Ankara Üniversitesi Veteriner Fakültesi Dergisi

Veterinary Journal of Ankara University

E-ISSN 1308-2817 Cilt/Volume 68 ● Sayı/Number 1 ● 2021

Ankara Univ Vet Fak Derg - vetjournal.ankara.edu.tr - Open Acsess

E-ISSN 1308-2817

Ankara Üniversitesi Veteriner Fakültesi Dergisi

Veterinary Journal of Ankara University

Cilt/Volume **68** • **Sayı**/Number **1** • **2021**

Ankara Üniversitesi Veteriner Fakültesi Dergisi

Cilt / *Volume*: 68 • Sayı / *Number*: 1 • 2021

Veterinary Journal of Ankara University

Üç ayda bir yayımlanır / *Published three monthly*

E-ISSN 1308-2817

Sahibi

Ankara Üniversitesi Veteriner Fakültesi Adına
Prof. Dr. Ender YARSAN
Dekan

Editörler Kurulu / *Editorial Board*

Sorumlu Yazı İşleri Müdürü
Baş Editör / *Editor-in Chief*
Doç. Dr. Levent Altıntaş, Türkiye

Editörler / *Editors*
Prof. Dr. Yılmaz Aral, Türkiye
Dr. Caner Bakıcı, Türkiye
Dr. Bülent Baş, Türkiye
Dr. Öğr. Üyesi Güzin İplikçioğlu Çil, Türkiye
Doç. Dr. Yasemin Salgırlı Demirbaş, Türkiye
Doç. Dr. Begüm Yurdakök Dikmen, Türkiye
Prof. Raphael Guatteo, Fransa
Prof. Dr. İ. Safa Gürcan, Türkiye
Prof. Shimon Harrus, İsrail
Associate Prof. Laura Hernández Hurtado, Portekiz
Dr. Nevra Keskin, Türkiye
Doç. Dr. Halit Kanca, Türkiye
Doç. Dr. Görkem Kısmalı, Türkiye
Assistant Professor Maria Graca Lopes, Portekiz
Prof. Erdoğan Memili, ABD
Dr. Ba Tiep Nguyen, Vietnam
Doç. Dr. Ömer Orkun, Türkiye
Prof. Dušan Palić, Almanya
Prof. Gonçalo Da Graça Pereira, Portekiz
Doç. Dr. Özge Sızmaz, Türkiye
Prof.Dr. Calogero Stelletta, İtalya
Dr. Yusuf Şen, Türkiye
Dr. Öğr. Üyesi Koray Tekin, Türkiye
Prof. Angel Vodenicharov, Bulgaristan
Dr. Nuh Yıldırım, Türkiye
Doç. Dr. Ayşe Zeynep Akkutay Yoldar, Türkiye

Sekreterya: Dr. Caner Bakıcı

Yönetim Yeri
Adres / *Address*

Ankara Üniversitesi Veteriner Fakültesi Dekanlığı
Yayın Alt Komitesi
06 110 Ankara, Türkiye
Tel: 90 312 317 03 15, **Fax:** 90 312 316 44 72
Sürdürüm ve iletişim: vfdergi@veterinary.ankara.edu.tr
URL: http://vetjournal.ankara.edu.tr

Yayın Türü: Yaygın süreli ve hakemli

Danışma Kurulu / *Advisory Board*
Prof. Dr. Mehmet Akan, Ankara University
Prof. Dr. Çiğdem Altınsaat, Ankara University
Prof. Dr. Wolfgang Bäumer, Berlin Freie University
Prof. Dr. Alev Gürol Bayraktaroğlu, Ankara University
Prof. Dr. Gerhard Breves, Hannover Veterinary Medicine University
Prof. Dr. Heiner Bollwein, Zurich University
Prof. Dr. Ali Bumin, Ankara University
Prof. Dr. R. Teodor Cristina, Banat's University
Prof. Dr. Ahmet Çakır, Ankara University
Prof. Dr. Roman Dabrowski, Lublin Life Science University
Prof. Dr. Ali Daşkın, Ankara University
Prof. Dr. Cornelia Deeg, Münih Ludwig Maximilian University
Prof. Dr. İbrahim Demirkan, Afyon Kocatepe University
Prof. Dr. Bilal Dik, Selçuk University
Prof. Dr. Levent Dirikolu, Louisiana University
Prof. Dr. Marc Drillich, Vienna Veterinary Medicine University
Prof. Dr. Bülent Ekiz, Istanbul-Cerrahpaşa University
Prof. Dr. Emel Ergün, Ankara University
Prof. Dr. Frank Gasthuys, Gent University
Prof. Dr. Tamay Başağaç Gül, Ankara University
Dr. Paweł Górka, Krakow Agriculture University
Prof. Dr. Berrin Kocaoğlu Güçlü, Erciyes University
Prof. Dr. Rıfkı Hazıroğlu, Ankara University
Assoc. Prof. Dr. Jia-Qiang He, Virginia Polytechnic Institute and State University
Prof. Dr. Şeref İnal, Selçuk University
Prof. Dr. M. Taner Karaoğlu, Ankara University
Prof. Dr. Abdullah Kaya, Selçuk University
Prof. Dr. Arif Kurtdede, Ankara University
Prof. Dr. Mariusz P. Kowalewski, Zurich University
Prof. Dr. Osman Kutsal, Ankara University
Prof. Dr. A. Serpil Nalbantoğlu, Ankara University
Prof. Dr. Ceyhan Özbeyaz, Ankara University
Prof. Dr. A. Serpil Nalbantoğlu, Ankara University
Prof. Dr. Hatice Öge, Ankara University
Prof. Dr. Hakan Öztürk, Ankara University
Prof. Dr. Lazo Pendovski, Skopje Ss. Cyril and Methodius University
Prof. Dr. H. P. Salmann, Hannover Veterinary Medicine University
Prof. Dr. Sabine Schäfer-Somi, Vienna Veterinary Medicine University
Prof. Dr. Franz Schwarzenberger, Vienna Veterinary Medicine University
Prof. Dr. Antti Sukura, Helsinki University
Prof. Dr. Mehmet Şahal, Ankara University
Prof. Dr. Adnan Şehu, Ankara University
Prof. Dr. Hamdi Uysal, Ankara University
Prof. Dr. Rıfat Vural, Ankara University
Prof. Dr. Sakine Yalçın, Ankara University
Prof.Dr. Hakan Yardımcı, Ankara University
Prof. Dr. Ender Yarsan, Ankara University

"Bu dergi Thomson Reuters®'in **SCI-EXP** ve **JCR** ile Uluslararası CABI yayınlarının **CAB Abstracts, Global Health**, **CAB Direct, Database Subsets; Scopus** ve **Ulakbim** (Yaşam Bilimleri) veri tabanları kapsamındadır.

This journal is covered by **SCI-EXP** and **JCR** of Thomson Reuters®, **CAB Abstracts**, **Global Health**, **CAB Direct, Database Subsets; Scopus** and **Ulakbim** (Life Sciences) database systems.

© Ankara Üniversitesi Veteriner Fakültesi Dergisi

Tüm hakları saklıdır. Bu Derginin tamamı ya da Dergide yer alan bilimsel çalışmaların bir kısmı ya da tamamı 5846 sayılı yasanın hükümlerine göre Ankara Üniversitesi Veteriner Fakültesi Dekanlığının yazılı izni olmaksızın elektronik, mekanik, fotokopi ya da herhangi bir kayıt sistemiyle çoğaltılamaz, yayımlanamaz.

İnternet Adresi / *Web Address*
http://vetjournal.ankara.edu.tr
Yayım Tarihi: 25.12.2020

Ankara Üniversitesi Veteriner Fakültesi Dergisi
Cilt / *Volume*: 68 • Sayı / *Number*: 1 • 2021
Veterinary Journal of Ankara University

İÇİNDEKİLER / *CONTENTS*

Research Article/*Araştırma Makalesi*

Treatment of dermatophilosis with oxytetracycline and tylosin combination in Saanen goat kids
Saanen oğlaklarında oksitetrasiklin ve tilosin kombinasyonu ile dermatofilozisin tedavisi
Uğur Aydoğdu, Ersoy Baydar, Mustafa Usta, Banu Dokuzeylül, Musa Karaman, Ziya İlhan **1**

Effect of adding humate to the ration of dairy cows on yield performance
Süt ineklerinde rasyona humat ilavesinin verim performansı üzerine etkisi
Songül Yüca, Mehmet Gül **7**

Characterization of *Pasteurella multocida* isolates recovered from the oral flora of cats
Kedilerin ağız florasından izole edilen *Pasteurella multocida* izolatlarının karakterizasyonu
Tuğçe Tınmaz, Baran Çelik, Barış Halaç, Arzu Funda Bağcıgil **15**

An investigation on the renal portal system in long-legged buzzard (*Buteo rufinus*)
Kızıl şahin'de (*Buteo rufinus*) renal portal sistem üzerine bir araştırma
Sedef Selviler Sizer, Murat Kabak, Burcu Onuk **21**

Effects of epidural bupivacaine, bupivacaine-butorphanol and bupivacaine-morphine on postoperative analgesia for hind limb and pelvic orthopedic operations in dogs
Köpeklerde arka ekstremite ve pelvisin ortopedik operasyonlarında epidural bupivakain, bupivakain-butorfanol ve bupivakain-morfin uygulamasının postoperatif analjezi üzerine etkisi
Birkan Karslı, Zeynep Pekcan, Ali Kumandaş, Barış Kürüm, Miyase Çınar **27**

Improvement of bovine *in vitro* embryo production by fetal calf serum and cysteamine supplementation and investigation of freezability
Sığır *in vitro* embriyo üretiminin fetal buzağı serumu ve sisteamin katkısı ile iyileştirilmesi ve dondurulabilirliğinin araştırılması
Asiye İzem Sandal, Hatice Şenlikci, Tuğba Elgün, Ramazan Arıcı, Sinem Özlem Enginler, Alper Baran, Kemal Ak, Tülay İrez, Özen Banu Özdaş **33**

Effects of genotype on the biomechanical parameters and composition of bone in the laying hen embryos
Yumurtacı tavuk embriyolarında genotipin kemik biyomekanik özellikleri ve bileşimine etkileri
Fatma Kübra Erbay Elibol, Esin Ebru Onbaşılar, Tuğba Karakan, Süleyman Taban, Teyfik Demir **39**

Treatment outcomes of using paraosseous clamp and cerclage stabilisation technique in long bone fractures of cats: a retrospective study
Kedilerde karşılaşılan ekstremite uzun kemik kırıklarının paraosseöz klemp ve serklaj ile stabilizasyon tekniği kullanılarak sağaltımları üzerine retrospektif bir çalışma
İlker Şen, Mehmet Sağlam **47**

Molecular presence of *Felis catus gammaherpesvirus* -1 in cats with ocular disorders in Turkey
Türkiye'de oküler bozukluğu olan kedilerde *Felis catus gammaherpesvirus* -1'in moleküler varlığı
Bahattin Taylan Koç, Müge Akkartal **53**

Long-term prospective assessment of subconjunctival triamcinolone acetonide in addition to topical therapy in the management of chronic superficial keratitis
Florin Beteg, Cristina Alexa Lelescu, Andrada Elena Urdă-Cîmpean, Marian Aurel Taulescu, Cosmin Mureșan **61**

Detection of SARS-CoV-2 using five primer sets
Alper Karagöz, Hidayet Tutun, Tutku Arslantaş, Özlem Altıntaş, Nadir Koçak, Levent Altıntaş **69**

Case Report / *Olgu Sunumu*

Clinicopathologic evaluation of oral squamous cell carcinoma in a young dog
Genç bir köpekte oral skuamöz hücreli karsinomun klinikopatolojik değerlendirilmesi
Hazal Öztürk Gürgen, Evrim Egeden, Gülbin Şennazlı **77**

Uterine papillary adenocarcinoma in a Pit-bull dog
Pitbull ırkı bir köpekte uterusta papiller adenokarsinom
Yanad Abou Monsef, Tuncer Kutlu, Osman Kutsal **83**

Priapism and its surgical treatment in a cat
Bir kedide priapizm ve cerrahi sağaltımı
Zülfükar Kadir Sarıtaş, Musa Korkmaz, Fatma Görücü, Sefa Çelik **87**

Short Communication / *Kısa Bilimsel Çalışma*

Slaughter and carcass characteristics of Kivircik lambs in different rearing seasons
Farklı büyüme mevsimlerinde Kıvırcık ırkı kuzuların kesim ve karkas özellikleri
Hülya Yalçıntan, Nurşen Öztürk, Pembe Dilara Keçici, Bülent Ekiz, Ömür Koçak, Alper Yılmaz **91**

Ankara Univ Vet Fak Derg, **68**, 1-5, 2021
DOI: 10.33988/auvfd. 610876

Treatment of dermatophilosis with oxytetracycline and tylosin combination in Saanen goat kids

Uğur AYDOĞDU[1,a,✉], Ersoy BAYDAR[1,b], Mustafa USTA[2,c,] Banu DOKUZEYLÜL[3,d], Musa KARAMAN[2,e,] Ziya İLHAN[4,f]

[1]Balıkesir University, Faculty of Veterinary Medicine, Department of Internal Medicine, Balıkesir; [2]Balıkesir University, Faculty of Veterinary Medicine, Department of Pathology, Balıkesir; [3]İstanbul University–Cerrahpaşa, Faculty of Veterinary Medicine, Department of Internal Medicine, İstanbul; [4]Balıkesir University, Faculty of Veterinary Medicine, Department of Microbiology, Balıkesir, Turkey.
[a]ORCID: 0000-0002-9828-9863; [b]ORCID: 0000-0002-2565-1796; [c]ORCID: 0000-0002-3346-9097; [d]ORCID: 0000-0003-3086-4726; [e]ORCID: 0000-0001-6721-6111; [f]ORCID: 0000-0003-3638-9196

✉Corresponding author: ugur.aydogdu@balikesir.edu.tr
Received date: 26.08.2019 - Accepted date: 23.06.2020

Abstract: In this study, it was aimed to determine the treatment efficacy of oxytetracycline and tylosin combination in Saanen goat kids with *Dermatophilus congolensis* infection. Samples, were collected from the 27 of 4-5-month-old Saanen goat kids, and analyzed from bacteriological, mycological and ectoparasitological aspects. *D. congolensis* was isolated from the samples as pure culture. In the mycological analysis of the samples, no microorganisms were found, while flea was detected in ectoparasitological examinations. A combination of oxytetracycline and tylosin was recommended for dermatophilosis treatment, and the lesions regressed 5 days after the treatment and disappeared over time. It was concluded that *D. congolensis* infection in Saanen goats kids were observed for the first time in Turkey and can be treated successfully with oxytetracycline and tylosin combination.
Keywords: *Dermatophilus congolensis*, goat kid, oxytetracycline, tylosin

Saanen oğlaklarında oksitetrasiklin ve tilosin kombinasyonu ile dermatofilozisin tedavisi

Özet: Bu çalışmada, *Dermatophilus congolensis* enfeksiyonlu Saanen oğlaklarında oksitetrasiklin ve tilosin kombinasyonunun tedavi etkinliğinin belirlenmesi amaçlandı. Saanen ırkı 4-5 aylık 27 oğlaktan toplanan örnekler bakteriyolojik, mikolojik ve parazitolojik açıdan analiz edildi. *D. congolensis* saf kültür olarak numunelerden izole edildi. Numunelerin mikolojik analizinde herhangi bir etkene rastlanmazken, ektoparazitolojik incelemeler sonucunda pire tespit edildi. Dermatofilozis tedavisi için oksitetrasiklin ve tilosin kombinasyonu önerildi ve lezyonlar 5. günden itibaren gerilemeye başlayarak zamanla tamamen kayboldu. Sonuç olarak, Saanen oğlaklarında *D. congolensis* enfeksiyonunun Türkiye'de ilk kez gözlendiği, oksitetrasiklin ve tilosin kombinasyonu ile başarılı bir şekilde tedavi edilebileceği kanısına varılmıştır.
Anahtar sözcükler: *Dermatophilus congolensis*, oğlak, oksitetrasiklin, tilosin

Introduction

Dermatophilosis is a dermatitis with acute, subacute and chronic course which is usually exudative and rarely proliferative and observed in animals such as cattle, sheep, goats and horses and sometimes in humans (8, 14). The disease is more prevalent especially in humid climates and in regions where ticks of the genus *Amblyomma* are endemic (2, 6). This infection, which is observed in different regions of the world, and also has been reported in sheep and goats in Turkey (5, 12). The agent is *Dermatophilus congolensis*, that is a Gram-positive bacteria with capnophilic, aerobic or facultative anaerobic. This agent has a filamentous structure (1-5 µm) consisting of coccoid zoospores (0.5-1.5 µm) (2, 5, 9). The bacterium shows activity in the epidermis layer without affecting the stratum corneum, which is the top layer of the epidermis. The infection can be transmitted through contact with infected animals, passive vectors (ectoparasites) and also through intradermal inoculation by contaminated thorny plants (1). The clinical appearance and the affected body parts vary by the host's individual sensitivity, nutritional and immunological status, heavy rainfall and mechanical trauma. Sporadic dermatophilosis outbreaks can be observed in rainy

periods when wool or hair remains wet for a long time (1, 6-8, 11).

Lesions are generally observed in the ear, nose, tail, and legs of sheep and goats and also in the back, head, neck and on the sides of the body in cattle. While vesicle, papule, edema or bonding of hair that occur in the early stages of infection are disregarded, the exudate that dries in later periods causes the formation of yellow-brown crusts firmly adhered to the skin (8). These crusts are granular and can often be removed by leaving the bleeding surface (7, 14). During healing phase, it is observed that the crusts are removed, new hairs come out or the lesional areas in the patient take a hairless appearance. Itching is not usually observed (8). In goats, lesions first spread over the lips and mouth and then to the feet and scrotum possibly by biting. Lesions may extend to all parts of the body, especially to the dorsal midline and inner parts of the thighs. In some cases, lesions begin in the external ear and may occlude the external auditory canal and the outward-facing part of the external nostrils due to severe crust formation (3). Antimicrobial therapy is used in the treatment of the disease. Long-acting oxytetracycline administration has been reported to be effective in cattle and sheep (7). Furthermore, it is stated that penicillin and streptomycin combination, erythromycin, lincomycin and spectinomycin combination can also be used in sheep (3). In a study (15), it was reported that topical povidone iodine combined with parenteral penicillin administration was successful in the treatment of severe cases. Similarly, Sekin et al. (12) reported that penicillin and streptomycin combinations in goats are effective in the treatment of dermatophilosis. However, Göçmen and Şen (5) reported that the application of injectable penicillin and streptomycin combination in goats with dermatophilosis did not cause a change in the course of the disease. Economic losses due to the disease are related to skin damage in cattle and wool yield in sheep (7). On the other hand, the infection may lead to significant economic losses due to a decrease in meat and milk yields and increases in treatment costs, death and mandatory slaughtering rates (16).

The aim of this study was to determine the treatment efficiency of oxytetracycline and tylosin combination in Saanen goat kids diagnosed with dermatophilosis.

Material and Methods

Sampling: This study was carried out on 27 Saanen goat kids in the 4-5 month age on a special goat farm in Bigadiç district of Balıkesir Province. It was reported that the lesions observed in goat kids began to form 10 days ago and were transmitted to 27 of 29 goat kids (93.1%) on the same farm. According to the owners' statement, goat kids were treated against scabies lesions however, no improvement was observed in those animals. The samples were collected from skin lesions (swab, skin and hair samples from lesion areas), performed bacteriological, mycological and ectoparasitological (scabies etc.) analyses.

Direct bacterioscopy: The samples prepared from skin scrapings were detected by the chemical method (methanol), stained with Diff-Quick and examined under an immersion microscope. In addition, in terms of mycotic agents, the samples collected from the superficial and deep areas of the lesional skin regions were treated with 10% NaOH and then examined under a microscope with a magnification of 40x.

Bacteriological analysis: The swab and skin scrapings were inoculated into defibrinated sheep blood agar(7%) (1.10886, Merck, Darmstadt, Germany) in two series. One of the series in an anaerobic medium and the other one in the medium with 5-10% CO_2 were incubated at 37°C for 5 days. The growing agents were identified according to conventional methods (5, 9). Catalase, lactose, dextrose, mannitol, maltose, sucrose and urease tests were used for the identification of *D. congolensis*.

Mycological analysis: The samples of skin scrapings and hair were parallelly inoculated into sabouraud dextrose agar (SDA) (1.05438, Merck) and one of the series was incubated at 25°C and the other one was incubated at 37°C for 5 weeks.

Ectoparasitic analysis: For this purpose, primarily the hair and skin of the animals were examined macroscopically for ectoparasites. In addition, in terms of mange mites in goat kids, the samples collected from the superficial and deep areas of the lesional skin regions were treated with 10% NaOH and then examined under a stereo-microscope.

Treatment: For treatment in goat kids, oxytetracycline (Primamycin LA®, Zoetis, USA) at a dose of 20 mg/kg was administered intramuscularly as a single dose and tylosin (Taylomisin® IE Ulugay, Turkey) at a dose of 15 mg/kg was administered intramuscularly for 3 days. Furthermore, a 10 mg/kg dose of cypermethrin pour-on (Cyperon® 5%, Hektaş, Turkey) was administered for treatment since flea infestation was observed in goat kids.

Results

Clinical findings: When the intensity of skin lesions was examined, it was observed that localizations were mainly around the head region and some of them were seen at the neck, back, and abdominal region. Appetite and physical examination findings of goat kids were found to be normal.

Direct bacterioscopy: Long filaments consisting of coccoid structures and similar to the tramline were observed in direct bacterioscopy (Figure 1).

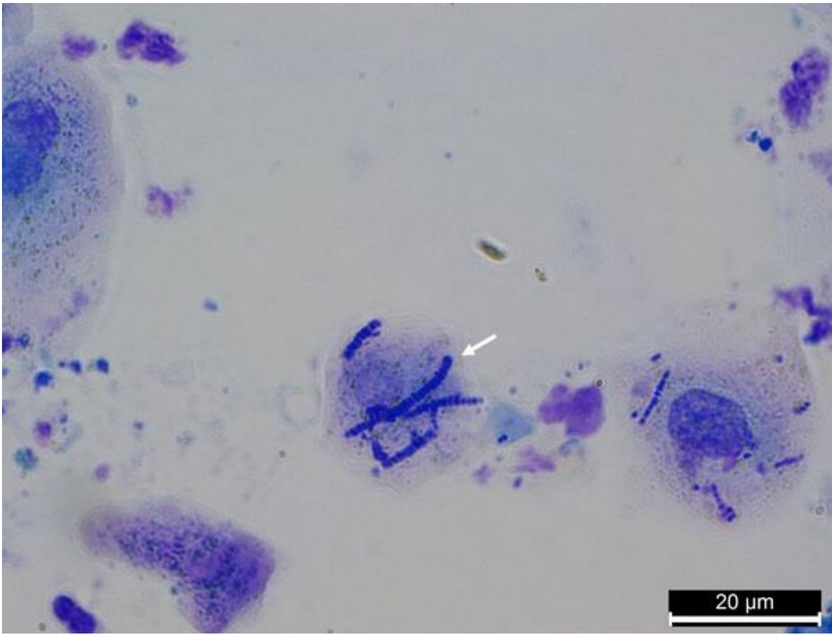

Figure 1. Long filaments (arrow) with multiple rows of cocci seen in scab materials stained with Diff-Quick.

Bacteriological analysis: R-type colonies were observed with 1-2 mm diameter at approximately 72 hours during bacteriological analysis. The identification of *D. congolensis* was performed according to various biochemical tests along with macroscopic and microscopic morphology. Catalase, urease and dextrose tests were determined as positive, but lactose, mannitol, maltose and sucrose tests were negative.

Mycological analysis: No growth was detected in SDA.

Ectoparasitic analysis: In the macroscopic examination, only flea infestation was determined. However, no species identification was done. In addition, scabies infestation was not encountered.

Treatment: The lesions began to heal in 5 days after treatment and completely disappeared over time in all goats.

Discussion and Conclusion

D. congolensis causes a disease process which mainly presents with skin lesions in various animal species all over the world. The infection is usually observed more intensively in tropical regions. This disease, which has been previously called mycotic dermatitis, is commonly called cutaneous streptothricosis in cattle, goats, and horses, and in sheep, it is also called lumpy wool disease when the wooled areas of the body are affected. Along with the formation of crust, exudative dermatitis, alopecia with the progression of infection are observed during the course of the disease. When the crusts are removed, sometimes there is a bleeding appearance on the skin. Furthermore, it can also be observed that new hair begins to emerge from under the crust (5, 7). In goats, it is reported that lesions are mostly localized in the head region and that lesions on the lips and mouth may spread to the feet and scrotum by biting. Moreover, lesions can be observed in the whole body, and also they may occur especially between the back and hind legs. It has been reported that severe crust formation around the ear and nose may sometimes occlude the ear canal and nostrils (3). The lesions have been reported to be limited to the head region in sheep and goats with *D. congolensis* infection (5). In this study, typical crust formations and lesions in goat kids were mostly localized in the head region and were commonly observed over the nose and in the external ear (Figure 2). However, a small number of lesions were also found in the back, abdomen, and legs of some goat kids in the study. Alopecic areas and new hair were detected when the crusts were removed.

Skin damage (ectoparasites, physical wear due to contaminated thorny bushes), excessive moisture, and disease and stress factors causing immunosuppression can be considered among the predisposing factors for clinical disease. It is reported that the loss of the layer of fat on the skin may contribute to the development of the disease. Extremely rainy weather conditions increase the likelihood of clinical disease by causing thinning of the layer of fat on the skin (6, 8, 11). The fact that the goat kids examined in this study were housed in the same place and intense flea infestation was observed in animals suggests that the flea factor may be effective in transmission. More studies are needed to be able to make a more reliable interpretation in this regard. On the other hand, the facts that the infection was observed in June and that Balıkesir region was partly rainy and had high moisture content during the relevant period support the information that climate conditions are effective in the epidemiology of the infection (7, 9).

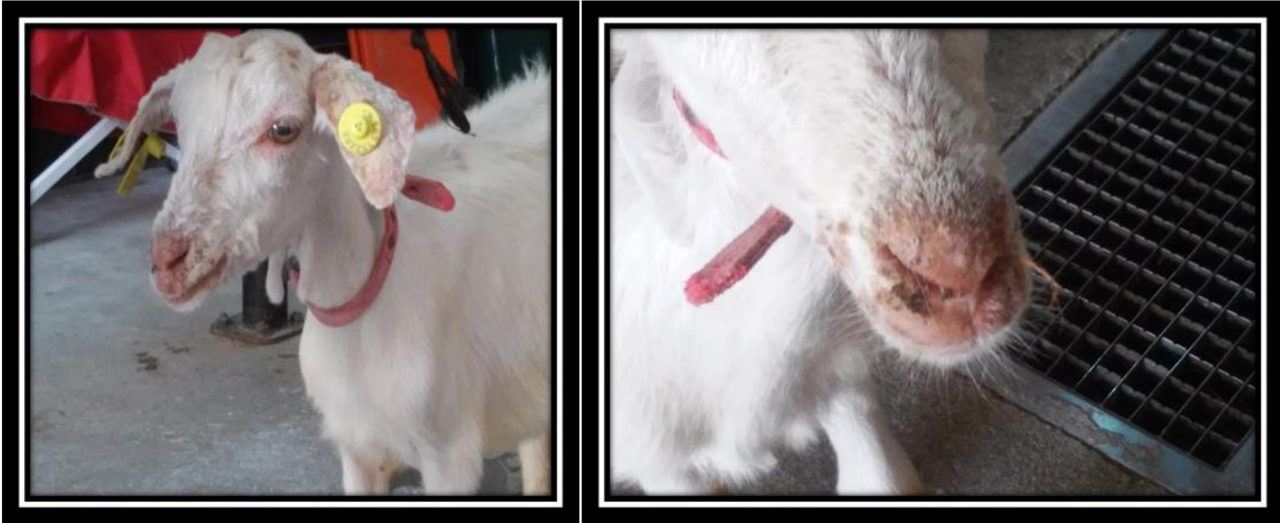

Figure 2. Lesions around the mouth and nose in goat kids with dermatophilosis.

There is no specific treatment for *D. congolensis* infection (2). It is stated that clinical symptoms may improve spontaneously in animals with a small number of agent input and developed immune system (7). However, the infection may cause significant economic losses since it is associated with high morbidity and mortality. Briefly, the agent leads to significant economic losses by causing decreases in milk and meat yield in various animal species. In this study, it was observed that there was no significant loss of weight in goat kids. This can be explained by the early diagnosis and treatment of the infection due to the high sensitivity of the local people on animal health.

In the studies carried out, *D. congolensis* has been reported to be susceptible to various antibiotics. These antibiotics include erythromycin, spiramycin, penicillin G, ampicillin, chloramphenicol, streptomycin, amoxicillin, tetracycline and novobiocin (7, 10, 13). Long-acting oxytetracycline administration in cattle and sheep has been reported to be effective in treatment (7). Furthermore, it is stated that penicillin and streptomycin combination, erythromycin, lincomycin and spectinomycin combination can also be used in sheep (3). Sekin et al. (12) reported that penicillin-streptomycin combinations have been to be effective in treatment in sheep and goats with *D. congolensis* infection. Göçmen and Şen (5) reported that there was no change in the course of the disease despite the administration of injectable penicillin-streptomycin combination for 5-6 days in animals with lesions among sheep and goats with dermatophilosis. They considered that the failure of penicillin-streptomycin administration was due to the fact that the treatment was performed in the advanced stages of the disease (5). Although there are studies on the use of long-acting tetracyclines and macrolides in sheep and cattle, studies in goats are scarce (or limitied). For this reason oxytetracycline and tylosin combination was used to test the efficacy and success of these drugs in the study. In this study, it was determined that the lesions regressed from day 5 and disappeared completely (Figure 3) over time along with the parenteral tylosin administration for 3 days with parenteral single dose long-acting oxytetracycline in goat kids. After the study, the herd was followed for 4 months and it was observed that no recurrence or a new case was detected.

Figure 3. The goat kid after treatment.

In conclusion, a few studies related the *in vitro* antibiotic susceptibility have been performed against *D. congolensis* (4). However, there are limited information in animals (*in vivo*). *D. congolensis* infection which was treated with oxytetracycline and tylosin combination successfully, was reported in Saanen goats for the first time in Turkey.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns.

Conflict of interest

The authors declared that there is no conflict of interest.

References

1. **Ambrose N, Lloyd D, Maillard JC** (1999): *Immune responses to Dermatophilus congolensis infection.* Parasitol Today, **15**, 295-300.
2. **Chitra MA, Jayalakshmi K, Ponnusamy P, et al** (2017): *Dermatophilus congolensis infection in sheep and goats in Delta region of Tamil Nadu*. Vet World, **10**, 1314-1318.
3. **Constable PD, Kenneth W, Hinchcliff KW, et al** (2017): Veterinary Medicine. A textbook of the diseases of cattle, horses, sheep, pigs and goats. WB Saunders Ltd, Missouri.
4. **Domingues PF, Guerra ST, de Paula CL, et al** (2017): *Successful therapy in unusual generalized Dermatophilus congolensis infection in a calf based on modified in vitro disk diffusion test.* Arq Inst Biol, **84**, 1-7 (e0382017).
5. **Göçmen H, Şen A** (2013): *Bir koyun-keçi sürüsünde gözlenen Dermatophilus congolensis infeksiyonu*. Uludag Univ J Fac Vet Med, **32**, 63-66.
6. **Loria GR, La Barbera E, Monteverde V, et al** (2005): *Dermatophilosis in goats in Sicily*. Vet Rec, **156**, 120-121.
7. **Moriello KA** (2019): Overview of dermatophilosis. Available at https://www.msdvetmanual.com/integumentary-system/dermatophilosis/overview-of-dermatophilosis (Accessed May 30, 2019).
8. **Or ME, Bakırel U** (2006): Skin diseases. 431-470. In: Y Gül (Ed), Geviş Getiren Hayvanların İç Hastalıkları. Medipress, Malatya.
9. **Quinn PJ, Markey BK, Leonard FC, et al** (2011): Veterinary Microbiology and Microbial Disease. Wiley-Blackwell, West Sussex.
10. **Ranjithkumar M, Saravanan M, Krishnakumar S, et al** (2018): *Dermatophilosis in a buffalo: A case report*. Buffalo Bull, **37**, 253-258.
11. **Roberson JR, Baird AN, Pugh DG** (2012): Diseases of the Integumentary System. 256-290. In: DG Pugh, AN Baird (Eds), Sheep and Goat Medicine. WB Saunders, Missouri.
12. **Sekin S, Elitok ÖM, Elitok B, et al** (2002): *Natural ovine dermatophilosis: Clinical aspects and efficacy of penicillin/streptomycin treatment*. Turk J Vet Anim Sci, **26**, 1013-1019.
13. **Tresamol PV, Saseendranath MR** (2013): *Antibiogram of Dermatophilus congolensis isolates from cattle*. Int J Live Res **3**, 117-121.
14. **Van Tonder EM, Horner RF** (1994): Dermatophilosis. 1472-1481. In: JAW Coetzer, GR Thomson, RC Tustin (Eds), Infectious Diseases of Livestock with Special Reference to Southern Africa. Oxford University Press, New York.
15. **Yeruham I, Elad D, Perl S (2003):** *Dermatophilosis in goats in the Judean foothills*. Revue Méd. Vét., **154**, 785-788.
16. **Zaria LT** (1993): *Dermatophilus congolensis infection (dermatophilosis) in animals and man! An update*. Comp Immunol Microbiol Infect Dis, **16**, 179-222.

