı olup kayıt esnasında dikkat edilecek hususlar belirtilmiştir. Klinik mastitisin tespitinde yetiştirici ve/veya veteriner hekimlerin müdahalede bulunduğu vakalar dikkate alınmıştır. Son işletme ziyaretinde yetiştirici ve işletme özellikleri ile ilgili koruyucu hekimlik tercihlerine yönelik bilgiler veri temin formu aracılığıyla temin toplanmıştır. Araştırmanın son işletme ziyaretinde bazı işletme sahiplerine ulaşılamamış, bazılarının verileri ise güvenilir olarak kabul edilemediğinden 63 işletmeye ait 683 adet postpartum dönem klinik mastitis verisi ile analizler gerçekleştirilmiştir.

PKM dönem olarak doğum sonrası 30 günlük periyot dikkate alınmış olup insidens aşağıdaki formül kullanılarak hesaplanmıştır (Beaglehole ve ark., 2006):

$$\text{Postpartum klinik mastitis insidens oranı (\%)} = \frac{\text{Vaka sayısı}}{\text{Postpartum dönemdeki hayvan sayısı}} \times 100$$

Hedef insidens 1/3'lük dilime göre hesaplanmıştır.

Verilerin normalite varsayımlarını yerine getirememesi nedeniyle (basıklık ve çarpıklık değerlerinin yüksek oluşu, Shapiro-Wilk değeri) grupların karşılaştırılmasında nonparametrik testler tercih edilmiştir. Çoklu grupların karşılaştırılmasında Kruskal Wallis, ikili grupların karşılaştırılmasında Mann Whitney U testi ile yapılmıştır. Bu testlerde ve tanımlayıcı istatistik analizlerinde işletme ölçeklerinde farklılıkların insidens oranlarına etkisini azaltmak için aritmetik ortalama yerine ağırlıklı ortalama kullanılmıştır.

İstatistiki analizlerde SPSS 15.0 programı kullanılmıştır.

Bu araştırmada "Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik" kapsamında etik kurul alınması gerekmemektedir.

Bulgular

Araştırma kapsamındaki işletmelerde bulunan ineklerin %64,3'ü simental ve melezi, %18,9'u montofon ve melezi, %16,8'i holştayn ırkı ineklerden oluşmaktadır. PKM insidensi en yüksek %15,65 ile holştayn ırkı ineklerde görülürken, en az %6,2 ile montofon ve melezi ırkı ineklerde meydana geldiği tespit edilmiştir. Tüm ineklerin dikkate alındığı hesaplamada PKM insidens değerinin %10,68 olduğu belirlenmiştir.

Hedef insidens; 1/3'lük dilimde %5 olarak hesaplanmıştır (21 işletme). Bir başka ifade ile 63 işletmenin 1/3'lük diliminde yer alan 21 işletmede hastalığın insidens değeri %5 ve altında olup 42 işletmedeki 429 hayvanda bu oranın ortalama %15,85 olduğu tespit edilmiştir

Mevcut araştırma içerisinde yer alan işletmelere ait özellikler ve bu özelliklere göre PKM insidens oranına ilişkin bilgiler Tablo 1'de verilmiştir.

Tablo 1'de görüldüğü üzere, araştırma kapsamındaki işletmelerin %73'ü küçük ölçekli, %63,5'i kapalı tip barınağa sahip olup, %92,1 oldukça yüksek bir oranda bağlı sistemde hayvanların yetiştirildiği tespit edilmiştir. İşletmelerin yaklaşık %90'a yakın bir kısmında revir ve doğumların gerçekleştiği padok bulunmamaktadır. İşletme ölçeği küçük olan, kapalı tip barınağa sahip, bağlı sistemin olduğu işletmelerdeki ineklerde PKM insidens değerinin sırasıyla %13,3, %11,4, %12,1 ile daha yüksek seyrettiği görülmektedir. Aynı şekilde işletmesinde revir bölümün bulunmadığı, doğumun ayrı bir bölme yerine ahırda gerçekleştiği işletmelerin ineklerinde insidens oranın %11,7 ve %12,6 ile daha yüksek olduğu dikkati çekmektedir.

Yetiştiricilere ait eğitim ve tecrübe düzeyi, yenilikleri takip etme durumu, danışmanlık hizmetinden faydalanma durumuna ilişkin bilgiler ve bu bilgilere göre PKM insidens oranının durumu ile ilgili veriler Tablo 2'te sunulmuştur.

Tablo 2'de görüldüğü üzere, işletme sahiplerinin %68,3'ünün ilköğretim seviyesinde resmi bir eğitimi bulunduğu, yetiştiricilerinin yaklaşık %85'inin süt

sığırcılığı ile ilgili özel bir eğitim almadığı ve işlerini baba mesleği olarak devam ettirdikleri tespit edilmiştir. Yetişicilerin yaklaşık yarısının 21 yıldan fazla bu iş ile iştigal etmelerine karşın, yenilikleri %68,3 oranında televizyonlardan takip ettikleri, ücret karşılığı danışmanlık hizmeti alan yetiştiricilerin oranının ise %4,8 olduğu belirlenmiştir.

Tablo 1. İşletme özellikleri ve bu özelliklere göre klinik mastitis insidens oranın değişimi*.

İşletme Özellikleri		İşletme sayısı	Oranı (%)	İnek sayısı	PKM insidensi (Ağırlıklı ortalama, %)	Std. Sapma
İşletme ölçeği	Küçük ölçekli (10 baş altı)	46	73,0	316	13,3	12,8
	Orta ve büyük ölçekli (10 baş üstü)	17	27,0	367	8,4	6,2
Barınak tipi	Kapalı	40	63,5	341	11,4	10,1
	Açık ve yarı açık	23	36,5	342	9,9	10,0
Barınak sistemi	Bağlı sistem	58	92,1	528	12,1	10,8
	Serbest sistem	5	7,9	155	5,8	4,2
Revir	Yok	56	88,9	538	11,7	10,8
	Var	7	11,1	145	6,9	5,6
Doğumların gerçekleştiği yer	Ahır	8	12,7	491	12,6	10,9
	Doğum padoğu	55	87,3	192	5,7	4,9

* Tablodaki tüm karşılaştırmalar P<0,05 düzeyinde anlamlı bulunmuştur.

Tablo 2. Yetiştiricilerin resmi ve mesleki eğitim düzeyi ile bu özelliklere göre PKM insidensinin aldığı değerler*.

Yetiştiricinin resmi ve mesleki eğitim düzeyi		İşletme sayısı	Oranı (%)	İnek sayısı	Ağırlıklı ortalama (%)	Std. Sapma
Resmi eğitim düzeyi	İlköğretim	43	68,3	347	14,5	10,7
	Lise	13	20,6	176	8,5	8,7
	Üniversite	7	11,1	149	5,4	5,3
Süt sığırcılığı eğitimi	Hayır	53	84,1	480	12,1	10,9
	Evet	10	15,9	203	7,4	6,8
Baba mesleği	Evet	54	85,7	536	11,2	10,1
	Hayır	9	14,3	147	8,8	9,9
Tecrübe Düzeyi	Az (1-10 yıl)	13	20,6	155	7,1	7,9
	Orta (11-20 yıl)	17	27,0	256	10,2	9,3
	Çok (21 yıl ve üzeri)	33	52,4	272	13,2	11,2
Yenilikleri takip etme yöntemi**	Televizyon	43	68,3	342	13,7	11,6
	İnternet	18	28,6	332	7,2	6,3
Danışmanlık hizmeti	Hayır	2	3,2	24	25,0	5,0
	Ücretsiz olarak veteriner hekimden	58	92,1	555	11,7	10,3
	Ücret karşılığı veteriner hekimden	3	4,8	116	4,3	4,2

* Tablodaki tüm karşılaştırmalarda istatistiki olarak farklılıklar P<0,05 düzeyinde anlamlı bulunmuştur.
** 2 işletmenin verisi eksik olduğu için hesaplamaya dahil edilmemiştir.

Resmi eğitim düzeyi ilköğretim olan, süt sığırcılığı eğitimi almamış, baba mesleği olarak işini sürdüren işletmelerdeki ineklerde insidens oranın sırasıyla %14,5, %12,1, %11,2 ile daha yüksek olduğu tespit edilmiştir. Yenilikleri internetten takip edip ücret karşılığı veteriner hekimlerden danışmanlık hizmeti alan işletmelerdeki ineklerde PKM insidensi sırasıyla %7,2 ve %4,3 ile oldukça düşük seviyede olduğu görülmektedir.

Yetiştiricilerin sağım ile ilgili bazı tercihleri ve bu tercihlere göre PKM insidens değerleri Tablo 3'te sunulmuştur. Tablo 3 incelendiğinde sağımların; %82,5 oranında sıralama yapılmadan rastgele yapıldığı, %15,9'unun elle gerçekleştirdiği, %77,8'inin aynı kişiler tarafından yapıldığı görülmektedir. Sağımda sıralamanın yapılmadığı, sağımın farklı kişiler tarafından yapıldığı işletmelerdeki ineklerde PKM insidensinin %13,8 ve %16,2 ile oldukça yüksek düzeyde olduğu belirlenmiştir. Sağımın ayrı ünitede yapıldığı işletmelerde PKM insidensinin %7,7 en düşük seviyede iken elle yapılan işletmelerdeki ineklerde bu oran %14,5'e çıkmaktadır. Sağımdan sonra yemlemenin yapılarak ineklerin sağım sonrası ilk yarım saat içinde yatmasının engellendiği işletmelerde de PKM insidensi %9,9 ile daha düşük bir düzeyde iken sağım öncesi daldırma kabı uygulamasının hiç kullanılmadığı, sağım makinesinin periyodik bakımlarının yapılmadığı işletmelerdeki ineklerde hastalığın insidensi sırasıyla %11,3 ve %11,7 ile daha yüksek olması dikkati çekmektedir.

Yetiştiricilerin bazı koruyucu hekimlik uygulama tercihleri, bilgi düzeyi ve bu özelliklere göre PKM insidensinin aldığı değerler Tablo 4'te verilmiştir.

Tablo 4'de görüldüğü üzere yetiştiricilerin %81'i kuru dönem meme içi antibiyotiği, %88,9'u mastitis aşısını, %95,2'si meme tıpasını hiç kullanmamaktadır. Sırasıyla bu işletmelerdeki ineklerde PKM insidensi %12,0; %11,3 ve %12,0 ile ortalamadan oldukça yüksek seviyededir. Yetiştiricilerin yaklaşık %40'ı CMT uygulamasını bilse de %85,7'si bu testi hiç kullanmamaktadır. Düzenli

Tablo 3. Yetiştiricinin sağım ile ilgili bazı tercihleri ve bu tercihlere göre PKM insidens değerleri*.

Yetiştiricinin sağım ile ilgili bazı tercihleri		İşletme sayısı	Oranı (%)	İnek sayısı	Ağırlıklı ortalama (%)	Std. Sapma
Sağımda sıra (öncelik) durumu	Evet	11	17,5	247	5,3	4,7
	Hayır	52	82,5	436	13,8	11,0
Sağımın yapılma şekli	Sağım ünitesi	6	9,5	182	7,7	6,6
	Ahırda makine ile	47	74,6	439	11,4	10,7
	Elle	10	15,9	62	14,5	11,9
Sağımı hep aynı kişi mi yapıyor?	Evet	49	77,8	572	9,6	9,8
	Hayır	14	22,2	111	16,2	10,0
Sağımcının düzenli eldiven kullanımı	Evet	13	20,6	162	10,5	9,7
	Hayır	50	79,4	521	10,7	10,2
Sağım sonrası ilk yarım saatte ineklerin yatma durumu	Evet	8	12,7	69	17,4	6,9
	Hayır	55	87,3	614	9,9	10,1
Sağım öncesi daldırma kabı kullanımı	Her zaman	8	12,7	114	8,8	10,8
	Bazen bazı mevsimlerde	7	11,1	93	9,7	11,9
	Hiç	48	76,2	476	11,3	9,5
Sağım makinesi kullananların makinenin periyodik bakımını yaptırma durumu	Evet	8	15,1	155	5,8	7,1
	Hayır	45	84,9	471	11,7	10,6

* Tablodaki karşılaştırmalarda sağımcının düzenli eldiven kullanımı dışındaki tercihlerde istatistiki olarak farklılıklar P<0,05 düzeyinde anlamlı bulunmuştur.

Tablo 4. Bazı koruyucu hekimlik uygulama tercihleri, bilgi düzeyi ve bu özelliklere göre PKM insidensin aldığı değerler*.

Bazı koruyucu hekimlik uygulama tercihleri ve bunlarla ilgili bilgi düzeyi		İşletme sayısı	Oranı (%)	İnek sayısı	Ağırlıklı ortalama (%)	Std. Sapma
Kuru dönem antibiyotik kullanımı	Kurudaki tüm ineklere	9	14,3	112	8,0	9,1
	Yok	51	81,0	507	12,0	10,5
	Mastitise sık yakalanan ve ithal hayvanlara	3	4,8	64	4,7	2,5
Mastitis aşısı kullanımı	Evet	7	11,1	163	8,6	7,9
	Hayır	56	88,9	520	11,3	10,6
Meme tıpası kullanımı	Evet	3	4,8	117	4,3	3,7
	Hayır	60	95,2	566	12,0	10,5
California Mastitis Testi *(CMT)* biliyor mu?	Evet	25	39,7	382	8,9	8,8
	Hayır	38	60,3	301	13,0	11,1
CMT uygulaması yapma/yaptırma durumu	Evet, düzenli olarak	2	3,2	57	3,5	5,2
	Bazen, Şüpheli durumlarda	7	11,1	175	9,7	8,1
	Hayır	54	85,7	451	12,0	10,8

* Tablodaki tüm karşılaştırmalarda farklılıklar istatistiki olarak P<0,05 düzeyinde anlamlı bulunmuştur.

olarak CMT uygulaması yapılan işletmedeki ineklerde PKM insidensinin %3,5 ile oldukça düşük sevide olması dikkat çekicidir.

İşletmelerin %19,0'ının verime göre yemlemenin yapıldığı, %9,5'inin düzenli yem katkısı kullandığı, %6,3'ünün konsantre yemi analiz ettirdiği, bu işletmelerdeki ineklerde PKM insidensinin sırasıyla %8,1, %3,6, %4,4 ile oldukça düşük seviyede olduğu tespit edilmiştir.

Tartışma ve Sonuç

Klinik mastitis insidensi literatürde genelde yıllık bazda incelenmekte olup postpartum döneme özgü çalışmalar nispeten azdır. PKM insidens oranı Erdogan ve ark. (2004) tarafından yapılan çalışmada 40 işletmedeki 1052 inekte %3 olarak hesaplanmış, Suthar ve ark. (2013) tarafından bir başka araştırmada da 24 işletmedeki 872 inekte %4,6 olarak bildirilmiştir. Suthar ve ark. (2013) çalışmada PKM insidens oranını İtalya, Hırvatistan, Macaristan, Polonya, Sırbistan, Slovenya, Portekiz, İspanya ve Almanya'da sırasıyla %8,7, %8,1, %7,4, %7, %29,9, %4,1, %4,6, %2,4, %1,8 olarak hesaplamıştır. PKM ile ilgili olarak Ribeiro ve ark. (2013) tarafından ABD'de yapılan bir çalışmada klinik mastitis insidens oranı %15,3, Sepulveda-Varas ve ark. (2015) tarafından Şili'de yapılan çalışmada klinik mastitis insidens oranı %11,7 olarak bildirilmiştir. Bu bulgularda bile PKM insidens değerleri minimum ile maksimum değerler arasında 17 kat farklılığın olması oldukça dikkati çekmektedir. Yildiz (2018) literatür taraması sonucu elde ettiği sekonder verilerin ortanca değerini hesaplayarak PKM insidenslerinin %7'ye karşılık geldiğini hesaplamıştır. Bu durum insidensin; ülkeye, bölgeye, işletme ölçeğine, barınak tipine, hayvan ırkına ve yaşına, hesaplama yöntemine, işletmenin türüne (özel işletme, halk elinde işletmeler vb), teşhis yöntemine, şiddetine, hastalık kontrol yöntem ve tercihlerine göre farklılık göstermesinden kaynaklanmaktadır (Çam ve İnal, 2021; Oliveira ve ark., 2013; Rişvanlı ve ark., 2021; Suresh ve ark., 2017; Yalcin ve ark., 2008). Öyle ki Ankara ilinde şahıslara ait 45 işletmede yıllık ortalama klinik mastitis insidensi %42, laktasyon insidensi %32,5, ağırlıklı aylık insidens yönteminde bu değerin %34,9 olduğu hesaplanmıştır (Yıldız, 2008; Yıldız ve Yalçın, 2014). Bir başka ifade ile aynı materyalde sadece insidens hesaplama yönteminin değişmesi halinde bile insidens değerlerinde rakamsal olarak yaklaşık %10'luk farklılığın bile olabilmesi literatür bulguların karşılaştırılmasındaki zorluğu göstermesi açısından oldukça önemlidir. Bu nedenle farklı ülke, bölge, işletme, ırk, bakım ve besleme gibi unsurlarda çok farklı sonuçların çıkması kaçınılmazdır.

Bu araştırmada PKM insidensi holştayn ırkı ineklerde (%15,65), simental ve montofon melezlerinden daha yüksek düzeyde seyrettiği tespit edilmiş olup, Çam ve İnal (2021) tarafından yapılan araştırmada da holştayn ırkı sığırlarda hastalığın simental ırkı sığırlara göre daha yüksek olduğu belirtilmektedir.

Bu araştırmada; küçük ölçekli, kapalı tip barınağın olduğu, yetiştiricinin resmi ve mesleki eğitiminin düşük olduğu, sağımın farklı kişiler tarafından yapıldığı işletmelerdeki ineklerde klinik mastitis insidensinin daha yüksek olduğu belirlenmiş bu bulgular Suresh ve ark. (2017)'nın Hindistan'da yaptığı araştırma bulgularıyla örtüşmektedir. Bu çalışmada, sağımcı hijyeni, sağım yöntemi, işletme zemininin türünün, düzenli tırnak kesiminin yapılmasının, yetiştirme şekli ve emziren buzağıların varlığının da mastitis insidensi üzerine etkisi olduğu belirtilmiştir (Suresh ve ark., 2017). Sağım yöntemi ve hijyenin kötü olduğu işletmelerde klinik mastitis insidensinin daha yüksek olduğu bildirilmektedir (Emre ve ark., 2011). Hollanda'da yapılan çalışmada da *E. coli*'nin neden olduğu klinik mastitis vakalarının çoğunlukla barınma koşulları, hijyen ve sağım makinesi ile ilişkili olduğu belirtilmektedir (Barkema ve ark., 1999). Kanada'da bağlı duraklı barınak tipine sahip işlemelerde insidensin daha yüksek olduğu tespit edilmiştir (Levison ve ark., 2016).

Senturk ve ark. (2014) tarafından yapılan çalışmada; sağımcıların eldiven kullanım oranın %8,8, teat dipping uygulamasının %14,7, sıralı sağımın %80,9, kuru dönem tedavi uygulamasının %30,9 olduğu bildirilmiştir. Konya, Burdur ve Kırklareli illerindeki süt sığırcılık işletme sahiplerinin %21'inin lise ve üzeri eğitim düzeyine sahip olduğu, %79'unun süt sığırcılığı ile ilgili herhangi bir eğitim almadığı, %46'sının hiçbir şekilde yenilikleri takip etmediği, %93'ünün subklinik mastitis hakkında bilgi sahibi olmadığı elle sağımın %6,6, sağımda eldiven kullanımının %18,3 oranında olduğu, sağım sonrası teat dipping, kuru dönem antibiyotik tedavisi, mastitis aşısı, CMT uygulayan ve klinik mastitis vakalarını düzenli olarak kaydeden üretici oranları sırasıyla %18, %62, %29, %15 ve %20 olarak tespit edilmiştir (Yalçın ve ark., 2010). Rişvanlı ve ark. (2021) büyük ölçekli işletmelerde (50 baş üstü) teat dipping uygulama oranının %70,4, kuru dönem antibiyotik uygulamalarının %72,8, mastitis aşısının %55,6 oranında uygulanırken, küçük ölçekli işletmelerde (50 baş altı) bu oranın sırasıyla %19,2, %11,9 ve %1,1 olduğu bildirilmektedir. Süt sığırcılığının daha bilinçli yapıldığı ülkelerde bu oranların oldukça yüksek olduğu görülmektedir. Öyle ki Kanada'da ticari işletmelerde yapılan çalışmada yetiştiricilerin kuru dönem mastitis tedavisinin %93,

sağım sonrası dezenfeksiyonun %97 oranında uyguladıkları belirtilmektedir (Aghamohammadi ve ark., 2018).

Araştırmamızda işletmelerin %76'sında teat dipping uygulanmadığı, kuru dönem mastitis tedavisin %81, mastitis aşısının %89, CMT'nin %86, sağım makinesinin periyodik bakımının %84,9 oranında hiç yapılmadığı, PKM insidensinin sırasıyla %11,3, %12, %11,3, %13, %11,7 ile daha yüksek bir düzeyde olduğunun tespiti literatür bilgileriyle örtüşmektedir. Mastitis kontrol yöntemleri ile ilgili yetersizliklerin temelinde yetiştiricilerin %84'ünün süt sığırcılığına özgü bir eğitim almamalarının etkili olduğu düşünülmektedir. Bu çalışmada olduğu gibi, Türkiye süt sığırcılığının daha bilinçli yapıldığı Burdur, Kırklareli ve Konya illerinde bile yetiştiricilerin %75'inden fazlasının yaptıkları iş ile ilgili herhangi bir eğitim almamaları (Yalçın ve ark., 2010) oldukça düşündürücüdür.

Yetiştiricilerin mesleki tecrübelerinin artmasında iş ile iştigal ettikleri sürenin yanında hayat boyu edindikleri bilgilerin toplamının önemli olduğu bir gerçektedir. Genel olarak iş tecrübesinin yükselmesi ile hastalık oranının daha düşük olacağı düşünülürken bu araştırmada yetiştiricilerin tecrübeleri artıkça hastalık insidensinin de arttığı görülmüştür. Bu duruma; tecrübesi daha az olanların bu işi geleneksel olarak baba mesleği olarak yapmadığı, yenilikleri ve güncel gelişmeleri daha etkin bir şekilde takip edip danışmanlık hizmeti almalarının etkili olduğunu düşündürmektedir. Hayvancılığı gelişmiş ülke örnekleri incelendiğinde üreticilerin yenilikleri takip etmesi ve modern gelişmelere çeşitli eğitimler aracılığıyla uyum sağlaması ile işletme verimliliği ve kârlılığına olumlu katkılar sağlamaktadır.

İrlanda'da süt kotalarının gevşetilmesiyle birlikte sektöre girecek girişimcilerin profiline bakıldığında girişimcilerin; % 7'si temel tarım sertifikalı (180 saat eğitim), %72'si ileri tarım sertifikalı (24 hafta eğitim, bunun yarısı çiftlik uygulamalı), %21'i lisans dereceli sertifikalı (4 yıllık eğitim) olduğu görülmektedir (McDonald ve ark. 2014).

Sonuç olarak düşük kâr marjının söz konusu olduğu sektörde özellikle Covid 19 pandemisi ve sonrası oluşan global ekonomik krizlerde yetiştiricilerin maliyetlerine hakim olmasının önemi daha da ön plana çıkmıştır. PKM insidensinin yüksek seyrettiği klinik mastitis kontrol yöntemlerin yeterince uygulanmadığı, hedef insidensin üzerinde hastalığın seyrettiği işletmelerde oluşan sakınılabilir kayıpların azaltılması gerekmektedir. Bu çalışmada da görüldüğü üzere süt sığırı işletmelerinde; yetiştirici, sağımcı ve bakıcıların mesleki yeterlilik düzeyleri artırılmalı, sürü sağlığı, bakım besleme, işletme ve hastalık yönetimi ile ilgili danışmanlık hizmetleri alınmalı, işletmelerin hijyen ve fiziki şartları iyileştirilmeli, sağım hijyeni ve mastitis kontrol protokollerinin oluşturularak özenli bir şekilde uygulanmalıdır. Süt sığırcılığı işletmelerinin hastalık maliyetlerini düşürecek koruyucu hekimlik hizmetlerinin teşvik edildiği desteklemelerin ön plana çıkması gerekmektedir. Endemik hastalıkların ekonomi perspektifiyle yönetimini odak haline getirecek şekilde yapılacak desteklemelerin ülke hayvancılığına önemli kazanımlar sağlayacağı düşünülmektedir.

Çıkar çatışması

Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan ederler.

Etik izin

Bu çalışma "Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik" Madde 8 (k) gereği HADYEK iznine tabi değildir. **"**Etik Beyan formu" doldurulup tüm yazarlarca imzalanarak sisteme yüklenmiştir.

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Benzerlik oranı

Makalenin benzerlik oranının sisteme yüklenen raporda belirtildiği gibi %8 olduğunu beyan ederiz.

Açıklama

Bu çalışma I. Uluslararası Gap Tarım ve Hayvancılık Kongresi 25-27 Nisan 2018 sözlü bildiri olarak sunulmuştur.

Yazar katkıları

Fikir/Kavram: AŞY
Tasarım: AŞY, OA
Denetleme/Danışmanlık: AŞY
Veri Toplama ve/veya İşleme: AŞY
Analiz ve/veya Yorum: AŞY, OA
Kaynak Taraması: AŞY, OA
Makalenin Yazımı: AŞY, OA
Eleştirel İnceleme AŞY, OA

Kaynaklar

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Essential and Non-Essential Metal Concentrations in Shrimps from Iskenderun Bay, Türkiye

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Abstract: Contamination of the aquatic environment and living things with pollutants is increasing daily. Among these pollutants, heavy metals come to the forefront regarding toxicological and public health due to their widespread use, toxicity, and resistance to degradation, as well as accumulation and biomagnification in the food chain. The study aimed to determine the essential (Cr, Cu, Fe, Mn, Ni, Zn) and non-essential (As, Al, Cd, Pb) metal concentrations by ICP-OES of *Metapenaeus monoceros, Penaeus japonicus, Penaeus semisulcatus* (n=30) caught from the Northeastern Mediterranean, Iskenderun Bay. The concentration of Cd, Cr, Ni, and Pb were under LOD. The concentration (ppm) ranges were Al: 0.73-38.89, As: 2.18-9.68, Cu: 28.96-69.01, Fe: 7.85-241.36, Mn: 0.44-1.28, and Zn: 51.71-108.51 for all species. Except for the As concentrations, the differences between mean concentrations of metals in shrimp species were not statistically significant. When the results of the study are compared with the findings of other studies on shrimps caught from the Iskenderun Bay, it shows that Cd, Cr, Ni, and Pb contamination levels decreased, while Cu, Fe, and Zn contamination levels increased.
Keywords: *Essential metals, Non-essential metals, Metapenaeus monoceros, Penaeus japonicus, Penaeus semisulcatus, Iskenderun Bay.*

Türkiye İskenderun Körfezi, Karideslerinde Esansiyel ve Non-Esansiyel Metal Konsantrasyonları

Özet: Akuatik ortam ve canlıların kirleticilerle kontaminasyonu gün geçtikçe artmaktadır. Bu kirleticiler arasında ağır metaller yaygın kullanım, toksisite ve bozunmaya karşı dayanıklılığı yanında besin zincirinde birikim ve biyomagnifikasyonu nedeniyle toksikolojik ve halk sağlığı açısından ön plana çıkmaktadır. Çalışmanın amacı Kuzeydoğu Akdeniz, İskenderun Körfezi'nden avlanan *Metapenaeus monoceros, Penaeus japonicus, Penaeus semisulcatus* karideslerinde (n=30) esansiyel (Cr, Cu, Fe, Mn, Ni, Zn) ve non-esansiyel (As, Al, Cd, Pb) metal konsantrasyonlarının ICP-OES ile belirlenmesiydi. Cd, Cr, Ni ve Pb konsantrasyonu LOD'un altında tespit edilmiştir. Tüm türler için konsantrasyon (ppm) aralıkları Al: 0.73-38.89, As: 2.18-9.68, Cu: 28.96-69.01, Fe: 7.85-241.36, Mn: 0.44-1.28 ve Zn: 51.71-108.51 olarak tespit edildi. As konsantrasyonları dışında, karides türlerinde ortalama metal konsantrasyonları arasındaki farklar istatistiksel olarak önemsizdi. Çalışmanın sonuçları İskenderun Körfezi'nden avlanan karidesler üzerinde yapılan diğer çalışmaların bulguları ile karşılaştırıldığında, Cd, Cr, Ni ve Pb kontaminasyon düzeylerinin azaldığını, Cu, Fe ve Zn kontaminasyon düzeylerinin ise arttığını göstermektedir.
Anahtar Kelimeler: *Esansiyel metaller, Esansiyel olmayan metaller, Metapenaeus monoceros, Penaeus japonicus, Penaeus semisulcatus, İskenderun Körfezi.*

Introduction

Aquatic environments and living organisms in Turkey, like the rest of the world, are contaminated with toxic pollutants such as heavy metals, pesticides, and endocrine disruptors (Cucu et al., 2019; Esfahani et al., 2020; Jia et al., 2016; Nyantakyi et al., 2021; Yarsan and Yipel, 2013; Yipel and Yarsan, 2014). Because of their widespread use, toxicity, and resistance to biodegradation, as well as their accumulation and biomagnification in living beings in the food chain, heavy metals are significant among aquatic pollutants (Altınok-Yipel et al., 2022; Esfahani et al., 2020; Jia et al., 2016; Liu et al., 2019). Heavy metal contamination of aquatic environments thus poses potential risks to regional ecology, humans, and other living things at the top of the food chain (Altınok-Yipel et al., 2022; Esfahani et al., 2020; Yarsan and Yipel, 2013).