Ankara Univ Vet Fak Derg, **68**, 7-14, 2021
DOI: 10.33988/auvfd. 626066

Effect of adding humate to the ration of dairy cows on yield performance

Songül YÜCA[1,a,✉], Mehmet GÜL[2,b]

[1]Ağrı İbrahim Çeçen University, Celal Oruç Animal Production School, Department of Animal Husbandry and Nutrition, Ağrı,
[2]Atatürk University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Erzurum, Turkey.
[a]ORCID: 0000-0003-4507-9800; [b]ORCID: 0000-0001-5477-1773

✉Corresponding author: syuca@agri.edu.tr
Received date: 27.09.2019 - Accepted date: 16.06.2020

Abstract: The aim of this study is to investigate the effects of different levels of humate addition to colostrum quality, milk composition, somatic cell count, some blood metabolites and reproductive performance in the period from prepartum 40th to postpartum 60th day. In this study, dairy cows from prepartum 40th to postpartum 60th day were given humate additive which was according to dosed on dry matter consumption basis from (75 g, 150 g). In the study, 26 Swiss Brown cows in the same care and feeding conditions were used in the second lactation. The feeds were weighed to determine the dry matter intake of the individual cows daily. Blood was collected from the *vena jugularis* at 40, 30, 20 and 10 days before the estimated date of birth, at calving (day 0) and on days 5, 10, 20, 30, 45, and 60 after birth. Colostrum and milk samples were taken in the study. As a result of the study, it has been determined that the use of humate additives has no effect on body condition score, body weight, in milk non-fat milk solid, density, protein, lactose, freezing point, somatic cell count, in blood triglicerides, phosphorus, magnesium, albumin, glucose, blood urea nitrogen. It was found that with the addition of humate additive to the feed, colostrum specific gravity increased, prepartum and postpartum dry matter intake increased, milk yield and the fat percentage increased, serum non-esterified fatty acid and blood beta-hydroxybutyric acid levels decreased, and postpartum serum calcium level increased. It was concluded that 75 g of humate, which is determined based on dry matter consumption, can be added to the rations of the dairy cows at this dose and has positive effects on colostrum quality, milk yield, milk fat ratio, and negative energy balance.
Keywords: Dairy cow, humate, yield performance

Süt ineklerinde rasyona humat ilavesinin verim performansı üzerine etkisi

Özet: Bu çalışmanın amacı, doğum öncesi 40. günden doğum sonrası 60. güne kadar olan dönemde rasyona farklı seviyelerde humat ilavesinin kolostrum kalitesi, süt bileşimi, somatik hücre sayısı, bazı kan metabolitleri ve üreme performansı üzerine etkilerini araştırmaktır. Bu çalışmada, doğumdan önce 40. günden doğumdan sonraki 60 güne kadar olan dönemdeki süt ineklerine kuru madde tüketim esasına göre (75 g, 150 g) belirlenen dozlarda humat katkı maddesi verilmiştir. Çalışmada, ikinci laktasyonda aynı bakım besleme şartlarında olan 26 İsviçre Esmeri inek kullanıldı. Her bir ineğin kuru madde tüketimini günlük olarak belirlemek için yemler tartıldı. Doğumdan önceki 40, 30, 20 ve 10. beklenen doğum tarihinde, doğum anında (0. gün) ve doğumdan sonraki 5, 10, 20, 30, 45 ve 60. günlerde *vena jugularis'*ten kan alındı. Çalışmada kolostrum ve süt örnekleri alındı. Çalışma sonucunda humat katkı maddesi kullanımının vücut kondisyon skoru, canlı ağırlık, sütte; yağsız kuru madde, yoğunluk, protein, laktoz, donma noktası, somatik hücre sayısı, kanda; trigliserid, fosfor, magnezyum, albümin, glukoz, kan üre azotu üzerine etkisinin olmadığı tespit edilmiştir. Ancak kolostrum özgül ağırlığının arttığı, prepartum ve postpartum kuru madde tüketiminin arttığı, süt veriminin ve sütteki yağ oranının arttığı, serum esterleşmemiş yağ asiti ve kan beta-hidroksi bütirik asit düzeylerinin azaldığı, postpartum serum kalsiyum düzeyinin arttığı bulunmuştur. Kuru madde tüketim esasına göre belirlenen 75 g humatın süt ineklerinin rasyonlarına bu dozda eklenebileceği, kolostrum kalitesi, süt verimi, sütte yağ oranı, negatif enerji dengesi üzerine olumlu etkilerinin olduğu sonucuna varılmıştır.
Anahtar sözcükler: Humat, süt ineği, verim performansı

Introduction

As a result of genetic and breeding studies carried out for years with the implementation of scientific advancements, high-quality products can be obtained from a small number of animals. The increased yield capacity of dairy cows made care feeding conditions difficult and increased their sensitivity to metabolic diseases. The most problematic period for dairy cows is the peripartum

period, and feeding errors in this period adversely affect the health and productivity of the cow during the lactation period. In terms of pregnancy and lactation characteristics, the peripartum period can be categorized into the distant dry period (between day −60 and day −20), near dry period (between day −20 and birth), early postpartum period (between birth and day 14), and late postpartum period (between day 14 and day 60) (12).

Prebiotics, probiotics, enzymes, organic acids, plant extracts, and humates have been used as feed additives because they are environmentally friendly, they do not adversely affect on animal and human health, and they increase the quality and quantity of products obtained after the prohibition of the use of antibiotics as feed additives (13, 14).

Humates are composed of substances such as carbohydrates, amino acids and phenols, which are formed by decomposition and decomposition of plant and animal residues in the soil. Humates include humic, fulvic, ulmic acid and some microminerals originating from humus, and humates are organic substances capable of chelating with some metal ions and making electron transfer. Histopathological and histochemical studies have shown that humates are harmless on blood, cardiovascular system, endocrine system, and other important organ systems (1). Studies have also shown that humates have a protective effect against diseases, they can be used safely even in pregnant animals, and they have no embryotoxic effect (1, 11).

Studies on humates have found that they have a positive effect on average daily gain in calves and fattening cattle (6, 16, 19); however, they have no effect on dry matter intake (DMI) in fattening cattle (4). Although they increase milk yield in Saanen goats, they do not change milk fat, non-fat milk solid, milk protein, lactose contents, and the total number of somatic cells and bacteria in milk (7, 8). Moreover, they increase dairy cow milk yield and ratio of milk fat to milk protein (20).

The aim of this study is to investigate the effects of different levels of humate addition to colostrum quality, milk composition, somatic cell count, some blood metabolites and reproductive performance in the period from prepartum 40th to postpartum 60th day

Material and Methods

The study included 26 Swiss Brown dairy cows in the second lactation with similar BCS; these cows were housed at Ağrı İbrahim Çeçen University Celal Oruç Animal Production School Education, Research and Application Farm. This study was approved by Atatürk University Animal Experiments Local Ethics Committee dated 18.09.2017 and numbered 36643897-000-E.1700250736. The cows were randomly divided into three groups; nine of them as control, eight of them as consuming 75 g of humate daily, and nine of them consuming 150 g of humate daily. The humic acid material used for this study was purchased from the Natural Feed Company. The humate product (Bovifarm, Turkey) used for this study was dark black. The certified composition of humic acid produced by Bovifarm contains activated leonardite at a rate of 400.000 mg/kg. The ration content used in the research is shown Table 1. The chemical composition of the feeds used in the research is shown in Table 2 and the chemical composition of total mix ration (TMR) is shown in Table 3. Animals were included in the experiment in the last 40 days before the estimated date of calving. The study groups were randomized to the control group, 75 g humate group, and 150 g humate group based on the randomized complete block design. In this study, two doses were studied. The dose of humate supplementation was determined according to dry matter consumption during dry period and lactation period. From the beginning of the study to the day of calving (-40-0) 75 g of humate additive, which was calculated based on dry matter consumption (2%), was administered. After the calving (0th-60th), depending on the increase in dry matter consumption (3.8%) calculated 125 g humate additive was given. 75 g and 150 g were used from the beginning of the study to calving, 125 g and 250 g were used from the day of calving until the end of the study. Humate additive was

Table 1. Ingredient composition of diet.

Ingredient	Amount (kg)
Concentrate feed	10
Corn silage	20
Alfalfa hay	2
Fescue	5

Table 2. Chemical composition of dietary ingredients, %.

Ingredient	DM	CP	CF	Fiber	Ash	Starch	ADF	NDF
Concentrate feed	89.91	19.35	2.58	9.80	7.04	23.91	31.21	16.48
Fescue	89.32	9.56	1.58	26.80	6.19	–	32.65	46.85
Alfalfa hay	87.35	13.46	2.27	22.60	8.26	–	32.98	41.21
Corn Silage	21.70	7.40	1.43	21.70	6.96	–	33.44	55.61

DM: Dry matter; CP: Crude protein; CF: Crude fat; ADF: Acid detergent fibre; NDF: Neutral detergent fibre.

Table 3. The chemical composition of the total mix ration (TMR), %.

Chemical composition	As feed	Dry matter
Dry matter (65°C)	51.81	
Dry matter (105°C)	98.13	100.00
Ash	4.43	8.55
Crude fat	0.80	1.55
Crude protein	6.60	12.74
Starch	6.32	12.19
NDF	25.52	49.27
ADF	17.05	32.90
ADL	4.34	8.37
NFC	17.20	33.20
RUP % CP[1]	39.00	39.00
RDP % CP[1]	61.00	61.00
RUP Digest[1]	62.00	62.00
TDN[1]	30.10	58.10
N Fraction A, CP[1]	20.19	20.19
N Fraction B, CP[1]	59.44	59.44
N Fraction C, CP[1]	39.00	39.00

NDF: Neutral detergent fibre; ADF: Acid detergent fibre; ADL: Acid detergent lignin; NFC: Non-fiber carbohydrate; RUP: Rumen undegradable protein; RDP: Rumen degradable protein; CP: Crude protein; TDN: Total digestible nutrient; [1]: Obtained by calculation (17).

given to animals in the form of individual feed in individual paddocks. Humate additive was absorbed into 2 kg of silage and mixed into 5 kg of individual morning ration as a way for cows to consume the additive. The standard ration used in the study was prepared in TMR twice a day. The ration prepared to calculate the feed consumption of the animals in the experimental group was given by weighing individually in the morning and evening. The morning feeding was done at 8 am after milking, and the evening feeding was done at 6 pm. For monitoring feed consumption, the ration which was prepared at 8 in the morning and 6 in the evening was individually weighed using a weighing scale (TS500, TEM, Turkey) with a capacity of 25 kg. The remaining feeds in front of the animals in the individual paddocks were collected and weighed. Morning ration was given to the animals who finished the silage mixed additive.

Postpartum vaginal discharge and odor controlled on day 5 and day 15, and morphological contraction of the uterus was checked by rectal examination. On postpartum day 20, the presence of corpus luteum (CL) in the ovary was detected by ultrasonography. Artificial insemination was performed on the postpartum day 60 using presynch+ovsynch synchronization method, and the study was completed in 100 days. In addition, pregnancy examination was performed by ultrasonography on the 35th day after artificial insemination.

To determine the milk yield, milking was performed with Milkline's 2×7 herringbone model (Italy) milking system at 7 am and 5 pm, and milk volume was recorded at the end of each milking session using automatic lactometers. The body weights of the animals were measured on the days −40, 0, 30, and 60 using a weight scale (ST-1500H; Densi, Turkey). The body condition score (BCS) values of animals were averaged on the same day by two persons using Ferguson model 1-5 scoring inspection.

Blood samples were taken from *vena jugularis* at 40, 30, 20, and 10 days before the expected date of calving and on days 0, 5, 10, 20, 30, 45, and 60 after calving into two vials of non-anticoagulant 10 mL vacuum tubes after milking and before feeding in the morning. The sera were separated by centrifugation at 4000 rpm for 10 min (NF-800, Nuve, Turkey) and stored at −20°C until analysis. To determine the specific gravity of colostrum, colostrum samples were taken in a beaker at the first hour after the cow after calving, and the specific gravity was determined by the Kruuse Colostrometer. Milk samples of individually dairy cows in the morning on days 10, 20, 30, 45, and 60 after birth were placed in 100 mL and 50 mL disposable sample containers. It were kept at 4°C until analysis. The dry matter, crude protein, crude fat and ash contents of the diets were determined according to the AOAC (2). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined according to Van Soest (18). Dietary feed ingredients were analysed at Lalahan Ministry of Agriculture and Forestry International Livestock Research and Training Center Laboratory and chemical compositions were presented in Table 2. Rumen-degradable protein (RDP), rumen-undegradable protein (RUP) and total digestible nutrient (TDN) values were calculated according to the methods specified in NRC (17). TMR analysis shown in Table 3 was performed in a special food and feed analysis laboratory (NutriLab, Turkey). Beta-hydroxybutyric acid (BHBA) analysis in blood samples was performed with blood ketone meter (Vet TD-4235, Taidoc, Taiwan), a B-Ketone measuring device, and test strips at the time of blood collection. Albumin (Alb), total protein (Tp), Triglicerides (Tg), glucose, calcium (Ca), magnesium (Mg), and phosphorus (P) parameters of sera were estimated using Beckman Coulter AU5800 auto-analyzer (Beckman Coulter, USA) by the chemiluminescence immunoassay method at Atatürk University Research Hospital Biochemistry Laboratory. Non-esterified fatty acid (NEFA) from sera was read on Biotek ELISA reader using the bovine NEFA ELISA kit (SunRed brand catalog number 201-04-0186, China) at Atatürk University Faculty of Veterinary Science Department of

Biochemistry Laboratory, and the results were shown in µmol/L unit. The composition of milk samples was determined based on the ultrasound working principle using a milk analyzer (Milkotester brand Master Pro-P2 model, S/N: 22837, Bulgaria) at the Livestock Research and Application Center, Atatürk University. Somatic cell counting was performed using the Bentley model device (S/N: 7145, USA) using flow cytometry technique in the laboratory at Atatürk University Faculty of Veterinary Science Department of Obstetrics and Gynecology. On the postpartum days 5 and 15, the uterus was checked for vaginal discharge and odor; in addition, the involution process was controlled by rectal examination on day 15. On day 20, the presence of CL was checked by ultrasonography (Hasvet USG device, S/N: 838, China).

All data obtained from the research were analysed using SAS (2009 version). The model included humate level and time as fixed factors and cows within humate levels as random factor. The linear model included the main effects of humate level and time as well as humate level by time interaction, separately for the prepartum and postpartum periods. Differences among humate levels and time were attained by the Least Squared Differences option. Differences were indicated by superscripts ($P<0.05$).

Results

When the effect of humate addition on Body weight (BW) and Body condition score (BCS) was examined, no significant difference was found between the groups in both prepartum and postpartum periods as shown in Table 4. We examined DMI was prepartum and postpartum periods; Table 5 shows that the addition of humate significantly increased the DMI in both periods and milk yield in humate groups compared with the control group ($P<0.05$). It is shown in Table 6 that the addition of humate significantly increased the specific gravity of colostrum ($P<0.05$). Table 7 shows that when the effect of humate addition on milk composition and milk somatic cell count was examined, fat content, salt content, and conductivity in milk significantly increased in the treatment groups compared to the control group ($P<0.05$), and this addition had no influence on other milk composition components and somatic cell count. Table 8a and Table 8b shows that when the effect of humate addition on some blood parameters was examined for prepartum and postpartum periods, humate addition significantly increased prepartum and postpartum albumin levels, postpartum Ca levels ($P<0.05$), significantly decreased prepartum and postpartum BHBA and NEFA levels ($P<0.05$), and had no influence on prepartum and postpartum Tg, P, Mg, glucose, and BUN levels. Table 9 shows that humate addition to the ration did not significantly decrease vaginal odor on postpartum days 5 and 15; however, CL formation on day 20 was statistically significantly affected by humate addition.

Taken together, humate addition did not have a statistically significant impact on postpartum problems and pregnancy; however, shown in Table 10 uterine disorders numerically decreased and pregnancy ratio numerically increased with humate addition.

Table 4. The effect of humate addition to the ration on body weight and body condition score.

Parameter	Group			Group	Time	Group*Time	Linear	Quadratic
	0	H75	H150					
Prepartum								
BW (kg)	625±10.1	673±6.32	645±5.02	0.6373	-	-	0.8205	0.3660
BCS	3.72±0.07	3.68±0.05	3.52±0.03	0.6745	-	-	0.3962	0.8192
Postpartum								
BW (kg)	614±16.9	613±11.47	586±8.10	0.2172	0.0233	0.9668	0.1557	0.2814
BCS	3.32±0.10	3.22±0.06	3.11±0.06	0.1830	0.0160	0.9408	0.0673	0.8771

BW: Body weight; BCS: Body condition score; Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Table 5. The effect of humate addition to the ration on dry matter intake in the prepartum-postpartum period and milk yield

Parameter	Group			Group	Time	Group*Time	Linear	Quadratic
	0	H75	H150					
DMI pre (kg)	13.00±0.12^{c}	13.37±0.14^{b}	13.80±0.11^{a}	0.0001	0.0002	1.0000	0.0001	0.8738
DMI post (kg)	15.02±0.15^{b}	16.10±0.12^{a}	15.90±0.10^{a}	0.0001	0.0001	0.9986	0.0001	0.0001
MY (L)	18.01±0.24^{c}	22.50±0.25^{a}	21.00±0.22^{b}	0.0001	0.0001	1.0000	0.0001	0.0001

DMI pre: Dry matter intake in the prepartum period; DMI post: Dry matter intake in the postpartum period; MY: Milk yield; *: Different exponential letters on the same line indicate differences between groups ($P<0.05$); Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Table 6. The effect of humate addition to the ration on specific gravity of colostrum.

Parameter	Group			Group	Linear	Quadratic
	0	H75	H150			
Colostrum specific gravity (g/mL)	1041±3.61[b]	1056±1.34[a]	1058±0.81[a]	0.0001	0.0001	0.0463

*: Different exponential letters on the same line indicate differences between groups (P<0.05); Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Table 7. The effect of humate addition to the ration on milk composition and somatic cell count.

Parameter	Group			Group	Time	Group*Time	Linear	Quadratic
	0	H75	H150					
Fat (%)	3.31±0.09[b]	3.71±0.13[a]	3.72±0.15[a]	0.0290	0.2120	0.7148	0.0253	0.1524
NFMS (%)	9.05±0.13	9.19±0.06	9.23±0.10	0.4473	0.7970	0.8409	0.2314	0.6846
Density (kg/m^3)	30.60±0.48	31.41±0.33	31.13±0.38	0.3783	0.7198	0.5994	0.3794	0.2816
Protein (%)	3.24±0.05	3.32±0.02	3.32±0.04	0.2464	0.7444	0.8267	0.1536	0.3861
Lactose (%)	4.90±0.07	5.03±0.04	5.02±0.05	0.2231	0.8315	0.8313	0.1736	0.2863
Salt (%)	0.68±0.01[b]	0.71±0.01[a]	0.70± 0.01[ab]	0.0681	0.3561	0.8765	0.1256	0.0815
FP (°C)	−0.587±0.01	−0.592±0.00	−0.594±0.01	0.7657	0.1398	0.9377	0.4927	0.8079
Conductivity (mS/cm)	4.74±0.03[b]	4.79±0.03[ab]	4.81±0.02[a]	0.1084	0.4843	0.9402	0.0420	0.5944
Lnscc	10.42±0.18	10.42±0.20	10.46±0.20	0.9954	0.0032	0.7839	0.9978	0.9238

NFMS: Non-fat milk solid; SCC: Somatic cell count; FP: Freezing point; *: Different exponential letters on the same line indicate differences between groups (P<0.05); Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Table 8a. The effect of humate addition on blood parameters in the prepartum period.

Parameter	Group			Group	Time	Group*Time	Linear	Quadratic
	0	H75	H150					
Tg (mg/dl)	19.4 ± 0.80	20.81 ± 0.92	19.81 ± 0.83	0.5073	0.8126	0.9635	0.6955	0.2682
Ca (mg/dl)	9.03 ± 0.06	9.13 ± 0.07	8.95 ± 0.06	0.1625	0.8553	0.7935	0.5919	0.0704
P (mg/dl)	5.85 ± 0.10	5.93 ± 0.10	5.82 ± 0.01	0.6319	0.0454	0.8400	0.8508	0.3519
Mg (mg/dl)	2.44 ± 0.03	2.40 ± 0.03	2.44 ± 0.0.04	0.5193	0.9711	0.1941	0.7245	0.2823
Tp (mg/dl)	7.58 ± 0.08	7.55 ± 0.09	7.57 ± 0.09	0.9444	0.0067	0.9325	0.8917	0.7598
Alb (mg/dl)	3.1 ± 0.04[a]	3.22 ± 0.04[a]	2.93 ± 0.04[b]	0.0001	0.7608	0.9975	0.0023	0.0001
Glucose (mg/dl)	57.32 ± 0.9	57.98 ± 1.28	56.63 ± 1.31	0.7226	0.8824	0.8649	0.6775	0.4995
BUN (mg/dl)	10.85 ± 0.54	11.26 ± 0.50	12.18 ± 0.67	0.2663	0.7737	0.9988	0.1161	0.7187
BHBA (mg/dl)	9.19 ± 0.46	8.45 ± 0.26	8.42 ± 0.36	0.2940	0.2972	0.9595	0.1617	0.4280
NEFA (µmol/L)	25.35 ± 1.90[a]	20.17 ± 2.35[a]	14.72 ± 2.29[b]	0.0010	0.5593	0.6621	0.0002	0.9501

*: Different exponential letters on the same line indicate differences between groups (P<0.05); Tg: Triglyceride; Ca: Calcium; P: Phosphorus; Mg: Magnesium; Tp: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; BHBA: Beta-Hydroxybutyric acid; NEFA: Non-esterified fatty acid; Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Table 8b. The effect of humate addition on blood parameters in the postpartum period.

Parameter	Group			Group	Time	Group*Time	Linear	Quadratic
	0	H75	H150					
Tg (mg/dl)	5.47 ± 0.30[a]	4.55 ± 0.28[b]	4.85 ± 0.25[ab]	0.469	0.0001	0.9956	0.0972	0.0689
Ca (mg/dl)	8.21 ± 0.06[b]	8.66 ± 0.08[a]	8.38 ± 0.09[b]	0.0001	0.0001	0.0222	0.0418	0.0001
P (mg/dl)	4.81 ± 0.12	5.05 ± 0.13	5.04 ± 0.14	0.3311	0.0014	0.6750	0.1958	0.4772
Mg (mg/dl)	2.35 ± 0.03	2.41 ± 0.04	2.35 ± 0.07	0.5796	0.0001	0.1089	0.8199	0.3106
Tp (mg/dl)	7.54 ± 0.14	7.47 ± 0.09	7.31 ± 0.10	0.3037	0.0001	0.4795	0.1234	0.9910
Alb (mg/dl)	2.99 ± 0.05[b]	3.18 ± 0.04[a]	2.82 ± 0.05[c]	0.0001	0.2402	0.8238	0.0109	0.0001
Glucose (mg/dl)	43.86 ± 3.02	42.96 ± 2.44	45.95 ± 3.33	0.6681	0.0001	0.9718	0.4250	0.6723
BUN (mg/dl)	8.81 ± 0.50	9.86 ± 0.57	9.59 ± 0.66	0.3397	0.0067	0.5386	0.3418	0.2672
BHBA (mg/dl)	9.27 ± 0.61[a]	7.47 ± 0.35[b]	7.48 ± 0.45[b]	0.0031	0.0001	0.5629	0.0019	0.1214
NEFA (µmol/L	31.45 ± 1.89[a]	14.96 ± 1.35[b]	16.1 ± 1.25[b]	0.0001	0.1444	0.0496	0.0001	0.0001

*: Different exponential letters on the same line indicate differences between groups (P<0.05); Tg: Triglyceride; Ca: Calcium; P: Phosphorus; Mg: Magnesium; Tp: Total protein; Alb: Albumin; BUN: Blood urea nitrogen; BHBA: Beta-Hydroxybutyric acid; NEFA: Non-esterified fatty acid; Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Table 9. The effect of humate addition on postpartum uterus and ovary.

	Group			χ^2	P value
	0	H75	H150		
Day 5th					
Smelling	3	0	4	4.54	0.1000
Clean	6	8	5		
Day 15th					
Smelling	2	0	1	1.94	0.3789
Clean	7	8	6		
Day 20th					
Corpus luteum +	3	8	5	7.3965	0.0248
Corpus luteum −	5	0	2		

Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Tablo 10. The effect of humate supplementation on postpartum problems and pregnancy.

Problem	Group			χ^2	P value
	0	H75	H150		
Endometritis	1	0	0	14.4926	0.0698
Inactive ovarium	2	0	0		
Mastitis	0	2	0		
Retencio secundinarium	2	0	4		
Healty	4	6	5		
Artifical insemination	8	8	7		
Pregnancy +	1	5	2	8.6133	0.0715
Pregnancy -	7	3	5		

Group 0: Control group; Group H75: Adding 75 g of humate to the ration; Group H150: Adding 150 g of humate to the ration.

Discussion and Conclusion

Body condition score is related to milk yield, nutritional status, health status, and fertility and should thus be continuously monitored. It is desirable to perform both BCS and BW monitoring in herd management; however, it is a disadvantage that the equipment is insufficient in the facilities and live weight monitoring requires more labor force than the BCS. In this study, humate addition was not found to have a statistically significant effect on BCS and BW. McMurphy et al. (16) conducted a study where they added humate in the ration of different sexes; consequently, they reported that humate provided 13.4% more live weight gain in female calves and 21.2% in male calves compared to the control group calves. Teravita et al. (19) and Cusack et al. (6) reported that humic and fulvic acid improved the increase in daily live weight of cows. Tunç (21) reported that humate had no significant effect on the live weight. The reason for the results not matching may be because of different humate doses, ages, periods, and durations. DMI is an important parameter to be emphasized, particularly in the transition period in ruminants. In the studies, it was seen that humate addition increased DMI in prepartum and postpartum periods. According to Covington et al. (5) and McMurphy et al. (16) humate addition to the ration increased DMI in lambs and Holstein cows, respectively. It was reported that humate addition did not significantly affect DMI in fattening cattle by McMurphy et al. (15) in calves by Chirase et al. (4), and in Saanen goats by Degirmencioglu (7). These contradictory results may be due to the different amounts of dose and different periods.

Colostrum is a good source of immunoglobulin for calves to be born and its quality is very important. It was found that humate addition significantly increased the specific gravity of colostrum. On reviewing the literature, we found no study on the effect of humate addition on colostrum quality. The increase in specific gravity of colostrum is considered to be due to the immunomodulatory effect of humate.

In the present study, it was seen that humate addition increased milk yield and its fat content. Humate addition did not have a statistically significant impact on non-fat milk solid, milk protein, lactose content, density and freezing point. Degirmencioglu (7) reported that humate addition in Saanen goats increased milk yield but did not change fat content, non-fat dry matter content, protein content, lactose ratios, and somatic cell count in milk. Tomassen et al. (20) reported that humate addition

increased milk yield and fat:protein ratio in milk and decreased somatic cell count by 50%. Griban et al. (10) and Xiaowang et al. (23) reported that fulvic acid significantly reduced somatic cell count in intervention group cows compared to the control group ones. The reason for obtaining different results among the studies could be differences in individuals, sources, doses, durations of humate use, cattle breed and feed materials.

Blood parameters are an indicator of the metabolic profile, and the effects of humate addition can also be seen in the blood. Among the parameters examined, particularly Ca, NEFA, and BHBA levels are very important during the transition period. In the present study, it was observed that humate addition significantly increased prepartum and postpartum albumin levels and postpartum Ca levels, and it significantly decreased prepartum and postpartum BHBA and NEFA levels. Further, it did not affect triglyceride, P, Mg, glucose, and BUN levels in prepartum or postpartum. Galip et al. (9) reported that humate addition in curly rams reduced serum albumin, glucose, Ca, and P levels and increased total protein levels. Tunç and Yörük (22) reported that it did not change serum albumin, total protein, triglyceride, glucose, Ca, and P levels in Red Karaman sheep. Budağ and Kara (3) reported that humate addition decreased serum total protein and increased serum BUN levels in Norduz sheep. It was reported that the humate addition did not change serum albumin, total protein, and Ca levels but increased Mg levels in bronze calves. Differences in the results among studies may be due to the different dose levels and different ages and races. In the review of literature, no study was found on the effects of humate addition on serum NEFA and BHBA; for this reason, they could not be sufficiently discussed. Humate use has been shown to decrease NEFA and BHBA levels before and after birth. Humate preparation may be thought to lead to relaxation in energy metabolism, especially in postpartum period. The low BHBA and NEFA values also led to an increase in dry matter consumption and an increase in milk yield in animals. This information supports each other.

In the present study, humate addition had no significant effect on vaginal odor on days 5 and 15 after birth, and it had a positive effect on CL formation on day 20. Humate addition has been shown to numerically reduce postnatal problems and numerically increase of pregnancy. In the review of literature, studies on the effects of humate on postnatal problems and pregnancy formation could not be found and therefore could not be sufficiently discussed.

According to our results, the addition of 75 g of humate had a positive effect on colostrum specific gravity, milk yield, milk composition, DMI, serum Ca and albumin levels, and serum NEFA and blood BHBA levels. Besides, 75g humate addition also had a positive effect on postpartum problems and pregnancy.

Acknowledgement

This study is produced from the first author's PhD thesis.

Financial Support

This study was supported by the Scientific Research Project Fund Atatürk University (Project number: TDK-2017-6297).

Ethical Statement

This study was approved by Atatürk University Animal Experiments Local Ethics Committee dated 18.09.2017 and numbered 36643897-000-E.1700250736.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Anonymous** (2017): Effect of humic acid on animals and humans an overview of literature and a review of current research. Available at http://www.humichealth.info/ effects.html (Accessed March 10, 2017).
2. **Association of Official Analytical Chemists-AOAC** (1990): Official Methods of Analysis. 15th ed. AOAC, Arlington, VA, USA.
3. **Budağ C, Kara U** (2016): *The effect of use of humic acid in some blood parameters and rumen protozoa in Norduz lambs.* Iğdır Univ J Inst Sci & Tech, **6**, 185-190.
4. **Chirase NK, Greene LW, McCollum FT, et al** (2000): *Effect of bovipro on performance and serum metabolites concentrations of beef steers*. Proc West Sec Amer Soc Anim Sci, **51**, 415-418.
5. **Covington BR, Ramsey WS, Greene LW, et al** (1997): Effects of humate on feedlot performance and carcass characteristics in feedlot lambs. J Anim Sci, 75: 270.
6. **Cusack PMV** (2008): *Effect of a dietary complex of humic and fulvic acids (FeedMAX15TM) on the health and production of feedlot cattle destined for the Australian domestic market*. Aust Vet J, **86**, 46-49.
7. **Degirmencioglu T** (2012): *Possibilities of using humic acid in diets for Saanen goats.* Mlijekartsvo, **62**, 278-283.
8. **Degirmencioglu T, Ozbilgin S** (2013): *Effect of administration of humic acid on somatic cell count and total bacteria in Saanen goats*. Inter J Vet Sci, **2**, 151-154.
9. **Galip N, Polat U, Biricik H** (2010): *Effects of supplemental humic acid on ruminal fermentation and blood variables in rams.* Ital J Anim Sci, **9**, 390-393.
10. **Griban VG, Stepchenko LM, Zhorina LV** (1988): *The live weight gain and disease resistence of young cattle and poultry stock as influenced by physiologicalyactive peat preperation.* 45-50. In: Proceedings of VIII Inter Peat Congress. Leningrad, Russia.

11. **Golbs S, Fuchs V, Kühnert M, et al** (1982): *Prenatal toxicologial testing of humic acids on laboratory rat*. Arch Exp Veterinarmed, **36**, 179-185.
12. **Grummer RR** (1995): *Impact of changes in organic nutrient metabolism on feeding the transition dairy cow.* J Anim Sci, **73**, 2820-2813.
13. **Karademir G, Karademir B** (2003): *Yem katkı maddesi olarak kullanılan biyoteknolojik ürünler.* Lalahan Hay Araşt Enst Derg, **43**, 61-74.
14. **Kutlu HR, Serbester U** (2014): *Ruminant beslemede son gelişmeler.* TURJAF, 2, 18-37.
15. **McMurphy CP, Duff GC, Harris MA, et al** (2009): *Effects of humic/fulvic acid in beef cattle finishing diets on animal performance, ruminal ammonia and serum urea nitrogen concentration*. J Appl Anim Res, **35**, 97-100.
16. **McMurphy CP, Duff GC, Sanders SR, et al** (2011): *Effects of supplementing humates on rumen fermentation in Holstein steers.* S Afr J Anim Sci, **41**, 134-140.
17. **National Research Council-NRC** (2001): Requirements of Dairy Cattle. Natl Acad Press, Washington DC.
18. **Robertson JB, Van Soest PJ** (1981): The Detergent System of Analysis. 123-158. In: WPT James, O Theander (Eds), The Analysis of Dietary Fiber in Food. Marcel Dekker, New York.
19. **Teravita TM** (2018): Humates in poultry and stock farming. Available at http://www.teravita.com/humates/chapter9.htm. (Accessed December 24, 2018).
20. **Tomassen BPH, Faust RH** (2018): *The use of a processed humic acid product as a feed supplement in dairy production in the Netherlends*, 339, In: Proceedings of The World Grows Organic International Scientific Conference, August, Basle, Switzerland.
21. **Tunç MA** (2012): *Süt emme dönemindeki buzağılarda humat ve probiyotiklerin performans, rumen fermentasyonu ve kan parametreleri üzerine etkisi*. Doktora Tezi. Atatürk Üniversitesi Sağlık Bilimleri Enstitüsü, Erzurum.
22. **Tunç MA, Yörük MA** (2012): *Humik asitlerin koyunlarda rumen ve kan parametreleri ile protozoon sayısı üzerine etkisi.* Kafkas Üniv Vet Fak Derg, **18**, 55-60.
23. **Xiaowang X, Shaohaua S, Lixia H** (2010): *Study on the effect of biochemical fulvic acid on somatic cell count and milk performance of dairy cows*. J Chin Dairy Cattle, **5**, 1-7.

Ankara Univ Vet Fak Derg, **68**, 15-20, 2021
DOI: 10.33988/ auvfd. 666779

Characterization of *Pasteurella multocida* isolates recovered from the oral flora of cats

Tuğçe TINMAZ[1,a,✉], Baran ÇELİK[1,b], Barış HALAÇ[1,c], Arzu Funda BAĞCIGİL[1,d]

[1]İstanbul University - Cerrahpaşa, Faculty of Veterinary Medicine, Department of Microbiology, İstanbul, Turkey.
[a]ORCID: 0000-0002-3446-6031; [b]ORCID: 0000-0001-9122-0284; [c]ORCID: 0000-0002-3067-9937;
[d]ORCID: 0000-0002-8838-7291.

✉Corresponding author: tugcetinmaz@yahoo.com
Received date: 29.12.2019 - Accepted date: 12.06.2020

Abstract: In this study, it was aimed to investigate the presence of *Pasteurella multocida* in the swab samples taken from the oral cavity of cats, and to determine the capsular type and antimicrobial susceptibility of the isolates. For this purpose, swab samples taken from 300 cats were inoculated onto Knight's selective enriched medium and blood agar for isolation of *P. multocida*. Following after capsular typing of the isolates by PCR, the susceptibilities of the isolates to ceftiofur, clindamycin, erythromycin, enrofloxacin, amoxicillin+clavulanic acid, trimethoprim/ sulfamethoxazole, tetracycline, and chloramphenicol were examined by the disk diffusion method. The relationships between oral colonization and various physiological and behavioral variables were evaluated, statistically. *P. multocida* was isolated from 48 (16%) of the samples and all strains were typed as capsular type A. While all of the isolates were resistant to clindamycin and susceptible to enrofloxacin, 8,33% tetracycline resistance was also remarkable. Multiple antibiotic resistance (MDR) was detected in 27% of the isolates. The colonization of *P. multocida* was found to be statistically significant in cats under the age of 1 and in cats living outdoors. The importance of antibiotic resistance observed in bacteria with a zoonotic character such as *P. multocida* should not be ignored since it poses a threat to public health. It was concluded that, in this study determination of tetracycline resistance, which has started to be reported in feline *Pasterurella multocida* isolates in recent years, and detection of multiple antibiotic resistance in 27% of isolates, was extremely important for public health.
Keywords: Antimicrobial susceptibility, capsular typing, cat, oral swab, *Pasteurella multocida*.

Kedilerin ağız florasından izole edilen *Pasteurella multocida* izolatlarının karakterizasyonu

Özet: Bu çalışmada kedilerin ağız boşluğundan alınan svap örneklerinde *Pasteurella multocida* varlığının araştırılması, kapsül tiplerinin belirlenmesi ve antibiyotik duyarlılıklarının saptanması amaçlandı. Bu amaçla, 300 kediden oral svap örnekleri toplandı ve *P. multocida* izolasyonu için selektif zenginleştirilmiş Knight's besiyeri ve kanlı agara ekimleri yapıldı. İzolatların kapsül tiplendirmeleri PCR ile yapıldıktan sonra seftiofur, klindamisin, eritromisin, enrofloksasin, amoksisilin klavulonik asit, trimetoprim/sulfametokasazol, tetrasiklin, kloramfenikola karşı duyarlılıkları disk difüzyon yöntemi ile incelendi. Oral kolonizasyon ile çeşitli fizyolojik ve davranışsal değişkenler arasındaki ilişkileri istatistiksel olarak değerlendirildi. İncelenen örneklerin 48 (%16)'inden *P. multocida* izole edildi ve tümü kapsüler tip A olarak belirlendi. İzolatların tamamı klindamisine dirençli ve enrofloksasine duyarlı bulunurken, % 8,33 oranında tetrasiklin direnci de dikkat çekici olarak belirlendi. İzolatların %27'sinde çoklu antibiyotik direnci saptandı. İstatistiksel analiz sonucu, bir yaş altı kediler ve dış ortamda yaşayan kedilerde kolonizasyon istatistiksel olarak anlamlı bulundu. *P. multocida* gibi zoonoz karakterdeki bakterilerde gözlenen antibiyotik direnci, halk sağlığı açısından tehdit oluşturduğundan gözardı edilmemelidir. Son yıllarda kedi *P. multocida* izolatlarında rapor edilmeye başlanan tetrasiklin direncinin bu çalışmada da saptanması ve tüm izolatlarda %27 oranındaki çoklu antibiyotik direncinin gözlemlenmesinin, halk sağlığı açısından son derece önemli olduğu sonucuna varıldı.
Anahtar sözcükler: Antimikrobiyal duyarlılık, kapsüler tiplendirme, kedi, oral svap, *Pasteurella multocida*.

Introduction

Pasteurella species are usually found in the normal flora of the oral, nasopharyngeal, and upper respiratory tract in animals, and they are also opportunistic pathogens associated with endemic outbreaks. *P. multocida* is a species that can be isolated from the oral flora of cats and dogs up to 90%. It also has a zoonotic character. Infection to people is usually caused by biting, scratching, licking or contact with nasal secretions (3,8,12,17,19). *P. multocida,* has 5 capsular serogroups such as A, B, D, E and F, and 16 somatic serotypes. The capsule plays the most important role in the identification of serogroup type.

Different serotypes and serogroups tend to cause certain diseases (5,14,33). Healthy cats are usually defined as the carriers of *P. multocida*. Cats living in nature spread bacteria in cat populations as a result of hunting and fighting. However, although cats are often reported as the carriers of *P.multocida* and the agent is mentioned to be a zoonotic, isolated from cat bites, there is no study on the prevalence of *P. multocida* in oral colonization of cats, the presence of virulence genes, and antimicrobial susceptibility profiles in Turkey.

The aim of this study was to isolate *P. multocida* from the oral flora of cats with a different clinical picture and to perform the capsular typing of isolates and to determine antimicrobial resistance profiles.

Material and Methods

Oral swab samples from 300 cats which were clinically healthy or were taken to veterinary clinics with various clinical symptoms in Istanbul between March 2017 and October 2018 were examined. This study was approved by Istanbul University Animal Experiments Local Ethics Board (Decision date: 23.02.2017). Swabs samples were collected from the oral mucosa to cover the inner cheek edge mucosa, gingiva and the top of the tongue, were transferred in Cary-Blair medium and stored at room temperature and brought to the laboratory within 5 days (16, 30). The data on breed, age, gender, antimicrobial treatment history, living-conditions of the sampled cats were recorded. According to these data, it was recorded that no antimicrobial agent had been used for last month in 286 of the cats, antibiotic therapy was performed for different reasons in 14 of them. The rest of the data collected from sampling population were showed in Table 1.

The swabs were streaked onto Nutrient Agar including 7% defibrinated sheep blood and onto selective medium contained defibrinated horse blood and antibiotics described by Knight et al. (18), and they were incubated for 24 hours at 37°C under aerobic conditions. Catalase and oxidase-positive isolates with Gram-negative coccobacillus morphology were considered as *P. multocida* suspected. The identification of the isolates and subsequently, capsular typing were performed according to the methods described previously (32,33). The DNA extraction was performed using the commercial kit (Quick-DNA Fungal/Bacterial Miniprep, Zymo Research, CA, USA). Identification and capsular typing of *P. multocida* isolates were performed (32) with ready-to-use mix (MyTaq HS mix, Zymo Research, CA,USA), and amplification protocol was applied as preliminary denaturation at 95°C for 5 minutes, and denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 30 seconds and final extension at 72°C for 5 minutes in total of 30 cycles. A field isolate obtained from Samsun 19 Mayıs University, Faculty of Veterinary Medicine, Department of Microbiology, and the strains coded PM492 (Capsule type A), PM 498 (Capsule type B) obtained from Dr. Conny Turni from the University of Queensland were used as positive controls in the study.

The antimicrobial susceptibilities of the isolates were examined by the disk diffusion method according to The European Committee on Antimicrobial Susceptibility Testing [amoxicillin/clavulanic acid (20/10 µg), tetracycline (30µg), trimethoprim-sulfamethoxazole (1.25/23.7µg)] (31), and The Clinical and Laboratory Standards Institute [ceftiofur (30µg), chloramphenicol (30µg), enrofloxacin (5µg)] (7) [clindamycin (2µg) and erythromycin (15µg)](27) criteria. *Escherichia coli* ATCC 25922 was used as quality control (QC) strain.The isolates which were found to be resistant to three or more antibiotic classes were defined as multidrug resistance (MDR) (20).

Table 1. Sample characteristics and statistical data: all cats' age, living conditions, gender, drug history information, respectively, and number of cats found to be positive and negative in terms of *P. multocida* by PCR.

Variables	Total (%)	PCR positive (%)	PCR negative (%)	P-value
Age (years)				
<1	51 (17)	2 (3.9)	49 (96.0)	**0.0045382***
1-6	217 (72)	39 (17.9)	178 (82.1)	0.131188
>6	32 (11)	7 (21.8)	25 (78.2)	0.337494
Living condition				
Indoor	161 (53.6)	31 (19.3)	130 (80.7)	0.097945
Outdoor	71 (23.6)	4 (5.6)	67 (97.4)	**0.0031971***
Indoor/Outdoor	68 (22.6)	13 (19.1)	55 (80.9)	0.425195
Gender				
Female	167 (55.6)	28 (16.8)	139 (83.2)	0.684907
Male	133 (44.3)	20 (15.03)	113 (84.97)	
Drug History				
Not used	286 (95)	47 (16.4)	239 (83.6)	0.3126667*
Used	14 (5)	1 (7.1)	13 (92.9)	

CI 95%, Fisher's exact test was used in the analyses with * mark, Pearson's chi-square test was used in others. P<0.05 was considered statistically significant in all analyses. Significant values are shown in bold characters.

Colonization rates (prevalence= number of colonized cats/number of cats examined x 100) were calculated and colonization relation with demographic variables analyzed by SPSS for Windows, Version 17.0 (SPSS Inc. Chicago, USA, Released 2008). The prevalence rates (PR) and the relevant 95% confidence interval values (95% CI) were calculated for the categorical variables associated with colonization for the control of infection. The relationship between oral colonization and age (below the age of 1, 1-6 years, over the age of 6), living conditions (indoor, outdoor, indoor/outdoor), gender (female/male), and antibiotic usage was investigated. The chi-square test was used for the estimation of independence of categorical variables, and Fisher's exact test was used when the expected cell value was below 5. A P-value <0.05 was considered statistically significant in all analyses performed.

Results

P. multocida was recovered from 48 of the samples, the isolation rate was found to be 16%. All isolates were belonged to capsular type-A. It was observed that no drug was used in the last month in 47 of the cats from which *P. multocida* was isolated, and an antibiotic was used for different reasons in only one of them. It was determined that 28 of the cats were female and 20 of them were male, 31 of them lived only indoor, 4 of them lived outdoor and 13 of them lived both indoor and outdoor.

As a result of antimicrobial susceptibility tests, while all isolates were found to be resistant to clindamycin, it was determined that the other most resistance were against to trimethoprim+sulfamethoxazole (co-trimoxazole) combinations in 15 isolates and amoxicillin+clavulonic acid combinations in 14 isolates. Antibiotics with the least resistance were found as chloramphenicol (2 isolates) and tetracycline (4 isolates). All isolates were susceptible to enrofloxacin. While resistance to a single antimicrobial agent was found in 35.42% (n=17) of isolates, resistance to two antimicrobial agents was found in 37.50% (n=18) of them, resistance to three antimicrobial agents was found in 20.83% (n=10) of them, and resistance to four antimicrobial agents was found in 6.25% (n=3) of them. In this case, the prevalence of multiple antibiotic resistance was found to be 27%. Distribution of antimicrobial susceptibilities and antimicrobial resistance profiles of the isolates were presented in Table 2 and 3 respectively.

When gender and the presence of colonization were evaluated, the ratios between males and females were found to be 15.03% and 16.8%, respectively, and the difference between them was not found to be statistically significant (Pearson's= 0.165, P=0.684907 95% CI: -0.061 <0.016> 0.092). Similarly, colonization was not found to be statistically significant in the samples taken from the animals with (7.1%) and without drug use (16.4%) (Fisher's exact test=0.857, P=0.3126667, 95% CI: 0.327 >2.556> 20.015). Colonization rates were found to be 3.9%, 17.9%, and 21.8% in the age groups of under age of 1, 1-6 years and over the age of 6, respectively. The colonization rate was found to be statistically significant in animals under the age of 1; at value 95% CI: -0.262 <-0.149> -0.036, P = 0.0045382 (Fisher's exact test). Similarly, when living conditions were compared, colonization was found to be statistically significant in animals living outdoor (95% CI: -0.263 <-0.153> -0.043 P(O>=E): 0.0031971, Fisher's exact test) (Table 1).

Table 2. Antimicrobial resistance prevalence of isolates.

Antimicrobial agent	Number of resistant bacteria	%
FUR	5	10.42
AUG	14	29.17
SXT	15	31.25
E	7	14.58
TE	4	8.33
C	2	4.17
CD	48	100.00
ENR	0	0.00

P: Prevalence, FUR: Ceftiofur, AUG: Amoxicillin+Clavulonic acid, SXT: Trimethoprim + Sulfamethoxazole, E: Erythromycin, TE: Tetracycline, C: Chloramphenicol, CD: Clindamycin, ENR: Enrofloxacin.

Table 3. Antimicrobial resistance profiles of isolates.

Antimicrobial agent	Number of isolates	%
CD	17	35.42
FUR/CD	1	2.08
AUG/CD	6	12.50
SXT/CD	10	20.83
TE/CD	1	2.08
FUR/ SXT/CD	2	4.17
AUG/TE/CD	3	6.25
AUG/E/CD	3	6.25
SXT/C/CD	1	2.08
SXT/E/CD	1	2.08
FUR/SXT/E/CD	1	2.08
FUR/AUG /E/CD	1	2.08
AUG/E/C/CD	1	2.08

P: Prevalence, CD: Clindamycin, FUR: Ceftiofur, AUG: Amoxicillin+Clavulonic acid, SXT: Trimethoprim + Sulfamethoxazole, TE: Tetracycline, E: Erythromycin, C: Chloramphenicol.

Discussion and Conclusion

P. multocida is defined as a part of the oropharyngeal microbiota of cats, dogs, and other animals. The infections caused by *P. multocida*, which is considered to be zoonotic, are associated with biting, scratching, or licking of the injured tissue. However, infections that occurred in humans without animal contact were also reported (3,10,12,19,28,29). In this study, it was aimed to investigate the isolation of *P. multocida* from intraoral swabs in cats since it is found in the oral microbiota of cats and is a bacterium with zoonotic character. Along with the determination of antibiotic resistance profiles of isolates, it was aimed to update treatment options in case of possible transmission and infection. The prevalence of oral *P. multocida* colonization in cats varies between 10.4% and 89.9% (11, 12,13,15,22,25). However, it is scientifically misleading to compare these studies without standards on population demographics, sampling sites, sampling methods, transport conditions of samples, analysis methods, and evaluation criteria. In this study, the *P. multocida* isolation rate was found to be 16%, which was consistent with previous studies.

One of the most important virulence factors of *P.multocida* which is thought to be commensal is polysaccharide capsule. It was reported that there was a relationship between the capsule types of the agent which they changed geographically, the host and the disease it caused. While serogroups B and E were isolated from bovine hemorrhagic septicemia disease around the world, serogroup E was never isolated in Europe, and serogroup B was isolated only in Eastern Europe. Serogroup F was isolated from cats, ducks, and poultry, type A and D strains were isolated from many domestic and wild animals, and it was reported that human infections were usually caused by serogroup A (2,6,9). While Arumugam et al. (2) reported that all cat and dog isolates in their study were serogroup A, Ferreira et al. (11) reported that 75.6% of cat isolates were serogroup A. In this study, it was determined that all of 48 *P. multocida* strains were determined as serogroup A, and it was observed that the results were parallel with the limited number of studies conducted on cats.

Few studies on *P. multocida* and oral colonization were usually limited to the determination of capsule serogroups, subspecies, and antibiotic susceptibility. The relationships between the population in which sampling was performed, the physiological, demographic, and behavioral data of this population, and colonization were not investigated. Since the colonization rates of *P. multocida* were not investigated extensively, it was unclear whether the agent was a commensal microorganism of the natural flora in cats or from an external source. In a study carried out by examining 1-week-old kittens, it was revealed that the agent was not naturally found in the oral flora, but passed during licking or milking from the mother (10). It was reported that the oral microbiota in cats varied with diet (1). However, in the literature, there is no data indicating the relationship between the presence of oral colonization and age, housing/care conditions, or gender. In this study, the presence of colonization in cats under the age of 1 was found to be significant compared to other age groups. The intestinal microbiota is known to change with age in cats and humans. However, the change of the oral microbiota with age has not yet been fully clarified (21,26). It is considered that long-term cohort studies involving the same animals with larger samples should be carried out to clarify the change on oral microbiota with age, the phenomenon of "microbiota aging." Similarly, in our study, the presence of *P. multocida* was found to be significantly higher in cats with contact the outside compared to cats that not. It was considered that this significant difference was based on behavioral, due to the fact that cats living outside infected each other during hunting, regional and sexual fights, and limited access to food and water resources. However, more extensive cohort studies were considered necessary to prove this assumption.

The infections associated with *P. multocida* are generally attempted to be treated empirically with broad-spectrum antibiotics. In previous studies carried out on *P. multocida* isolates originating from cats and dogs, penicillin (12,22), tetracycline (11,22,34), ampicillin (22,34), amoxicillin+clavulanic acid (11,12), ceftiofur (11,22,34), enrofloxacin (34), azithromycin (12), co-trimoxazole (11) antibiotics were reported to be susceptible. The results of this study indicated that enrofloxacin was the most effective antimicrobial agent followed by chloramphenicol, ceftiofur, and tetracycline. These results are consistent with previous studies. Resistance to clindamycin (34), cotrimoxazole (11,34) and erythromycin (34) antibiotics was reported. Clindamycin resistance is known to be common in *P. multocida* strains (34). In this study, resistance to clindamycin was detected in all strains. This result is consistent with previous studies. Tetracycline is one of the recommended agents for use in infections caused by *P. multocida* strains resistant to clindamycin and sulfanamides in humans (34). The evaluation criteria such as break points of many antibiotics for canine or feline *P. multocida* isolates have not been published yet. Nevertheless, in two studies published in recent years, where only MIC values were given, high MIC values for tetracycline were reported in two isolates (23,24). Ujvari et al. (34) stated that tetracycline resistance was not detected in clinical samples in cats, while another surveillance study in the same year reported 4.1% tetracycline resistance (4).In this study, it was determined

4 (8.33%) of the cat isolates were resistant to tetracycline, and 9 (18.75%) of them were moderately susceptible. Tetracycline resistance is routinely reported in *P.multocida* isolates of pig and bovine origin. In the current study, it was determined that 7 of the isolates were resistant to erythromycin, and 5 of them were moderately susceptible. The result related to erythromycin resistance is consistent with the results obtained previously (11). It was considered that the fact that the studies reporting the result of decreased susceptibility related to erythromycin were associated with *P. multocida* strains isolated from poultry and pet animals could be attributed to the active use of this antibiotic for a long time as a performance enhancer in animals since the 1960s.

In this study, resistance to three and more antibiotic classes was detected in 27% of the isolates. The MDR patterns detected reveal the developing resistance to some commonly used classes of antibiotics such as lincosamides, cephalosporins, sulphonamides in *P. multocida* isolates. In this study, 13 resistance patterns were detected, and clindamycin and trimethoprim + sulfamethoxazole + clindamycin were observed to be the most common patterns among them. Since resistance/multiple resistance patterns were not reported in previous studies, the results obtained in the current study could not be compared.

Cats are behaviorally in close contact with cohabitants. The results of our study provided data on the prevalence of *P.multocida* strains colonized in cats, its capsule types, antibiotic resistance and some significant relationships between colonization and population dynamics. In particular, the potential threat related to relatively high rates of MDR *P.multocida* isolates like any other zoonotic bacteria, should not be underestimated. Horizontal transmission of resistance genes and epidemiological shift observed in susceptible populations lead to an increase in epidemics and MDR bacteria waiting for the right genes to come together. Empirical antibiotic treatments are frequently preferred in animal and human practice, so antibiotic resistance in animal isolates, especially zoonotic bacteria such as *P. multocida*, provides a strong insight of potential resistance in future human infections. It does not appear possible to cease the development of antibiotic resistance with today's technologies. However, selective pressure and spread of resistance can be slowed down by reasonable and responsible antibiotic use and surveillance studies throughout the country.

Acknowledgments

This work was prepared from the first author's PhD thesis. The authors would like to thank to Dr. Conny Turni from the University of Queensland and to Prof. Dr. Alper Çiftçi and Assoc. Prof. Dr. Arzu Fındık from Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Microbiology for the positive controls.

Financial Support

This study was supported by Scientific Research Project Coordination Unit of Istanbul University-Cerrahpaşa. Project code: TDK-2017-25374.

Ethical Statement

This study was approved by Istanbul University Animal Experiments Local Ethics Board (23.02.2017).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Adler CJ, Malik R, Browne G V, et al** (2016): *Diet may influence the oral microbiome composition in cats.* Microbiome, **4**, 23.
2. **Arumugam ND, Ajam N, Blackall PJ, et al** (2011): *Capsular serotyping of Pasteurella multocida from various animal hosts-a comparison of phenotypic and genotypic methods*. Trop Biomed, **28**, 55–63.
3. **Björkholm B, Eilard T** (1983): *Pasteurella multocida osteomyelitis caused by cat. bite* J Infect, **6**, 175–177.
4. **Bourély C, Cazeau G, Jouy E, et al** (2019): *Antimicrobial resistance of Pasteurella multocida isolated from diseased food-producing animals and pets*. Vet Microbiol, **235**, 280–284.
5. **Boyce JD, Chung JY, Adler B** (2000): *Pasteurella multocida capsule: Composition, function and genetics*. J Biotechnol, **83**, 153–160.
6. **Chen Y, Sun E, Song J, et al** (2018): *Complete Genome Sequence of a Novel T7-Like Bacteriophage from a Pasteurella multocida Capsular Type A Isolate*. Curr Microbiol, **75**, 574–579.
7. **CLSI** (2018): *Performance standarts for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; Informational Supplement. 4th ed. CLSI supplement VET08.*
8. **Dolieslager SMJ, Riggio MP, Lennon A, et al** (2011): *Identification of bacteria associated with feline chronic gingivostomatitis using culture-dependent and culture-independent methods*. Vet Microbiol, **148**, 93–98.
9. **Donnio PY, Lerestif-Gautier AL, Avril JL** (2004): *Characterization of Pasteurella spp. strains isolated from human infections*. J Comp Pathol, **130**, 137–142.
10. **Fernandez-Esparrach G, Mascaro J, Rota R, et al** (1994): *Septicemia, peritonitis, and empyema due to Pasteurella multocida in a cirrhotic patient*. Clin Infect Dis, **18**, 486.
11. **Ferreira TS, Moreno LZ, Felizardo MR, et al** (2016): *Pheno- and genotypic characterization of Pasteurella multocida isolated from cats, dogs and rabbits from Brazil*. Comp Immunol Microbiol Infect Dis, **45**, 48–52.
12. **Freshwater A** (2008): *Why your housecat's trite little bite could cause you quite a fright: a study of domestic felines*

on the occurrence and antibiotic susceptibility of Pasteurella multocida. Zoonoses Public Heal, **55**, 507–513.

13. **Ganiere JP, Escande F, Andre G, et al** (1993): *Characterization of Pasteurella from gingival scrapings of dogs and cats*. Comp Immunol Microbiol Infect Dis, **16**, 77–85.
14. **Hatfaludi T, Al-Hasani K, Boyce JDJD, et al** (2010): *Outer membrane proteins of Pasteurella multocida*. Vet Microbiol, **144**, 1–17.
15. **Ivana S, Câmpeanu G, Bogdan AT, et al** (2010): *Characterization of Pasteurella sp. strains by macrorestriction profiles of PFGE bands from oral swab samples*. Rom Biotechnol Lett, **15**, 5034–5041.
16. **Kawamoto E, Sawada T, Maruyama T** (1997): *Evaluation of transport media for Pasteurella multocida isolates from rabbit nasal specimens*. J Clin Microbiol, **35**, 1948–1951.
17. **Kimura R, Hayashi Y, Takeuchi T, et al** (2004): *Pasteurella multocida septicemia caused by close contact with a domestic cat: Case report and literature review*. J Infect Chemother, **10**, 250–252.
18. **Knight DP, Paine JE, Speller DC** (1983): *A selective medium for Pasteurella multocida and its use with animal and human specimens*. J Clin Pathol, **36**, 591–594.
19. **Kouppari G, Garoufi A, Stamos G, et al** (1999): *Pasturella multocida septicemia and meningitis in an infant*. Clin Microbiol Infect, **5**, 101–102.
20. **Magiorakos A, Srinivasan A, Carey RB, et al** (2012): *Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance*. Clin Microbiol Infect, **18**, 268–281.
21. **Masuoka H, Shimada K, Kiyosue-Yasuda T, et al** (2017): *Transition of the intestinal microbiota of cats with age*. PLoS One, **12**, 1-9.
22. **Mohan K, Kelly PJ, Hill FWG, et al** (1997): *Phenotype and serotype of Pasteurella multocida isolates from diseases of dogs and cats in Zimbabwe*. Comp Immunol Microbiol Infect Dis, **20**, 29–34.
23. **Morrissey I, Moyaert H, de Jong A, et al** (2016): *Antimicrobial susceptibility monitoring of bacterial pathogens isolated from respiratory tract infections in dogs and cats across Europe: ComPath results*. Vet Microbiol, **191**, 44–51.
24. **Moyaert H, de Jong A, Simjee S, et al** (2019): *Survey of antimicrobial susceptibility of bacterial pathogens isolated from dogs and cats with respiratory tract infections in Europe: ComPath results*. J Appl Microbiol, **127**, 29–46.
25. **Moyaert H, de Jong A, Simjee S, et al** (2014): *Antimicrobial resistance monitoring projects for zoonotic and indicator bacteria of animal origin: common aspects and differences between EASSA and EFSA*. Vet Microbiol, **171**, 279–283.
26. **Nakanishi H, Furuya M, Soma T, et al** (2019): *Prevalence of microorganisms associated with feline gingivostomatitis*. J Feline Med Surg, **21**, 103–108.
27. **NCCLS** (2004): *Performance Standarts for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals; Informational Supplement. NCCLS document M31-S1*.
28. **Orsini J, Perez R, Llosa A, et al** (2013): *Non-zoonotic Pasteurella multocida infection as a cause of septic shock in a patient with liver cirrhosis: a case report and review of the literature*. J Glob Infect Dis, **5**, 176.
29. **Tamaskar I, Ravakhah K** (2004): *Spontaneous bacterial peritonitis with Pasteurella multocida in cirrhosis: case report and review of literature*. South Med J, **97**, 1113–1115.
30. **Tefera G, Smola J** (2002): *Modification of Cary-Blair Transport Medium for Pasteurella multocida and Mannheimia haemolytica*. Acta Vet Brno, **71**, 229–233.
31. **The European Committee on Antimicrobial Susceptibility Testing.** (2017): Breakpoint tables for interpretation of MICs and zone diameters. Version 7.0. http://www.eucast.org. (Accessed March 12,2020).
32. **Townsend KM, Boyce JD, Chung JY, et al.** (2001): *Genetic organization of Pasteurella multocida cap loci and development of a multiplex capsular PCR typing system*. J Clin Microbiol, **39**, 924-929.
33. **Townsend KM, Frost AJ, Lee CW, et al.** (1998): *Development of PCR assays for species-and type-specific identification of Pasteurella multocidaisolates*. J Clin Microbiol, **36**, 1096–1100.
34. **Ujvári B, Weiczner R, Deim Z, et al.** (2019): *Characterization of Pasteurella multocida strains isolated from human infections*. Comp Immunol Microbiol Infect Dis, **63**, 37–43.

Ankara Univ Vet Fak Derg, **68**, 21-25, 2021
DOI: 10.33988/auvfd.678172

An investigation on the renal portal system in long-legged buzzard (*Buteo rufinus*)

Sedef SELVİLER SİZER[1,a,✉], Murat KABAK[2,b], Burcu ONUK[2,c]

[1]Ondokuz Mayıs University, Graduate School of Health Sciences, Samsun; [2]Ondokuz Mayıs University, Faculty of Veterinary Medicine, Department of Anatomy, Samsun, Turkey.
[a]ORCID:0000-0002-1990-4507; [b]ORCID: 0000-0003-4255-1372; [c]ORCID: 0000-0001-8617-3188.

✉Corresponding author: sedef.selviler@omu.edu.tr
Received date: 21.01.2020 - Accepted date: 15.06.2020

Abstract: This study was carried out for the morphological examination of renal portal system in the long-legged buzzard. A total of nine adult long-legged buzzards were used in the study. The latex procedure was performed for the macroanatomic detection of the vessels forming the renal portal system. Dissections were performed under the stereomicroscope and later photographs were taken. The renal portal system vessels formed by the cranial renal portal vein and caudal renal portal vein in the long-legged buzzard. Afferent renal branches were separated from these vessels and efferent renal branches were observed to join renal veins. The anastomosis of the left cranial renal portal vein and right cranial renal portal vein was not observed at the cranial side, besides the presence of interiliac anastomosis was determined between the right internal iliac vein and left internal iliac vein at the caudal side. The findings of the renal portal system of long-legged buzzard *(Buteo rufinus)* were generally similar to those noted in other birds.
Keywords: Anatomy, long-legged buzzard, renal portal system.