Using crustaceans and other aquatic organisms as bioindicators to investigate the level of heavy metal contamination in the environment is a common practice (Jia et al., 2016; Yarsan and Yipel, 2013; Yüzereroğlu et al., 2009). Moreover, human health risk assessments on aquatic organisms in the food chain are essential and current public health research topics (Baki et al., 2018; Ezemonye et al., 2019; Liu et al., 2019; Yu et al., 2020). Shrimp and other crustaceans are ideal bioindicators for aquatic contamination studies because of their hypersensitivity and rapid response to contaminants such as heavy metals (Maraschi et al., 2020; Suami et

al., 2019). Since shrimps have necessary amino acids, minerals, vitamins, and long-chain unsaturated fatty acids, they have a significant position in the food chain regarding nutrition (Liu et al., 2019). About 13.3% of all seafood caught in Turkey is shrimp (Ministry of Agriculture and Forestry, 2021). Iskenderun Bay, located in the Northeast Mediterranean, is an important ecological and economic region due to its aquatic biodiversity and aquaculture potential (Aytekin et al., 2019; Yipel and Tekeli, 2016). In addition, there are studies on the levels of metals reaching the Gulf of Iskenderun in aquaculture as a result of increased agricultural, urban, and industrial activity, as well as the potential health risks associated with human consumption of these products (Aytekin et al., 2019; Duysak and Ersoy, 2014; Kaya and Turkoglu, 2017; Kaymacı and Altun, 2016). Therefore, in similar studies, monitoring the metal levels of seafood products periodically and carrying out risk assessments based on their consumption is recommended (Asare et al., 2018; Rakib et al., 2021; Sobihah et al., 2018; Yipel et al., 2016).

The study aimed to determine the essential (Cr, Cu, Fe, Mn, Ni, Zn) and non-essential (As, Al, Cd, Pb) metal concentrations in *Metapenaeus monoceros*, *Penaeus japonicus*, *Penaeus semisulcatus* shrimps caught from Iskenderun Bay in the Northeast Mediterranean.

Materials and Methods

Materials

Shrimp samples of *Metapenaeus monoceros* (n=10), *Penaeus japonicus* (n=10), and *Penaeus semisulcatus* (n=10) species were collected from the local seafood markets, which shrimping in Iskenderun Bay, Northeast Mediterranean region and kept at -20 °C until analysis. The chemicals (Nitric acid, hydrogen peroxide) used in the study were of analytical purity. This study is not subject to HADYEK permission by Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Methods

Samples (0.5 g) were burned with microwave digestion in nitric acid (8 mL, 65%) and hydrogen peroxide (2 mL, 30%) with a 15-minute ramp and hold procedure at 1200 W, 100% power, 800 psi, and 200 °C (Cem X Press, USA). Inductively coupled plasma-optical emission spectroscopy (ICP-OES) was used to determine essential (Cr, Cu, Fe, Mn, Ni, and Zn) and non-essential (Al, As, Cd, and Pb) metal concentrations (Spectro, Germany). Method validation was carried out on parameters limit of detection (LOD) (ppb; Al: 0.49, As: 11.46, Cd: 1.43, Cr: 2.66, Cu: 2.19, Fe: 2.77, Mn: 1.76, Ni: 4.49, Pb: 9.97, Zn: 2.41), recovery (74.23-98.65%), relative standard deviation (RSD) (Al: 1.243, As: 0.599, Cd: 0.753, Cr: 1.177, Cu: 0.861, Fe: 0.752, Mn: 1.022, Ni: 0.770, Pb: 0.428 and Zn: 0.625), and correlation coefficient (R^2) (<0.999) parameters.

Statistical analysis: The data were statistically analyzed using one-way analysis of variance (ANOVA) and the post hoc Duncan test. P values less than <0.05 were considered statistically significant.

Results

The arithmetic and geometric mean, median, standard error, minimum and maximum concentrations (ppm) of essential (Cr, Cu, Fe, Mn, Ni, and Zn) and non-essential (Al, As, Cd, and Pb) elements in *Metapenaeus monoceros, Penaeus japonicus,* and *Penaeus semisulcatus* tissues were presented in Table 1 and Table 2. The concentration of Cd, Cr, Ni, and Pb were under LOD. The concentration (ppm) ranges of essential elements were Cu: 28.96-69.01, Fe: 7.85-241.36, Mn: 0.44-1.28, Zn: 51.71-108.51, and the concentration (ppm) ranges of non-essential elements were Al: 0.73-38.89 and As: 2.18-9.68 for three shrimp species. The mean concentrations are sorted for essentials as Zn>Cu>Fe>Mn and for non-essential as Al>As. Except for the As concentrations ($p<0.05$), the differences between mean concentrations of selected metals in shrimp species included in the study were not statistically significant.

Discussion and Conclusion

The consumption of shrimp and other seafood products is increasing because of their high nutritional value (protein, fat, vitamins, minerals, etc.). However, this increase also raises some concerns for public health due to the increased risk of contamination in the food chain brought on by the increased discharge of metals and other potentially toxic pollutants into the aquatic environment (Aytekin et al., 2019; Baki et al., 2018; Yarsan and Yipel, 2013; Yipel et al., 2014). According to recent studies, metal pollution in Iskenderun Bay, an important shrimp fishing area, has increased due to intense urban, agricultural, and industrial activities (Aytekin et al., 2019; Duysak and Uğurlu, 2020). Ag, Cu, Cr, Fe, Ni, Pb, and Zn concentrations in the tissues of *Penaeus semisulcatus* collected from the Iskenderun Bay were measured in the study published by Yılmaz and Yılmaz in 2007. Even though Yılmaz and Yılmaz stated that the amounts of Cr, Ni, and Pb (ppm) in the muscle tissues of *Penaeus*

Table 1. Essential metal concentrations (ppm) in tissues of *Metapenaeus monoceros, Penaeus semisulcatus,* and *Penaeus japonicus.*

Species		Cr	Cu	Fe	Mn	Ni	Zn
Metapenaeus monoceros	**Mean**	<LOD	45.29	24.71	0.92	<LOD	69.32
	Geometric Mean	<LOD	43.37	17.22	0.87	<LOD	67.83
	Std. Error of Mean	<LOD	5.99	7.38	0.13	<LOD	7.01
	Minimum	<LOD	29.67	2.28	0.57	<LOD	56.15
	Maximum	<LOD	65.51	50.45	1.28	<LOD	102.79
Penaeus semisulcatus	**Mean**	<LOD	43.43	29.13	0.87	<LOD	69.01
	Geometric Mean	<LOD	41.27	22.50	0.82	<LOD	67.02
	Std. Error of Mean	<LOD	6.47	8.88	0.12	<LOD	8.20
	Minimum	<LOD	28.96	7.85	0.53	<LOD	53.46
	Maximum	<LOD	69.01	64.78	1.26	<LOD	108.51
Penaeus japonicus	**Mean**	<LOD	31.72	96.17	0.74	<LOD	61.91
	Geometric Mean	<LOD	31.69	40.81	0.69	<LOD	60.54
	Std. Error of Mean	<LOD	0.58	46.16	0.13	<LOD	7.11
	Minimum	<LOD	30.14	4.97	0.44	<LOD	51.71
	Maximum	<LOD	33.27	241.36	1.16	<LOD	89.89
P value		-	0.231	0.114	0.613	-	0.303

Table 2. Non-essential metal concentrations (ppm) in tissues of *Metapenaeus monoceros, Penaeus semisulcatus,* and *Penaeus japonicus.*

Species		Al	As	Cd	Pb
Metapenaeus monoceros	**Mean**	15.46	7.69[a]	<LOD	<LOD
	Geometric Mean	7.77	7.59	<LOD	<LOD
	Std. Error of Mean	6.64	0.56	<LOD	<LOD
	Minimum	1.64	6.16	<LOD	<LOD
	Maximum	38.89	9.68	<LOD	<LOD
Penaeus semisulcatus	**Mean**	2.37	4.45[b]	<LOD	<LOD
	Geometric Mean	1.94	4.32	<LOD	<LOD
	Std. Error of Mean	0.64	0.52	<LOD	<LOD
	Minimum	0.73	3.40	<LOD	<LOD
	Maximum	4.97	6.90	<LOD	<LOD
Penaeus japonicus	**Mean**	2.32	3.42[b]	<LOD	<LOD
	Geometric Mean	2.02	3.31	<LOD	<LOD
	Std. Error of Mean	0.68	0.44	<LOD	<LOD
	Minimum	1.14	2.18	<LOD	<LOD
	Maximum	4.92	4.78	<LOD	<LOD
P value		0.108	0.005	-	-

semisulcatus were <LOD-13.1, 0.6-3.6, and 0.2-0.6, respectively, in their research, these metals could not be found in any of the three shrimp species in the current study (<LOD). In the same study, the range of muscle Cu concentration (ppm) was reported to be 17.2-42.4; however, in the current study, high average levels were found in the species *Penaeus semisulcatus* (43.43) and *Metapenaeus monoceros* (45.29). Muscle Fe concentration (ppm) was reported as 5.9-33.1 in the study, and the mean level determined in *Penaeus japonicus* (96.17) species was higher than in this study. Muscle Zn concentrations (ppm) have been reported to range between 4.3 and 10.3, with average levels determined in all three shrimp species being higher than in this study (Yılmaz and Yılmaz, 2007). In a study by Fırat et al. (2008) that Cu, Cd, Cr, Fe, and Zn concentrations (ppm) were found to be 34.24, 16.72, 60.38, 18.69, and 27.75, respectively, in *Penaeus semisulcatus* tissues that were caught in the Iskenderun Bay and metal levels other than Cd and Cr were lower than the current study results. Cu, Cd, Fe, Pb, and Zn concentrations were measured in the tissues of *Penaeus semisulcatus* caught in the Iskenderun Bay in the study published by Aytekin et al. (2019). While Cd and Pb concentrations (ppm) in the muscle tissues of *Penaeus semisulcatus* were reported by Aytekin et al. to range from 6.52 to 8.33 and 22.18 to 62.75, respectively, in the current findings, these two metals were not found in any shrimp species (<LOD). The same study showed the average amounts of Cu, Fe, and Zn in muscle as 19.35-34.23, 16.15-24.23, and 37.43-61.42, respectively. The current study found that all shrimp species had higher average concentrations of these three metals (Aytekin et al., 2019). Table 3 presents the research study on the shrimp species used in this study that was not conducted in Iskenderun Bay.

When the current study's findings are compared to those of other studies on shrimp caught in various parts of the world, it is clear that Cd, Cr, Ni, and Pb contamination levels are decreased, while Cu,

Table 3. Other studies that determined metal concentration in tissues of *Metapenaeus monoceros, Penaeus semisulcatus,* and *Penaeus japonicus*

Country	Area	Species	Method	Metal	Result ppm	Literature	Current study ppm
Democratic Republic of the Congo	Atlantic Coast	*Penaeus* spp.	ICP-MS	Cu	16.01 -18.71	Suami et. al., 2019	28.96 - 69.01
				Cr	0.08 -0.10		<LOD
				Ni	<LOD		<LOD
				Zn	55.90 -85.44		51.71 - 108.51
Iran	Persian Gulf	*Penaeus semisulcatus*	Neutron activation analysis	As	8.28	Heidarieh et. al., 2013	4.45
				Fe	288		29.13
				Mn	25.43		0.87
				Zn	68.73		69.01
Saudi Arabia	Red Sea Coast	*Penaeus semisulcatus*	AAS	Cu	5.33	El Gendy et. al., 2015	43.43
				Cd	1.57		<LOD
				Ni	0.8		<LOD
				Pb	2.33		<LOD
				Zn	17.33		69.01
Türkiye	Mersin	*Penaeus semisulcatus*	ICP-MS	Cu	2.98	Korkmaz et. al., 2019	43.43
				Cd	<LOD		<LOD
				Fe	6.64		29.13
				Pb	0.29		<LOD
				Zn	14.18		69.01
Egyptian	Mediterranean Coast	*Metapenaeus monoceros*	AAS	As	0.39	Abd-Elghany et. al., 2020	7.69
				Cd	0.24		<LOD
				Pb	1.13		<LOD
Mozambique	Maputo Bay (Bembe)	*Metapenaeus monoceros*	ICP-MS	As	9.9	Sturve et. al., 2021	7.69
				Cd	0.07		<LOD
				Cu	33		45.29
				Cr	<LOD		<LOD
				Mn	3.2		0.87
				Ni	0.19		<LOD
				Pb	<LOD		<LOD
				Zn	43.5		69.32
China	Beibu Gulf	*Penaeus japonicus*	AAS	As	0.65	Gu et. al., 2018	3.42
				Cu	3.78		31.72
				Cd	0.09		<LOD
				Cr	0.15		<LOD
				Pb	0.04		<LOD
				Zn	10		61.91

Fe, and Zn contamination levels are generally increased.

The Iskenderun Bay, which has significant ecological diversity and aquaculture potential, is known to be under high heavy metal contamination stress because of intensive anthropogenic activities. As a result, metal concentrations in gulf-caught shrimp and other fisheries are important environmental and public health parameters that should be monitored regularly in terms of both marine ecosystem health and human health through the food chain.

Early detection of the adverse effects of metals and other pollutants at the organism level is essential protecting the ecosystem's health in the region. On the other hand, risk assessment for consumers regarding metal and different pollutant concentrations in fisheries, which play an important role in the food chain, is vital to protecting public health.

Conflict of Interest

The authors stated that they did not have anyreal, potential or perceived conflict of interest.

Ethical Approval

This study is not subject to HADYEK permission in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics Committees".

Similarity Rate

We declare that the similarity rate of the article is 6% as stated in the report uploaded to the system.

Author Contributions

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Data Collection and / or Processing: MY, IOT
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Özet: Hayvan popülasyonundaki kontrolsüz artış üremenin denetlenmesi konusunu daha da önemli hale getirmiştir. Tüm dünyada kontrasepsiyon amacıyla halen kullanılmakta olan cerrahi ve hormonal yöntemlerin birçok dezavantajı bulunmaktadır. İmmünokontrasepsiyon bu dezavantajları ortadan kaldırmak amacıyla yeni bir kontrasepsiyon yöntemi olarak düşünülmüştür. Görevi çeşitli etkenlere karşı vücudu korumak olan immün sistemin fizyolojik tolerans etkisi gebeliğin devam etmesini sağlamaktadır. İmmünokontrasepsiyon ile immün sistemin bu etkisi tersine çevrilerek gebelik ile sonuçlanan reprodüktif süreci bozmaktadır. Farklı deyişle immün sistemin vücudun kendi reprodüktif sürecinde belli aşamalara/dokulara saldırması ile sağlanmaktadır. Bunun için bir antijen-antikor kompleksinin oluşturulması gereklidir. 1970'li yıllardan bu yana çalışılmakta olan immünokontraseptiyon alanında hali hazırda zona pelusida (ZP), gonadotropin salgılatıcı hormon (GnRH) ve lüteinleştirici hormon (LH) reseptörlerine karşı aşılamalar geliştirilmiştir. Sunulan derleme immünokontrasepsiyon ve günümüzde kullanılan aşılar hakkındaki bilgilerin güncellenmesi amacıyla hazırlanmıştır.
Anahtar Kelimeler: *GnRH aşısı, İmmünokontrasepsiyon, Üremenin denetlenmesi, ZP aşısı.*

Immunocontraception in Veterinary Medicine

Abstract: The uncontrolled increase in the animal population has made the issue of controlling reproduction even more critical. Worldwide surgical and hormonal methods still used for contraception have many disadvantages. Immunocontraception has been considered a new method of contraception to eliminate these disadvantages. The physiological tolerance effect of the immune system, whose task is to protect the body against various factors, ensures the continuation of pregnancy. Immunocontraception reverses this effect on the immune system and disrupts the reproductive process that results in pregnancy. In other words, it is provided by the immune system attacking certain stages/tissues in the body's reproductive function. For this, it is necessary to form an antigen-antibody complex. Vaccinations against zona pellucida (ZP), gonadotropin-releasing hormone (GnRH), and luteinizing hormone (LH) receptors have already been developed in the field of immunocontraception, which has been studied since the 1970s. The presented review has been prepared to update the information about immunocontraception and currently used vaccines.
Keywords*: Control of reproduction, GnRH vaccine, Immunocontraception, ZP vaccine.*