Kızıl şahin'de (*Buteo rufinus*) renal portal sistem üzerine bir araştırma

Özet: Bu çalışma kızıl şahinde renal portal sistemin morfolojik olarak incelenmesi amacıyla yapılmıştır. Çalışmada toplam 9 adet yetişkin kızıl şahin kullanıldı. Renal portal sistemi oluşturan damarların makroanatomik tespiti için latex uygulandı. Diseksiyonlar steromikroskop altında yapıldı ve daha sonra fotoğrafları çekildi. Kızıl şahin'de renal portal sistem damarları, vena portalis renalis cranialis ve vena portalis renalis caudalis'ten oluşmaktaydı. Bu damarlardan afferent renal dalların ayrıldığı ve efferent renal dalların vena renalis'e katıldığı gözlendi. Cranial tarafta vena portalis renalis cranialis dexter ve vena portalis renalis cranialis sinister arasında anastomoz görülmezken, caudal tarafta, vena iliaca interna dexter ve vena iliaca interna sinister arasında (interiliac anastomosis) anastomozun varlığı belirlendi. Kızıl şahin (*Buteo rufinus*) renal portal sisteminin bulguları genel olarak diğer kuşlarda belirtilenlerle benzer bulundu.
Anahtar sözcükler: Anatomi, kızıl şahin, renal portal sistem.

Introduction

The kidneys are responsible for providing body homeostasis by filtering the waste products as a result of metabolism (13, 18). They generally weigh about 1% of body weight and their color changes according to the amount of blood they contain (1, 13). The kidneys of birds which are different from the mammals consist of three lobes symmetrically lying on the renal fossa of the synsacrum (6, 10, 12, 14, 16). Another difference is that, renal portal system in birds consists of a lot of vessels and carries venous blood to the proximal tubule (2, 8, 10, 12, 13). This system plays an important role in nourishing the nephrons (3, 10). Thus, venous blood is transported to the tubules responsible for the creation of the urine (3, 13). Kidneys receive afferent blood that comes from high pressure renal arteries and from low pressure renal portal system (8, 9). Cranial and caudal renal portal veins located in the renal portal system are branches of external iliac vein. While cranial renal portal vein receives venous blood only from the hind limb, the caudal renal portal vein receives venous blood from the hind limb, pelvis, coccyx and caudal mesenteric vein (2). The cranial renal portal vein supplies venous blood to the cranial lobe. Caudal renal portal vein supplies venous blood to the middle and caudal lobes (2). The caudal mesenteric vein is particularly important due to providing a direct connection between the renal portal system and the hepatic portal system (5, 12, 13). The renal portal valve located in the

lumen of the common iliac vein provides the blood flow in the renal portal system. When this valve is open, the portal blood bypasses the kidney and goes to the caudal vena cava (2, 9). When the valve is closed, the blood flows into the renal parenchyma (5, 13).

Although the knowledge about renal portal system in different species is presented, no literature information about the subject has been encountered in long-legged buzzard. Therefore, this study aimed to determine the anatomical structure of renal portal system in long-legged buzzard.

Material and Methods

In this study, 9 adult long-legged buzzards (*Buteo rufinus*), weighing between 900-1520 gr, were examined. The material of our study were collected from long-legged buzzards that were brought to the faculty clinic due to various injuries and died during treatment. The vessels were washed with 0.9% saline solution. Via a cannula, latex colored with red and blue dyes were injected into descending aorta and caudal vena cava, respectively. The materials were left at room temperature 24 h and then fixated with 10% formalin. Veins that belong to the renal portal system were determined by dissection under the stereomicroscope (Model SZ6, Olympus Corporation, Japan). The photographs were taken with the digital camera (Model C-5060, Olympus Corporation, Japan).

Results

In the long-legged buzzard, the kidneys located in renal fossa that extend from the caudal of the lungs to the synsacrum were determined to be composed of three lobes that include cranial, middle and caudal (Figure 1). The length-width of the cranial (17.82±1.79 mm-11.75±1.5 mm), middle (9.63±2.16 mm-5.13±0.70 mm) and caudal (12.13±1.93 mm-9.72±1.84 mm) lobes of the right side and the length- width of the cranial (17.44±2.33 mm-12.51±1.4 mm), middle (8.85±1.47 mm-6.43±3.07 mm) and caudal (12.24±1.39 mm-8.75±2.17 mm) lobes of the left side were measured.

The renal portal system vessels were formed by cranial and caudal renal portal veins in the long-legged buzzard. The cranial and caudal renal portal veins were branches of right and left external iliac veins (Figure 2). The caudal renal portal vein was first removed from the external iliac vein. After average 1.67 mm, cranial renal portal vein was separated from external iliac vein. Immediately after this separation, the external iliac vein formed anastomosis with the caudal renal vein. A narrowing was detected in this area during latex applications. This narrowing was defined as renal portal valve (Figure 2). The external iliac vein continued as the common iliac vein after the renal portal valve and then opened to the caudal vena cava. Afferent renal branches were separated from both cranial renal portal vein and caudal renal portal vein and spread into the kidneys (Figure 2). While the afferent branches of cranial renal portal vein entered only to cranial kidney lobe, the afferent branches of caudal renal portal vein entered to both middle and caudal kidney lobes. Efferent branches were observed to join renal veins. The caudal renal vein was continued side by side with the ureter (Figure 3). It was determined that the ischiadic vein joined the caudal renal portal vein at the cranial level of the caudal renal lobes (Figure 3). And then, it was observed that this vessel, which has gone through the caudal lobe, was anastomosed with the internal iliac vein at the caudal level of the renal lobe. Right and left internal iliac veins joined at caudal level of the caudal lobe and formed an interiliac anastomosis (Figure 4). It was observed that the caudal mesenteric vein was opened to this anastomosis region (Figure 4).

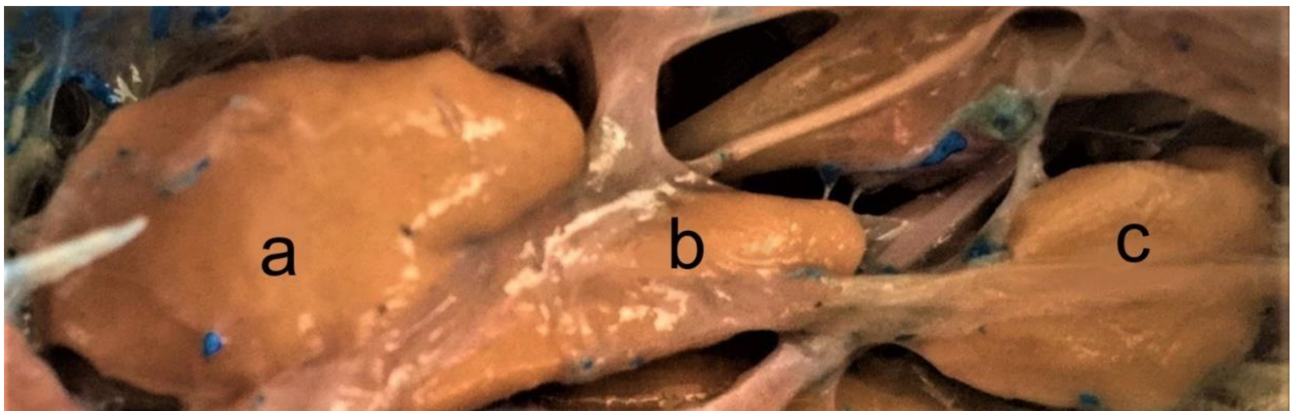

Figure 1. Ventral view of the kidneys. a: left cranial lobe; b: left middle lobe; c: left caudal lobe.

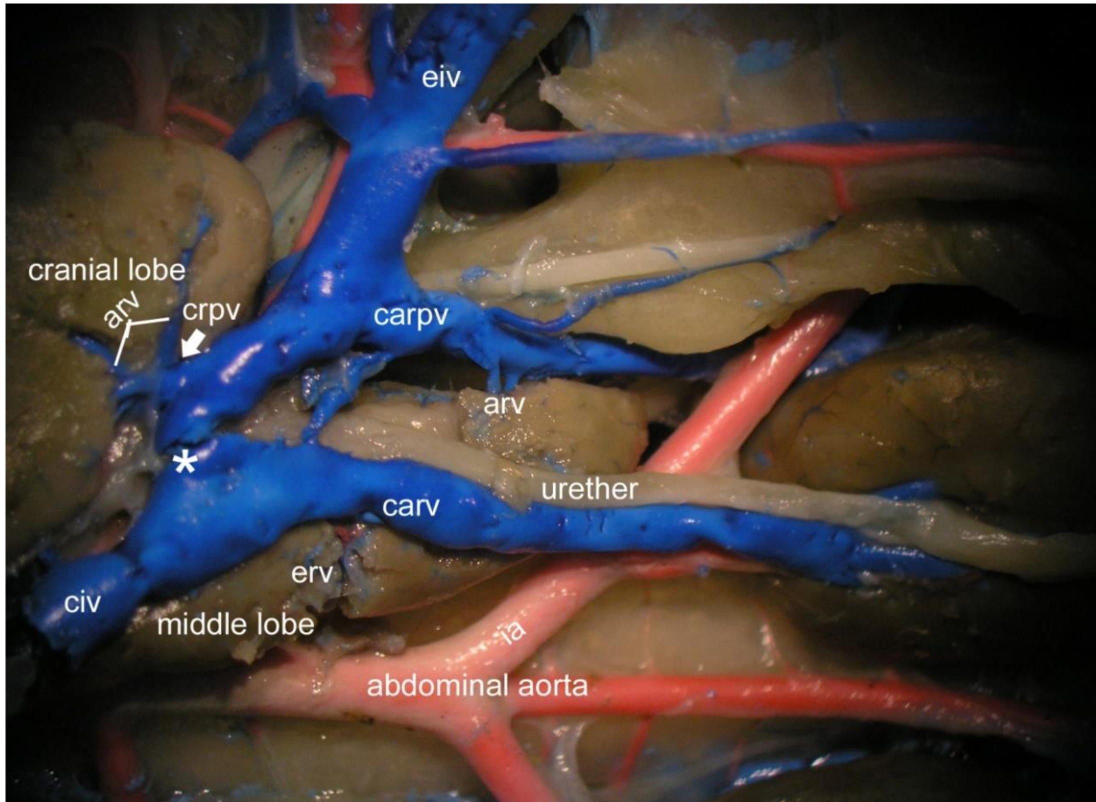

Figure 2. A view of the vessels associated with the kidneys. eiv: external iliac vein; civ: common iliac vein; crpv: cranial renal portal vein; carpv: caudal renal portal vein; carv: caudal renal vein; erv: efferent renal vein; arv: afferent renal vein; ia: ischiadic artery; *: renal portal valve.

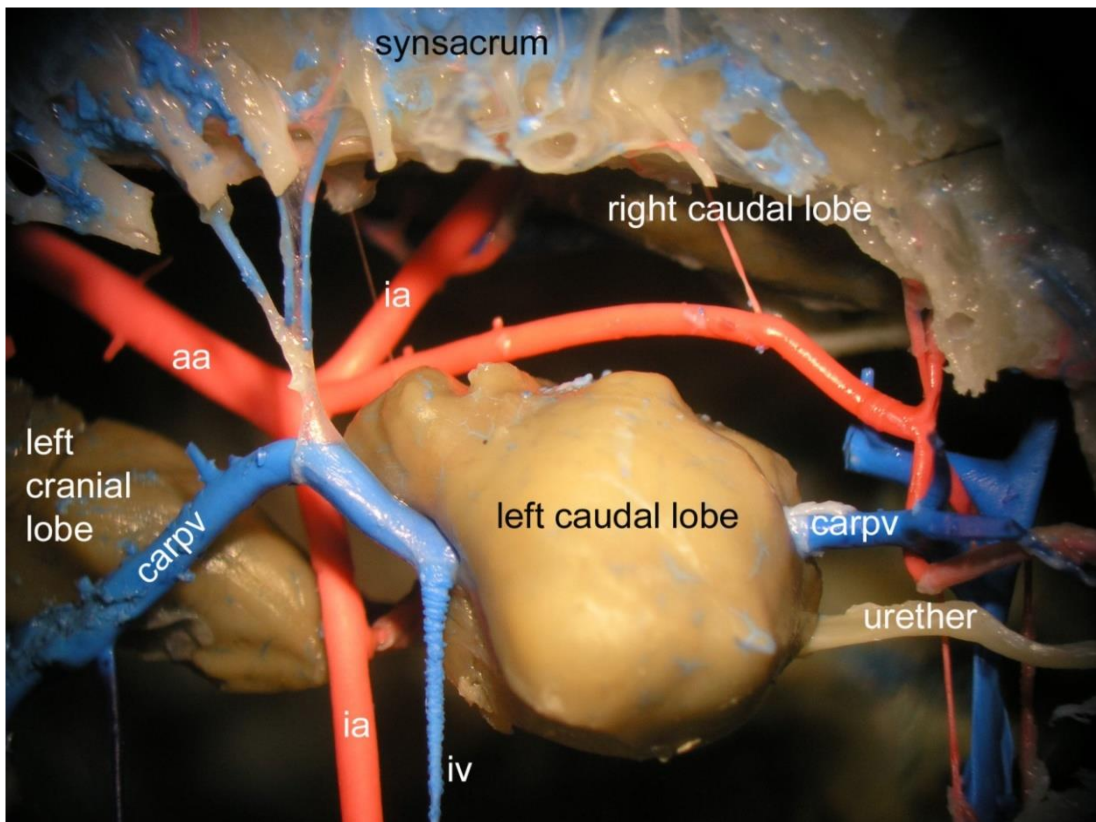

Figure 3. Lateral view of the vessels associated with the kidneys. carpv: caudal renal portal vein; aa: abdominal aorta; ia: ischiadic artery; iv: ischiadic vein.

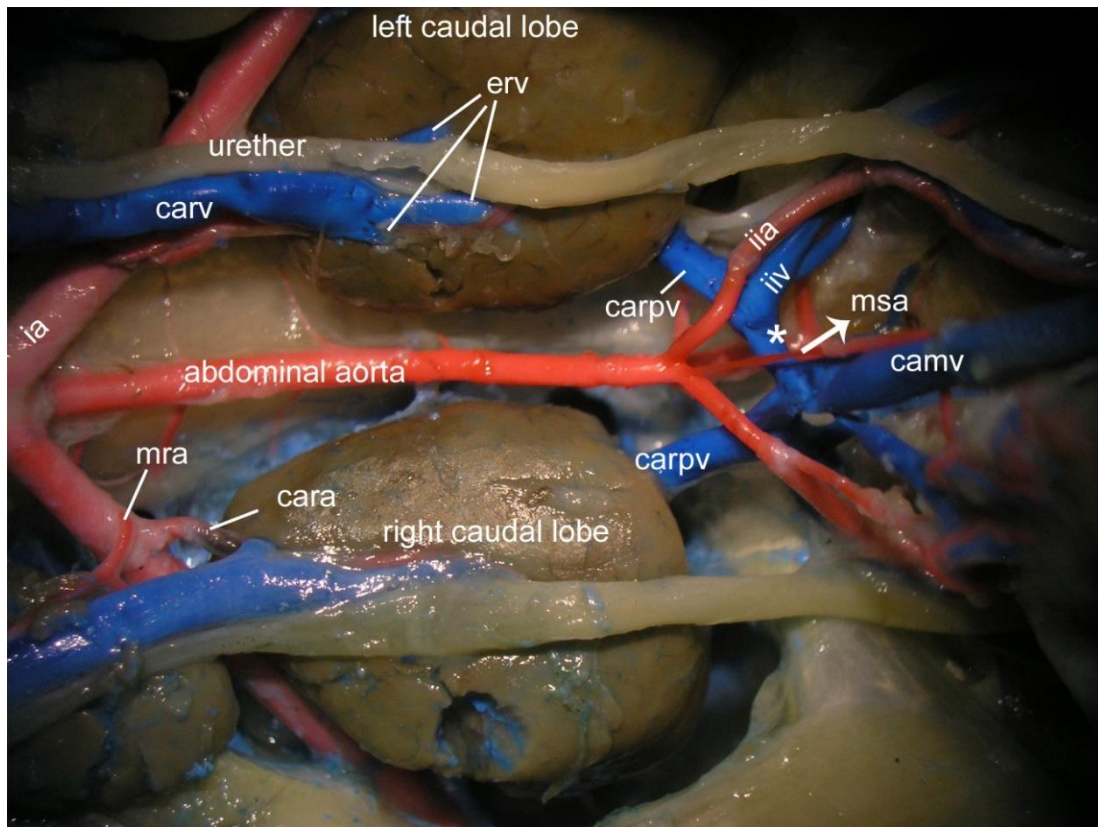

Figure 4. Ventral view of the vessels associated with the kidneys. carv: caudal renal vein; erv: efferent renal vein; carpv: caudal renal portal vein; camv: caudal mesenteric vein; ia: ischiadic artery; mra: middle renal artery; cara: caudal renal artery; iia: internal iliac artery; iiv: internal iliac vein; msa: middle sacral artery; *: interiliac anastomosis.

Discussion and Conclusion

Although the number of kidney lobes was reported as two lobes in hornbill (11), in many species, such as coot bird (7), domestic fowl (15), emu (14), harrier (1), mallard duck (1), and golden eagle (4), it has been reported that it consists of three lobes. In our study, the kidneys of the long-legged buzzard were composed of three lobes were similar to many other species. It was mentioned that kidneys were located in the renal fossa, which extends from the caudal of lungs to the synsacrum, in some species such as rock dove (16), flamingos (17), coot bird (7), owl (16) and harrier (1). This localization of the kidneys was compatible with the above-mentioned literatures.

The renal portal system in avian, which is different from mammals, allows the excretion of uric acid by tubular secretion (10, 13, 17). The vessels in this system may differ between species (5, 8, 12). The cranial and caudal renal portal veins were separated from the external iliac vein on both the right and left sides in pigeon (5) and domestic fowl (3). There were differences at the right and left sides in cape griffon vulture (12). At left side, the cranial renal portal vein, the caudal renal portal vein and caudal renal vein arose from the common iliac vein. At right side, the caudal renal vein and the caudal renal portal vein were firstly joined and then entered to the common iliac vein. In our study, cranial and caudal renal portal veins and caudal renal veins were similar to pigeon and domestic fowl when looking at the exit points. There was no differences at the right and left sides. It has been reported that, while afferent renal branches are separated from renal portal veins, efferent renal branches join the renal veins (12, 13). In the long-legged buzzard, it was found similar to those mentioned in Havenga (12) and King and McLelland (13). King and McLelland (13) stated that the presence of a venous ring occurs by anastomosis of the left and right cranial renal portal veins in the cranial side. In pigeon (5), there is no mention of such a venous ring. In this study, the venous ring was not observed, so it was similar to that mentioned in the pigeon. It has been reported that the renal portal valve provides blood flow into the renal portal system (2, 9, 13). There are differences in the literature about the localization of the renal portal valve. It has been stated that this valve is located in the lumen of the common iliac vein by Havenga (12) in cape griffon vulture and Canny (9) in avian. In our study, same valve found in lumen before forming anastomosis between the external iliac and renal veins as stated in domestic fowl by Akester (2).

The caudal renal portal vein which is separated from the external iliac vein forms anastomosis with the internal

iliac vein on both sides and in caudal side. Then, both internal iliac veins merge to form the interiliac anastomosis. This interiliac anastomosis is involved with the caudal mesenteric vein (cocygeomesenteric) that has a direct connection between the renal portal system and the hepatic portal system (5, 12). The course and connections of the caudal renal portal vein mentioned above were compatible with our study.

With this study, for the first time, the morphological structure of the renal portal system in the long-legged buzzard was examined in details. The renal portal system consisted of cranial and caudal renal portal veins. The results of this study will contribute to the anatomy literature and will help researchers to work on the subject.

Acknowledgements

A part of this study is presented as oral presentation at the "5th International Group Congress & 8th International Scientific Meeting Vet-Istanbul Days of Veterinary Medicine" on the 23-27th September 2018.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Abood DA, Ali FR, Azhar SK, et al** (2014): *Comparative anatomical and histological features of the kidney in harrier (Circus aueroginosus), chicken (Gallus domesticus) and mallard duck (Anas platyrhynchos).* The Iraqi J Vet Med, **38**, 107-113.
2. **Akester AR** (1964): *Radiographic studies of the renal portal system in the domestic fowl (Gallus domesticus).* J Anat, **98**, 365-376.
3. **Akester AR** (1967): *Renal portal shunts in the kidney of the domestic fowl.* J Anat, **101**, 569-594.
4. **Al-Agele RA** (2012): *Study the anatomical descriptions and histological observations of the kidney in golden eagles (Aquila Chrysaetos).* The Iraqi J Vet Med, **36**, 145–152.
5. **Al-Ajeely RA, Mohammed FS** (2012): *Morpho-histological study on the development of kidney and ureter in hatching and adulthood racing pigeon (Columba livia domestica).* Int J Sci Nature, **3**, 665-677.
6. **Bahadır A, Yıldız H** (2015): Veteriner Anatomi. Ezgi Kitabevi, Bursa.
7. **Batah AL** (2012): *Morphological and histological study for the kidneys of coot bird (Fulica atra).* Bas J Vet Res, **11**, 128-136.
8. **Blackburn R, Prashad D** (1990): *The avian renal portal system: A model for studying nephrotoxicity of xenobiotics.* Toxicol Lett, **53**, 219-221.
9. **Canny C** (1998): *Gross anatomy and imaging of the avian and reptilian urinary system.* Semin Avian Exot Pet Med, **7**, 72-80.
10. **Dursun N** (2007): Evcil kuşların anatomisi. Medisan Yayın Serisi, Ankara.
11. **Feinstein B** (1962): *Additional cases of bilobated kidneys in the hornbills.* The Auk, **79**, 709-711.
12. **Havenga LN** (2015): *A morphological study of the kidney and renal portal system of the Cape griffon vulture (Gyps coprotheres).* MSc dissertation. University of Pretoria, South Africa.
13. **King AS, McLelland J** (1984): Birds, Their Structure and Function. Bailliere Tindall Ltd, London.
14. **Michalek K, Szczerbinska D, Grabowska M, et al** (2016): *Anatomical and morphological study of the kidneys of the breeding emu (Dromaius novaehollandiae).* Turk J Zool, **40**, 314-319.
15. **Morild I, Bohle A, Christensen JA** (1985): *Structure of the avian kidney.* Anat Rec, **212**, 33-40.
16. **Nabipour A, Alishahi E, Asadian M** (2009): *Some histological and physiological features of avian kidney.* J Appl Anim Res, **36**, 195-198.
17. **Reshag AF, Abood DA, Dawood MS** (2016): *Anatomical and histological study of the kidneys and salt glands in great flamingos (Phoenicopterus roseus).* The Iraqi J Vet Med, **40**, 140-146.
18. **Simon E** (1982): *The osmoregulatory system of birds with salt glands.* Comp Biochem Physiol, **71**, 547–556.

Ankara Univ Vet Fak Derg, **68**, 27-32, 2021
DOI: 10.33988/auvfd.680443

Effects of epidural bupivacaine, bupivacaine-butorphanol and bupivacaine-morphine on postoperative analgesia for hind limb and pelvic orthopedic operations in dogs

Birkan KARSLI[1,a,✉], Zeynep PEKCAN[1,b], Ali KUMANDAŞ[1,c], Barış KÜRÜM[1,d], Miyase ÇINAR[2,e]

[1]University of Kırıkkale, Faculty of Veterinary Science, Department of Surgery, Kırıkkale; [2]University of Kırıkkale, Faculty of Veterinary Science, Department of Biochemistry, Kırıkkale, Turkey.
[a]ORCID: 0000-0003-4208-3134; [b]ORCID: 0000-0003-1047-5280; [c]ORCID: 0000-00027679-2126;
[d]ORCID: 0000-0002-5559-7815; [e]ORCID: 0000-0003-3806-9938.

✉Corresponding author: birkankarsli@gmail.com
Received date: 27.01.2020 - Accepted date: 16.06.2020

Abstract: The aim of this study was to compare the postoperative analgesic effects of epidural bupivacaine, bupivacaine-butorphanol and bupivacaine-morphine in hind limb and pelvic orthopedic operations in dogs. Three groups each containing 10 dogs were formed. Through the lumbosacral space, group B was administered bupivacaine, 1 mg kg^{-1}; group BB was administered bupivacaine, 1 mg kg^{-1} and butorphanol, 0.25 mg kg^{-1}; and group BM was administered bupivacaine, 1 mg kg^{-1} and morphine, 0.1 mg kg^{-1}. Anaesthesia was induced with diazepam (0.2 mg kg^{-1}) and propofol (5 mg kg^{-1}) and maintenance with isoflurane in oxygen. Pain assessment was performed with the University of Melbourne Pain Scale (UMPS) by a blinded observer. Plasma cortisol levels were measured preoperatively and postoperatively. Postoperative pain scores were significantly lower at 2 hours in the group BM, and at 8 and 12 hours in the groups BB and BM compared to the group B (P=0.007). Group BM had a lower plasma cortisol level than the group B at 2 hours (P=0.002). Bupivacaine-butorphanol and bupivacaine-morphine use were shown to provide longer analgesia in the postoperative period; and the epidural administration of bupivacaine-butorphanol and bupivacaine-morphine was considered to be potentially useful in clinical practice.
Keywords: Analgesia, dog, epidural, opioid, pain assessment.

Köpeklerde arka ekstremite ve pelvisin ortopedik operasyonlarında epidural bupivakain, bupivakain-butorfanol ve bupivakain-morfin uygulamasının postoperatif analjezi üzerine etkisi

Özet: Bu çalışmanın amacı, köpeklerde arka ekstremite ve pelvisin ortopedik operasyonlarında epidural bupivakain, bupivakain-butorfanol ve bupivakain-morfin uygulamasının postoperatif analjezik etkilerinin karşılaştırılmasıdır. Her grupta 10 köpek olacak şekilde 3 farklı çalışma grubu oluşturuldu. Lumbosakral aralıktan grup B'ye bupivakain 1 mg kg^{-1}; grup BB'ye bupivakain 1 mg kg^{-1} ve butorfanol 0,25 mg kg^{-1}; grup BM'ye ise bupivakain 1 mg kg^{-1} ve morfin 0,1 mg kg^{-1} uygulaması yapıldı. Anestezi indüksiyonu diazepam (0,2 mg kg^{-1}) ve propofol (5 mg kg^{-1}) ile yapıldıktan sonra idame izofloran ile sağlandı. Ağrının değerlendirilmesi kör gözlemci tarafından Melbourne Üniversitesi ağrı skalası (UMPS) kullanılarak yapılmıştır. Preoperatif ve postoperatif plazma kortizol seviyeleri ölçüldü. BM grubunun postoperatif 2. saatte plazma kortizol seviyesi B grubuna göre daha az olarak tespit edildi (P=0,002). Postoperatif ağrı skorları 2. saatte BM grubunda, 8. ve 12. saatte BB ve BM grubunda B grubuna göre daha az olarak tespit edildi (P=0,007). Çalışma sonucunda bupivakain-butorfanol ve bupivakain-morfin kullanımının postoperatif dönemde sadece bupivakain kullanımına göre daha uzun süreli analjezi sağladığı ve bupivakain-morfine göre daha düşük yan etkiye sahip olan bupivakain-butorfanolun epidural uygulamasının pratikte kullanılmasının faydalı olabileceği düşünülmüştür.
Anahtar sözcükler: Ağrı değerlendirmesi, analjezi, epidural, köpek, opioid.

Introduction

Multimodal analgesia with the combined use of opioid, local anesthetics and nonsteroidal anti-inflammatory drugs (NSAIDs) is the current approach for pain management related to orthopedic surgical interventions. In veterinary practice, epidural applications of local anesthetics and opioids are widely used to maintain analgesia in pelvic and hind limb operations (17). Local anesthetics block nerve conduction, affecting both tactile and pain perception while opioids affect opioid

receptors in the medulla spinalis to reduce or eliminate pain (1). Therefore, anesthetic-analgesic effect produced by the combined use of a local anesthetic and an opioid is greater than the use of either agent alone (5,17).

Fractures may induce very severe pain, and opioids are the most suitable option for this purpose (6). All opioid analogs' analgesic efficacy and side effects depend on the ratio of the opioid receptors and their drug-binding capacity. For example, epidural use of μ-receptor agonist opioids like morphine provides postoperative analgesia (16-24 hours) but may also induce side effects like pruritus, nausea, vomiting, urinary retention and respiratory depression (17). On the other hand, epidural use of butorphanol, a strong κ-receptor agonist and a weak μ-receptor agonist-antagonist, produces a morphine-like analgesic effect but less side effects (15). The duration of action of epidural butorphanol is 80-240 minutes in dogs but it lasts longer when used in combination with local anesthetics (16,18).

Several subjective pain scoring methods are widely used in veterinary medicine on the interpretation of animal behavior by the observer. University of Melbourne Pain scale is also one of them which is regarded as more sensitive and more accurate than many descriptive and numerical rating scale reliable method of clinical pain assessment and has been used with success in dogs (8, 9). Cortisol production increase in pain and stress. Despite its limitation, serum cortisol concentration is recognized as an indicator and most objective criteria pain assessment in dogs (3, 10).

This study aimed to investigate the analgesic effects of bupivacaine, bupivacaine-butorphanol and bupivacaine-morphine combined with bupivacaine for pelvic and hind limb orthopedic operations in dogs.

Material and Methods

This study was approved by Kırıkkale University Clinical Practice Ethics Committee (15/19) and the animal owners were informed about the study design.

***Animals*:** The study included 30 dogs (mean age 20.67±18, mean weight 22.43±20) older than 8 months admitted to Veterinary Faculty Animal Hospital for hind limb or pelvic fractures or hind limb joint problems. According to the physical examination and biochemical test results, the dogs with abnormal blood results, coagulopathy, dermatitis on lumbosacral region were excluded from the study. The sample size of the study was calculated with the G*Power (ver. 3.1.9.2, Franz Faul, Universität Kiel, Germany) statistical analysis. The required sample size for 85% power, α = 0.05 type I error, β = 0.147 and sample size was calculated as 30.

Study protocol: The animals were randomly assigned the animals into three groups of 10 animals each, based on a computer-generated randomization schedule. The groups were nor based on gender.

Group B: 1 mg kg^{-1} bupivacaine 0.5% (Marcaine; AstraZeneca, England) and 0.22 ml kg^{-1} isotonic sodium chloride 0.9% (Isotonic NaCl; Eczacıbaşı Baxter, Turkey) were mixed.

Group BB: 1 mg kg^{-1} bupivacaine 0.5% (Marcaine; AstraZeneca, England), 0.25 mg kg^{-1} butorphanol 1% (Butomidor; Richterpharma, Austria), and 0.22 ml kg^{-1} isotonic sodium chloride 0.9% (Isotonic NaCl; Eczacıbaşı Baxter, Turkey) were mixed.

Group BM: 1 mg kg^{-1} bupivacaine 0.5% (Marcaine; AstraZeneca, England), 0.1 mg kg^{-1} morphine %1 (Morphine; Galen İlaç, Turkey), and 0.22 ml kg^{-1} isotonic sodium chloride 0.9% (Isotonic NaCl; Eczacıbaşı Baxter, Turkey) were mixed.

Epidural injections were carried out using a Tuohy needle (Kairos, Turkey) by the same anesthetist. During injections, the patients were sternally positioned, and the accuracy of injection site was determined using loss of resistance and hanging drop technique. Maximum epidural injection volume was determined as 12 ml (9).

General anaesthesia procedure: Oral feeding was stopped 12 hours prior to anaesthesia but no water restriction was applied. All patients had a catheter placed to *vena cephalica antebrachium* for anaesthesia induction and fluid support during operation. Anaesthesia induction was performed via intravenous (IV) route using 0.2 mg kg^{-1} diazepam (Diazem; Deva İlac, Turkey) and 5 mg kg^{-1} propofol (Propofol 2% Fresenius; Fresenius Kabi, Austria). After endotracheal tube intubation, anaesthesia maintenance was achieved using volatile isoflurane in 100% oxygen and vaporizer set at 1.5-2.5% (Isoflurane; Adeka İlac, Netherlands), using a semicircle system (SMS company, Turkey) and a fresh gas flow of 2 L minute^{-1}. Balanced electrolyte solution (Ringesol; Vilsan, Turkey) was administered at a rate of 10 ml kg^{-1} hour^{-1} by IV route. Meloxicam (Maxicam; Sanovel, Turkey) (0.2 mg kg^{-1}) was administered via IV route 5 minutes prior to the operation.

Perioperative heart rate (HR) and rhythm, respiratory rate (RR), end-tidal CO_2 ($P_E{'}CO_2$), rectal body temperature, arterial haemoglobin saturation (SpO2), and systolic (SAP), diastolic (DAP), mean arterial blood pressure (MAP) were monitored (Petaş KMA 900, Turkey) non-invasively to ensure anaesthesia safety.