Giriş

Hayvan popülasyonundaki kontrolsüz artış tüm dünyada biyolojik çeşitliliği ve biyolojik güvenliği tehdit etmektedir. Araştırıcılar her yıl 8 ile 10 milyon arasında kedi ve köpeğin barınaklara bırakıldı, bu hayvanlardan 4 ile 5 milyonuna ise ötenazi yapıldığını tahmin etmektedir. Ayrıca sahipsiz sokak hayvanı popülasyonundaki bu artış, insan ve diğer evcil hayvanlarla aynı habitatın kullanılmasından kaynaklanan sürekli temas nedeniyle hem zoonoz hem de değerli gen kaynağı olan ve/veya ekonomik değeri bulunan diğer hayvan türleri için rezervuar ve/veya vektör olarak çeşitli hastalıkların yayılmasında da etkin rol oynamaktadır (Kutzler ve Wood, 2006). Vahşi yaşamda ise üremenin kontrolü kuduz ve brusellozis gibi bulaşıcı hastalıkları kontrol altına almada etkilidir. Saldırganlık ve temas ile yayılan bu hastalıklarda amaç hayvanların bir araya gelmesini önlemektir ve özellikle çiftleşme döneminde hayvanlar bir araya gelmekte ve saldırganlıkları artmaktadır. Günümüzde kullanılan itlaf ve aşılama yöntemlerinin hayvanların birbirleri ile temasını azaltmada yeterli olmaması nedeniyle yeni yöntemler aranmaktadır (Killian ve ark., 2009). Hayvan popülasyonunun kontrolsüz artışı ve yol açtığı zararları engellemek üremelerinin denetlenerek hayvan sayılarını kontrol altına tutmakla mümkündür. Üremenin denetlenmesinde cerrahi ve hormonal kontrasepsiyon yöntemleri kullanılır. Cerrahi kontrasepsiyon hem pet hayvanlarında hem de vahşi yaşamda en sık kullanılan yöntemdir. Ovaryohisterektomi, ovarektomi, histerektomi, prepubertal gonadektomi, kastrasyon ve vazektomi gibi birçok tekniği bulunmaktadır (Khatal ve ark., 2019). Ancak cerrahi kontrasepsiyon yöntemi, geri dönüşümsüz olması, yüksek maliyetli olması, iş gücünü arttırması,

hayvanların yakalanması, taşınması ve postoperatif süreçteki bakım ihtiyacının getirdiği zorluklar, sahipli hastalarda bazı hasta sahiplerinin bu yöntemi istememesi, anestezi ve/veya operasyon risklerinin olması yanında yol açtığı diğer lojistik sorunlar nedeniyle üremenin denetlenmesi konusunda yetersiz kalmaktadır (Soto ve ark., 2005).

Hormonal kontrasepsiyonda progestinler, androjenler veya GnRH analogları gibi çeşitli hormonlar kullanılır (Asa, 2018). Bu yöntemin belki de en büyük avantajı etkisinin geri dönüşümlü olmasıdır. Yöntemin etki süresi ve şekli kullanılan hormona, uygulamanın yapıldığı seksüel siklus dönemine ve bireysel özelliklere bağlı olarak değişir. Dolayısıyla kontraseptif etkinin uzatılması için tekrarlayan uygulamalara gereksinim duyulmaktadır ki bu da hormonal kontrasepsiyonun dezavantajlarından birisidir. Bunun yanında hormonların kendilerine has dezavantajları da bulunmaktadır. Steroid hormonlar kilo artışı, uyuşukluk veya huzursuzluk, reprodüktif ve meme dokusunda hiperplastik veya neoplastik değişiklikler ile diyabetes mellitus gibi yan etkilere yol açmaktadır (Kutzler ve Wood, 2006). Androjenler ise vücut kokusunda artış, üriner inkontinens, idrar püskürtme, servikal dermiste kalınlaşma ve epiforaya neden olmaktadır (Plumb, 2002). Cerrahi ve hormonal kontrasepsiyonun yukarıda kısaca belirtilen dezavantajları nedeniyle alternatif yöntem arayışına girilmiştir.

İmmünokontrasepsiyon bu amaçla, alternatif olarak geliştirilmeye başlanmıştır. Yöntem reprodüktif süreçte etkili olan hormon veya yapılara karşı vücudun antikor üretmesini sağlamak üzerine kuruludur (Naz ve Saver, 2016). Sunulan derlemede, geleneksel kontrasepsiyon yöntemlerine alternatif olarak geliştirilen ve gelecekte hayvan popülasyon kontrolünde çok önemli yeri olacağı öngörüldüğü için konu üzerinde çalışmaların artarak devam ettiği immünokontrasepsiyon yöntemine ait güncel yaklaşımlar özetlenmiştir.

1. İmmünokontrasepsiyon: En genel ifadesi ile immün sistem, vücudu bakteri, virüs, parazit, mantar, kanser hücreleri ve toksinler gibi yabancı materyallerden koruyan savunma sistemidir (Riaz ve ark., 2022). İmmünokontrasepsiyon ise bakteriyel, viral ve toksik ajanlar içeren aşılarla, bireyin kendi immün sisteminin gebelikten korunmaya yönelik olarak aktive edilerek, fertilitenin engellendiği bir kontrasepsiyon yöntemidir (Gupta ve ark., 2004). Bir başka ifade ile immünokontrasepsiyon üreme ile ilgili özgün proteinlere veya dokulara karşı fizyolojik tolerans/self toleransı ortadan kaldırarak hümöral ve hücresel bağışıklık tepkileri oluşturmaktır. Burada immünolojik tolerans devreye girmektedir. İmmünolojik tolerans antijenlere özgün bir immünolojik yanıt oluşturulmasıdır (Munks, 2012). Oluşan bu immün yanıtta ise self tolerans söz konusudur. Self tolerans kendinden olan ile olmayanı ayırt etme durumudur ve vücut kendinden olana tepki vermemelidir. Diğer bir deyişle yabancı antijenlere tolerans (immün sistemin vücuda yabancı antijenlere tepki vermemesi) ve fizyolojik tolerans (vücudun kendi antijenlerine tepki vermemesi) söz konusudur. Yabancı antijenlere tolerans istenmeyen bir durumdur zira vücudu enfeksiyonlara açık hale getirerek hayati tehlikeye kadar varabilen sonuçlara yol açar. Fizyolojik tolerans ise istenen bir durumdur ve özellikle fertilite ile ilgilidir. Gebeliğin şekillenmesi veya dişi reprodüktif sistemin spermatozoaya reaksiyon göstermemesi fizyolojik toleranstır. İşte immünokontrasepsiyon söz konusu olduğunda, infertiliteye neden olmak için bu self toleransın kırılması gerekmektedir (Turvey ve Broide, 2010). Bu da immün sistemini reprodüktif sistemin belirli bir bileşenine karşı immün yanıt oluşturmaya teşvik eden adjuvanlar yardımıyla kırılır (Munks, 2012; Turvey ve Broide, 2010). Hümöral ve hücresel immün sistemin uyarılması ile elde edilen antikorlar kendilerine has bölgelere bağlanarak reseptörleri bloke ederek veya hormon salgılanmasını engelleyerek reprodüktif süreci durdurmaktadır. Dolaşımdaki antikor düzeyinin düşmesi durumunda ise reprodüktif süreç tekrar başlar (Kustritz, 2018). Fertil döneme geri dönüşe izin veren immünokontraseptif aşıların etkili bir yöntem oluşu, sistemik yan etkisinin olmaması, tek uygulamada başarı olanağının bulunması ve diğer yöntemlere kıyasla ucuz bir yöntem olması immünokontrasepsiyonun avantajları arasında sayılmaktadır (Gupta, 2022).

İdeal bir immünokontrasepsiyon aşısı aşağıdaki özellikleri sağlamalıdır. Herhangi bir sağlık riski taşımamalı, uzun etkili ve etkisi geri dönüşümlü olmalıdır (Naz ve Saver, 2016). Hedefi sadece reprodüktif sistem olmalıdır. Reprodüktif sistem dışında önemli bir fizyolojik ya da patolojik etkinliği bulunmamalıdır (Munks, 2012). Hem dişi hem de erkekte etkili olmalı her iki cinsiyet için farklı aşıya gereksinim duyulmamalıdır. Seksüel davranışları azaltmalıdır. Bu etki hayvanat bahçesi, çiftlik ve evcil hayvanlarda avantaj iken sürü hiyerarşisinin gerekli olduğu vahşi hayvanlarda dezavantajdır (Naz ve Saver, 2016). Vahşi hayvanlarda ve çiftlik hayvanlarında vücutta kalıntı madde bırakmamalı dolayısıyla etkisi besin zincirinin diğer halkasına geçmemelidir. Diğer fertilite kontrol yöntemlerine kıyasla maliyeti daha uygun olmalıdır (Munks, 2012).

İlk çalışmalar memeli oositini hem ovülasyon öncesi hem de ovülasyon sonrası saran ZP üzerine yoğunlaşmıştır (Skinner ve ark., 1996). Daha sonra, endojen reprodüktif hormonlar hedef alınmıştır. İmmünokontrasepsiyon için en umut verici hormonal antijenin ise GnRH olduğu bildirilmiştir (Robbins ve

ark., 2004). GnRH aktivitesi engellenerek, tüm reprodüktif süreç engellenebilmektedir (Levy ve ark., 2004). LH ve LH reseptörünü (LH-R) hedefleyen immünokontrasepsiyon girişimleri de evcil karnivorlarda başarılı olmuştur (Saxena ve ark., 2002; Saxena ve ark., 2003).

1.1.Zona Pelusida Aşıları: Zona pelusida türe özgü sperm-oosit bağlanmasında, akrozom reaksiyonunun başlatılmasında ve implantasyondan önce oositin korunmasında önemli rol oynamaktadır (Moros-Nicolás ve ark., 2018). Bu nedenle, 1970'li yıllardan bu yana immünokontrasepsiyon için ZP hedef haline gelmiştir. Mekanik olarak, reseptör bölgelerinin bozulması, reseptörlerin bloke edilmesi, ZP'nin kimyasal modifikasyonu ya da ZP'ye özel antikorların üretilmesi infertiliteye neden olarak immünokontraseptif etki göstermektedir (Liu ve ark., 1989).

Zona pelusida membranında ZP1, ZP2 ve ZP3 olarak adlandırılan farklı moleküler ağırlığa sahip üç ana bölgesel glikoprotein vardır. ZP1, diğer iki dimer (ZP2-ZP3) arasında bağlayıcı görev yapar. ZP2, akrozom reaksiyona giren sperm için bir reseptördür ve polispermiyi önler. ZP3 ise akrozom reaksiyonunun indüksiyonundan sorumlu birincil sperm reseptörüdür (Kirkpatrick ve ark., 2012). ZP antikorlarının, ZP dışında herhangi bir hücre ya da dokuya bağlanmaması beklenmektedir. Bu nedenle ZP'ye özel antikor üretimi yapılabilmektedir (Barber ve Fayrer-Hosken, 2000). ZP aşısı dolaşımda bulunan antikorların spermin ZP'ye bağlanmasını engelleyerek etki gösterir (Kirkpatrick ve ark., 2012). Birçok memeli türünde ZP antikorlarının fertilizasyonu engellediği belirlenmiştir (Mask ve ark., 2015). Mezbahalarda rahatlıkla bulunabilir olması ve insan ZP'si ile benzerliği nedeniyle, immünokontrasepsiyon araştırmalarında sıklıkla domuz ZP'si (pZP) kullanılmaktadır. Domuz ZP3'ü, kedi, köpek, inek ve fare ZP3'ü ile %65,6 ile %83,6 arasında amino asit sekans homolojisine sahiptir (Moros-Nicolás ve ark., 2018).

Günümüzde pZP ile hazırlanan üç adet ZP aşı preparatı bulunmaktadır: ZonaStat-H (Human Society Of The United States, Washington, Amerika Birleşik Devletleri), pZP-22 (Iowa Üniversitesi Eczacılık Fakültesi, Iowa, Amerika Birleşik Devletleri) ve SpayVac® (ImmunoVaccine Technologies, Yeni İskoçya, Kanada). Bu üç aşı arasındaki fark, pZP'nin elde edilme şekli, saflaştırma yöntemi ve kullanılan adjuvanlardır (Naz ve Saver, 2016).

Zona pelusida aşıları, birçok vahşi hayvan türünde başarılı sonuç vermiştir. Amerika Birleşik Devletleri'ndeki vahşi bir at sürüsünde 13 yıldan uzun süreli devam eden çalışmada, sürüdeki atlar pZP aşısı ile yıllık olarak aşılanmıştır. Sürüde %92,4-100 kontraseptif etki elde edilirken aşılamayı takiben 2 yıl içerisinde sürüde nüfus artışı gözlenmemiştir (Kirkpatrick ve Turner, 2008). İlginç bir şekilde, aşılanmayan kısrakların ortalama ölüm yaşı 6,47 yıl ve art arda 3 yıldan fazla aşılanan kısraklarda ise ölüm yaşı 19,94 yıl olmuştur. Bu da pZP'nin yaşam süresini önemli ölçüde uzattığını göstermektedir (Kirkpatrick ve Turner, 2007).

Zona pelusida aşılarının etkili dozunun, özellikle yaban hayvanlarında belirlenmesi zordur. Yapılan çalışmalarda pZP'nin dozu, 50 ile 600 µg/hayvan arasında değişen geniş bir aralıkta farklılık göstermektedir. Geyik ve atlarda etkili doz 65 ile 100 µg/hayvan arasındadır.

Dozu etkileyen her zaman hayvanın kilosu değildir. Örneğin Turner ve ark., (2002) hem bizon (ortalama 408 kg) hem de dünyanın en küçük geyiği (ortalama 11,5 kg) olan pudu geyiğinde 100 µg pZP kullanılarak immünokontrasepsiyon sağlamıştır (Turner ve ark., 2002).

Diğer yandan ZP aşısı hayvan davranışlarını değiştirebilmekte hatta üreme sezonlarını uzatabilmekte ve östrüs siklusu sayısını arttırabilmektedir. pZP ile aşılanan kısrak ve geyiklerde üreme sezonunun uzadığı gözlenmiştir (Nuñez ve ark., 2010). Başka bir çalışmada 24 yıl boyunca aşılanan kısraklarda ve 11 yıldan fazla aşılanan Afrika fillerinde hiçbir davranış değişikliği gözlenmemiştir (Delsink ve ark., 2013; Kirkpatrick ve ark., 2012).

1.2.Gonadotropin Salgılatıcı Hormon Aşıları: Reprodüktif süreçteki rolü nedeniyle GnRH mükemmel bir immünokontrasepsiyon hedefidir. Omurgalılarda, yapısal olarak farklı 30 GnRH formu tanımlanmıştır ve çoğu omurgalıda GnRH-I, GnRH-II ve GnRH-III izomerleri bulunmaktadır (Kochman, 2012). GnRH kontraseptif aşıları için genellikle memeli GnRH-I izomeri kullanılmaktadır. GnRH küçük bir dekapeptid olması nedeniyle yeterli düzeyde immünojenik değildir yani tek başına yeterli antikor üretimi sağlayamaz. Bu yüzden GnRH molekülünü immünojenik hale getirmek için, keyhole limpet hemosiyanin (hemoglobin analoğu ve immün sistemi aktive edebilecek büyüklükte olan bir metalloprotein), difteri toksoidi veya tetanoz toksoidi gibi taşıyıcı bir proteinle konjuge edilerek immünolojik yanıtın uyarılması gereklidir (Meeusen ve ark., 2007).

Gonadotropin salgılatıcı hormon aşılarının dişi ve erkekte kontrasepsiyon amacıyla uygulanabilir olması önemli bir avantajdır. GnRH aşıları, GnRH molekülünün hipofiz kökenli gonadotroplarını uyarmasını engelleyerek folikül uyarıcı hormon (FSH) ve LH salgılanmasını önler. Bu hormonların salınımı engellendiği için seksüel davranışlar da engellenmektedir (Giriboni ve ark., 2020).

Günümüzde çalışmalarda kullanılan GonaCon™, GonaCon™-Equine, GonaCon™-Blue, Improvest®, Equity™, Bopriva, Repro-BLOC® olmak

üzere yedi farklı GnRH aşı formülasyonu bulunmaktadır (Naz ve Saver, 2016).

Gonadotropin salgılatıcı hormon aşılarında da doz ayarlaması zordur. Bu aşıların farklı adjuvan, cinsiyet ve zaman aralıkları ile denenmesinde aynı türde dahi belirli bir doz aralığının belirlenmesi güçtür. Örneğin erkek ve dişi domuzlarda 1000 veya 2000 µg GnRH aşısı yapıldığında farklı seviyelerde immünolojik yanıt oluşmuştur. Dişiler 2000 µg dozda, erkekler ise 1000 µg dozda daha yüksek antikor titresi üretmiştir (Killian ve ark., 2006). Dişi geyikler 1000 ile 2000 µg olmak üzere iki farklı dozda GonaCon™ ile aşılanmış ve 2000 µg dozun daha yüksek antikor titresi oluşturmasına rağmen iki dozun yol açtığı kontraseptif etkinin istatistiksel olarak önemi olmadığı belirlenmiştir (Killian ve ark., 2009). GnRH aşıları erkek kedilerde %67 oranında etkilidir (Levy, 2004). GnRH aşıları dişi kedilerde ise aşılama sonrası 5 ay ile 5 yıla kadar infertiliteye neden olmuştur (Robbins ve ark., 2004).

Diğer yandan, bazı türlerde GnRH aşısı yapılan hayvanların vücut kondisyon skorunun yapılmayanlardan daha iyi olduğu belirlenmiştir. Kanada geyiklerinde bir GonaCon™ enjeksiyonu ile 3 yıl süreli kontraseptif etki sağlanmıştır ve hayvanlar daha az bir araya gelmiştir. Bu da brusellozisin yayılmasını kontrol altına almada etkili olmuştur (Killian ve ark., 2009).

GnRH aşısı başka aşılar ile kombine edilebilmektedir. Köpeklerde GnRH ve köpek distemper virüsü içeren kombine aşıda, immünojenik uyarımda azalma olmadığı, yüksek antikor titreleri elde edildiği, testis boyutunda azalma olduğu ve spermatogenezisin baskılandığı görülmüştür (Jung ve ark., 2005; Vargas-Pino ve ark., 2013). GnRH aşılarının bu özelliği gelecekte vahşi yaşamda ve sahipsiz sokak hayvanlarında bulaşıcı hastalıklar ile mücadelede önemli bir avantaj olabilir.

Östrüs siklusu sayısını artıran ZP aşılarının tersine GnRH aşıları östrüs sikluslarının sayısını azaltmaktadır. Her iki cinsiyette GnRH aşılarının yan etkisi bulunmamaktadır (Curtis ve ark., 2008). GnRH aşılarının, özellikle gebeliğin devamı için LH molekülüne ihtiyaç duyan türlerde gebe hayvanlarda kullanılması abortuslara neden olabilmektedir (Ransom ve ark., 2014). Tablo 1'de ZP ve GnRH aşılarının karşılaştırılması daha detaylı olarak verilmiştir.

Tablo 1. ZP aşılarının ve GnRH aşılarının karşılaştırılması (Naz ve Saver, 2016'dan uyarlanmıştır).

Özellik	ZP Aşıları	GnRH Aşıları
Geri dönüşüm	> 5 yıl	1- 6 yıl
Etkilediği cinsiyet	Sadece dişi	Dişi ve erkek
Gebelikte kullanım	+	-
Gonadal hormonlara etkisi	-	+
Seksüel davranışlara etkisi	-	+
Ovaryum dokusuna özgüllük	+	-
Gonadal patoloji	+	+
Soğuk zincir gereksinimi	+	-
Besin zinciri geçişi	-	-

1.3. Lüteinleştirici Hormon Reseptör Aşıları: Aşı olarak LH reseptör (LH-R) proteinlerinin kullanıldığı çalışma sayısı oldukça azdır. İmmun sistem LH reseptörlerini, yabancı bir protein olarak görmediğinden GnRH ve ZP'ye karşı bir immün yanıt geliştirilmesinde karşılaşılan benzer zorluklar LH reseptör aşıları için de geçerlidir (Katherine ve Linda, 2013).

Lüteinleştirici hormon reseptör aşısı uygulanan dişi kedilerin östrojen konsantrasyonlarında önemli oranda değişiklik şekillenmediği belirlenmiştir. Bu da aşılanmış kedilerde bazal foliküler büyümenin ve östrojen eksikliğinin olmadığını göstermektedir. Benzer şekilde aşılanmış kedilerin serum LH konsantrasyonlarında da çok az bir fark tespit edilmiştir. Ancak östrojen ve LH konsantrasyonlarının aksine aşılanan dişi kedilerde serum progesteron konsantrasyonları azalmaktadır. Bu veriler, dolaşımdaki LH-R antikorlarının ovaryumdaki LHR'lerine bağlanarak doğrudan progesteron üretimini engellediğini göstermektedir. Kedilerde LH-R aşılaması sonucunda düşük antikor titresi elde edilse de progesteron üretimini baskılayabilmektedir. LH-R aşıları ile 11 aydan uzun süreli kontrasepsiyon sağlanmıştır (Saxena ve ark., 2003).

Lüteinleştirici hormon reseptör aşısı uygulanan köpeklerde yüksek antikor titreleri ve düşük serum progesteron seviyeleri tespit edilmiş ve bu köpeklerin vajinal kanama, vulvada ödem gibi östrüs siklusu belirtileri göstermediği gözlenmiştir. Aşı, uygulama sonrası 12 aya kadar infertiliteye neden olmuştur. Uygulamanın yaklaşık 501. gününden itibaren antikor titrelerindeki düşüşle birlikte köpekler siklik aktivitelerinin başladığına dair dış belirtiler göstermeye başlamıştır (Saxena ve ark., 2002).

İmmünokontrasepsiyon çalışmalarında LH ve/veya FSH reseptörlerini hedeflemenin pratik olmayacağı çünkü bu moleküllerin reprodüktif sistem dışında da reseptörlerini içeren ve aşılamadan olumsuz etkilenebilecek birçok doku ve organ bulunduğu belirtilmektedir (Katherine ve Linda, 2013). Tiroid uyarıcı hormon (TSH) ve LH aynı alfa alt birimine sahiptir. Bu da konraseptif aşılarda LH molekülünün hedeflenmesini sorunlu hale getirir. LH molekülünün alfa alt birimi ile etkileşime giren antikorlar, TSH molekülünün de alfa alt birimi ile etkileşime girebilir (Munks, 2012). Ayrıca bu yöntemin ticari olarak bulunabilirliği, üretim ve pazarlama maliyetleri de immünokontrasepsiyon aşı çalışmalarında LH ve LH-R'nin hedeflenmesini zorlaştırmaktadır (Munson, 2006).

Sonuç

İmmünokontrasepsiyon etik, geri dönüşümlü, dokuya özgü etki mekanizması, tek bir uygulama ile uzun süreli infertiliteye neden olması, yan etkilerinin az olması ve kolay uygulanabilmesi gibi avantajlarından dolayı özellikle gelecekte ön plana çıkması beklenen bir kontrasepsiyon yöntemidir. Ancak her hayvanda gerekli antikor titresinin sağlanamaması, infertilite üzerindeki etki süresinin aynı olmaması, antikor titresinin ne zaman düşmeye başlayacağının belirlenememesi gibi nedenlerden dolayı immünokontraseptif aşı çalışmaları halen devam etmektedir. Araştırmalar ZP ve GnRH aşıları üstüne yoğunlaşmıştır. Ancak ZP aşılarının östrüs süresini kısaltması, her iki cinsiyette de kullanılamaması gibi dezavantajlarından dolayı gelecekteki çalışmaların GnRH aşılarının üstüne yoğunlaşacağı gözükmektedir.

Çıkar çatışması

Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Etik izin

Yazarlar Araştırma ve Yayın Etiğine uyulduğunu beyan etmişlerdir.

Benzerlik Oranı

Makalenin benzerlik oranının sisteme yüklenen raporda belirtildiği gibi %3 olduğunu beyan ederiz.

Yazar Katkıları

Fikir/Kavram: SSA, AGA
Tasarım: SSA, AGA
Denetleme/Danışmanlık: SSA, AGA
Veri Toplama ve/veya İşleme: SSA, AGA
Analiz ve/veya Yorum: SSA, AGA
Kaynak Taraması: SSA, AGA
Makalenin Yazımı: SSA, AGA
Eleştirel İnceleme: SSA, AGA

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Tavuklarda Sindirim Sistemi Mikrobiyotası ve Önemi

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Özet: Tavukların gastrointestinal kanalı, besinlerin sindirilmesinde, emiliminde, bağışıklık sisteminin gelişmesinde ve patojen mikroorganizmaların uzaklaştırılmasında hayati rol oynayan mikrobiyotayı barındırır. Dokuz yüzden fazla bakteri türünü içeren mikrobiyotanın; tavuğun yaşı, cinsiyeti, sindirim sisteminde bulunduğu yer ve tükettiği yemden etkilendiği düşünülmektedir. Son yıllarda bu konu hakkında yapılan araştırmalar, mikrobiyotanın konağın sağlık ve hastalık durumu üzerine büyük öneme sahip olduğunu göstermiştir. Sindirim sistemi mikrobiyatasının fonksiyonunun daha iyi anlaşılması, gelecekte kanatlı sağlığı ve üretiminin iyileştirilmesi için yeni fırsatlar sağlayacaktır. Bu makalede, tavuk gastrointestinal mikrobiyotasının oluşumu, fonksiyonu, çeşitliliği ve mikrobiyotayı etkileyen faktörler hakkında genel bilgiler mevcut literatürden yararlanılarak derlenmiştir.
Anahtar Kelimeler: *Bağırsak sağlığı, Mikrobiyom, Mikrobiyota, Tavuklar.*

Digestive System Microbiota in Chickens and Its Importance

Abstract: The gastrointestinal tract of chickens harbors the microbiota, which plays a vital role in the digestion and absorption of nutrients, the development of the immune system, and the exclusion of pathogenic microorganisms. This microbiota, containing more than 900 bacterial species, is thought to be affected by the age and sex of the chicken, its location in the digestive system, and the feed consumed by the chicken. Research on this subject in recent years has shown that microbiota significantly impacts the host's health and disease status. A better understanding of the function of the digestive microbiota will provide new opportunities for improving poultry health and production in the future. This article reviews general information about the formation, function, and diversity of the chicken gastrointestinal microbiota and the factors affecting the microbiota, referring to the existing literature.
Keywords: *Gut health, Microbiome, Microbiota, Chickens.*

Giriş

Mikrobiyota, insan ve hayvanlarda belirli bir sistemi ve/veya bölgeyi kolonize eden kommensal, simbiyotik ve patojenik mikroorganizmaları içeren mikrobiyal topluluklar olarak tanımlanır. Bu mikroorganizmaların tamamının genomu ise mikrobiyom olarak bilinmektedir (Sender ve ark., 2016). Tavuk mikrobiyotası içerisinde mantarlar, virüsler, protozoonlar ve arkealar da tanımlanmış olmasına rağmen, bakterilerin daha yoğun bulunduğu bildirilmiştir (Sood ve ark., 2020). ABD'de Ulusal Sağlık Enstitüsü (National Institutes of Health, NIH) ve Ulusal İnsan Genom Araştırma Enstitüsü (National Human Genome Research Institute, NHGRI) tarafından 2008 yılında başlatılan "İnsan Mikrobiyom Projesi" ve kısa süre sonra Avrupa'da başlatılan "İnsan Bağırsağı Metagenomiği Projesi" (METAgenomics of the Human Intestinal Tract, MetaHIT) konunun önemini göstermeleri açısından oldukça anlamlıdır. Aslında, özellikle rumen ve bağırsak florasının incelendiği çalışmalar nedeniyle, veteriner hekimlikte aynı kapsamdaki araştırmaların geçmişi daha köklüdür (Diker, 2017). Yakın zamana kadar kümes hayvanlarının mikrobiyotası üzerine yapılan araştırmalar, mikrobiyotanın sadece küçük bir kısmını kültüre edebilen geleneksel mikrobiyolojik tekniklere dayanmaktaydı. Son yirmi yılda ise, DNA dizi analizleri, kütle spektrometresi ve biyoinformatik alanındaki teknik ilerlemeler ve gerçekleştirilen araştırmalar neticesinde, sindirim sistemi mikrobiyotasının, konağın sağlığı ve hastalık durumu üzerinde büyük bir öneme sahip olduğu anlaşılmıştır (Kuda ve ark., 2017).