In order to determine perioperative plasma cortisol levels blood samples were drawn into lithium heparin tubes before anaesthesia induction (baseline), at the end of the operation (T0), and postoperative 2nd (T2), 8th (T8) and 24th (T24) hours. Blood samples were stored at -70°C and analyzed within 6 months. Canine cortisol ELISA kit (Cusabio; Cusabio Biotech Ltd, China) was used to determine cortisol concentration by assay procedure. The

patients were taken to the recovery room after the endotracheal tube was removed once swallowing reflex returned.

Pain assessment: Pain scores were assessed using a modified University of Melbourne Pain Scale (UMPS) 0-20 (9). All pain assessments and measurements were performed by the same individual and blinded observer. Pain observations were recorded before premedication in the preoperative (baseline, before premedication) and postoperative period; extubation time was considered the zero-point (T0), and at 30th minute (T0.5) and at 1st (T1), 2nd (T2), 4th (T4), 8th (T8), 12th (T12), 16th (T16), and 24th (T24) hours thereafter. The time of recovery of motor functions and first urination after epidural injection were recorded. Motor functions were assessed using positive withdrawal pedal reflex, strong resistance to flexion, anal sphincter reflex and tail motion. When any pain criterion on the scale was equal to or greater than 3 or the total pain score was equal to or greater than 9 (maximum score 20) analgesia failure was considered. In case of analgesia failure, an additional analgesic (1 mg kg^{-1} morphine) was administered by intramuscular route and that case was excluded.

Statistical analysis: The statistical analyses were performed using the SPSS v15 (SPSS Inc. IL, USA) statistical software. The data were tested for normality using a Shapiro–Wilk test and results showed that they did not meet the parametric test assumptions. Therefore nonparametric test was used. Age, body weight, baseline HR, RR, and rectal body temperature and intraoperative HR, RR, rectal body temperature, $P_E'CO_2$, and SpO2 levels as well as the time to recovery of postoperative motor functions, time to urination, and perioperative plasma cortisol levels were compared using the Kruskal-Wallis test and a $P < 0.05$ was considered statistically significant. For the UMPS levels, the nonparametric Kruskal-Wallis test was used for inter-group comparisons. Paired group comparisons were performed using the Mann-Whitney U test. Bonferroni correction was applied to paired comparisons and $P < 0.017$ was considered statistically significant.

Results

Our study included 21 Kangal dogs, 2 Labrador retrievers, 5 Setters, and 2 Golden retrievers. Of these animals, 14 animals underwent osteosynthesis for tibia and femur fracture; 14 excision arthroplasties for acetabulum fracture or chronic coxofemoral luxation; and 2 Paatsama procedure for rupture of anterior cruciate ligament.

One of the animals in the group BM required additional analgesia during recovery from anaesthesia and thus excluded from the study. All other animals recovered from anaesthesia without any problem.

No significant difference was noted among the animals with respect to age and weight ($P > 0.05$). Baseline HR, RR, rectal body temperature and total anaesthesia time were similar among the groups ($P>0.05$) (Table 1).

No significant differences were found among the groups with respect to intraoperative SpO2, $P_E'CO_2$ and rectal body temperature ($P>0.05$). There was a significant difference between the RR values ($P<0.01$) (Table 2).

There was no significant difference between the intraoperative HR with respect to baseline value ($P>0.05$) (Table 3).

The comparison of perioperative plasma cortisol levels was within the reference range (0-138 nmol L^{-1}). Inter-group analysis revealed that group BM had a significantly lower cortisol level than group B at postoperative second hour (T2) ($P=0.002$) (Table 4).

No significant difference was evident among the groups with regard to time of postoperative urination and recovery of motor function ($P > 0.05$).

Inter-group analysis of UMPS levels at prespecified time points revealed that group BM had a significantly less severe pain score than group B at T2 ($P=0.007$). In addition, group BB and BM had lower pain scores than group B at T8 and T12 ($P\leq0.007$). In this respect, no significant differences were found between the groups with respect to pain scores at other time points (Table 5).

Table 1. Characteristics and anaesthesia time of dogs (n = 10)

Variable	B	BB	BM	*P*-Value*
Age (months)	24.86±24	22.86±24	14.29±12	0.18
Body weight (kg)	21.86±21	22.14±20	23.29±18	0.98
Baseline HR (beats $minutes^{-1}$)	111.71±120	111.43±120	109.43±112	0.82
Baseline RR (breaths $minute^{-1}$)	29.57±25	23.71±24	24.57±24	0.56
Baseline rectal temp.(˚C)	38.78±38.7	38.13±38.3	38.62±38.6	0.14
Anaesthesia time (minutes)	95.71±105	98.71±92	95.00±90	0.93

Data are given as mean±median. B (Bupivacaine), BB (Bupivacaine-butorphanol), BM (Bupivacaine-morphine), RR (Respiration rate), HR (Heart rate).

Table 2. Intraoperative respiration rate (RR), arterial saturation of hemoglobin (SpO2), end tidal carbon dioxide ($P_E{'}CO_2$) and rectal body temperature values in dogs (n = 10)

Variable	B	BB	BM	*P*-Value*
RR (breaths minute^{-1})	20.86±20[a]	13.14±13[b]	15.43±16[b]	0.01
SpO2 (%)	94.57±95	93.43±93	94.86±96	0.48
$P_E{'}CO_2$ (mm Hg)	40.57±41	42.86±43	42.57±43	0.16
Rectal temp.(˚C)	37.6±37.7	36.84±36.7	37.38±37.4	0.06

[a,b] Differences between the groups denominated by different letters in the same line are significant. Data are presented as mean±median. B (Bupivacaine), BB (Bupivacaine-butorphanol), BM (Bupivacaine-morphine)

Table 3. Preoperative and intraoperative mean heart rate at various time points in dogs (n=10)

Time	B	BB	BM
Baseline	111.71±120	111.43±120	109.43±112
T0	103.14±110	107.29±100	111.57±112
T30	103.71±108	99.43±104	112.57±115
T60	108.14±108	101.14±102	115.14±114
Extubation	105.14±104	102.71±101	108.43±108
P*-Value	0.60	0.66	0.88

Data are presented as mean±median. T0 (before incision), T30 (30th minutes in operation), T60 (60th minutes in operation). B (Bupivacaine), BB (Bupivacaine-butorphanol), BM (Bupivacaine-morphine)

Table 4. Perioperative mean plasma cortisol concentration values (nmol L^{-1}) at various time points (n = 10)

Time	B	BB	BM	*P*-Value*
Baseline	49.91±67.74	49.47±50.20	39.10±45.30	0.87
T0	39.01±47.44	34.60±22.58	26.46±31.32	0.86
T2	47.74±52.68[a]	36.95±23.12[ab]	18.41±17.62[b]	0.002
T8	68.35±59.86	37.74±17.74	32.46±36.13	0.69
T24	52.38±57.76	43.56±36.64	36.14±42.23	0.68

[a, b] Differences between the groups denominated by different letters in the same line are significant. Data are presented as mean±median. B (Bupivacaine), BB (Bupivacaine-butorphanol), BM (Bupivacaine-morphine), T0 (extubation time), T2 (postoperative 2nd hours), T8 (postoperative 8th hours), T24 (postoperative 24th hours).

Table 5. Postoperative pain assessment for the University of Melbourne Pain Scale (UMPS) (range 0-20) (n = 10)

Time (hours)	B	BB	BM	*P*-Value
T0	0.00±1	0.00±1	0.00±1	P=1.00
T0.5	1±1	0.00±1	1±1	P=0.70
T1	1±1	1±1	1±1	P=0.25
T2	2±1[a]	1±1[ab]	1±1[b]	P=0.007
T4	3±1	2±0	2±1	P≥0.023
T8	3±1[a]	2±1[b]	2±1[b]	P≤0.007
T12	3±1[a]	2±1[b]	2±1[b]	P≤0.007
T16	3±1	2±0	2±0	P≥0.037
T24	2±0	2±1	2±1	P=0.36

[a, b]: Differences between the groups denominated by different letters in the same line are significant. Data were presented as median±interquartile range. B (Bupivacaine), BB (Bupivacaine-butorphanol), BM (Bupivacaine-morphine), T0 (extubation time), T0.5 (postoperative 30th minutes), T1 (postoperative 1st hour), T2 (postoperative 2nd hours), T4 (postoperative 4th hours), T8 (postoperative 8th hours), T12 (postoperative 12th hours), T16 (postoperative 16th hours), T24 (postoperative 24th hours)

Discussion and Conclusion

In animals, epidural injections are performed with spinal needles or Tuohy needle. The latter is specifically designed for epidural puncture and allows feeling the ligamentum flavum (10). In the present study, epidural injections were performed successfully with the hanging drop technique using a Tuohy needle. A dog in the group BM recovered from anaesthesia in an agitated state and was excluded from the study after administering additional analgesic with parenteral route. This was thought to have occured due to irregular distribution of the administered drug in the epidural space secondary to congenital or acquired problems affecting spinal cord, i.e. abnormal amount of epidural fat, stenosis of epidural space and to the effect of gravity (18).

Opioids are the most effective drugs for postoperative analgesia and it has been reported that epidural opioid administration had a longer duration of action than the parenteral route (11,14). It is also known that the combined use of opioids with local anesthetics provide higher quality and longer analgesia (5). As before mentioned epidurally administered bupivacaine, butorphanol, and morphine exert analgesic effect through different mechanisms. Although morphine use has been shown to reduce pain intensity in fracture cases, this effect has been reported to be inadequate. Therefore, the combined use of morphine with other analgesics as part of multimodal analgesia may be more beneficial (6). Some studies have reported that when preoperative analgesia was administered, patients had less pain and also required fewer analgesic substances in the postoperative period (10,17). In previous studies, it was shown that epidural butorphanol produced short analgesic effect when administered alone but had a prolonged analgesic effect when used in combination with local anesthetics (4). In the present study, we demonstrated that the use of bupivacaine alone had a shorter duration of analgesic action but the bupivacaine-butorphanol and bupivacaine-morphine combinations produced a more sustained analgesic effect. Although having a shorter duration of analgesic effect, butorphanol's longer analgesic action in the postoperative period is thought to be possibly related to the favorable effect of preemptive analgesia on postoperative pain. In line with the above data, we demonstrated that the combined use of butorphanol and bupivacaine in dogs also produced a longer analgesic effect (in current study 8-12 hours) than the use of butorphanol alone (80-240 min.) (18).

There are a limited number of studies about the epidural use of butorphanol in animals. It has been reported that after an epidural butorphanol administration, plasma butorphanol concentration was higher than cerebrospinal fluid concentration in dogs. It is thought that the reason for the low concentration of the butorphanol in cerebrospinal fluid is due to the fact that is diluted by the production of cerebrospinal fluid continuously. But it was shown that absence of sensitivity was observed in hind limbs for 3 hours after epidural administration of butorphanol. (16).

It is known that opioids are widely used for postoperative pain control in veterinary practice. However, parenteral opioid use produces side effects like sedation, bradycardia, respiratory depression, vomiting, defecation, urinary retention, pruritus and ileus (19). It has been reported that epidural opioid administration reduced the rate of opioid-induced side effects and produced a longer analgesic effect (7). In the present study the mean respiratory rate was 20 $minute^{-1}$ in the bupivacaine group but was 13 and 15 per minute in the bupivacaine-butorphanol and bupivacaine-morphine groups, respectively. Considering that dogs' respiratory range under anaesthesia is 6-12 per minute (13), it was evident that the intraoperative range of respiratory rate in the present cases was out of the reference limits, suggesting an inadequate anaesthesia depth; however, as intraoperative $P_E'CO_2$ and SpO2 levels as well as HR were within reference range, this condition was not attributed to analgesia and thus no extra analgesic was deemed necessary.

Preoperative plasma cortisol concentration being in the reference range may be considered as a sign of an animal being minimally distressed (10). It has been reported that plasma cortisol concentration is a nonspecific indicator of stress and pain in animals; several studies have specifically dealt with the effects of pain on plasma cortisol levels of dogs, but still, until better biomarkers are found about stress and pain, plasma cortisol concentrations remains important in studies about analgesia (5, 12). It has been reported that serum cortisol levels were lower among animals that were administered analgesics for pain control after orthopedic operations (2). Epidural morphine was used for analgesia in ovariohisterectomy operation in dogs and plasma cortisol level was found to return to normal by 6 hours postoperatively (10). The results of this study showed that plasma cortisol level at the postoperative second hour was lower in the group B compared to groups BB and BM. This was considered to be related to a delayed and longer analgesic effect of morphine.

University of Melbourne pain scale is widely used for postoperative pain assessment (2), takes into consideration an animal's general appearance, appetite, salivation status, whining, reaction to palpation in the operation site, pulse and respiratory rate with mental state, and body temperature (2, 6). However, some researchers have informed that fear, stress, and concerns may alter physiological variables like HR and RR considered in rating of pain. The animal's environment and season may

also be an important factor for body temperature shifts and while rating of pain these factors should also be considered for the sake of accuracy of the results (3). Odette and Smith (9), in a study of pelvic orthopedic operations in dogs, reported that epidural bupivacaine alone produced less analgesic effect than its combined use with morphine, and this difference was statistically significant at postoperative first hour. The present study pain severity was evaluated utilized UMPS pain scale and in agreement with the above-mentioned study (9), an animal's whining, excess salivation, and mental and physical state were recorded at specified intervals during intra and postoperative periods. As a result, it was demonstrated that pain severity did not significantly differ by the second hour but animals in the bupivacaine-morphine group had less severe pain at the second hour postoperatively. It was shown that pain was less severe in the groups bupivacaine-butorphanol and bupivacaine-morphine at the 8th and 12th hours postoperatively than the group bupivacaine.

In conclusion, epidural bupivacaine, bupivacaine-butorphanol, and bupivacaine- morphine administrations all were shown to provide adequate analgesia in hind limb or pelvic operations in dogs. It is noteworthy to mention that bupivacaine-butorphanol and bupivacaine-morphine combinations had more prolonged analgesic action than the stand-alone use of bupivacaine, which is regarded as a supporting point for the use of the combination. As a result, the combined use of local anesthetics and butorphanol, via epidural route was noted to be potentially more useful in clinical practice.

Financial Support

This research was supported by the Scientific Research Project Coordination Unit of Kırıkkale University (2015/126).

Ethical Statement

This study was approved by Kırıkkale University Clinical Practice Ethics Committee (15/19).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Gaynor JS, Muir WW** (2009): Handbook of Veterinary Pain Managmement (2nd ed). Mosby, USA.
2. **Grisneaux E, Pibarot P, Dupuis J**, et al (1999): *Comparison of ketoprofen and carprofen administered prior to orthopedic surgery for control of postoperative pain in dogs.* J Am Vet Med Assoc, **215**, 1105-1110.
3. **Holton LL, Scott EM, Nolan AM, et al** (1998): *Relationship between physiological factors and clinical pain in dogs scored using s numeric rating scale*. J Small Anim Pract, **39**, 469-474.
4. **Kar P** (2016): *A comparative study between epidural butorphanol with bupivacaine and bupivacaine alone for intra-operative and post-operative analgesia in lower limb orthopaedic surgeries*. Int J Res Med Sci, **4**, 4251-4255.
5. **Kona-Boun JJ, Cuvelliez S, Troncy E** (2006): *Evaluation of epidural administration of morphine or morphine and bupivacaine for postoperative analgesia after premedication with an opioid analgesic and orthopedic surgery in dogs*. J Am Vet Med Assoc, **229**, 1103-1112.
6. **Mathews KA** (2000): *Pain assessment and general approach to management*. Vet Clin North Am Small Anim Pract, **30**, 729-755.
7. **McMurphy R** (1993): *Postoperative epidural analgesia*. Vet Clin North Am Small Anim Pract, **23**,703-716.
8. **Mich PM, Hellyer PW** (2008): Objective, Categoric Methods for Assessing Pain and Analgesia. 78-109. In: JS Gaynor, WW Muir (Eds), Handbook of Veterinary Pain Management (2nd ed). Mosby, St. Louis.
9. **Odette O, Smith LJ** (2013): *A comparison of epidural analgesia provided by bupivacaine alone, bupivacaine + morphine, or bupivacaine + dexmedetomidine for pelvic orthopedic surgery in dogs*. Vet Anaesth Analg, **40**, 527-536.
10. **Pekcan Z, Koc B** (2010): *The post-operative analgesic effects of epidurally administered morphine and transdermal fentanyl patch after ovariohysterectomy in dogs*. Vet Anaesth Analg, **37**, 557-565.
11. **Plumb DC** (2002): Butorphanol tartrate. 116-119. In: DC. Plumb (Ed), Veterinary Drug Handbook (4th ed). Iowa State Press, USA.
12. **Rijnberk A, Kooistra HS** (2010): Adrenals. 61-93. In: A Rijnberk, (Ed), Clinical Endocrinology of Dogs and Cats: An Illustrated Text (2nd Ed). Schlütersche, Hannover.
13. **Smith LJ** (2015): Normal Values for Anesthetized Patients. 119-122. In: LJ Smith (Ed), Questions and Answers in Small Animal Anaesthesia (1st ed). Wiley Blackwell, UK.
14. **Smith N** (2008): *Butorphanol: a double-blind evaluation in postoperative patients with moderate or severe pain*. Can J Anaesth, **21**, 600-610.
15. **Trim CM** (1983): *Cardiopulmonary effects of butorphanol tartrate in dogs*. Am J Vet Res, **44**,329-331
16. **Troncy E, Besner GJ, Charbonneau R, et al** (1996): *Pharmacokinetics of epidural butorphanol in isoflurane anaesthetized dogs*. J Vet Pharmacol Therap, **19**, 268-273.
17. **Troncy E, Junot S, Keroack S, et al** (2002): *Resuts of preemptive epidural administration of morphine with or without bupivacaine in dogs and cats undergoing surgery: 265 cases (1997-1999)*. J Am Vet Med Assoc, **221**, 666-672.
18. **Valverde A** (2008): *Epidural analgesia and anaesthesia in dogs and cats*. Vet Clin North Am Small Anim Pract, **38**, 1205-1230.
19. **Wheeler M, Oderda GM, Ashburn MA, et al** (2002): *Adverse events associated with postoperative opioid analgesia: a systematic review*. J Pain. **3**, 159-180.

Ankara Univ Vet Fak Derg, **68**, 33-38, 2021
DOI: 10.33988/auvfd. 681452

Improvement of bovine *in vitro* embryo production by fetal calf serum and cysteamine supplementation and investigation of freezability

Asiye İzem SANDAL[1,a,✉], Hatice ŞENLİKCİ[1,b], Tuğba ELGÜN[2,c], Ramazan ARICI[1,d], Sinem Özlem ENGİNLER[3,e], Alper BARAN[1,f], Kemal AK[1,g], Tülay İREZ[2,h], Özen Banu ÖZDAŞ[1,i]

[1]İstanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Reproduction and Artificial Insemination, İstanbul; [2]Biruni University, Medical School, Department of Histology and Embryology, İstanbul; [3]İstanbul University-Cerrahpasa, Faculty of Veterinary Medicine, Department of Obstetrics and Gynecology, İstanbul, Turkey.
[a]ORCID: 0000-0002-4952-7861; [b]ORCID: 0000-0002-9568-2088; [c]ORCID: 0000-0002-1311-6892;
[d]ORCID: 0000-0002-2239-2526; [e]ORCID: 0000-0002-2352-876X; [f]ORCID: 0000-0001-7053-3337;
[g]ORCID: 0000-0002-4053-9655; [h]ORCID: 0000-0001-8272-4931; [i]ORCID: 0000-0002-6867-5915

✉Corresponding author: izem@istanbul.edu.tr
Received date: 30.01.2020 - Accepted date: 17.07.2020

Abstract: The aim of this study was to investigate the effects of cysteamine (Cys) and fetal calf serum (FCS) in synthetic oviduct fluid (SOF) and charles and rosenkrans (CR1aa) on the bovine in vitro embryo production and its ability of freeze. The oocytes were divided into two groups and allowed to mature in TCM-199, with and without cysteamine. They were divided into 4 subgroups according to whether they contain Cys and FCS in 2 different culture media (SOF and CR1aa). Accordingly, 8 groups were formed as SOF+FCS+Cys, SOF+Cys, SOF+FCS, SOF, CR1aa+FCS+Cys, CR1aa+Cys, CR1aa+FCS and CR1aa. Embryos were cultured for 7-9 days and were frozen using vitrification method. Development of embryos was observed during the first 24 hr post-thaw period. The highest rate of compact morula was 20% in SOF+FCS+Cys and 19.4% in CR1aa+FCS+Cys. The rate of blastocyst in SOF+FCS and CR1aa+FCS were found 17.9% and 15.4% respectively and the difference between groups was statistically significant (P<0.05). During the 12 hr vitality assessment, the highest rate was determined in SOF+FCS (47%). The results indicate that FCS has a positive effect in reaching compact morula and blastocyst regardless of the presence of the culture medium or antioxidant used. There was no statistically difference between the values of post-thaw embryos.
Keywords: Bovine, cysteamine, embryo, fetal calf serum, vitrification.

Sığır in vitro embriyo üretiminin fetal buzağı serumu ve sisteamin katkısı ile iyileştirilmesi ve dondurulabilirliğinin araştırılması

Özet: Bu çalışmanın amacı, sisteamin (Cys) ve fetal buzağı serumu (FCS)'nun sentetik oviduct sıvısı (SOF) ve charles ve rosenkrans (CR1aa) medyumlarında in vitro sığır embriyo üretimi ve donma kabiliyeti üzerindeki etkilerini araştırmaktır. Toplanan oositler iki gruba ayrıldı ve sisteaminli ve sisteaminsiz TCM-199 medyumunda maturasyonuna izin verildi. 2 farklı medyumda (SOF ve CR1aa) kültüre edilmek üzere Cys ve FCS içerip içermediklerine göre 4 alt gruba ayrıldı. Buna göre 8 alt grup; SOF+FCS+Cys, SOF+Cys, SOF+FCS, SOF, CR1aa+FCS+Cys, CR1aa+Cys, CR1aa+FCS ve CR1aa olarak oluşturuldu. Embriyolar 7-9 gün boyunca kültüre edildi ve vitrifikasyon yöntemi kullanılarak donduruldu. Çözdürme sonrası ilk 24 saat boyunca embriyonik gelişim gözlendi. Kompakt morula oranı en yüksek SOF+FCS+Cys'de %20 ve CR1aa+FCS+Cys'de %19,4 olarak bulundu. SOF+FCS ve CR1aa+FCS'deki blastosist oranı sırasıyla %17,9 ve %15,4 olarak saptandı ve gruplar arasındaki fark istatistiksel olarak anlamlıydı (P<0,05). En yüksek 12 saatlik canlılık değerlendirme oranı SOF+FCS'de (%47) belirlendi. Elde edilen sonuçlar, kullanılan kültür ortamının veya antioksidanın varlığına bakılmaksızın FCS'nin kompakt morula ve blastosiste ulaşmada olumlu bir etkiye sahip olduğunu göstermektedir. Çözdürme sonrası embriyonik gelişimleri arasında istatistiksel olarak bir fark yoktu.
Anahtar sözcükler: Embriyo, fötal buzağı serumu, sığır, sisteamin, vitrifikasyon.

Introduction

The success of reproductive technologies widely used in animals mainly depends on the cryopreservation of gamete cells and the embryo. Due to advances in technology and science, the vitrification method has gained popularity instead of slow freezing, which has been a standard method used in various animal species (27).

During the period of in vitro mammalian embryo culture, atmospheric oxygen tension is routinely used, and this leads to the generation of reactive oxygen species (ROS) (14, 31). DNA damage, lipid peroxidation, oxidative modifications of proteins, and inhibition of oocyte and spermatozoon fusion are among the known detrimental effects of ROS (2). Moreover, ROS may develop under some conditions physiologically as an important factor in programmed cell death (apoptosis) (31, 34). ROS may originate directly from male and female gametes or embryos in different stages of embryonic development, but it may also originate as a result of environmental conditions (9). The most important endogenous source of ROS is oxidative phosphorylation. Inhibition of oxidative phosphorylation reduces ROS generation and has a positive effect on in vitro embryo development (29). Oxygen tension is the most important factor that increases ROS generation. The oxygen tension within the oviduct only equals to ¼ of the atmospheric oxygen tension. It was reported that the synthesis of glutathione (GSH), one of the non-protein sulfhydryl compounds, and freezing resistance in in vitro-produced bovine embryos increased under low oxygen tension (5-7%) (21, 22). Antioxidants such as β-mercaptoethanol, cysteamine, cystine, cysteine, N-acetyl-L-cysteine (NAC), resveratrol and superoxide dismutase (SOD) were used to protect in vitro-produced bovine embryos against oxidative stress (4, 15, 32).

Essential amino acids, especially those that cannot be synthesized by the body, play a crucial role in embryo development (12, 33). However, although it has been reported that protein supplements used in bovine in vitro embryo production such as L-glutamine, fetal calf serum, fetal bovine serum, and bovine serum albumin were useful in the embryonic implantation stage (16, 19). In addition, some scientists have also stated that adding serums into in vitro culture medium resulted in some changes in the metabolism of embryos, which lead to accumulation of fatty acids (23) and increased the number of cytoplasmic lipid droplets (1) and eventually caused a decrease in cryotolerance of the cells (18). In particular, ammonia released by the metabolism of glutamine has some negative effects on the embryo (11, 24). Most importantly, ammonia is one of the main factors causing “large calf syndrome” (17). Therefore, serum-free media have been preferred in recent studies.

The ovarian tissues or cells are basically frozen by two methods. The first one is the traditional slow-freezing method, and the second one is the rapid freezing (vitrification) method. The vitrification method includes flash-freezing of the intracellular and extracellular water, forming a glass-like structure. The major advantage of this method is that it forms smaller ice crystals due to rapid and flash freezing, unlike the traditional slow freezing method (27).

There are many difficulties in genital cell freezing studies as known. It is up to new scientific studies to overcome all previously mentioned positive or negative situations. In our study bovine species preferred for the material, especially considering the contribution to the country livestock. Based on this information, we decided to investigate the effect of cysteamine and fetal calf serum on the production and freezability of in vitro bovine embryos.

Material and Methods

All chemicals are obtained from Sigma-Aldrich (St. Louis, Mo, USA).

Oocyte collection and selection: Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory within 2-3 h in dulbecco's phosphate buffered saline (D-PBS / FTTS) at 30-35 °C. Oocytes collected by slicing method were assessed and only those with smooth cell walls, homogenous cytoplasm, undamaged zona pellucida and those with at least three or more layers of cumulus oophorus cells were selected.

Oocyte maturation: After 3 passages in HEPES-buffered TCM-199 (M5017) medium, with and without 100 µM cysteamine (M9768), selected oocytes were washed in maturation medium (IVM-5% FCS (F0804)) and transferred to 700 µl IVM medium (20-30 oocytes/well). The cells were allowed to mature at 38.8 °C in a 5% CO_2 incubator for 24 h.

Fertilization of maturated oocytes: The oocytes with scattered cumulus oophorus cells around them were classified as mature. They were transferred to in vitro fertilization (IVF-TALP) medium and allowed to adapt to the new environment. The frozen straws of 2 Holstein bulls were thawed at 37 °C for 30 sec. The sperm motility was examined and those of at least 45-60% motility were detected. Swim down method was preferred for sperm preparation. Washed spermatozoons were added on top of the oocytes (400-600x10^3 spermatozoon/well). The motility of oocytes and spermatozoon was then checked and allowed to fertilize for 18-22 h in 5% CO_2 at 38.8 °C.

Transfer of fertilized oocytes into the culture medium: The fertilized oocytes were transferred into 1 ml of washing medium at 38.8°C (HEPES-buffered TCM-199). After 1-minute mixing, fertilized zygotes were then cultured at 38.8 °C in 5% CO_2, 5% N_2 and 90% humidity for 7-9 days. 8 groups were formed such as SOF+FCS+Cys, SOF+Cys, SOF+FCS, SOF, CR1aa+FCS+Cys, CR1aa+Cys, CR1aa+FCS, and CR1aa. 5% FCS was used in the serum groups.

Freezing mature embryos: At the end of the culture period, the healthy embryos reaching the blastocyst and expanded blastocyst stage in all groups in the presence of

SOF and CR1 medium were selected and frozen using the vitrification method in straws. Equilibration solution (VS1) was 1.5 M ethylene glycol (EG-E9129) and 1 M dimethylsulfoxide (DMSO-D2650), and the vitrification solution (VS2) was 2.5 M EG, 2 M DMSO and 0.5 M sucrose (S1888). For adaptation, selected embryos were kept in PBS droplets at room temperature. Firstly, the embryos were passaged in VS1 solution prepared as 3 separate 70 µl drops for 3 min. Then, they were placed in VS2 solution using the same volume and repetition count for 40 sec. 1 to 3 embryos were placed in pre-prepared embryo straws for freezing and sealed with polyvinyl alcohol (PVA). After the straws were sealed, they were immediately immersed into liquid nitrogen (-196 °C) and frozen.

Thawing of frozen embryos: Frozen embryo straws were pulled out of the liquid nitrogen after at least 1 week. After being kept at room temperature for 10 sec, they were directly thawed in the 37 °C water-bath in 30 sec. The embryos were stored in a petri of 1 M sucrose solution prepared with PBS containing 20% FCS for 3 min. They were then taken into a 0.5 M sucrose solution and allowed to stand for 1 minute there. Subsequently, they were transferred into HEPES-buffered TCM-199 for the removal of cryoprotectants.

The culture of thawed embryos: Thawed embryos were washed 3 times in HEPES-buffered TCM-199 medium and then cultured in SOF and CR1aa culture media of their groups. At the 12th and 24th, the embryos were monitored to see whether they continued to develop after thawing.

Statistical analysis: Chi-square test in SPSS 13.0 package software was used for statistical analysis. P-value < 0.05 was accepted statistically significant.

Results

The number of oocytes undergoing maturation during the study period was recorded as 1247. A total of 1019 cells proceed to in vitro culture immediately after in vitro fertilization. The mean cleavage rate of all groups was found to be 62.5%. At the end of 7-9 days, the average rate of blastocyst stage was 20.2%. The evaluation of the study results reveals that the highest cleavage rate belonged to the CR1aa+Cys group with 69.9%, which was followed by the SOF+Cys group with 67.8% The group with the lowest rate was CR1aa+FCS with 58.2%. Similarly, the SOF+FCS group showed a low cleavage rate of 57.8% (Table 1). The evaluation of other experimental groups revealed no statistical difference although there was a percentage difference between the rates of cleavage. Although there was no statistical difference between the groups in terms of division rates, in the blastocyst stage, the effects of FCS and cysteamine appeared differently in both cultures. While the highest cleavage rate of CR1aa medium with cysteamine (69.9%) was achieved in the cleavage stage, the rate of reaching blastocysts decreased to 2.3%. The lowest cleavage rate in the CR1aa medium with FCS was 58.2% and increased to

Table 1. Development of bovine embryos from cleavage to blastocyst stages after vitrification and IVC

	In Vitro Culture n (%)					Cryopreservation n		Viability n (%)	
Groups	Cleavaged /cultured cell	Compact morula/ cleavaged	Early blastocyst/ cleavaged	Blastocyst/ cleavaged	Expanded blastocyst/ cleavaged	Vitrified embryos	Thawed* embryos	12h/ vitrified	24h/ vitrified
SOF	78/120 (65.0)	4^{b}/78 (5.1)	11/78 (14.1)	5^{b}/78 (6.4)	0 (0)	16	15	7/16 (43.7)	0/16 (0)
SOF+FCS	67/116 (57.8)	8^{ab}/67 (11.9)	4/67 (6.0)	12^{a}/67 (17.9)	1/67 (1.5)	17	15	8/17 (47.0)	1/17 (5.8)
SOF+Cys	78/115 (67.8)	10^{ab}/78 (12.8)	3/78 (3.8)	10^{ab}/78 (12.8)	1/78 (1.3)	14	13	5/14 (35.7)	1/14 (7.1)
SOF+FCS+Cys	90/144 (62.5)	18^{a}/90 (20.0)	12/90 (13.3)	12^{ab}/90 (13.3)	2/90 (2.2)	26	23	11/26 (42.3)	2/26 (7.6)
CR1aa	67/115 (58.3)	4^{bc}/67 (6.0)	4/67 (6.0)	3^{b}/67 (4.5)	0/67 (0)	7	7	2/7 (28.5)	0/7 (0)
CR1aa+FCS	78/134 (58.2)	11^{ab}/78 (14.1)	8/78 (10.3)	12^{a}/78 (15.4)	0/78 (0)	20	18	8/20 (40.0)	1/20 (5.0)
CR1aa+Cys	86/123 (69.9)	3^{c}/86 (3.5)	10/86 (11.6)	2^{b}/86 (2.3)	0/86 (0)	12	11	3/12 (25.0)	0/12 (0)
CR1aa+FCS+Cys	93/152 (61.2)	18^{a}/93 (19.4)	7/93 (7.5)	8^{b}/93 (8.6)	3/93 (3.2)	18	14	6/18 (33.3)	1/18 (5.5)

*Number of cultured embryos after thawing
a,b,c There is a significant difference between the values in the same column with no common letters ($P < 0.05$)

15.4% when reaching blastocyst. In the same perspective, the low rate of cleavage in the SOF medium with FCS (57.8%) and increased to 17.9% when reaching blastocyst stage. This suggests that the use of cysteamine and fetal calf serum in different culture media have no effects on the cleavage or blastocyst stages. A total of 130 embryos were obtained, which were frozen using vitrification and found to be at different developmental stages at the end of in vitro culture after the thawing process. The viability assessment of embryos was performed in 12 h and 24 h after the thawing. From the 12 h onwards, degenerations were observed. By the end of the 24 h, embryonic development stopped in all groups. The rates of reaching all of embryonic stages and viability rates between groups are given in Table 1.

Discussion and Conclusion

In a study, Sovernigo et al. (28) investigated the effects of different antioxidant supplements on the development of embryos and found that quercetin, vitamin C, resveratrol, and carnitine did not affect the rate of cleavage and reaching the blastocyst stage. These results are in parallel with the results of the research conducted by Sandal and Ozdas (26), Enginler et al. (7), Anchordoquy et al. (3) and Kharche et al. (13) also reported that the supplementation of 100 µM cysteamine did not result in a statistically significant in cleavage rates. Although many studies have reported that FCS has a positive effect on embryo development, it is known that the incidence of large calf syndrome is high among offsprings born as a result of the transfer of embryos, which were developed in a high-protein-containing medium (25). Therefore, serum-free media have been preferred in recent studies (8, 10, 30). Some researchers argue that the use of FCS in the in vitro culture stage leads to high lipid accumulation in blastocysts, and deteriorates embryo quality (5). According to the results obtained in the study, the low rates of cleavage in the serum and cysteamine-free groups (SOF+FCS and CR1aa+FCS), regardless of the culture medium used, seems to support this argument (11, 17). In a study, Murillo et al. (20) developed bovine embryos both (5%) in and without FCS and did not detect a statistical difference in their development stages up to morula, however, they found a statistical difference in the early blastocyst stage ($P<0.005$). In this study, a statistical difference was found in the compact morula and blastocyst stages in favor of the groups that used FCS compared to the other groups. Gomez et al. (8) in their study, they preferred FCS (5%) and BSA (20 g L^1) in SOF medium of bovine embryo culture. The researchers achieved the best blastocyst development rate in the FCS group (21.2%) and found a statistical difference compared to other groups ($P<0.05$). It is seen that this result and our result (17.9%) are parallel to each other. Duque et al. (6) reported the rate of bovine embryos that they produced in vitro in the SOF medium containing 5% FCS as 21.1% and found a statistical difference between the blastocyst rate (6.6%) found in the culture medium they did not add serum. In this study, the rate of reaching blastocyst in the SOF group (6.4%) and the rate of blastocyst in the SOF+FCS group (17.9%) is similar to Duque et al. (6)'s results. When the SOF+FCS, SOF+Cys and SOF+FCS+Cys groups of the study were examined, there was no statistical difference even in the percentage of achieving the blastocyst and compact morula stages. This result suggests that the combination of FCS and Cys may have an antagonistic effect on embryo development. Considering the CR1aa+FCS (15.4%), CR1aa+Cys (2.3%) and CR1aa+FCS+Cys (8.6%) groups of the study, it shows similar results in terms of the rate of reaching blastocyst compared to the same SOF groups. This situation gives us the positive effects of FCS and Cys on embryo development when used separately; it suggests that when used in combine, the effect turns into either a negative or antagonist type. The presence of antagonist effect is evident from the statistical difference between CR1aa+FCS and CR1aa+ FCS+Cys groups ($P<0.05$). These results show that FCS has a positive effect on the development of embryos even in the absence of antioxidants in the blastocyst stage. This appears to be consistent with the study carried out by Murillo et al. (20), which argued that the use of low concentration of FCS has a positive effect on the development of embryos. To make a comparison in terms of culture media, which are frequently preferred in bovine in vitro embryo culture; CR1aa appears to need more serum or protein addition than SOF (30). The fact that the blastocyst rate obtained in a study of Wan et al. (30) was higher in the CR1aa+BSA+FCS group compared to other groups and statistically different from other groups (25.5%) is an indicator of this. Likewise, in this study the blastocyst ratios of embryos developed in CR1aa medium are lower than the SOF medium supports that SOF is more preferable in bovine in vitro embryo culture.

As a result of the study, it can be concluded that SOF+FCS can support bovine IVC embryos developing to blastocyst with the same efficiency as SOF+FCS+Cys or SOF+Cys, but combination of 5% FCS and 100 µM cysteamine in CR1aa medium show an antagonist effect and affect the development of bovine embryos negatively.

Financial Support

This study was supported by Istanbul University Research Fund with the project number 55727.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Abe H, Yamashita S, Itoh T, et al** (1999): *Ultrastructure of bovine embryos developed from in vitro matured and fertilized oocytes: Comparative morphological evaluation of embryos cultured either in serum free medium or serum supplemented medium.* Mol Reprod Dev, **53**, 325-335.
2. **Aitken RJ, Harkiss D, Buckingham D** (1993): *Relationship between iron-catalysed lipid peroxidation potential and human sperm function.* J Reprod Fertil, **98**, 257-265.
3. **Anchordoquy JM, Anchordoquy JP, Testa JA, et al** (2015): *Influence of vascular endothelial growth factor and Cysteamine on in vitro bovine oocyte maturation and subsequent embryo development.* Cell Biol Int, **39**, 1090-1098.
4. **Balasubramanian S, Rho GJ** (2007): *Effect of cysteamine supplementation of in vitro matured bovine oocytes on chilling sensitivity and development of embryos.* Anim Reprod Sci, **98**, 282-292.
5. **Do VH, Walton S, Taylor-Robinson AW** (2016): *Improvements to in vitro culture media for use in bovine IVF*. J Vet Sci Anim Husb, **4** , 205.
6. **Duque P, Gomez E, Diaz E, et al** (2003): *Use of two serum during bovine embryo culture in vitro.* Theriogenology, **59**, 889-899.
7. **Enginler SO, Ozdaş OB, Sandal AI, et al** (2016): *The effect of cysteamine and oviductal cells in different culture media on the development of sheep embryos.* Kafkas Univ Vet Fak, **22**, 139-145.
8. **Gomez E, Rodriguez A, Munoz M, et al** (2008): *Serum free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification.* Theriogenology, **69**, 1013-1021.
9. **Guerin P, El Mouatassim S, Menezo Y** (2001): *Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings.* Hum Reprod Update, **7**, 175-189.
10. **Hajarian H, Aghaz F, Karami Shabankareh H** (2017): *Replacement of serum with sericin in in vitro maturation and culture media: Effects on embryonic developmental competence of Sanjabi sheep embryo during breeding season.* Theriogenology, **92**, 144-148.
11. **Hammon DS, Wang S, Holyoak GR** (2000): *Effects of ammonia during different stages of culture on development of in vitro produced bovine embryos.* Anim Reprod Sci, **59**, 23-30.
12. **Hong J, Lee E** (2007): *Intrafollicular amino acid concentration and the effect of amino acids in a defined maturation medium on porcine oocyte maturation, fertilization, and preimplantation development.* Theriogenology, **68**, 728-735.
13. **Kharche SD, Agrawal S, Pathak J, et al** (2016): *Influence of cysteamine supplementation during in vitro culture of early stage caprine embryos on blastocyst production.* Indian J Anim Sci, **86**, 304-306.
14. **Kitagawa Y, Suzuki K, Yoneda A, et al** (2004): *Effects of oxygen concentration and antioxidants on the in vitro developmental ability, production of reactive oxygen species (ROS), and DNA fragmentation in porcine embryos.* Theriogenology, **62**, 1186-1197.
15. **Kobayashi M, Lee ES, Fukui Y** (2006): *Cysteamine or β-mercaptoethanol added to defined maturation medium improves blastocyst formation of porcine oocytes after intracytoplasmic sperm injection.* Theriogenology, **65**, 1191-1199.
16. **Kuran M, Robinson JJ, Staines ME, et al** (2001): *Development and de novo protein synthetic activity of bovine embryos produced in vitro in different culture systems.* Theriogenology, **55**, 593-606.
17. **Lane M, Gardner DK** (1994): *Increase in postimplantation development of cultured mouse embryos by amino acids and induction of fetal retardation and exencephaly by ammonium ions.* J Reprod Fertil, **102**, 305-312.
18. **Moore K, Rodriguez-Sallaberry CJ, Kramer JM, et al** (2007): *In vitro production of bovine embryos in medium supplemented with a serum replacer: Effects on blastocyst development, cryotolerance and survival to term.* Theriogenology, **68**, 1316-1325.
19. **Murillo-Ríos A, Maillo V, Muñoz M, et al** (2017): *Short- and long-term outcomes of the absence of protein during bovine blastocyst formation in vitro.* Reprod Fert Develop, **29**, 1064-1073.
20. **Murillo A, Muñoz M, Martín-González D, et al** (2017): *Low serum concentration in bovine embryo culture enhances early blastocyst rates on Day-6 with quality traits in the expanded blastocyst stage similar to BSA-cultured embryos.* Reprod Biology, **17**, 162–171.
21. **Oyamada T, Fukui Y** (2004): *Oxygen tension and medium supplements for in vitro maturation of bovine oocytes cultured individually in a chemically defined medium.* J Reprod Develop, **50**, 107-117.
22. **Raty M, Ketoja E, Pitkanen T, et al** (2011): *In vitro maturation supplements affect developmental competence of bovine cumulus-oocyte complexes and embryo quality after vitrification.* Cryobiology, **63**, 245-255.
23. **Reis A, Rooke JA, McCallum GJ, et al** (2003): *Consequences of exposure to serum, with or without vitamin E supplementation, in terms of the fatty acid content and viability of bovine blastocysts produced in vitro.* Reprod Fert Develop, **15**, 275-284.
24. **Rooke JA, Ewen M, Mackie K, et al** (2004): *Effect of ammonium chloride on the growth and metabolism of bovine ovarian granulosa cells and the development of ovine oocytes matured in the presence of bovine granulosa cells previously exposed to ammonium chloride.* Anim Reprod Sci, **84**, 53-71.
25. **Sağırkaya H, Mısırlıoğlu M, Kayaç A, et al** (2007): *Developmental potential of bovine oocytes cultured in different maturation and culture conditions.* Anim Reprod Sci, **101**, 225-240.

26. **Sandal AI, Ozdaş OB** (2015): *Vitrification of in vitro-produced bovine embryos matured in modified TCM-199 medium.* Turk J Vet Anim Sci, **39**, 688-692.
27. **Saragusty J, Arav A** (2011): *Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification.* Reproduction, **141**, 1-19.
28. **Sovernigo TC, Adona PR, Monzani PS, et al** (2017): *Effects of supplementation of medium with different antioxidants during in vitro maturation of bovine oocytes on subsequent embryo production*. Reprod Domest Anim, **52**, 561-569.
29. **Thompson JG, McNaughton C, Gasparrini B, et al** (2000): *Effects of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro.* J Reprod Fertil, **118**, 47-55.
30. **Wan P, Hao Z, Zhou P, et al** (2009): *Effects of SOF and CR1 media on developmental competence and cell apoptosis of ovine in vitro fertilization embryos.* Anim Reprod Sci, **114**, 279-288.
31. **Valk JVD, Brunner D, Smet KD, et al** (2010): *Optimization of chemically defined cell culture media - Replacing fetal bovine serum in mammalian in vitro methods.* Toxicol in Vitro, **24**, 1053-1063.
32. **Van Soom A, Yuan YQ, Peelman LJ, et al** (2002): *Prevalence of apoptosis and inner cell allocation in bovine embryos cultured under different oxygen tensions with or without cysteine addition.* Theriogenology, **57**, 1453-1465.
33. **Zabihi A, Shabankareh HK, Hajarian H, et al** (2019): *Resveratrol addition to in vitro maturation and in vitroculture media enhances developmental competence of sheep embryos.* Domest Anim Endocrin, **68**, 25-31.
34. **Zolini AM, Carrascal-Trianaa E, Ruiz de Kinga A, et al** (2019): *Effect of addition of L-carnitine to media for oocyte maturation and embryo culture on development and cryotolerance of bovine embryos produced in vitro.* Theriogenology, **133**, 135-143.

Ankara Univ Vet Fak Derg, **68**, 39-46, 2021
DOI: 10.33988/auvfd.690049

Effects of genotype on the biomechanical parameters and composition of bone in the laying hen embryos

Fatma Kübra ERBAY ELIBOL[1,a,✉], Esin Ebru ONBAŞILAR[2,b], Tuğba KARAKAN[3,c], Süleyman TABAN[4,d], Teyfik DEMİR[5,e]

[1]TOBB University of Economics and Technology, Department of Biomedical Engineering, Ankara; [2]Ankara University, Faculty of Veterinary Medicine, Department of Animal Breeding and Husbandry, Ankara; [3]Ankara University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Ankara; [4]Ankara University, Faculty of Agriculture, Department of Soil Science and Plant Nutrition, Ankara; [5]TOBB University of Economics and Technology, Department of Mechanical Engineering, Ankara, Turkey.
[a]ORCID: 0000-0002-4117-1098; [b]ORCID: 0000-0002-1321-0280; [c]ORCID: 0000-0001-8868-5291;
[d]ORCID: 0000-0002-7997-9412; [e]ORCID: 0000-0001-6352-8302

✉Corresponding author: fatmakubra.erbay@gmail.com
Received date: 17.02.2020 - Accepted date: 09.07.2020

Abstract: Bone problems are highly prevalent in laying hens. These problems affect the welfare, production and economic losses. Bone development begins in the embryonic period, and if the skeletal system develops well at that time, the subsequent production period can be affected positively. The present experiment aimed to investigate the effect of genotype on biomechanical parameters and composition of bone in the laying hen embryos. For this purpose, 360 fertilized eggs were obtained from two brown (Atak-S and Brown Nick) and two white (Atabey and Nick) layer breeders and incubated. Metatarsus, tibia and femur properties were examined on the embryonic d 19 and 21. Results showed that genotype played an important role in determining the biomechanical properties and mineral composition of the metatarsus, tibia and femur in the embryonic period. Examined bone characteristics improved with embryonic age. The least mineralization was observed in the metatarsus bone. In conclusion, bone properties were infleunced from the genotype. However, these differences were not related with laying hens being white or brown. The effect of the interaction between genotype and embryonic age on the bone properties should be considered.
Keywords: Bone, embryo, genotype, laying hen,

Yumurtacı tavuk embriyolarında genotipin kemik biyomekanik özellikleri ve bileşimine etkileri

Özet: Yumurtacı tavuklarda kemik sorunları oldukça yaygındır. Bu sorunlar refahı, üretimi ve ekonomik kayıpları etkiler. Kemik gelişimi embriyonik dönemde başladığından bu dönemde iskelet sistemi iyi gelişirse, sonraki üretim periyodu olumlu yönde etkilenebilir. Bu çalışmada genotipin yumurtacı tavuk embriyolarında kemiğin biyomekanik özellikleri ve bileşimine etkisini incelemek amaçlanmıştır. Bu amaçla iki kahverengi (Atak-S ve Kahverengi Nick) ve iki beyaz (Atabey ve Nick) yumurtacı damızlıklardan elde edilen 360 adet döllü yumurta toplanmış ve inkübe edilmiştir. Embiyonik dönemin 19 ve 21. günlerinde metatarsus, tibia ve femur özellikleri incelenmiştir. Sonuçlar, embriyonik dönemde genotipin metatarsus, tibia ve femurun biyomekanik özellikleri ve mineral bileşiminde önemli bir rol oynadığını göstermiştir. Embriyonik yaşın artmasıyla incelenen kemik özellikleri iyileşmiştir. En az minerilizasyon metatarsus kemiğinde gözlenmiştir. Sonuç olarak, kemik özellikleri genotipten etkilenmiştir. Fakat bu farklılıklar beyaz veya kahverengi yumurtacı tavuk olmasıyla ilişkili değildir. Genotip ve embriyonik yaş arasındaki etkileşimin kemik özellikleri üzerindeki etkisi dikkate alınmalıdır.
Anahtar sözcükler: Embriyo, genotip, kemik, yumurtacı tavuk

Introduction

Laying hens have a skeleton to provide mobility support, protection and store for essential minerals especially calcium. Welfare, health and performance of hen and economic implications are related to skeletal development (10). The skeleton of birds is composed of a mineral part (70%), organic part (20%) and water (10%). Most of the bones' mineral structure is composed of calcium and phosphorus (10, 22). In modern table egg production, companies use different brown and white laying hybrids depending on breeding methods. There are significant differences between white and brown laying

hybrids in terms of body weight, feed consumption, egg weight and egg production (14, 15). However, genetic selection for higher production rate has come with unintended consequences; in particular, bone problems, depending on the rearing system, diet and hen's age. By the end of egg production the hens are susceptible to osteoporosis (11, 23). Bone fractures caused by osteoporosis are rated as serious welfare problems (18) resulting in increased economic losses through increased mortality and decreased eggshell quality. The skeletal problems involve mainly leg problems (25).

In fact, the basis of bone development takes place in the incubation. Femur and tibia start developing at 3.5 days of incubation, however, calcification begins at 10th day of incubation (17). Around the 10th day of incubation, calcium from the eggshell is transported to the embryo via chorioallantoic membrane (9). Bone calcium content also increases sharply from day 14 of incubation and starts to plateau at day 19 of incubation (12). The last phase of embryo development is marked by dramatic physiological and metabolic changes (13). Only few studies are available investigating the differences in bone development in the embryonic period of the different layer hybrids. Well-formed skeleton within the egg might increase the chick's healthiness in the layer period. Therefore, the aim of this study was to examine the differences regarding development and properties of leg bones during the embryonic age in different brown and white layer hybrids.

Material and Methods

The animal experimental protocol was approved by the Ankara University Animal Care and Use Committee (2015/5/102). When determining the sample to be used in the study the Power of test (1-β) was 0.80 calculated by G. Power statistical packet software. A total of 360 fertilized eggs were obtained from Atak-S, Atabey, Brown Nick and Nick layer breeders at 28 weeks of age. All eggs were numbered and weighed. Eggs were loaded to the incubator (Çimuka Incubator, Ankara, Turkey) set to 37.7ºC and 53% RH with 45^0 rotation every hour. On d 18, the eggs were transferred to the hatcher (Çimuka Incubator, Ankara, Turkey) set to 37.5ºC and 70% RH. Eggs were placed in individual boxes in the hatcher allowing specific identification of each hatched chick on d 21 (15).

At the beginning of 19 and 21 d of incubation, twelve eggs from each genotype were randomly selected, weighed and opened. Embryos were sacrificed by cervical dislocation. Metatarsus, tibia and femur bones were dissected and cleared of all soft tissues. Bones were stored at -20 ° C until analysis and dissolved at room temperature just before testing. Each bone was weighed using an analytical scale to the nearest 0.01 mg. Length was determined from the proximal end to distal end, and the width at the medial diaphysis (1). Leg bones from each embryo were subjected to the three-point bending test until failure occurred. Test was performed on Instron 5944 testing frame (Instron, Norwood, MA, USA). Loading rate was 5 mm/min. Span length was 10 mm for tibias. In bending test, span length should be about 16 times the thickness of the specimen generally. However, due to the nonuniform structure of bone, length-to-width ratio of 16:1 cannot be achieved. Therefore, 10 mm is the maximum span length which we can measure flexure of the bone. Load was applied to the midpoint of the shaft. Load vs displacement data was collected for each sample. Stiffness values were calculated from the slope of linear region of the load displacement curves. Breaking force was determined from the load displacement curves as well. Breaking force was defined as load at failure. Yield load is the load where permanent deformation of the system begins. Displacement at yield load is the displacement at which permanent deformation begins.

Dry matter and ash in the bones were determined according to the AOAC (3) methods. For the determination of mineral levels (Zn, Cu, Mn, Fe, Na, K, Ca, P and Mg), bones were analyzed (5) using an ICP-OES (Perkin Elmer Optima™ DV 2100 Model, Dual View, Perkin Elmer Life and Analytical Sciences, Shelton, CT, USA). The values of these minerals were shown as per kg DM.

Statistical analyses: All of the experimental results are presented as mean±SEM. Two-way ANOVA was used for all data in order to test for main and interactive effects of genotype and embryonic age. If ANOVA revealed significant effects, it was followed by Tukey test. Statistical significance was accepted at $P \leq 0.05$ (6).

Results

All geometrical and biomechanical parameters of metatarsus measured were affected by genotype except displacement at yield (Table 1). The major difference among genotype groups was observed in Nick embryos. Metatarsus of Nick embryos was found to be the heaviest and the longest comparing with other genotypes ($P<0.001$). Width, breaking force, stiffness and yield load of metatarsus in the Nick and Brown Nick embryos were found higher than those of the metatarsus in the Atabey and Atak-S embryos. As the embryonic age progressed, metatarsus properties were also increased except displacement at yield ($P<0.001$). Two way interactions between hybrid and embryonic age were found in the weight, stiffness and yield load of metatarsus ($P<0.01$). Because, the increases in the weight, stiffness and yield load of metatarsus in Nick embryos were the highest from embryonic d (E) 19 to E21 among the other embryos.

Table 1. Effect of genotype and embryonic age on geometrical and biomechanical parameters in the metatarsus

Genotype	Embryonic age	Weight (g)	Length (mm)	Width (mm)	Breaking force (N)	Stiffness (N/mm)	Yield load (N)	Displacement at yield (mm)
White layer								
Atabey		0.16^{c}	19.95^{ab}	1.38^{b}	4.73^{b}	5.68^{b}	3.00^{b}	0.87
Nick		0.22^{a}	20.72^{a}	1.55^{a}	6.30^{a}	9.83^{a}	4.52^{a}	0.78
Brown layer								
Atak-S		0.15^{c}	18.67^{c}	1.32^{b}	4.82^{b}	7.08^{b}	2.72^{b}	0.73
Brown Nick		0.19^{b}	19.65^{b}	1.54^{a}	5.90^{a}	9.31^{a}	4.07^{a}	0.81
	E19	0.15	19.22	1.38	4.83	6.17	2.95	0.85
	E21	0.21	20.28	1.52	6.04	9.78	4.21	0.75
Atabey	E19	0.14	19.49	1.34	4.01	3.93	2.51	0.88
	E21	0.19	20.41	1.42	5.46	7.42	3.48	0.86
Nick	E19	0.18	20.26	1.48	5.27	6.78	3.08	0.83
	E21	0.26	21.18	1.63	7.33	12.88	5.96	0.73
Atak-S	E19	0.12	17.88	1.18	4.56	6.48	2.87	0.76
	E21	0.18	19.45	1.45	5.08	7.68	2.58	0.71
Brown Nick	E19	0.19	19.23	1.51	5.49	7.48	3.33	0.92
	E21	0.20	20.06	1.58	6.31	11.15	4.81	0.70
SEM		0.003	0.108	0.020	0.105	0.222	0.132	0.031
					P-value			
Genotype		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.113
Embryonic age		<0.001	<0.001	0.001	<0.001	<0.001	<0.001	0.430
Genotype X Embryonic age		0.003	0.628	0.313	0.061	0.004	0.001	0.652

a,b,c Means within a column with different superscript letters differ.

The values of weight, length, breaking force and stiffness of tibia were found to be the highest in the Nick embryos (P≤0.001) as in the metatarsus (Table 2). Width of tibia values were found as 1.48, 1.55, 1.53 and 1.60 mm in the Atabey, Nick, Atak-S and Brown Nick embryos, respectively. Brown Nick had the widest tibia in the embryonic period (P<0.01). Yield loads were 4.29, 5.14, 3.74 and 5.44 N for Atabey, Nick, Atak-S and Brown Nick embryos, respectively; tibia of Nick and Brown Nick embryos had higher yield load values than the other examined embryos (P<0.001). Displacement at yield was not found different in the genotype groups. Weight (P<0.001), length (P<0.001), width (P<0.05), breaking force (P<0.001), stiffness (P<0.001) and yield load (P<0.001) increased with the increase in the embryonic age from E19 to E21. But, displacement at yield did not differ between embryonic age groups. Interaction was found only for length and yield load of tibia (P<0.05).

Nick embryos had the heaviest (P<0.01) and the longest (P<0.001) femur than the others in the examined embryonic period (Table 3). Femur widths were found as 1.48, 1.57, 1.43 and 1.58 mm in the Atabey, Nick, Atak-S and Brown Nick embryos, respectively. The lowest breaking force value was found in the Atak-S embryos. Stiffness values were 11.10, 14.38, 9.86 and 12.52 N/mm in the Atabey, Nick, Atak-S and Brown Nick embryos, respectively. The highest stiffness (P<0.001) and yield load (P<0.05) of femur were found in the Nick embryos. Displacement at yield did not differ among the genotype groups. Examined geometrical and biomechanical parameters of femur except of displacement at yield increased from E19 to E21 (P<0.01). Increase in the femur stiffness from E19 to E21 was lowest in the Atabey embryos, and this situation caused the genotype and embryonic age interaction.

Mineral levels of metatarsus, tibia and femur are presented in Table 4, 5 and 6. Fe, Zn, Na and Ca levels in the metatarsus; ash, Fe, Zn, Na and Ca levels in the tibia, and ash, Fe, Zn, Na and Ca levels in the femur were affected by the genotype (P<0.05). All examined minerals in the metatarsus, tibia and femur except Mg in the metatarsus and tibia increased with embryonic age (P≤0.05). The highest Fe content in the metatarsus, tibia and femur was found in the Nick and Brown Nick embryos (P<0.001). The highest Ca level was found as 223.49 (P<0.01), 272.85 (P<0.001) and 276.34 g/kg DM (P<0.001) in the metatarsus, tibia and femur bones, respectively in the Brown Nick embryos.

In metatarsus, interaction effects between genotype and embryonic age were observed for Fe and Na concentrations. This was due to the higher increment in Fe level in the Brown Nick embryos and lower increase in Na level in the Atabey embryos from E19 to E21. In the tibia bone, increase in the ash, Cu, Fe and P concentrations from E19 to E21 varied by genotype and this resulted in interaction. Same trend was also observed for ash, Cu, Fe, Na and P levels in the femur from E19 to E21.

Table 2. Effect of genotype and embryonic age on geometrical and biomechanical parameters in the tibia

Genotype	Embryonic age	Weight (g)	Length (mm)	Width (mm)	Breaking force (N)	Stiffness (N/mm)	Yield load (N)	Displacement at yield (mm)
White layer								
Atabey		0.23^{bc}	27.06^{b}	1.48^{b}	7.03^{b}	16.16^{bc}	4.29^{b}	0.45
Nick		0.27^{a}	28.53^{a}	1.55^{ab}	7.71^{a}	20.52^{a}	5.14^{a}	0.42
Brown layer								
Atak-S		0.21^{c}	26.28^{b}	1.53^{ab}	6.22^{c}	14.80^{c}	3.74^{b}	0.63
Brown Nick		0.26^{ab}	27.06^{b}	1.60^{a}	7.50^{ab}	18.27^{ab}	5.44^{a}	0.44
	E19	0.20	26.20	1.51	6.36	14.42	4.03	0.45
	E21	0.28	28.27	1.57	7.87	20.32	5.27	0.52
Atabey	E19	0.18	25.81	1.44	6.06	13.77	3.80	0.40
	E21	0.28	28.30	1.53	8.00	18.55	4.77	0.50
Nick	E19	0.23	28.02	1.54	6.74	15.18	3.73	0.48
	E21	0.31	29.04	1.55	8.68	25.33	6.54	0.36
Atak-S	E19	0.17	24.78	1.48	5.62	13.24	3.65	0.49
	E21	0.26	27.79	1.58	6.82	16.35	3.83	0.78
Brown Nick	E19	0.22	26.19	1.58	7.02	15.50	4.94	0.42
	E21	0.29	27.93	1.62	7.98	21.04	5.94	0.46
SEM		0.004	0.127	0.012	0.092	0.491	0.125	0.051
				P-value				
Genotype		<0.001	<0.001	0.007	<0.001	0.001	<0.001	0.410
Embryonic age		<0.001	<0.001	0.012	<0.001	<0.001	<0.001	0.453
Genotype X Embryonic age		0.471	0.036	0.467	0.137	0.079	0.003	0.549

[a,b,c] Means within a column with different superscript letters differ.

Table 3. Effect of genotype and embryonic age on geometrical and biomechanical parameters in the femur

Genotype	Embryonic age	Weight (g)	Length (mm)	Width (mm)	Breaking force (N)	Stiffness (N/mm)	Yield load (N)	Displacement at yield (mm)
White layer								
Atabey		0.15^{b}	19.22^{b}	1.48^{bc}	6.49^{a}	11.10^{b}	4.54^{ab}	0.72
Nick		0.18^{a}	20.40^{a}	1.57^{ab}	7.31^{a}	14.38^{a}	4.90^{a}	0.68
Brown layer								
Atak-S		0.13^{b}	18.44^{b}	1.43^{c}	5.34^{b}	9.86^{b}	3.32^{b}	0.62
Brown Nick		0.15^{b}	19.18^{b}	1.58^{a}	6.48^{a}	12.52^{ab}	4.36^{ab}	0.74
	E19	0.14	18.75	1.47	5.61	9.21	3.31	0.70
	E21	0.17	19.87	1.55	7.21	14.72	5.25	0.68
Atabey	E19	0.13	18.87	1.44	5.91	10.53	3.71	0.65
	E21	0.17	19.57	1.51	7.06	11.66	5.37	0.80
Nick	E19	0.16	19.82	1.49	6.49	10.61	4.20	0.75
	E21	0.20	20.97	1.65	8.14	18.14	5.50	0.60
Atak-S	E19	0.12	17.65	1.42	4.53	6.78	2.08	0.69
	E21	0.15	19.23	1.44	6.16	12.93	4.56	0.55
Brown Nick	E19	0.14	18.67	1.54	5.50	8.90	3.23	0.71
	E21	0.16	19.69	1.61	7.46	16.14	5.50	0.77
SEM		0.004	0.114	0.013	0.113	0.363	0.186	0.029
				P-value				
Genotype		0.002	<0.001	<0.001	<0.001	<0.001	0.024	0.491
Embryonic age		0.001	<0.001	0.002	<0.001	<0.001	<0.001	0.749
Genotype X Embryonic age		0.698	0.630	0.249	0.671	0.015	0.692	0.212

[a,b,c] Means within a column with different superscript letters differ.

Table 4. Effect of genotype and embryonic age on metatarsus composition

Genotype	Embryonic age	Ash (g/100g DM)	Cu (mg/kg DM)	Fe (mg/kg DM)	Mn (mg/kg DM)	Zn (mg/kg DM)	Na (g/kg DM)	Ca (g/kg DM)	P (g/kg DM)	Mg (g/kg DM)	K (g/kg DM)
White layer											
Atabey		40.87	0.33	163.23^{c}	0.49	221.87^{a}	2.71^{b}	206.51^{ab}	106.43	2.78	2.67
Nick		42.89	0.36	218.42^{a}	0.52	201.59^{a}	3.36^{a}	210.86^{ab}	105.01	3.35	2.83
Brown layer											
Atak-S		43.88	0.43	201.66^{b}	0.54	181.63^{b}	3.72^{a}	201.40^{b}	106.88	2.76	2.65
Brown Nick		41.78	0.46	256.29^{a}	0.58	207.59^{a}	3.40^{a}	223.49^{a}	113.21	3.23	2.45
	E19	40.21	0.33	165.53	0.45	147.19	2.71	198.70	95.89	2.95	2.14
	E21	44.51	0.46	254.27	0.61	259.15	3.88	222.44	119.87	3.37	3.16
Atabey	E19	39.60	0.22	141.13	0.41	151.77	2.65	192.52	94.03	2.72	2.16
	E21	42.15	0.45	185.32	0.56	291.95	2.77	220.49	118.83	2.84	3.18
Nick	E19	39.60	0.29	165.06	0.41	145.75	2.57	199.69	92.13	3.25	2.33
	E21	46.18	0.43	271.78	0.64	257.43	4.15	216.33	117.88	3.45	3.34
Atak-S	E19	42.36	0.39	166.29	0.45	134.61	3.08	186.48	99.06	3.07	2.30
	E21	45.40	0.46	237.03	0.63	228.66	4.35	216.33	114.70	3.45	3.00
Brown Nick	E19	39.27	0.41	189.65	0.55	156.63	2.55	216.10	98.34	2.76	1.77
	E21	44.29	0.50	322.92	0.61	258.54	4.25	230.89	128.09	3.69	3.13
SEM		0.426	0.017	4.675	0.036	4.996	0.102	2.306	1.311	0.116	0.112
							P-value				
Genotype		0.074	0.087	<0.001	0.837	0.041	0.008	0.006	0.124	0.319	0.692
Embryonic age		<0.001	<0.001	<0.001	0.034	<0.001	<0.001	<0.001	<0.001	0.075	<0.001
Genotype X Embryonic age		0.340	0.328	0.006	0.883	0.390	0.036	0.637	0.253	0.599	0.774

^{a,b,c} Means within a column with different superscript letters differ.