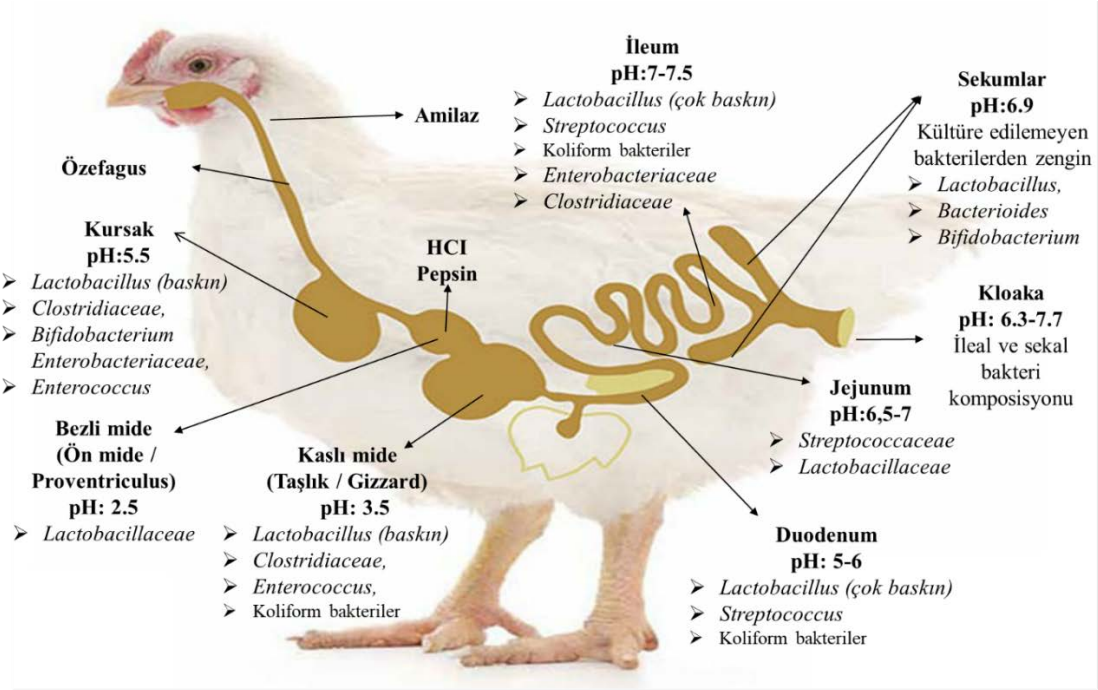
Tavuklarda Sindirim Sistemi Mikrobiyotasının Oluşumu ve Gelişimi: Sindirim sistemi kolonizasyonunun civcivlerin kuluçkadan çıktıktan hemen sonra başladığı ve kuluçka ortamının mikrobiyal çeşitlilik üzerinde büyük bir etkisi olduğu düşünülmektedir. Tavukların yaşamının 1. gününde sekumda; streptokoklar ve enterobakteriler, 3. gününde ise laktobasiller, streptokoklar, enterokoklar ve koliformlar gibi birçok bakterinin gastrointestinal sistemin farklı kısımlarından izole edilebildiği bildirilmiştir (Aruwa ve ark., 2021). Ayrıca tavuk ve hindilerde gerçekleştirilen bir araştırma, kanatlı

hayvan türleri arasında mikrobiyota çeşitliliğinde büyük farklılıklar olduğunu göstermiştir (Stanley ve ark., 2013). Bu çeşitlilik, insan ve diğer hayvanların mikrobiyotasında da bulunmuştur. Bu durum hem konakçıya hem de çevresel faktörlere bağlanmıştır (Simpson ve ark., 2000). Hayvansal üretim sistemleri arasında kümes hayvanları, yavruların annelerinden ayrılması nedeni ile farklılık göstermektedir. Bu nedenle, yumurtadan çıkan civcivlerin mikrobiyotalarının gelişimi üzerinde ebeveyn etkisinin belirgin bir şekilde azaltılmış olduğu düşünülmektedir. Bunun dışında, ticari kuluçkahanelerde yumurtadan çıkan civcivler, kuluçka çalışanları, altlık, yem ve taşıma kutuları gibi çevresel kaynaklara bağlı olarak çeşitli mikroorganizmalarla karşılaşırlar. Bu sebeplerle araştırmacılar tavuk mikrobiyotasının geniş çapta değişen profillere sahip olmasını, civcivlerin yaşamlarının ilk saatlerinde kanatlı kaynaklı olmayan mikroorganizma çeşitliliğine maruz kalması ve azaltılmış ebeveyn etkisine bağlı olduğunu düşünmektedir (Stanley ve ark., 2013).

Mikrobiyotanın Bileşimi: Genel olarak tavuklardaki mikrobiyota yaş, cinsiyet, yem içeriği gibi çeşitli faktörlere göre değişir ve bu nedenle, yayımlanan çalışmalarda taksonomik kompozisyon profilleri büyük ölçüde farklılık göstermiştir. Ayrıca, mikrobiyom konağın ömrü boyunca birçok değişikliğe uğrar. Tek bir şube, cins veya türün konak mikrobiyotası üzerindeki hakimiyetinin geçici olduğu bildirilmiştir (Marmion ve ark., 2021). Wei ve ark. (2013), mikrobiyotayı 13 şube olarak sınıflandırmış ve *Firmicutes* (%70), *Bacteroidetes* (%12.3) ve *Proteobacteria* (%9.3) şubelerine eşdeğer olan 915 operasyonel taksonomik birimin (OTU) varlığını belirlemişlerdir. Bu 915 OTU içerisinde genel olarak *Clostridium*, *Ruminococcus*, *Lactobacillus* ve *Bacteroides*'in baskın olduğu 117 cins tanımlanmıştır (Clavijo ve Flórez, 2018). *Firmicutes* şubesinde etanol üreten *Ethanoligenes* cinsinin yüksek prevalansa sahip olduğu gösterilmiştir. *Proteobacteria* şubesinde *Desulfohalobium* cinsinin de baskın olduğu tespit edilmiştir (Sood ve ark., 2020).

Sindirim Sistemi Konumuna Göre Mikrobiyota Kompozisyonu: Tavuk sindirim sisteminin bölümleri farklı bakteri çeşitliliğine sahiptir. Bu nedenle araştırmacılar her bir bölümün ayrı ekosistem olarak düşünülmesini önermiştir (Sekelja ve ark., 2012; Stanley ve ark., 2014). Bölümler arasındaki bakteri kompozisyonu; bireysel genetik, çevresel koşullar, yem ve antimikrobiyallerin kullanımı gibi faktörlere bağlı olarak değişim gösterdiği için tipik mikrobiyal profilleri tanımlamanın zor olduğu bildirilmiştir (Stanley ve ark., 2014). Tavuk sindirim sisteminde yem, ağızda amilaz, lipaz gibi enzimler ile sindirime tabi tutulur ve özofagustan geçerek kursağa aktarılır. Kursak, özofagusun genişlemesi ile oluşan yemlerin depolandığı organdır. Burada yem ıslatılarak yumuşatılır ve proventrikulusa geçer. Proventrikulusta, hidroklorik asit ve pepsin gibi sindirim enzimleri ile parçalanarak taşlığa iletilir. Taşlık, öğütülen, çözünmeyen tahılları biriktirir ve sindirim enzimleri aracılığı ile proteinlerin sindirimini tamamlayarak ince bağırsağa aktarır. İnce bağırsak yemin sindirimi ve emilimini sağladıktan sonra, yemin fermente edildiği ve her 24 saatte bir boşaltılan bir çift tüp olan sekum'a açılır. Burada polisakkaritler gibi ince bağırsakta sindirilmeyen yemin sindirimi sağlanır. Sindirim kanalının son kısmını oluşturan kalın bağırsakta son posa emilerek kloakadan dışarı atılır (Clavijo ve Flórez, 2018; Lu ve ark., 2003; Noy ve ark., 1995; Stanley ve ark., 2014; Uni ve ark., 1999). Tavuk sindirim sistemi profilleri **Şekil 1**'de özetlenmiştir. **Kursakta**, başta Gram pozitif fakültatif anaerobik bakteriler, özellikle de *Lactobacillus* türleri (en yaygın türler; *Lactobacillus salivarius*, *L. fermentum*, *L. reuteri* ve *L. acidophilus*) olmak üzere 10^8 ila 10^9 cfu/g (1 g'da koloni oluşturan bakteri sayısı) kadar yoğunlukta mikrobiyota barındığı bildirilmiştir (Rehman ve ark., 2007). **Taşlık**, *Lactobacillus spp.* (etlik piliçlerde önemli sayıda *L. aviarius* ve *L. salivarius*) ve *Enterococcus* spp.'nin yoğun olduğu bir kompozisyonu içermektedir (Bjerrum ve ark., 2005). **İnce bağırsakta**, en yoğun bakteri konsantrasyonları başlıca *Lactobacillus*, *Enterococcus* ve çeşitli *Clostridiaceae*'dır (Clavijo ve Flórez, 2018). İleum mikrobiyotası, ince bağırsak bölümleri arasında en çok incelenen bölümdür. *Lactobacillus* cinsinin baskın grubu oluşturduğu (%70) ve ardından *Clostridium* (%11), *Streptococcus* (%6.5) ve *Enterococcus* (%6.5) cinslerinin bulunduğu bildirilmiştir (Lu ve ark., 2003). Kanatlılarda **sekum**, kalın bağırsağın en büyük taksonomik çeşitlilik ve yoğunluğa sahip olan bölümüdür. Şüphesiz, sindirim kanalında yeminen uzun süre (12 ila 20 saat) tutulduğu organdır. Bu organı mikrobiyota için önemli bir niş yapan diğer özellikler, su emiliminin en yoğun olduğu sindirim bölümü olması, üre düzenlemesinden sorumlu olması ve karbonhidratların fermantasyonunu gerçekleştirmesidir. Sekum mikrobiyotasının zenginliği, ince bağırsakta bakteriyel sindirime dirençli olan selüloz, nişasta ve polisakkarid bakımından zengin yem maddelerinin sindirimi ile ilişkilendirilmektedir (Goldstein, 1989). Sekumda, en yoğun grubu *Clostridium türleri* (%65) oluştururken, bunu *Fusobacterium* (%14), *Lactobacillus* (%8) ve *Bacteroides* (%5) cinslerinin izlediği bildirilmiştir (Lu ve ark., 2003).

Mikrobiyotada Bulunan Patojenler: Tavuk mikrobiyatası, zoonoz hastalıklara neden olan bakteriyel toplulukları barındırır ve bunlardan en önemlileri Campylobacteriosis ve Salmonellosis'e



Şekil 1. Tavuk Sindirim Sistemi Profilleri (Stanley ve ark., 2014).

neden olan etkenleri içermektedir. Salmonellalar dünyada en sık görülen gıda kaynaklı hastalık etkenleridir. Bu nedenle, hayvan kaynaklı serotipler insanlar için potansiyel patojenler olarak kabul edilmektedir (Yapıcıer ve Sareyyüpoğlu, 2022). Salmonellaların tavuk bağırsak mikrobiyomunda küçük bir takson olduğu ve kümes hayvanlarında dağılımında sporadik bir yerleşim gösterdiği bildirilmiştir (Liljebjelke ve ark., 2005). *Salmonella*, kanatlı hayvan türlerinde hastalığa neden olabilmektedir. Ancak hastalığa duyarlılık; konağın yaşına, bağışıklık durumuna ve hastalığa neden olan *Salmonella* serotipi veya suş tipine bağlıdır (Barrow, 1991). Bir başka patojen olan *Campylobacter* türleri (çoğunlukla *C. jejuni* ve *C. coli*) bağırsaklarda 10^7 cfu /g'a kadar hemen hemen tüm tavuk bağırsaklarında bulunmuştur ve bu nedenle de genellikle tavuklar için patojenik olmadığı öne sürülmüştür (Stern ve ark., 1995). Ancak yapılan daha güncel bir çalışmada etkenin tavuk sağlığını etkilediği ve aynı zamanda gıda kaynaklı önemli bir patojen olduğu gösterilmiştir (Humphrey ve ark.,2014). Öte yandan, *Escherichia coli*, tüm yaşam döngüsü boyunca sağlıklı tavukların bağırsaklarında bol miktarda bulunan bir γ-proteobakteridir. Bazı *E. coli* suşları, kümeslerdeki yüksek amonyak miktarına bağlı olarak *Mycoplasma gallisepticum* kaynaklı solunum yolu infeksiyonlarını takiben kanatlılarda ikincil fırsatçı enfeksiyonlara neden olabilir. Ayrıca yumurtacı tavuklarda, yumurta peritoniti veya salpenjit gibi patolojik değişikliklere neden olduğu bildirilmiştir (Landman ve ark, 2013). Tavuklardan izole edilen bazı APEC (Avian Pathogenic *E. coli*) suşları, insan ekstra-intestinal *E. coli* patotipleriyle ortak virülans özellikleri olan P-pili, S-pili, CNF toksini, Ibe proteinleri veya K1 kapsülüne sahip olabilir. Ancak bu özellikler kanatlı izolatları arasında sporadik olarak bulunmuştur (Dziva ve ark., 2013). Bu virülans genlerine sahip APEC suşları, *E. coli* referans koleksiyonu B2 grubuna ait insan bağırsak *E. coli* suşları ile filogenetik olarak ilişkili görünmektedir. Bu durum, bazı APEC'lerin zoonotik olabileceğini düşündürmektedir (Ewers ve ark., 2007). Patojenler arasında yer alan clostridial popülasyon; *Clostridium perfringens*, *C. septicum* ve *C. colinum* gibi bazı türleri içerir. Bu türlerden *C. perfringens*'in tavuklarda nekrotik enterite neden olduğu ve insan patotiplerinin sahip olmadığı yeni bir toksin tipini içerdiği rapor edilmiştir (Keyburn ve ark., 2010).

Tavuklarda Sindirim Sistemi Mikrobiyotasının Rolü ve Önemi: Tavuk sindirim sistemi içerisinde yer alan karmaşık ve çeşitli mikrobiyal topluluklar, alınan yemin parçalanmasına ve sindirilmesine yardımcı olurlar. Yapılan çalışmalarda kanatlı mikrobiyotasının içeriği ile yemden yararlanma oranının ilişkili olduğu ifade edilmiştir (Huang ve ark., 2021). Bu nedenle, tavuk ile bakteriyel mikrobiyomu arasındaki etkileşimler kapsamlı bir şekilde birçok araştırma grubu tarafından incelenmiştir. Bunun sonucunda mikrobiyotanın; besin değişimi, immünolojik modülasyon, sindirim sistemi fizyolojisi ve patojenlerin dışlanması üzerine çeşitli etkileşimleri olduğu gösterilmiştir (Pan ve Yu, 2014).

Besin Değişimi: Tavukların sindirim sistemi mikrobiyotası, önemli olan besin bileşenleriyle hem doğrudan hem de dolaylı olarak etkileşimde

bulunurlar. Bu önemli besinler; kısa zincirli yağ asitleri (asetik asit, bütirik asit ve propiyonik asit), organik asitler (laktik asit,formik asit), antimikrobiyal bileşikler (bakteriyosinler) ve vitaminleri (K vitamini ve B vitamin gruplarını) içerir (Pan ve Yu, 2014). Tavuk sindirim sistemi bakterilerinin çoğu polisakkaritleri, oligosakaritleri ve disakaritleri birincil şekerler halinde hidrolize ederek asetat, butirat ve propiyonat gibi kısa zincirli yağ asitleri üretir. Ayrıca kan akışını düzenler, enterositlerin büyümesini ve çoğalmasını uyarır ve bağırsaklardaki mukozal bağışıklığı etkileyen müsin üretimini düzenler (Tellez ve ark., 2006). Bununla birlikte, azot metabolizmasına da katkıda bulunur. Tavuk sindirim sistemi, karşılıklı olarak bağırsak bakterilerine besin sağlayabilir. Örneğin, goblet hücrelerinin bağırsakta ürettiği müsin, benzer şekilde kommensal bakteriler ve patojenler için önemli bir karbon, azot ve enerji kaynağıdır (Tellez ve ark., 2006).

Mikrobiyota ve Bağışıklık Sistemi: Bağırsaklarla ilişkili bağışıklık sistemi, sağlam bir mukozal katman, sıkıca birbirine bağlanmış bağırsak epitel hücreleri, salgılanan antikorlar (immünoglobulin A) ile antimikrobiyal peptitleri içerir. Yararlı bir mikrobiyal topluluğun, normal fizyolojik homeostazı koruduğu ve konakçı bağışıklık sisteminin düzenlenmesinde önemli bir rol oynadığı bildirilmiştir (Sommer ve Bäckhed, 2013). Tavukların bağışıklık sistemi hem doğal hem de kazanılmış bağışıklık ile etkili olur. Doğal bağışıklık bağırsak mukozasının enfeksiyona karşı birinci savunma hattı olup bakterilerin bağırsak epiteline girmesini önleyen bir bariyer görevi üstlendiği bilinmektedir (Carter ve ark., 2009). Kazanılmış bağışıklık sistemi ile ilgili olarak; kommensal bakterilerin bağışıklık tepkisini düzenlediği ve yardımcı T hücrelerini uyararak mukozal membranda koruma sağladığı öne sürülmektedir (Oakley ve ark., 2014). Fakat bu mekanizmalar henüz tam olarak açıklığa kavuşturulmamıştır. Ayrıca mikrobiyotanın sitokin ve kemokinlerin üretimine rehberlik ettiği, B-hücre yanıtını ve IgA üretimini modüle ettiği düşünülmektedir (Macpherson ve Uhr, 2004).