Table 5. Effect of genotype and embryonic age on tibia composition

Genotype	Embryonic age	Ash (g/100g DM)	Cu (mg/kg DM)	Fe (mg/kg DM)	Mn (mg/kg DM)	Zn (mg/kg DM)	Na (g/kg DM)	Ca (g/kg DM)	P (g/kg DM)	Mg (g/kg DM)	K (g/kg DM)
White layer											
Atabey		50.03^{bc}	0.58	215.84^{c}	0.64	265.50^{a}	3.16^{b}	244.15^{b}	120.66	3.12	3.03
Nick		52.41^{a}	0.54	280.13^{a}	0.60	244.14^{a}	4.08^{a}	246.41^{b}	115.96	3.72	3.14
Brown layer											
Atak-S		51.34^{ab}	0.63	256.27^{b}	0.65	221.32^{b}	4.40^{a}	247.27^{b}	114.45	3.52	2.95
Brown Nick		48.17^{c}	0.63	307.12^{a}	0.77	271.77^{a}	3.82^{ab}	272.85^{a}	121.49	3.51	2.69
	E19	48.74	0.52	205.44	0.54	185.67	3.19	232.37	103.38	3.25	2.36
	E21	52.24	0.67	324.23	0.79	315.69	4.54	272.97	132.90	3.69	3.54
Atabey	E19	49.78	0.39	178.36	0.53	180.79	2.99	225.19	101.15	2.99	2.47
	E21	50.29	0.76	253.31	0.76	350.21	3.34	263.11	140.17	3.24	3.59
Nick	E19	51.37	0.46	209.81	0.45	182.76	3.29	222.10	103.35	3.62	2.53
	E21	53.45	0.61	350.44	0.75	305.52	4.87	270.72	128.58	3.82	3.76
Atak-S	E19	48.56	0.62	208.65	0.56	160.65	3.54	224.92	104.22	3.33	2.48
	E21	54.12	0.65	303.89	0.73	282.00	5.26	269.61	124.68	3.72	3.46
Brown Nick	E19	45.23	0.61	224.95	0.63	218.49	2.93	257.27	104.80	3.06	1.98
	E21	51.12	0.66	389.28	0.91	325.05	4.70	288.43	138.18	3.96	3.39
SEM		0.327	0.021	4.364	0.054	5.148	0.123	1.915	1.211	0.123	0.122
								P-value			
Genotype		<0.001	0.291	<0.001	0.721	0.002	0.006	<0.001	0.106	0.393	0.589
Embryonic age		<0.001	0.001	<0.001	0.028	<0.001	<0.001	<0.001	<0.001	0.082	<0.001
Genotype X Embryonic age		0.009	0.021	0.002	0.976	0.167	0.144	0.392	0.038	0.734	0.920

^{a,b,c} Means within a column with different superscript letters differ.

Table 6. Effect of genotype and embryonic age on femur composition

Genotype	Embryonic age	Ash (g/100 g DM)	Cu (mg/kg DM)	Fe (mg/kg DM)	Mn (mg/kg DM)	Zn (mg/kg DM)	Na (g/kg DM)	Ca (g/kg DM)	P (g/kg DM)	Mg (g/kg DM)	K (g/kg DM)
White layer											
Atabey		50.82[a]	0.62	219.22[b]	0.68	268.60[a]	3.31[b]	249.15[b]	124.32	3.39	3.28
Nick		50.87[a]	0.55	279.91[a]	0.63	246.10[a]	4.11[a]	249.98[b]	117.75	3.94	3.29
Brown layer											
Atak-S		52.30[a]	0.66	256.86[b]	0.66	226.28[b]	4.52[a]	249.40[b]	116.99	3.86	3.13
Brown Nick		48.89[b]	0.66	311.67[a]	0.78	273.20[a]	4.14[a]	276.34[a]	124.18	3.71	2.88
	E19	48.57	0.54	209.13	0.59	188.95	3.46	237.72	106.31	3.41	2.64
	E21	52.86	0.71	324.70	0.79	318.14	4.57	274.72	135.31	4.01	3.65
Atabey	E19	50.77	0.43	186.78	0.55	185.75	3.32	233.55	106.15	3.07	2.87
	E21	50.86	0.81	251.66	0.81	351.46	3.30	264.76	142.49	3.61	3.68
Nick	E19	47.37	0.46	208.57	0.51	183.44	3.20	226.13	105.18	3.83	2.76
	E21	54.37	0.65	351.25	0.76	308.75	5.01	273.83	130.32	4.05	3.83
Atak-S	E19	50.00	0.64	210.80	0.63	166.20	3.97	227.35	107.41	3.59	2.78
	E21	54.59	0.69	302.91	0.69	286.36	3.30	271.44	126.56	4.12	3.49
Brown Nick	E19	46.14	0.64	230.36	0.66	220.40	3.36	263.85	106.50	3.16	2.14
	E21	51.63	0.68	392.98	0.90	326.00	4.91	288.84	141.86	4.26	3.61
SEM		0.313	0.022	4.375	0.052	5.199	0.099	1.790	1.265	0.117	0.115
						P-value					
Genotype		0.002	0.278	<0.001	0.758	0.005	<0.001	<0.001	0.065	0.298	0.550
Embryonic age		<0.001	<0.001	<0.001	0.054	<0.001	<0.001	<0.001	<0.001	0.013	<0.001
Genotype X Embryonic age		0.002	0.021	<0.001	0.882	0.216	0.011	0.091	0.047	0.603	0.644

[a,b] Means within a column with different superscript letters differ.

Discussion and Conclusion

For poultry, skeletal system is one of the most important development systems in the embryonic period. Genetic selection as a tool to obtain higher production has undesirable effects on the skeletal system resulting in major problems such as osteoporosis, fracture, paralysis and death (24). The better bone development in the embryonic period results in less bone problems during the production period. In the present study, clear differences were observed for geometrical and biomechanical parameters of leg bones obtained from different genotypes in the embryonic period. It is well documented that commercial brown hybrid layers represent the heavy weight type with stronger bones than the light weight type such as white layers (7). However, results in this study showed that this idea does not apply to the embryonic period. Because, almost all geometrical and biomechanical parameters of leg bones were found to be highest in the Nick embryos and second highest level was found in the Brown Nick embryos. Nick embryos had heavier and longer metatarsus and femur along with longer tibia.

Bone biomechanical parameters such as breaking force, stiffness, yield load and displacement at yield load are commonly used to determine the bone quality. Breaking force is the amount of force required to cause a fracture (8). Stiffness is the resistance to elastic deformation and yield load is the flexibility limit point (8, 20). When excessive load is applied than the yield load, permanent deformation occurs in the bones impeding their proper functioning. This means that high breaking force, stiffness and yield load values are important for strong bones in the embryonic period. Metatarsus and tibia of Nick and Brown Nick embryos had the highest breaking force, stiffness and yield load. These results showed that the effect of being brown or white layer hens on examined bone parameters was not statistically significant in the embryonic period. However, genotype is an important parameter for development in the skeletal system in the embryonic period.

For all leg bones, neither genotype nor embryonic age had any significant effect on the displacement values at yield loads. Increased mechanical strength was observed in all genotypes with advancement in embryonic age. Age dependent increase in stiffness and yield load was greatest in metatarsus of Nick embryos, while lowest increase in the stiffness was observed in the femur of Atabey embryos. This means that Nick group had the greatest increment in the mechanical strength of bone with advancement in embryonic age. The embryonic age

efficiently improved the breaking force, stiffness and yield load in the tibia as compared to the other leg bones.

During embryonic period, minerals for bone development are acquired from the eggshell, albumen and yolk. The strength of the bones depends on their mineral density. In this study, differences were noted in the storage of minerals in different leg bones. The mineral accumulation in the bones of embryos did not differ being they were white or brown laying hens. The effects of the levels of Zn, Mn and Cu on bone development and bone strength have been illustrated in some studies (4, 19). Zn deficiency decreases bone collagen turnover and is accompanied by leg deformities (21, 26). In the present study it was observed that leg bones of Atak-S embryos had the lowest Zn content. However, Mn and Cu levels of leg bones were not found to be different among the genotype groups. Onbaşılar et al. (16) reported higher Zn content in the femur of embryos from hybrid type layers as compared to the pure breeds.

Angel (2) reported that tibia is one of the most mineralized bones in the skeleton in the production period. Results in this study showed that tibia and femur were more mineralized bones than metatarsus in the embryonic period. Mineral accumulation in the bones increased from E19 to E21. Only, Mg level in the metatarsus and tibia were not found statistically different from E19 to E21.

In conclusion, genotype is a factor determining bone development during embryonic period. Bone properties were affected by the genotype but these differences were not related with laying hens being white or brown. The effect of the genotype should be considered in the interaction between embryonic ages. Interaction between genotype and embryonic age should be considered.

Financial Support

This study was supported by the Ankara University Scientific Research Fund (BAP, 16B0239004).

Ethical Statement

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (2015/5/102).

Conflicts of Interest

Authors declare that they have no conflict of interests.

References

1. **Alfonso-Torres KA, Gargaglioni LH, Pizauro JM, et al** (2009): *Breeder age and bone development in broiler chicken embryos.* Braz J Vet. Res Anim Sci, **61**, 219-226.
2. **Angel R** (2007): *Metabolic disorders: limitations to growth of and mineral deposition into the broiler skeleton after hatch and potential implications for leg problems.* J Appl Poult Res, **16,** 138-149.
3. **AOAC** (2000): Association of Official Analytical Chemists. Official Methods of Analysis of AOAC International. 17th ed, Gaithersburg.
4. **Beattie JH, Avenell A** (1992): *Trace element nutrition and bone metabolism.* Nutr Res Rev, **5**, 167–188.
5. **Boss CB, Fredeen KJ** (2004): Concepts, Instrumentation and Techniques in Inductively Coupled Plasma Optical Emission Spectrometry, 3th ed. Perkin Elmer Life and Analytical Sciences, USA.
6. **Dawson B, Trapp RG** (2001): Basic and Clinical Biostatistics, 3rd ed. Lange Medical Books/McGraw-Hill Medical Publishing Division, New York.
7. **Fleming RH, McCormack HA, McTeir L, et al** (2006): *Relationships between genetic, environmental and nutritional factors influencing osteoporosis in laying hens.* Br Poult Sci, **47**, 742-755.
8. **Forestier-Zhang L, Bishop N** (2016): Bone strength in children: understanding basic bone biomechanics. *Arch Dis Child,* **101**, 2-7.
9. **Gabrielli MG** (2004): *Carbonic anhydrases in chick extra-embryonic structures: a role for CA in bicarbonate reabsorption through the chorioallantoic membrane.* J Enzym Inhib Med Ch, **19**, 283-286.
10. **Kierończyk B, Rawski M, Józefiak D, et al** (2017): *Infectious and non-infectious factors associated with leg disorders in poultry–a review.* Ann Anim Sci, **17,** 645-669.
11. **Korver DR, Saunders-Blades JL, Nadeau KL** (2004): *Assessing bone mineral density in vivo: Quantitative computed tomography.* Poult Sci, **83**, 222–229.
12. **Kubota M, Abe E, Shinki T, et al** (1981): *Vitamin D metabolism and its possible role in the developing chick embryo.* Biochem J, **194**, 103-109.
13. **Nasir Z, Peebles ED** (2018): A*vian embryo nutrition and incubation*. Poult Sci, **97**, 2994-2995.
14. **Onbaşılar EE, Ünal N, Erdem E, et al** (2015): *Production performance, use of nest box, and external appearance of two strains of laying hens kept in conventional and enriched cages.* Poult Sci, **94**, 559-564.
15. **Onbaşılar EE, Güngör ÖF, Taban S, et al** (2018): *Comparison of different brown and white layer hybrid embryonic development and uptake of nutrients in the egg.* Anim Reprod Sci, **198,** 57-64.
16. **Onbaşılar EE, Güngör ÖF, Demir T, et al** (2018): *Femur Properties of Embryo in the Layer Hybrid and Pure Breeds.* Braz J Poult Sci, **20,** 805-810.
17. **Pechak DG, Kujawa MJ, Caplan, AI** (1986): *Morphology of bone development and bone remodeling in embryonic chick limbs.* Bone*,* **7**, 459-472.
18. **Sandılands V, Sparks N, Wilson S, et al** (2005): *Laying hens at depopulation: The impact of the production system on bird welfare.* Br Poult Abst, 23- 24.
19. **Seo HJ, Cho YE, Kim T, et al** (2010): *Zinc may increase bone formation through stimulating cell proliferation, alkaline phosphatase activity and collagen synthesis in osteoblastic MC3T3-E1 cells.* Nutr Res Pract, **4**, 356-361.

20. **Sevil Kilimci F, Kara ME** (2013): *Basic concepts to assessment of mechanical properties of bones.* Animal Health Prod and Hyg, **2**, 235-239.
21. **Starcher BC, Hill CH, Madaras JG** (1980): *Effect of zinc deficiency on bone collagenase and collagen turnover*. J Nutr, **110**, 2095-2102.
22. **Turek SL** (1984): *Physiology and mineralization of bone.* Orthop. **1,** 136-190.
23. **Whitehead CC, Fleming RH** (2000): *Osteoporosis in cage layers.* Poult Sci, **79**, 1033-1041.
24. **Whitehead C** (2004): Skeletal Disorders in Laying Hens: The Problem of Osteoporosis and Bone Fractures. In: Welfare of the Laying Hen. Poultry Science Symposium Series. CABI Publishing, USA.
25. **Yair R, Cahaner A, Uni Z, et al** (2017): *Maternal and genetic effects on broiler bone properties during incubation period.* Poult Sci, **96**, 2301-2311.
26. **Yair R, Shahar R, Uni Z** (2013): *Prenatal nutritional manipulation by in ovo enrichment influences bone structure, composition, and mechanical properties.* J Anim Sci, **91**, 2784-2793.

Ankara Univ Vet Fak Derg, **68**, 47-52, 2021
DOI: 10.33988/auvfd.695818

Treatment outcomes of using paraosseous clamp and cerclage stabilisation technique in long bone fractures of cats: a retrospective study

İlker ŞEN [1,a,✉], Mehmet SAĞLAM [2,b]

[1]Sivas Cumhuriyet University, Faculty of Veterinary Medicine, Department of Surgery, Sivas; [2]Aksaray University, Faculty of Veterinary Medicine, Department of Surgery, Aksaray, Turkey.
[a]ORCID: 0000-0001-8288-4871; [b]ORCID: 0000-0001-8934-8529

✉Corresponding author: ilkersenn@yandex.com
Received date: 28.02.2020 - Accepted date: 01.07.2020

Abstract: The stabilisation technique of paraosseous clamp and cerclage has become one of the most widely used current techniques because of the easy of applicability and low cost. It is a relatively new method providing rigid fixation in the treatment of fractures, especially those which do not have sufficient cortex resistance and have a wide medullar canal in the period when the long bones of the extremity have not completed development. The technique is based on binding 3 double cerclage wires to each other to form a paracortical belt. The aim of this study was to evaluate the clinical and radiological results of fractures in the extremity long bones of cats treated with the new stabilisation method of paraosseous clamp and cerclage. The study group was formed of 14 cats of various breeds and ages, of both genders, diagnosed with a fracture of the extremity long bone. Clinical and radiographical follow-up examinations were made at 10, 30, and 45 days postoperatively. Clinical healing was achieved in 13 cases on postoperative day 45, and those cases could use the affected extremity. A correlation was determined between the functional healing results and low complication rates, including unstable fractures, and it is supported that this technique could be use of this technique in the treatment of long bone fractures. In conclusion, the results of this study demonstrated that the paraosseous clamp and cerclage stabilisation technique can be used as an alternative method in the treatment of long bone fractures, especially in indicated cases.
Keywords: Cat, cerclage, fracture, paraosseous clamp, stabilisation.

Kedilerde karşılaşılan ekstremite uzun kemik kırıklarının paraosseöz klemp ve serklaj ile stabilizasyon tekniği kullanılarak sağaltımları üzerine retrospektif bir çalışma

Özet: Paraosseöz klemp ve serklaj ile stabilizasyon tekniği, kolay uygulanabilirliği ve düşük maliyeti nedeniyle güncel teknikler arasında yer almaya başlamıştır. Ekstremite uzun kemiklerinin kemik gelişiminin tamamlanmadığı dönemde, özellikle korteksin yetersiz direnci ve medullar kanalın geniş olduğu kırıkların sağaltımında rijid fiksasyon sağlayan nispeten yeni bir metottur. Uygulama 3 çift serklaj telinin parakortikal olarak bir kemer oluşturacak şekilde birbirine bağlanması esasına dayanmaktadır. Bu çalışmada, kedilerin ekstremite uzun kemiklerinde oluşan kırıkların yeni bir stabilizasyon yöntemi olan "Paraosseöz klemp ve serklaj ile stabilizasyon tekniği" kullanılarak sağaltımının klinik ve radyolojik değerlendirme sonuçlarını aktarmak amaçlandı. Çalışma materyalini, klinik ve radyolojik muayeneler sonrasında, ekstremite uzun kemiklerinde kırık belirlenen değişik ırk, yaş ve cinsiyette 14 kedi oluşturdu. Hastaların postoperatif 10, 30 ve 45. gün klinik ve radyografik kontrolleri yapıldı. Postoperatif 45. günde 13 olguda klinik iyileşmenin sağlandığı ve olguların ilgili ekstremitelerini kullandıkları gözlendi. Stabil olmayan kırıklar da dahil olmak üzere, fonksiyonel iyileşme sonuçları ve düşük komplikasyon oranlarıyla ilişkilendirildiğinde, uzun kemiklerin kırıklarının sağaltımında bu tekniğin kullanımı desteklenmektedir. Sonuç olarak, "Paraosseöz klemp ve serklaj ile stabilizasyon tekniği"nin, kedilerin ekstremite uzun kemik kırıklarının sağaltımında, özellikle endike olan olgularda alternatif bir yöntem olabileceği kanısına varılmıştır.
Anahtar kelimeler: Kedi, kırık, paraosseöz klemp, serklaj, stabilizasyon.

Introduction

In the application of non-rigid stabilisation methods, such as intramedullary nailing, major complication rates are extremely high. More rigid fixation techniques, such as external fixator or plate osteosynthesis provide better clinical healing with a major complication rate of <6% (5).

As stabilisation with paraosseous clamp and cerclage is simple to apply and low-cost, veterinary surgeons have started to accept this technique in surgery (5).

This is a new method providing rigid fixation in the treatment of long bone fractures. It is indicated in particular in simple or fragmented diaphyseal fractures. The intact section in both fragments of the fracture must be of a length which can support the required number of double cerclage wires. The method is based on the binding together of 3 double cerclage wires to form a paracortical belt. In a study of two small case series, successful results were reported even in multi-fragmented fractures (3).

The body of the clamps, which are formed with pins or Kirschner wires (K-wires), assists in consolidation of the long axis of the bone by functioning as an extraosseous splint. The transcortical section of the applied pin or K-wire assists in providing rotational stability (10). At the same time, minimal movement is permitted in the fracture line due to the flexibility of the pins. Micro-movement of the fragments triggers callus formation (1, 2). The small implant dimensions assist in protecting bone tissue, blood circulation and surrounding soft tissues. Intracorporeal placement of the implant increases implant compatibility of the patient and the risk of the animal injuring itself can be avoided. In addition, the risk of infection, implant loosening and neuromuscular damage is reduced. Postoperative care requirements are at a minimal level, and there is no need for frequent bandage changing and pin cleaning. The costs of surgery and time, which is dependent on the operator's experience, are comparable with other techniques (8).

The advantages of this system are that it can be applied to every size of patient and the implant costs are low, whereas the major disadvantage is that the approach to the operated area is similar to that used in osteosynthesis provided with a plate (9).

The aim of this retrospective study was to present the clinical and radiological evaluation results of long bone fractures in cats treated with new osteosynthesis method that named paraosseous clamp and cerclage stabilisation.

Materials and Methods

A total of 14 cats of various breeds and ages, of both genders that were diagnosed with a long bone fracture of the extremity as a result of clinical and radiological examinations were included in this study. Information about the animals are presented in Table 1.

In clinical examination of the cases, first a detailed anamnesis was taken from the owner of the animal. In cases with a good general condition, sedation was administered and two-way radiographs were taken in the craniocaudal (Cr-Cd) and mediolateral (ML) positions. In preoperative period, the owners were instructed not to give any food for 8 hours before the operation and no water for 4 hours. As sedation, 80 μgr/kg (0.08 ml/kg) Medetomidine was administered, followed by a intramuscular injection of 5 mg/kg Ketamine HCl to provide general anaesthesia. In the operations, 1.5 mm K-wires and 0.5 mm cerclage wires were used.

Surgical procedure: Using an appropriate approach to the field of operation, the bone fragments were exposed. Two or three K-wires of 1.5 mm diameter were cut at various lengths, and then shaped in a U-form to be used as the clamp. The bone fragments were brought end to end to provide anatomic alignment. On the lateral, medial, cranial and caudal surfaces of the fragments, transcortical holes were opened as far from the fracture line as possible, K-wires were prepared accordingly the lengths and diameters. The transcortical holes were made perpendicular to the bone long axis on the lateral and cranial surfaces of the humerus, on the cranial and lateral or craniolateral surfaces of the antebrachium, on the cranial,

Table 1. The distribution of breed, age, gender, and fracture localisation of the cases.

Case no	Age (Year)	Breed	Gender	Lesion
1	1	Domestic cat	♀	Tibia
2	1,5	Domestic cat	♂	Tibia
3	2	Scottish fold	♂	Radius-ulna
4	9 months	Domestic cat	♂	Femur
5	3	Domestic cat	♂	Humerus
6	10 months	Domestic cat	♀	Radius-ulna
7	11 months	Ankara	♂	Radius-ulna
8	1	Domestic cat	♂	Tibia
9	1	Domestic cat	♀	Monteggia
10	7 months	Persian	♂	Tibia
11	1	Domestic cat	♂	Femur
12	8 months	Ankara	♀	Monteggia
13	2	Domestic cat	♀	Tibia
14	2	Domestic cat	♂	Radius-ulna

lateral and caudolateral surfaces of the femur, and on the medial, craniomedial and caudomedial surfaces of the tibia. The clamps were passed through the transcortical section holes matched with the holes opened appropriate to the dimensions of the clamps previously prepared on the same surfaces. The clamps were temporarily fixed using forceps. The same procedures were repeated for the other planes. Cerclage wires of 0.5 mm diameter were placed surrounding the clamps. A sufficient number of cerclage wires were applied to provide rigid stabilisation and were tightened. Thus, the fixation of the K-wires to the bone was increased (Figure 1 and Figure 2).

The operation procedure was completed with routine closure of the operated region. Postoperatively, a splint and bandage were applied and analgesia of meloxicam at 0.2 mg/kg was administered subcutaneously. Clinical and radiographical examinations were applied at 10, 30 and 45 days postoperatively.

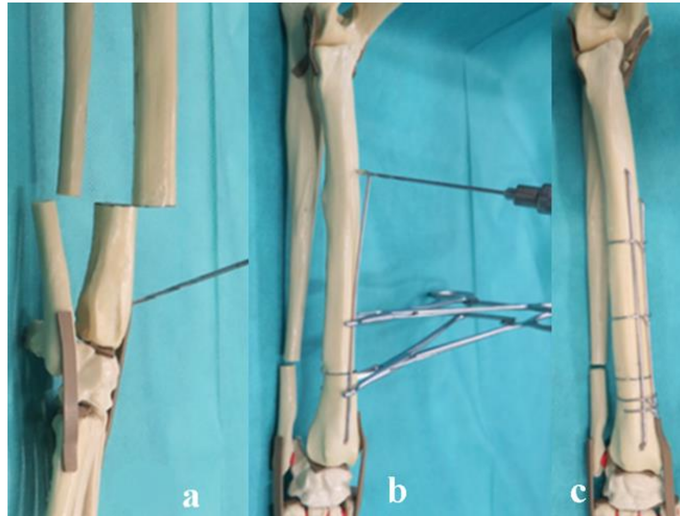

Figure 1. The application principles of the stabilisation technique with paraosseous clamp and cerclage. **a)** opening the hole on the distal fragment of the radius, **b)** temporarily fixing the pins to the bone cortex with locking forceps, **c)** applying the cerclage wires around the clamps. (5)

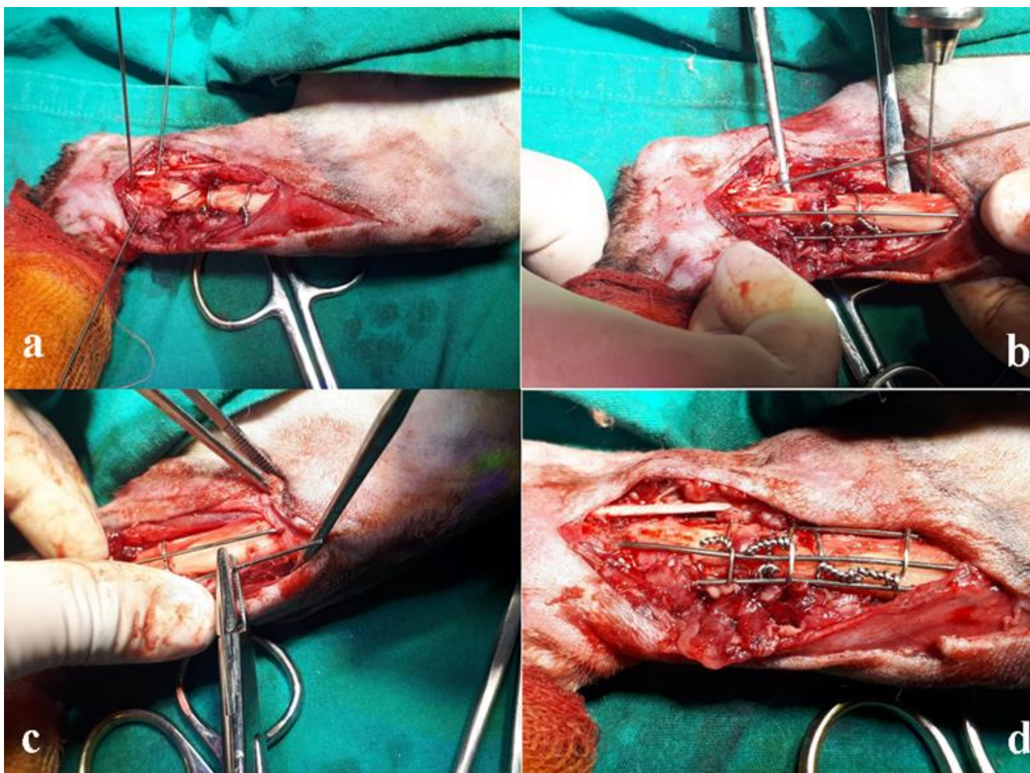

Figure 2. Application of the stabilisation technique with paraosseous clamp and cerclage to the tibia. **a)** transcortical application of the K-wires to various surfaces of the fracture fragment, **b-c)** bending the K-wires to give a U-shape, **d)** paraosseous fixation with cerclage wires by passing the U-shaped K-wire through the holes opened in the upper fragment.

Results

Nine of 14 cats various breeds and ages were male and 5 were female. The following breeds were included: 10 cross-breeds, 2 Ankara, 1 Persian and 1 Scottish Fold. The fracture localisation were included: 1 humerus, 4 radius-ulna, 2 proximal ulna fracture with dislocation of the radius head (Monteggia lesion), 2 femur, and 5 tibia. The clinical data of the cases are shown in Table 1.

In the follow-up examination on the postoperative 10th day, no complications were observed in 13 cases. In case no 4, angulation was observed in the fracture line, which was related to the metallic resistance of the K-wires used in the application, and a revision operation was performed on this case. For this, the fracture line was reached with a routine procedure. A 3 mm Steinmann pin was applied to the femur with a retrograde technique from the medullary opening, from where the angulation of the fragments originated, and the fragments were re-aligned by correcting the angulated pins. The Steinmann pin was not advanced as far as the distal femur because of the narrowing of the medullar canal by the transcortically applied K-wires (Figure 3). The operation area was closed routinely and a supportive bandage was applied. This case could not be followed up after the postoperative 20th day.

With the exception of case no 4, functional healing was obtained in all the other cases at the follow-up examination on the postoperative 45th day, and all the cases were seen to be able to comfortably use the affected extremity. As the implants used were biocompatible, they were not removed but left on the bone after clinical healing (Figure 4 and Figure 5).

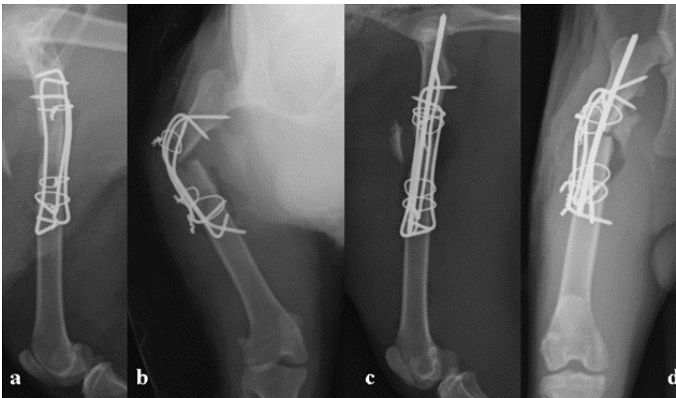

Figure 3. Craniocaudal ve mediolateral radiographs of case 4 of the fractured femur; **a)** postoperative 1st day, **b)** postoperative 10th day, **c-d)** postoperative 20th day radiographs.

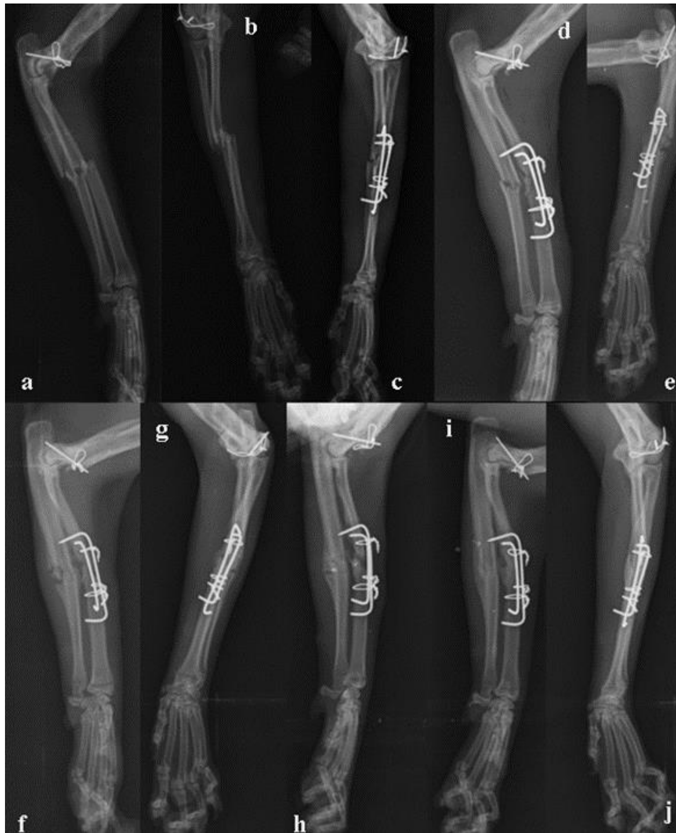

Figure 4. Craniocaudal ve mediolateral radiographs of case 6 of the fractured antebrachium; **a-b)** Immediate preoperative radiographs, **c-d)** postoperative 1st day, **e-f)** postoperative 10th day, **g-h)** postoperative 20th day, **i-j)** postoperative 45th day radiographs.