Mikrobiyotanın Sindirim Sistemine Etkisi: Yumurtadan çıkan civcivlerin sindirim sistemleri, yaşamın ilk saatlerinden itibaren mikroorganizmalar tarafından kolonize edilir. İlk yerleşen mikroorganizmalar zaman içinde istikrarlı ve farklı bir popülasyonun oluşması için temel bir ortam sağlar. Başlangıçta tavukların sindirim sistemi fakültatif aeroblar tarafından kolonize edilir ve daha sonra anaeroblar ile yer değiştirir. Aerobik bakterilerin çoğalması ve oksijen tüketimi, bağırsak ekosisteminde zorunlu anaerobların üreme ve kolonizasyonunu destekleyen indirgeyici koşulları sağlar (Aruwa ve ark., 2021). Tavuk bağırsağının iç yüzeyi kalsiform epitel hücreleri tarafından salgılanan bir mukoza tabakası ile kaplanmıştır. Yapılan bir çalışmada, geleneksel olarak yetiştirilen tavukların mukozalarındaki müsin miktarının, düşük bakteri yüküne sahip ticari tavuklarınkine göre daha yüksek olduğu bildirilmiştir. Bu durum, bağırsak mikrobiyotasının mukoza tabakasının oluşumunu düzenlemede etkili olduğunun göstergesidir (Mitsuhiro ve Jun-ichi, 1994). Bu hipotezi destekleyen diğer çalışmalar Chambers ve Gong (2011) tarafından derlenmiştir. Ayrıca patojen protozoon ve bakteriler morfolojik değişikliklere neden olmaktadır. Örneğin, *Eimeria* spp. ve *C. perfringens* ile birlikte enfekte olmuş tavuklarda, bağırsak villi uzunluğunun önemli ölçüde azaldığı görülmüştür ve aynı etki *Salmonella typhimurium* ile enfekte olmuş tavuklarda da görülmüştür (Golder ve ark., 2011).

Mikrobiyota ve Patojenlerin Dışlanması: Yarışmalı dışlama, aynı kaynak için rekabet eden iki türün istikrarlı bir şekilde bir arada bulunamayacağını belirtmektedir. Bu nedenle, rakiplerden biri her zaman diğerine üstünlük kurarak onu dışlar. Mikrobiyota, kolonileşen patojenik bakterilerle rekabet eder ve bağırsaktaki patojenlerin adhezyonu ve kolonizasyonunu azaltabilir. Bu azalma, ortamın fiziksel işgali, belirli bir alandaki kaynaklar için rekabet, doğrudan fiziksel veya kimyasal etkileşimin farklı mekanizmaları sonucu olabilir. Örneğin mikrobiyotadaki etkenler tarafından bakteriyosin üretimi özellikle patojenler tarafından kolonizasyon sürecine müdahale ile ilişkilendirilmiştir (Razmyar ve ark., 2017). Bu korumaya yol açan işleyişin kodu çözülmemiş olmasına rağmen rekabetçi dışlama işlemi, etlik tavuklarda *Salmonella*'nın bağırsak kolonizasyonunu önlemede en etkili yaklaşımlardan biri olmaya devam etmektedir (Chambers ve Gong, 2011).

Mikrobiyotayı Etkileyen Faktörler

Tavuğun Yaşı: Tavukların yaşı, sindirim sistemi bakteri kompozisyonunu etkileyen en önemli faktörlerden biridir. Mikrobiyotanın gelişimi ve zamansal dalgalanmalarının izlendiği bir çalışmada, 1 günlük civcivlerde ileum ve sekumda bakteri yoğunluklarının 10^8-10^{10} cfu/g olduğu, 3. günde 10^{11} cfu/g 'a ulaştığı ve sonraki 30 günlük izleme boyunca da nispeten sabit kaldığı bildirilmiştir (Apajalahti ve ark., 2004). Benzer bir çalışma, ileum mikrobiyotasının kuluçka ve büyüme aşamaları (0-16 haftalık) sırasında kademeli olarak değiştiğini, yumurtlama aşamasına (17 haftalık) geçtikten sonra değişimin daha yoğun olduğunu, yine sekum mikrobiyotasının, yumurtlama aşamasına (16-25 haftalık) geçtikten sonra büyük ölçüde değiştiğini göstermektedir (Ngunjiri ve ark., 2019). Genel olarak tavuklarda ileri yaşlarda mikrobiyota bileşiminde

daha stabil bakteri taksonlarının oluştuğu görülmektedir (Shang ve ark., 2018).

Cinsiyet: Kanatlılarda cinsiyet farklı üretim sisteminin bir parçasıdır. Çünkü yumurtacı tavuk sürüleri dişilerden oluşurken, etlik piliç sürülerinde erkekler ve dişiler genellikle birlikte yetiştirilir. Erkek ve dişi piliçlerin bakteri topluluklarındaki farklılıkların büyümeyle ilgili olmayan faktörlerden etkilendiği düşünülmektedir (Kers ve ark., 2018). Deneysel bir çalışmada, büyüme hızında 21. güne kadar hiçbir farklılık gözlenmezken, 3. günde bağırsak mikrobiyota bileşiminde farklılıklar tespit edilmiştir (Lumpkins ve ark.,2008). PCR ile güçlendirilmiş 16S ribozomal RNA (rRNA) gen fragmanlarının denatüre gradyan jel elektroforezi (DGGE) ile saptandığı bağırsak mikrobiyota toplulukları, erkek ve dişiler arasında %30'dan daha az benzerlik göstermiştir (Lumpkins ve ark., 2008). Dişi ve erkek piliçlerin (22 ve 42 günlük) kantitatif PCR (qPCR) kullanılarak karşılaştırıldığı başka bir çalışmada, sekumlarında *Lactobacillus salyarius*, *L. cripatus*, *L. aviarius* ve *E. coli* yoğunluğu bakımından farklılıklar tespit edilmiştir (Torok ve ark., 2013).

Beslenme: Tavukların beslenmelerinde yer alan yem maddeleri mikrobiyotanın oluşumu ve gelişimini düzenler. Bu nedenle mikrobiyotanın oluşumunda beslenmenin büyük etkiye sahip olduğu düşünülmektedir. Protein, yağ, nişasta gibi sindirilebilirliği düşük yem kullanımı veya bu maddelerin sindirilebilirliğini artıracak enzim ilavesi, ısıl işlemler gibi önlemlerin alınmamış olması sağlıklı mikrobiyota gelişimini olumsuz etkilemektedir (Marmion ve ark., 2021). Tam olarak sindirilememiş yemler, patojen bakterilerin gelişmesini ve kolonizasyonunu artırmaktadır. Bunun sonucu olarak, yemden yararlanma oranı düşmekte, büyümede gerileme görülmekte ve konakçı enfeksiyonlara karşı duyarlı hale gelmektedir. Örneğin soya yağı içeren diyetlerle beslenen tavukların, hayvansal kaynaklı yağlarla beslenen tavuklardan daha düşük *C. perfringens* yoğunluğuna sahip olduğu görülmüştür (Clavijo ve Flórez, 2018; Luo ve ark., 2016).

Antibiyotikler: Kümes hayvanı yemlerine subterapötik seviyelerde antibiyotiklerin dahil edilmesinin, yem verimliliğini ve büyüme performansını arttırdığı ve *C. perfringens* dahil enterik bakteriyel patojenlerin seviyelerini azalttığı gösterilmiştir (Neumann ve Suen, 2015). Bu amaçla, basitrasin metilen disalisilat ve salinomisin gibi iyonoforlar dahil olmak üzere klasik antibiyotik takviyelerin yaygın olarak kullanıldığı rapor edilmiştir (Johansen ve ark., 2007). Bununla birlikte, antibiyotik kalıntılarının insan sağlığı üzerindeki etkisi ve antibiyotiğe dirençli bakterilerin ortaya çıkması konusundaki halk sağlığı endişeleri nedeniyle, birçok ülke kanatlı yemlerinde belirli antibiyotiklerin kullanımını aşamalı olarak kaldırarak bu riskleri azaltmak için adımlar atmıştır (Agunos ve ark., 2019). Son yıllarda, yeni nesil dizileme teknolojisi kullanılarak gerçekleştirilen 16S rRNA araştırmaları, mikrobiyota profillerindeki değişiklikleri ayırt etmekte önemli derecede kolaylıklar sağlamıştır. Yapılan son çalışmalar, antibiyotik takviyeli yemlerin gastrointestinal sistemde yararlı bakterilerin çoğunluğunu oluşturan *Lactobacillus*, *Bifidobacteria* ve *Streptococcus* gibi Gram pozitif bakterilerin miktarında önemli azalmalara yol açtığı gösterilmiştir (Broom, 2017). Bu azalma sonrasında *Salmonella* ve *Campylobacter* benzeri Gram negatif bakterilerin miktarı artmıştır. Bu durumun bağırsaklarda rölatif sayısı azalan kommensal bakterilerin patojenler ile rekabet eksikliğinden kaynaklandığı düşünülmektedir (Kumar ve ark., 2019).

Probiyotikler: Probiyotikler, yeterli miktarlarda uygulandığında konakçı sağlığına yarar sağlayan canlı mikroorganizmalar olarak tanımlamıştır (Smith, 2014). Örneğin bazı mikroorganizmalar, yem liflerini ve diğer besin maddelerini konakçı için hazır hale getirerek, daha verimli bir şekilde sindirilebilmesini sağlar (Mahmood ve Guo, 2020). *Lactobacillus* konakçı için yararlı olan substratların sindirimi veya fermantasyonu sırasında, antimikrobiyal özellikteki laktik asitleri üretebilir. Bu üretimi doğal mikrobiyota veya mikrobiyota ortamının stabilizasyonu sayesinde başarırlar. *Lactobacillus acidophilus*'tan salgılanan bakteriyosinlerin, patojenlerin hücrelere bağlanma ve kolonizasyonunu rekabetçi bir şekilde önleyerek patogenezi azalttığı bilinmektedir (Alvarez-Sieiro ve ark., 2016). Ayrıca *Lactobacillus* takviyeli yemle beslenen tavuklarda *Salmonella* ve enteropatojenik *E. coli* (EPEC) popülasyonunda azalma ve antikor üretiminde artış olduğu bildirilmiştir (Brisbin ve ark., 2010).

Prebiyotikler: Prebiyotikler, konağın sağlığını geliştirmek için sindirim sisteminde faydalı mikrobiyal aktiviteyi uyaran ve büyümeyi teşvik eden cansız besin maddeleri olarak tanımlanmaktadır. Probiyotiklerle karşılaştırıldığında; üretilmesi daha ucuz, konakta istenmeyen yan etki riskleri daha düşük ve üretim sürecinin daha kolay olduğu bildirilmektedir (Clavijo ve Flórez, 2018). Çeşitli bitkilerde yaygın olarak bulunan inülin, ilgili hidrolizatları ve oligofruktoz, prebiyotiklerin klasik örneklerini temsil ettiği bildirilmiştir (Ricke, 2015). Tavuklarda prebiyotiklerle ilgili çeşitli çalışmalarda, *Salmonella*, *E. coli* ve *Eimeria* spp.gibi patojenlerin inhibisyonunda mannan oligosakkaritlerinin (maya hücre duvarları) etkili olduğu ve oligofruktozların performansı artırdığı bildirilmiştir (Stanley ve ark., 2014). Mannan oligosakkaritleri ve lignin ile beslenen tavuklar üzerine yapılan bir çalışma; sekumdaki yararlı bakteri popülasyonunun, jejunumdaki villus yüksekliğinin ve goblet hücrelerinin sayısında artış

olduğunu bildirmiştir. Ayrıca altlıktaki *E. coli* popülasyonunda azalma tespit edilmiştir (Baurhoo ve ark., 2007). Oligofruktozların kullanımı ile ilgili yapılan bir çalışmada, *Bifidobacterium* ve *Lactobacillus*'un üremesini arttırdığı, ince bağırsakta ve sekal sindirimde *Escherichia coli*'yi inhibe ettiği ancak büyüme performansı, sindirim enzimi aktiviteleri, bağırsak mikrobiyotası veya morfoloji üzerinde önemli bir etkisi olmadığı gösterilmiştir (Xu ve ark., 2003).

Sonuç

Son yıllarda sindirim sistemi mikrobiyotasının taksonomik yapısı ve bağırsak sağlığına katkılarını anlamada önemli ilerlemeler kaydedilmiştir. Yapılan çalışmalar, sindirim sistemi mikrobiyal ekosisteminin dinamiklerinin açığa çıkartılması açısından önemlidir. Tanımlama ve karakterizasyon teknolojilerinde devam eden gelişmelerle birlikte kanatlı mikrobiyomunun yeni üyelerinin keşfedilmesi muhtemeldir. Gelecekteki çalışmalarda mikrobiyomun rolüne ilişkin anlayışımızı arttırmak amacıyla, omik (Genomik, Transkriptomik, Proteomik, Metabolomik ve Metagenomik) yaklaşımların uygulanması anlamlı olacaktır. Bu alanda yapılan çalışmalar neticesinde tavukların çevre, yem ve fizyolojik değişikliklere bağlı olarak bağırsak mikrobiyotasının nasıl yönetileceği konusu daha iyi anlaşılacaktır.

Çıkar çatışması

Yazarlar bu yazı için gerçek, potansiyel veya algılanan çıkar çatışması olmadığını beyan etmişlerdir.

Etik izin

Bu çalışma "Hayvan Deneyleri Etik Kurullarının Çalışma Usul ve Esaslarına Dair Yönetmelik" Madde 8 (k) gereği HADYEK iznine tabi değildir. "Etik Beyan Formu" yazarlarca imzalanarak sisteme yüklenmiştir. Ayrıca yazarlar Araştırma ve Yayın Etiğine uyulduğunu beyan etmişlerdir.

Benzerlik Oranı

Makalenin benzerlik oranının sisteme yüklenen raporda belirtildiği gibi %5 olduğunu beyan ederiz.

Açıklama

Ankara Üniversitesi Sağlık Bilimleri Enstitüsü, Mikrobiyoloji Anabilim Dalı Doktora seminerinden özetlenmiştir.

Yazar Katkıları

Fikir/Kavram: KT, BS
Tasarım: KT
Denetleme/Danışmanlık: BS
Veri Toplama ve/veya İşleme: KT
Analiz ve/veya Yorum: KT, BS
Kaynak Taraması: KT
Makalenin Yazımı: KT
Eleştirel İnceleme: BS

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HARRAN ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ YAYIN KURALLARI *

1- Harran Üniversitesi Veteriner Fakültesi Dergisi (Harran Üniv Vet Fak Derg), özellikle Veteriner Hekimliği bilim alanı ile ilgili olmak üzere insan ve hayvan sağlığını kapsayan Türkçe ve İngilizce olarak hazırlanmış orijinal klinik ve deneysel araştırmalar, olgu sunumları, derlemeler (çağrılı veya sorumlu yazara ait derleme konusu ile ilgili en az 3 araştırma makalesinin referans listesinde olması gereklidir), kısa bilimsel makale ve editöre mektuplar yayınlayan hakemli bir dergidir. Dergide İngilizce hazırlanmış makalelerin yayımlanmasına öncelik verilir. Dergi 6 ayda bir, yılda 2 sayı olarak yayınlanır. Yayınlanan makalelerden ücret alınmamaktadır.

2- Dergiye kabul edilen yayınlar başka bir yerde yayınlanmamış olmalıdır. Eş zamanlı olarak incelenmek üzere başka dergilere gönderilmiş olmamalıdır. Yayınlanan makalelerden doğacak her türlü hukuki ve cezai sorumluluk yazarlara aittir. Yazarlara yayın hakkı bedeli ödenmez. Gönderilen makaleler ve ekleri makale yayınlansın veya yayınlanmasın geri iade edilmez.

3- Daha önce kongrelerde tebliğ edilmiş ve özeti yayımlanmış çalışmalar, bu durum kapak sayfasında belirtilmek üzere kabul edilir. Bununla birlikte yayın, tezden üretilmiş ise ve destekleyen kuruluş var ise yayında belirtilmelidir.

4- Dergi Editörlüğüne ulaşan makale, dergi editörlüğünce ön değerlendirmeye tabi tutulur. Editörlük, ön değerlendirme sonucuna göre makaleyi reddetme veya hakem değerlendirmesine tabi tutmadan önce düzeltme isteme hakkına sahiptir.

5- Makaleler değerlendirme için en az iki hakeme gönderilir. Makale kabul sürecinde, iki hakemin görüşlerinin farklı olması durumunda editör, üçüncü bir hakemin veya danışma kurulunun görüşünü alarak karar verir.

6- Harran Üniversitesi Veteriner Fakültesi Dergisi, etik ilkelere saygı çerçevesinde, TÜBİTAK ULAKBİM tarafından Türkiye'de tüm üniversitelerin kullanımına açmış olduğu "ithenticate" intihal tespit programı aracılığıyla gönderilen tüm makale, olgu sunumu ve derlemelerin ön değerlendirmesinin yapılması ve sonuçların gönderilmesi gerekmektedir. Bu ön değerlendirme sonuçlarına göre, makale, olgu sunumu veya derlemelerin başka kaynaklarla benzerlik oranının **%15'i** (özet, abstract ve kaynaklar hariç) aşmaması gerekmektedir. "ithenticate" programı aracılığı ile yapılacak öndeğerlendirmede benzerlik oranının %15 değerini aşması durumunda yayımlanmak üzere dergimize gönderilen makale, olgu sunumu veya derlemeler değerlendirilmeye alınmayacaktır.

7- Gönderilen herhangi bir makalenin (tüm makale kategorileri için) referanslarının en az % 20'sinin son beş yılda yayınlanan referansları içermesi gerekir. Anonim kaynaklar asgari düzeyde tutulmalıdır.

8- Makale yayına kabul edildiği takdirde her türlü yayın hakkının devredildiğine dair beyanları kapsayan Telif Hakkı Devir Sözleşmesinin tüm yazarlar tarafından imzalanarak basımdan önce elektronik olarak dergi editörlüğüne gönderilmesi gerekmektedir. Telif Hakkı Devir Sözleşmesi gönderilmeyen makaleler yayımlamaya kabul edilmiş olsalar bile basılmazlar.

9- Harran Üniversitesi Veteriner Fakültesi Dergisi'ne gönderilecek makale, olgu sunumu, derleme vb. çalışmalar, https://dergipark.org.tr/tr/pub/huvfd adresinden gönderildiğinde değerlendirme sürecine alınmaktadır.

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YAZIM KURALLARI

Yazılar, MS Word formatında, Times New Roman yazı tipinde, 12 punto, çift satır aralıklı ve her kenardan 2.5 cm boşluk bırakılarak hazırlanmalıdır. Makaleye satır numaraları (makalenin 2. sayfasından başlamak üzere sürekli olacak şekilde) eklenmelidir. Bu şekildeki yazılar, şekil ve tablolar dâhil olmak üzere orijinal bilimsel araştırmalar ve derlemelerde 15, kısa bilimsel makale ve olgu sunumlarında 5 sayfayı geçmemelidir.

Birimler ve ölçüler için Uluslararası Standart birimleri (SI-sistem) kullanılmalıdır.

Araştırma Makaleleri: Orijinal araştırma makaleleri aşağıdaki ana konu sıralamasına göre dizilmelidir: Başlık, Yazar adları (Sorumlu yazar (*) ile işaretlenmeli), Yazar adresleri, Yazar ORCID numaraları, Özet ve Anahtar kelimeler (3 - 6 kelime), İngilizce başlık, Abstract ve Keywords ile Giriş, Materyal ve Metot, Bulgular, Tartışma ve Sonuç, Teşekkür veya Bilgilendirme ile Kaynaklar. Her bir Tablo ve Şekil ayrı sayfalarda yer almalıdır.

YAZIM DÜZENİ

Özet: Orijinal araştırma makalelerinde 250, diğer makale türlerinde 200 kelimeyi geçmeyecek şekilde hazırlanmalıdır.

Anahtar Kelimeler: En fazla 6 tane olmak üzere her iki dildeki özetin altında alfabetik sırayla verilmelidir. Anahtar kelimeler, Türkiye Bilim Terimleri arasından seçilmelidir. Anahtar kelimelerin seçiminde Türkiye Bilim Terimleri internet adresinden (http://www.bilimterimleri.com) yararlanılmalıdır.

Giriş: Sonuçların anlaşılabilmesi ve yorumlanabilmesi için o konu ile ilgili yapılmış olan çalışmalar hakkında bilgilere yer verilmelidir. Giriş'te çalışmanın hipotezi belirtilmelidir. Çalışmanın amacı bu bölümün en sonunda açık olarak yazılmalıdır.

Materyal ve Metot: Bu bölümde deneysel çalışmalar diğer araştırmacılar tarafından tekrarlanabilecek yeterlilikteki detayı ile verilmelidir. Uluslararası indeksli dergilerde yayınlanmış bir makalede açıklanan bir teknik kullanıldığında, metodun çok kısa açıklanması ve ilgili orijinal makaleye atıf yapılması gereklidir. Makalede etik kurul izini ve/veya yasal/özel izin alınmasının gerekip gerekmediği bu bölümde belirtilmelidir. Materyal olarak hayvan kullanılan orijinal araştırma makalelerinde (klinik, deneysel, saha çalışmaları vb.); etik kurul onayı alınmış olmalıdır. Etik kurul onay/izin belgesinin "alındığı etik kurulun ismini, sayısını ve tarihini" içeren açıklayıcı bilgiler materyal ve metot bölümüne yazılmalıdır. Yayın kurulu etik kurul onay belgesini isteme hakkına sahiptir.

Bulgular: Araştırma bulguları açık ve anlaşılabilir şekilde verilmelidir. Bulgular, gerektiğinde tablo ve şekillerle desteklenmeli ve kısa olarak sunulmalıdır.

Tartışma ve Sonuç: Bulgular gereksiz ayrıntıya girmeden literatürler ışığında tartışılmalı ve bulguların önemi vurgulanmalıdır. Sonuç ya da öneri cümlesi ile bitirilmelidir.

Teşekkür: Çalışma veya makaleye kişisel katkı ve parasal destek burada belirtilmelidir.

Derleme: Derginin yayın alanlarındaki konularda yenilikleri içeren, güncel kaynaklardan yararlanılarak hazırlanmış makaleler olup, yazarların konu ile doğrudan ilişkili en az 3 adet çalışmalarının olması ve bunların derleme içinde kullanılması durumunda yayınlanmak üzere kabul edilebilecektir. Sorumlu yazar, derlemesini gönderirken konu ile ilgili makalelerinin de künye bilgilerini dergi editörlüğüne göndermelidir (makale künyeleri, makale metninin en son sayfasında sunulmalıdır). Harran Üniversitesi Veteriner Fakültesi Dergisi'nde değerlendirmeye alınan ve yayınlanan derlemeler **çağrılı derleme**lerden oluşmaktadır. Derlemelerde; Özet, Giriş, Sonuç ve Kaynaklar bölümleri bulunmalıdır.

Olgu Sunumu: Yazarların, karşılaştıkları yeni veya ender gözlemlenen olguların ele alındığı, bilimsel değere sahip bilgileri içeren eserlerdir. En fazla 15 kaynak kullanılmalı ve bu kaynakların güncel olmasına özen gösterilmelidir. Olgu sunumları; Özet, Giriş, Olgu tanımı, Tartışma ve Sonuç ile Kaynaklar bölümlerinden oluşmalıdır.

Kısa Bilimsel Makale: Kısa bilimsel makalelerde dar kapsamlı olarak ele alınmış, yeni bilgi ve bulgular sunulmalıdır. Araştırma makalesi formatında hazırlanmalı ve en fazla 5 sayfa olmalıdır. En fazla 2 tablo veya şekil içermelidir.

Kaynaklar

Metin içinde atıf yapılırken;

1. Yazar veya yazarların soyadından sonra parantez içinde kaynağın yayın yılı belirtilmelidir; Adams (1998) tarafından; Wilkie ve Whittaker (1997) tarafından; Doyle ve ark. (2007) tarafından….
2. Cümlenin sonunda atıf yapıldığında ise yazar ismi ve yayın yılı parantez içinde belirtilmelidir; ... bildirilmiştir (Adams, 1998); .... bildirilmiştir (Wilkie ve Whittaker, 1997); ..... bildirilmiştir (Doyle ve ark., 2007).
3. Birden çok kaynağa atıf yapılması durumunda önce alfabetik sonra kronolojik sıralama yapılmalıdır;
   …. bildirilmiştir (Adams, 1998; Adams, 2008; Doyle ve ark., 2007; Wilkie ve Whittaker, 2006).
4. Aynı yazarın aynı yıl yayınları söz konusu ise her biri "a" harfinden başlayarak küçük harflerle işaretlenmelidir;
   …. (Adams, 2005a; Adams, 2005b;…).

Kaynak listesi aşağıdaki şekilde hazırlanmalıdır:

1. **Kaynak listesi yazar soyadına göre alfabetik olarak sıralanmalıdır.**
2. **Kaynaklarda yer alacak dergi adları ISI web of Science'a göre kısaltılmalı ve italik yazılmalıdır.**
3. **Kaynakların yazın şekli aşağıdaki şekilde olmalıdır.**

Makale; Sullivan JC, Sasser JM, Pollock JS, 2007: Sexual dimorphism in oxidant status in spontaneously hypertensive rats. *Am J Phsiol Integr Comp Physiol,* 292 (1), 64-68.

Kitap; Cadenas E, Packer L, 2001: Handbook of Antioxidants. 2nd ed., Marcel Dekker Inc., New York, USA.

Kitaptan bir bölüm: Bahk J, Marth EH (1990). Listeriosis and Listeria monocytogenes In: Foodborne Diseases, Cliver DO (Ed), 248-256, Academic Press, San Diego. **Web sayfası:** Anonim (1) http://www.emea.europa.eu, Erişim tarihi; 01.04.2010.

Tez: Er A, 2009: Makrolid grubu antibiyotiklerin endotoksemide sitokin düzeylerine etkisi. Doktora tezi, SÜ Sağlık Bilimleri Enstitüsü, Konya.

Bilimsel toplantıda sunulan bildiri: Allen WR, Wilsher S, Morris L, Crowhurst JS, Hillyer MH, Neal HN, 2006: Re-establishment of oviducal patency and fertility in infertile mares. In: Proceedings of the Ninth International Symposium on Equine Reproduction, Kerkrade, Holland, pp. 27-28.

Tablo ve Şekiller: Her bir tablo ve şekil ayrı sayfalara yerleştirilmelidir. Kullanım sırasına göre numaralandırılmalı, kısa başlıklarla ifade edilmeli ve metin içinde tablo numarası verilerek atıfta bulunulmalıdır. Tablo başlıkları makalenin yazım dilinde tablonun üst bölümüne yazılmalıdır. Tabloda kullanılan kısaltmalar ve gerekli açıklamalar tablo altında verilmelidir. Şekil başlıkları makalenin yazım dilinde şeklin alt bölümüne yazılmalıdır.

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4- The article that reaches the Journal Editor is subjected to preliminary evaluation by the journal editor. Editing has the right to reject the article according to the preliminary evaluation result or to request correction before subjection to the reviewer.

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International Standard Units (SI-system) should be used for units and dimensions.

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STYLE AND FORMAT

Abstract: It should be prepared not to exceed 250 words in original research articles and 200 words in other types of articles.

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Introduction: In order for the results to be understood and interpreted, information about the studies done on that subject should be included. In the introduction, the hypothesis of the study should be specified. The purpose of the study should be clearly written at the end of this section.

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Compilation: These are articles that contain innovations on the subjects of the journal's publications and are prepared by using current references. If the authors have at least 3 works directly related to the subject and they can be accepted for publication. When submitting his review, the responsible author should send the imprint information of the articles related to the subject to the editor of the journal (article tags must be presented on the last page of the article text). Reviews compiled and published in Harran University Veterinary Faculty Journal are invited reviews. in the compilation; Summary, Introduction, Conclusion and References sections should be available.

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3. In case of reference to more than one reference, first alphabetical and chronological order should be done;

.... reported (Adams, 1998; Adams, 2008; Doyle et al., 2007; Wilkie & Whittaker, 2006).

4. If the same author has publications in the same year, each should be marked in lowercase letters, starting with the letter "a";

.... (Adams, 2005a; Adams, 2005b;…).

The list of references should be prepared as follows:

1. Reference list should be listed alphabetically by author surname.

2. The names of the journals in the references should be shortened according to the ISI web of Science and should be written in italics.

3. Type of references should be as follows.

Journal article; Sullivan JC, Sasser JM, Pollock JS, 2007: Sexual dimorphism in oxidant status in spontaneously hypertensive rats. Am J Phsiol Integr Comp Physiol, 292 (1), 64-68.

Book; Cadenas E, Packer L, 2001: Handbook of Antioxidants. 2nd ed., Marcel Dekker Inc., New York, USA.

Chapter in a book: Bahk J, Marth EH (1990). Listeriosis and Listeria monocytogenes In: Foodborne Diseases, Cliver DO (Ed), 248-256, Academic Press, San Diego. Web page: Anonymous (1) http://www.emea.europa.eu, Access date; 01.04.2010.

Thesis: Er A, 2009: Effect of macrolide antibiotics on cytokine levels in endotoxemia. PhD thesis, SU Health Sciences Institute, Konya.

Paper presented at the scientific meeting: Allen WR, Wilsher S, Morris L, Crowhurst JS, Hillyer MH, Neal HN, 2006: Re-establishment of oviducal patency and fertility in infertile mares. In: Proceedings of the Ninth International Symposium on Equine Reproduction, Kerkrade, Holland, pp. 27-28.

Tables and Figures: Each table and figure should be placed on separate pages. It should be numbered according to the order of use, expressed in short titles, and should be cited by giving the table number in the text. Table titles should be written in the writing language of the article in the upper part of the table. Abbreviations and necessary explanations used in the table should be given under the table. Figure titles should be written at the bottom of the figure in the writing language of the article.

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