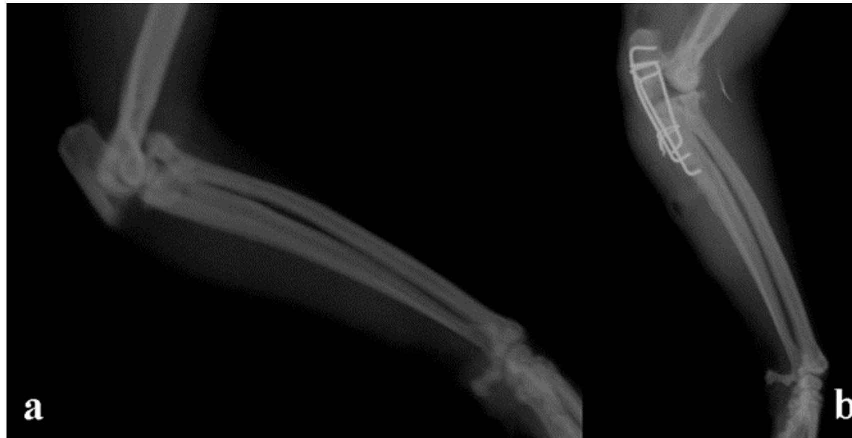

Figure 5. Mediolateral radiographs of case 9 of the fractured antebrachium; **a)** Immediate preoperative radiography, **b)** postoperative 30th day radiograph.

Discussion and Conclusion

The method described here is a new method which provides rigid fixation in the treatment of long bone fractures. It can be used in the treatment of simple or fragmented fractures. For the application, the intact sections of both fragments must be of a length which can support a sufficient number of double cerclage wires for fixation of the clamps to the bone. This method is based on the attachment to each other of 3 double cerclage wires to form a paracortical belt (3). In previous studies that have reported the results of the paraosseous clamp and cerclage stabilisation technique, stabilisation has been obtained with double cerclage wires applied according to the method described with pins used in the treatment of various extremity long bone fractures. In addition, the use of a single layer cerclage wire in the application of paraosseous clamp and cerclage stabilisation technique has been reported to be successful in cats and small dog breeds with fractures of the antebrachium and tibia because of the small diameter and length of the bones (10). In the current study, the paraosseous clamp and cerclage stabilisation technique was applied to 14 cats of varying age and breed for fractures in the extremity long bones. Paraosseous stabilisation to the bone was obtained with 1.5mm diameter K-wires and 0.5mm diameter cerclage wires bent to form a single layer around the K-wire and bone. In one of the current cases (case no 4), follow-up could not be continued after the postoperative 20th day, and in the other 13 cases (1 humerus, 3 antebrachium, 2 Monteggia lesion, 2 femur and 5 tibia fractures), functional healing was obtained. According to these clinical healing results, this technique can be applied with single layer cerclage wire in the treatment of extremity long-bone fractures in cats.

Stress protection occurs when metal implants such as bone plate and screws are used in fracture treatment or joint replacement surgery. Although hard plates create rigid stabilisation in the fracture region, preserves contact between the fracture fragments and allows early weight-bearing and movement, resorption occurs as a result of the high rate of hardness of the implant and the reduced physiological burden of the bone (6). In previous studies, resorption has been reported in 7 of 20 fractures (7) and in 10 of 15 fractures (4). In a study by Manchi et al. (5), ulna resorption was determined in 2 cases and non-union in 1 ulna. The stress protection formed as a result of the rigid fixation created by the paraosseous clamp and cerclage stabilisation technique was concluded by the authors to have prevented the healing process of the ulna. In the 13 cases of the current study that completed the postoperative follow-up examinations, no complications of bone resorption, delayed union or non-union were observed. In addition to the fracture fixation provided by the 1.5 mm diameter K-wires and 0.5 mm cerclage wires used in the application, union was stimulated by the micro-movement at the degree permitted between the fragments and it was thought that the physiological burden on the affected bone was not completely neutralised.

Manchi et al. (5) reported implant-associated complications in 4 of 17 dogs, and emphasised that healing was obtained as a result of the paraosseous clamp and cerclage stabilisation technique applied in the revision surgery of these cases. In the current study, implant-related complications developed in one case (case no 4). On the postoperative 10th day clinical and radiological examinations, angulation was determined in the fragments with weight-bearing on the extremity, so revision surgery was performed on this case. In this operation, after reaching the femur, a 3 mm Steinmann pin was applied with the retrograde method from the medullar opening which had formed between the fragments because of angulation. At the same time, the K-wires and cerclage wires applied paraosseously were corrected manually without removing them from the area. After routine closure of the operated area, the fragments were seen to

have been sufficiently re-aligned on the radiograph. However, after this procedure, this case could not be followed up.

Reasons for preferring the paraosseous clamp and cerclage stabilisation technique in veterinary orthopaedic surgery are easy application together with internal fixation, especially in developing animals with large medullary canals, and it is a low-cost technique. It can be applied in the treatment of simple and fragmented fractures of extremity long bones. With the exception of case no 4, which could not be followed up, functional clinical healing was obtained in the other 13 cases with no complications.

Compared to other techniques defined for the treatment of long bone fractures, this is a new technique and data and study results in literature are extremely limited. Further studies would be able to contribute to this subject in respect of the advantages and disadvantages of the technique, overcoming complications originating from potential implant errors, and the evaluation of long-term results. As the functional healing results, including in unstable fractures, were correlated with low complication rates, the use of this technique in the treatment of long bone fractures is supported.

In conclusion, the paraosseous clamp and cerclage stabilisation technique provides successful results in the treatment of extremity long bone fractures in cats, and as the costs of application are lower compared to other treatment techniques, it can be considered to be a good alternative treatment method.

Finacial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Bartolomaeus E, Niebauer GW, Schmiedmayer HB, et al** (2007): *Untersuchungen zum Biegeverhalten einer Parakortikalen-Klammer-Cerclage-Konfiguration im Vergleich zu einer 3,5 mm Dynamischen Kompressionsplatte*. Wien Tierärztl Mschr, **94**, 184–191.
2. **Behrend A** (2007): *Biomechanische und klinische Studie zur chirurgischen Frakturversorgung langer Röhrenknochen der Katze*. Mensch & Buch Verlag, 68–69.
3. **Fossum TW** (2013): Radial and ulnar fractures. 1140-1154. In: Small Animal Surgery 4th Edition. Mosby Inc, St. Louis.
4. **Hamilton MH, Langley Hobbs SJ** (2005): *Use of the AO veterinary mini 'T'-plate for stabilisation of distal radius and ulna fractures in toy breed dogs*. Vet Comp Orthop Traumatol, **18**, 18–25.
5. **Manchi G, Brunnberg MM, Shahid M, et al** (2017*): Radial and ulnar fracture treatment with paraosseous clamp-cerclage stabilisation technique in 17 toy breed dogs*. Vet Rec Open, **4**, e000194.
6. **Millis DL, Levine D** (2014): Responses of Musculoskeletal Tissues to Disuse and Remobilization. 92-153. In: Canine Rehabilitation and Physical Therapy. 2nd Edition. Elsevier Inc. Saunders, Philadelphia.
7. **Piras L, Cappellari F, Peirone B, et al** (2011): *Treatment of fractures of the distal radius and ulna in toy breed dogs with circular external skeletal fixation: a retrospective study*. Vet Comp Orthop Traumatol, **24**, 228–235.
8. **Slunsky P, Halter L, Florczak S, et al** (2017): *Repair of a femoral fracture in a Congo African Grey parrot (Psittacus erithacus erithacus) with a paracortical-clamp-cerclage technique.* J Zoo Wildl Med, **48**, 1204–1209.
9. **Şen İ, Sağlam M, Kibar B** (2015): *Kedilerde karşılaşılan radius-ulna kırığının sağaltım sonuçlarının klinik ve radyolojik değerlendirilmesi.* Vet Hek Der Derg, **86**, 25–33.
10. **Wanivenhaus G** (2001): *Paraossäre Klammer-Cerclage-Stabilisierung: eine Biologische osteosynthesemethode*. Wien Tierärztl Mschr, **88**, 123–128.

Ankara Univ Vet Fak Derg, **68**, 53-59, 2021
DOI: 10.33988/auvfd. 714110

Molecular presence of *Felis catus gammaherpesvirus* -1 in cats with ocular disorders in Turkey

Bahattin Taylan KOÇ[1,a,✉] Müge AKKARTAL[2,b]

[1]Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Virology, Aydın; [2]Nova Veterinary Clinic, Balıkesir, Turkey.
[a]ORCID: 0000-0002-4279-6233; [b]ORCID: 0000-0002-1499-7652

✉Corresponding author: btkoc@adu.edu.tr
Received date: 03.04.2020 - Accepted date: 17.06.2020

Abstract: *Felis catus gammaherpesvirus*-1 (FcaGHV-1) is a newly discovered feline virus, and its effects on the health of cats are not certainly known. Known feline viruses play a huge role in ocular disorders in domestic and wild cats. Also, emerging viruses may be responsible for ophthalmic diseases of cats. We aimed to investigate the presence of FcaGHV-1, *Feline calicivirus* (FCV), *Feline herpesvirus* -1 (FHV-1), *Feline immunodeficiency virus* (FIV), *Feline infectious peritonitis virus* (FIPV), *Feline leukemia virus* (FeLV), *Feline panleukopenia virus* (FPV) in cats in Turkey by molecular methods, and to examine the relationship between the virus and ocular disorders by obtained data. Swab, blood and biopsy samples were collected from 45 owned cats with ocular disorders. Molecular techniques including viral genome amplification and sequencing were used in this study. Two out of 45 cats (2/45; 4.4%) found to be FcaGHV-1 and FIV positive. Our two sequences and a reference sequence from Japan (LC437925) have constructed a separated subgroup in the molecular phylogenetic tree. In this study, we have not found a close relationship between ocular disorders and the presence of FcaGHV-1. This is the first study exhibiting the presence of FcaGHV-1 and its molecular status in Turkey. Acquired knowledge suggests performing further studies on FcaGHV-1.
Keywords: Cats, gammaherpesvirus, molecular, ophthalmic, phylogeny.

Türkiye'de oküler bozukluğu olan kedilerde *Felis catus gammaherpesvirus* -1'in moleküler varlığı

Özet: *Felis catus gammaherpesvirus*-1 (FcaGHV-1), kedilerin sağlığı üzerindeki etkileri bilinmeyen yeni keşfedilmiş bir kedi virusudur. Bilinen kedi virusları, evcil ve vahşi kedilerdeki oküler bozukluklarda büyük rol oynamaktadırlar. Ayrıca, yeni ortaya çıkan viruslar kedilerin oftalmik hastalıklarından sorumlu olabilir. Bu nedenle hem Türk kedilerinde FcaGHV-1, *Feline calicivirus* (FCV), *Feline herpesvirus* -1 (FHV-1), *Feline immunodeficiency virus* (FIV), *Feline infectious peritonitis virus* (FIPV), *Feline leukemia virus* (FeLV), *Feline panleukopenia virus* (FPV) varlığını moleküler yöntemlerle araştırmayı, hem de virus ve oküler bozukluklar arasındaki ilişkiyi elde edilen verilerle incelemeyi amaçladık. Swap, kan ve biyopsi örnekleri, oküler bozukluğu olan 45 kediden toplandı. Bu çalışmada viral genom amplifikasyonu ve sekanslama dahil moleküler teknikler kullanılmıştır. 45 kediden ikisinde (2/45; %4,4) FcaGHV-1 ve FIV pozitif olarak bulundu. İki sekansımız ve Japonya'dan bir referans sekans (LC437925), moleküler filogenetik ağaçta ayrı bir alt grup oluşturmuştur. Bu çalışmada, oküler bozukluklar ile FcaGHV-1 arasında sıkı bir ilişki saptanmamıştır. Bu, Türkiye'de FcaGHV-1 varlığını ve moleküler durumunu gösteren ilk çalışmadır. Edinilen bilgi, FcaGHV-1 hakkında daha fazla çalışma yapılmasını önermektedir.
Anahtar Kelimeler: Kediler, gammaherpesvirus, moleküler, oftalmik, filogeni.

Introduction

Ocular diseases caused by feline viruses are frequently observed in domestic cats. Clinical manifestations can occur in many variable forms including chemosis, epiphora conjunctivitis, keratitis, keratoconjunctivitis, uveitis, etc. Viruses can be either the main factor or cofactor for these ocular disorders (9, 10, 13, 19, 23). *Feline herpesvirus*-1 (FHV-1) and *Feline calicivirus* (FCV) are more prominent agents compared to other viruses causing ocular infections (9, 13, 23). FHV-1 belongs to the genus of *Varicellovirus*, the subfamily of *Alphaherpesvirinae*, the family of *Herpesviridae* (13). FCV is taxonomically classified in the genus of *Vesivirus*, the family of *Caliciviridae*. Both are highly contagious viruses and cause respiratory and ophthalmic infections in *felids* (10, 13, 23).

Other feline viruses, such as *Feline immunodeficiency virus* (FIV), *Feline leukemia virus* (FeLV), *Feline*

panleukopenia virus (FPV), and *Feline adenovirus* (FAdV), may also cause similar ocular symptoms, in addition to their specific symptoms (9, 19, 23). However, these agents are less important compared to FHV and FCV for ocular disorders. Mostly, mentioned agents can be the cofactors for FHV-1 and FCV. Especially, FIV and FeLV are immunosuppressive agents for cats, which can be the underlying essential factors in some ocular disorders by other relevant agents (9, 19, 23). In addition to all these, emerging viruses might have been already acting an efficient role in ocular infections due to their pathological reflections, that have not been unknown.

Felis catus gammaherpesvirus -1 (FcaGHV-1) is a recently discovered virus in domestic cats (2). FcaGHV-1 is classified in the genus of *Percavirus*, the subfamily of *Gammaherpesvirinae*, the family of *Herpesviridae* (2, 4). FcaGHV-1 is claimed as a novel virus that might cause immunosuppressive symptoms in cats in studies that have been conducted so far, although it has been assumed as asymptomatic in the early stage of discovery (2-4, 15-18, 24). There have already been some studies on its presence, epidemiology, prevalence and comparison to risk factors in various countries (2-4, 15-18, 24). However, the interaction of the ocular system and FcaGHV-1 has not been specifically considered.

In this study, we hypothesized that FcaGHV-1 would be responsible for ocular infection in case of the absence of FHV-1 and FCV. Accordingly, we have intended to investigate the molecular presence of FcaGHV-1 and its related phylogenetic analysis, in addition to other viral agents (FHV-1, FCV, FIV, FIPV, FeLV, FPV) in client-owned cats with ocular symptoms in Turkey.

Materials and Methods

Sampling: In this study, conjuctival swabs, whole blood, and biopsy samples have been collected from 45 clinically symptomatic cats had at least an ocular disorder including corneal opacity, conjunctivitis, keratoconjunctivitis, chemosis, epiphora, symblepheron in 2019. The biopsy samples, including different parts of the eye – globe, conjunctiva, were only taken from operated cats during operation by veterinary ophthalmology surgeon. Individual properties were recorded by declaration and approval of the client during sample collection. All samples from the vet clinics in Balıkesir and Aydın were stored at -20°C during transport to Aydın Adnan Menderes University Faculty of Veterinary Medicine Department of Virology laboratory.

Molecular detection analysis of feline viruses: Nucleic acids from all samples were isolated using the method of "Phenol:Chloroform:Isoamyl Alcohol (25:24:1)" as described by Chomczynski and Sacchi (5). Reverse transcriptase-polymerase chain reactions were conducted to each isolated RNA, belonging to FCV and FIPV according to kit producer's protocol (RevertAid, First Strand cDNA Synthesize Kit, Thermo®, USA). Isolated DNAs and obtained cDNAs were kept at -80 ºC until molecular analyses, and densities of nucleic acids were measured by nanodrop (Thermo Scientific™ NanoDrop™ One, CA, USA) immediately before polymerase chain reaction. In polymerase chain reactions, different primer sets, previously reported, were used to amplify specific partial gene regions belonging to each mentioned virus (Table 1). PCR conditions were optimized and performed according to the suggested protocol by the recombinant enzyme manufacturer (VitaProof® Polymerase, Procomcure, Austria). PCR products were run in 1% agarose gel in "Tris:Acetic Acid:EDTA" solution under electrophoresis. Amplified products were screened under blue light transilluminator.

Phylogenetic analysis of FcaGHV-1: Novel FcaGHV-1 gB partial sequences were cleaned from noisy and error readings using Tracer software implemented in a molecular and phylogenetic analysis tool, MEGA X (11) after sequencing process. Cleaned sequences and other reference sequences from GenBank were aligned by using MEGA X (11). A maximum-likelihood tree was conducted by estimating based Kimura-2 parameter and 1000 bootstrap replicates.

Results

We have not detected any positivity in terms of FCV, FHV, FIPV, FeLV, and FPV. Two out of 45 cats with ophthalmic symptoms were detected both FcaGHV-1 positive (2/45; 4.4%), both also FIV positive. We have obtained amplified products in the length of 350 bp. and 859 bp. for FcaGHV-1 and FIV, respectively. Of two dual infection cases (FIV/FcaGHV-1), in the first one, a cat, named "Şirine", exhibited the worst clinical condition compared to other cats in the study (Figure 1). This cat is one-year old and female. Chemosis, blepharospasm, symblepheron, and deep corneal ulcers were observed in clinical examination and the cat suffered by both the pain and physical limitation. The eyes of cat "Şirine" were operated to relief by an ophthalmology surgeon and a conjunctival biopsy material was taken to investigate the presence of mentioned viruses (FcaGHV-1, FHV, FIV, FCV, FIPV, FeLV, and FPV) during operation. After the first operation, as a complication, conjunctival tissue had covered on the corneal surface. Conjunctival tissue was excised using the keratectomy method as a second operation after the active symptomatic period. Another cat named "Sokak Kedisi" was male and two-months old and had corneal ulcer and conjunctivitis in clinical examination.

Phylogenetic analysis of FcaGHV-1 was conducted with downloaded sequences from GenBank. After phylogenetic tree construction, sequences cumulated on two main separated branches which have been colored purple and blue (Figure 2). Both Turkish FcaGHV-1 sequences found to be close to 4484 from Japan (LC437925). These three strains constituted a separate subgroup which had 93.5% similarity rates to other taxa in purple colored cluster (Figure 2).

Table 1. Primer sets used for this study.

Virus	Primer ID	Sequence (5'→3')	Target gene	Ref.
FIV	VE-1S	GAGTAGATACWTGGTTRCAAG	env	(7)
	VE-1R	CATCCTAATTCTTGCATAGC		
	VE-2S	CAAAATGTGGATGGTGGAAY		
	VE-2R	ACCATTCCWATAGCAGTRGC		
FHV-1	FH737-f	GCACACGACCGGCTAATACAGG	gB	(26)
	FH737-r	CAGCTTTCGAGAGGCACATACCC		
FCV	8F	CACSTTATGTCYGACACTGA	C (capsid)	(10)
	8R	CTRGADGTRTGCARRATTT		
FIPV	212	TAATGCCATACACGAACCAGCT	M	(22)
	1179	GTGCTAGATTTGTCTTCGGACACC		
FPV	P1	ATGAGTGATGGAGCAGTTC	VP2	(1)
	VP	TTCTAGGTGCTAGTTGAG		
FcaGHV-1	FeGH-1f	CCTCCCAGGTTCARTWYGCMTAYGA	gB	(6)
	FeGH-1r	CCGTTGAGGTTCTGAGTGTARTARTTRTAYTC		
	FeGH-2f	AAGATCAACCCCACNAGNGTNATG		
	FeGH-2r	GTGTAGTAGTTGTACTCCCTRAACATNGTYTC		
FeLV	I2-xU3-F1	ATTTCACAAGGMATGGAAAATTAC	U3	(20)
	I2-xU3-R1	TAGTTYAAATGAGGCGGAAGGT		

Figure 1. A FcaGHV-1 positive cat, named "Şirine", with the ocular disorders. The cat clinically displayed multisymptomatic ocular disorders including keratoconjunctivitis, symblepheron, and corneal ulcer.

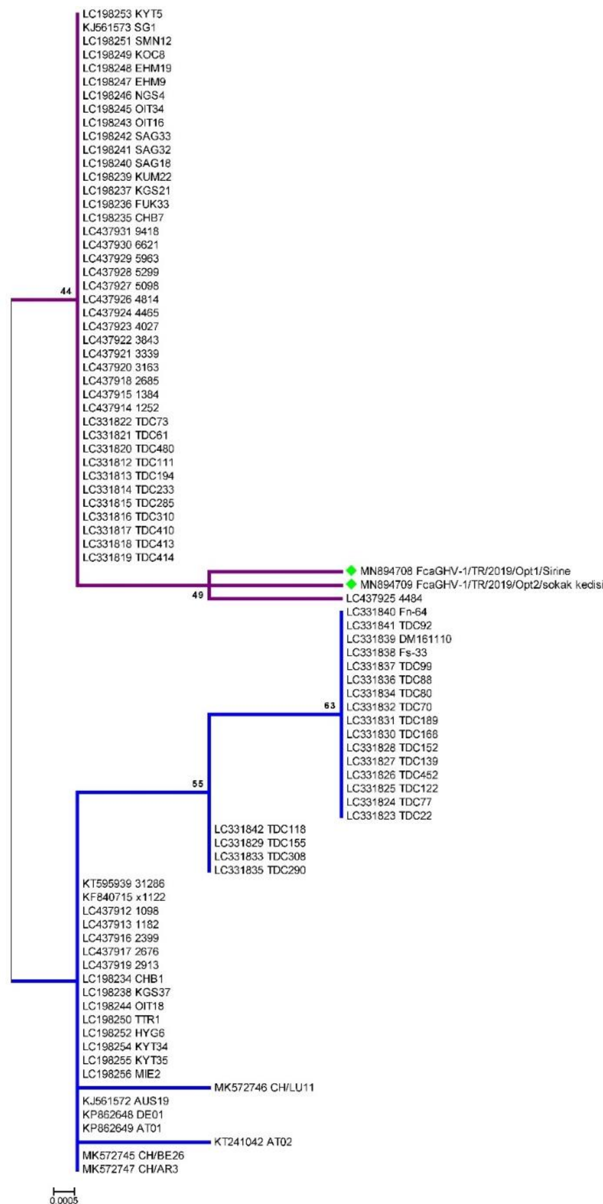

Figure 2. A maximum-likelihood tree of FcaGHV-1. The phylogenetic tree was constructed in MEGA X. Two main branches appeared which one is blue-colored and another is purple colored. The minor subgroup also appeared into purple-colored taxa in which our Turkish FcaGHV-1 sequences existed. Our sequences (GenBank Accession no. MN894708 and MN894709) were marked with "♦".

Discussion and Conclusion

FcaGHV-1 is a newly discovered virus in cats (2). There is not a quite knowledge about the virus's pathogenesis mechanism and its reflection on clinic conditions (2-4, 15-18, 24). Performed studies on FcaGHV-1 have mostly focused on its presence, prevalence, and distribution based on individual features (2-4, 15-18, 24). Accordingly, we, also, have investigated the presence of FcaGHV-1 and its clinical status by appeared symptoms in ocular tissues.

To conclusions of the other studies of FcaGHV-1 that have been performed so far, it has been considered this agent mostly infects to chronically FIV infected cats (2, 3, 16-18). Potential immunodeficiency triggered by FIV might be leading to the predisposition condition for FcaGHV-1 as similar to the pathogenesis mechanism of Human gammaherpesvirus and HIV (2, 17, 25). We have detected proviral DNA of FIV in same individuals infected by FcaGHV-1. It has strengthened the hypothesis that FIV infected cats are more predisposed to FcaGHV-1 up to five times (2, 25). Specifically, FcaGHV-1 infection is more likely to occur as ocular disorders in FIV infected cats. Perhaps, *Equine herpesvirus -5* (EHV-5), a gammaherpesvirus causing ocular disease in equine, should to be paid attention as research on FcaGHV-1 pathogenesis strategy continues (21).

To our knowledge, this report exhibits the first molecular presence of FcaGHV-1 in Turkey. Other

worldwide studies on FcaGHV-1 are available, however, presented molecular data in that studies to be evaluated are limited (2-4, 15-18, 24). Nevertheless, we have found sequences of FcaGHV-1 in GenBank database and downloaded all overlapping gene regions of FcaGHV-1 with our sequences. As the different from other phylogenetic studies, we compared sequences FcaGHV-1 between themselves without sequences other genera of gammaherpesviruses. In the phylogenetic tree, two main clusters occurred which is seemed as purple and blue colored. With this appeared phylogenetic tree, "temporary" genotypes classification might be assumed as an effector for large-scaled and detailed molecular studies in the future. Molecular phylogenetic analysis revealed that Turkish FcaGHV-1 sequences drew a separated branch in purple colored genogroup in the phylogenetic tree. We have assessed alignment at amino acid levels why the possibility of genogroup has appeared. Two critical point changes at 530th (G ↔ R) and 584th (T ↔ I) amino acids of gB have been remarked and accordingly, it has been brought to mind that these changes might be responsible for genogroup diversification although our sequences covering to partial gB (ORF8) of FcaGHV-1 in BLAST (Figure 3). Nevertheless, this would be a speculative approach to decide genotyping or genogrouping. Detailed analysis such as next-generation sequencing (NGS) should be performed. After that, a more accurate decision would appear on genotyping.

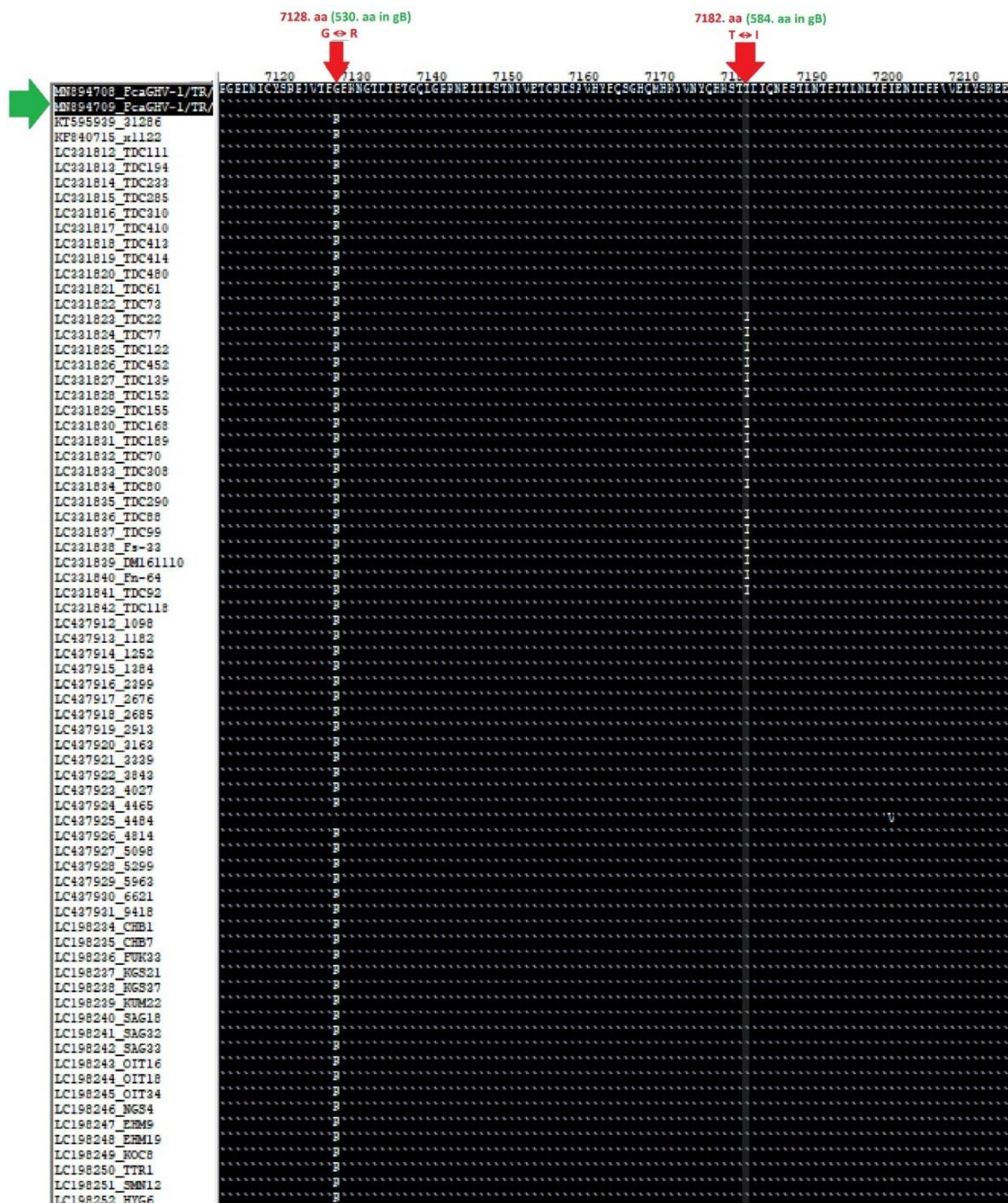

Figure 3. Amino acids changes on partial gB. Point mutations, where 530th (G ↔ R) and 584th (T ↔ I) amino acids, can be considered to lead phylogenetic diversity.

We performed molecular detection and sequencing using conventional PCR primers previously described by Tateno et al. (24). More recently, Real-Time quantitative PCRs (RT-qPCR) have been performed into two different studies from Japan and Italy. RT-qPCR technique was claimed to be more reliable to diagnose FcaGHV-1 than conventional PCR in these studies. We have not yet been provided RT-qPCR facilities in our laboratory, therefore we could not compare outputs with mentioned studies. Detailed prevalence evaluation is not appropriate to compare data from other studies due to the minority of the population, however, only overall rates of presence of FcaGHV-1 could be paid attention. In our study, we found a positive rate to be 4.4 % in the cohort. This result is not compatible with the majority of other prevalence studies which have reported between 6% and 23% (2, 8, 12, 14, 15, 18, 25). Only a few studies exhibited similar positivity rates, for example, Tateno et al. (23) and Caringella et al. (4) found the prevalence percentages as 1.3% (27/2659) and 1.01% (23/1738), respectively. Further analysis would be performed to evaluate both genetic status and both the prevalence of virus according to multivariate knowledge.

In sum, it has been thought by researchers that FcaGHV-1 causes a long-lasting and chronic infection, although it has not been exactly known how its molecular dynamics and pathology work. As related to this, performing only molecular studies on FcaGHV-1 is not enough to assess completely. We should consider in point-of-care, both molecular and serological studies should be performed. Data revealed from this application would leverage to elucidate the pathogenesis mechanism and interaction with immunity and other viruses.

This is the first report presenting molecular presence and phylogenetic of FcaGHV-1 in Turkey. Additionally, through our study, it was verified that FcaGHV-1 might have existed in cats with ocular disorders even though it had been declared being asymptomatic before. Regarding the conclusion of this study, it should be enlightened by further analysis, whether FcaGHV-1 in cats with ophthalmic symptoms were randomly detected in relevant tissues.

Acknowledgements

We thank the staff of Nova Veterinary Clinic, and cat owners' who provide the approval for this study.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns. All client approved and signed the informed consent form that allowing to use of collected samples as materials for this study. The publishing of this study was approved by the Local Ethical Committee for Experimental Animal of the Aydın Adnan Menderes University (No:64583101/2020/035).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Battilani M, Balboni A, Ustulin M, et al** (2011): *Genetic complexity and multiple infections with more Parvovirus species in naturally infected cats*. Vet Res, **42**, 1-9.
2. **Beatty JA, Troyer RM, Carver S, et al** (2014): *Felis catus gammaherpesvirus 1; a widely endemic potential pathogen of domestic cats.* Virology, **460-461**, 100-107.
3. **Beatty JA, Sharp CR, Duprex WP, et al** (2019): *Novel feline viruses: Emerging significance of gammaherpesvirus and morbillivirus infections.* J Feline Med Surg, **21**, 5-11.
4. **Caringella F, Desario C, Lorusso E, et al** (2019): *Prevalence and risk factors for Felis catus gammaherpesvirus 1 detection in domestic cats in Italy.* Vet Microbiol, **238**, 108426.
5. **Chomczynski P, Sacchi N** (2006): *The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on.* Nat Protoc, **1**, 581-585.
6. **Ehlers B, Dural G, Yasmum N, et al** (2008): *Novel mammalian herpesviruses and Lineages within the gammaherpesvirinae: cospeciation and interspecies transfer*. J Virol, **82**, 3509-3516.
7. **Endo Y, Cho KW, Nishigaki K, et al** (1997): *Molecular characteristics of malignant lymphomas in cats naturally infected with feline immunodeficiency virus*. Vet Immunol Immunopathol, **57**, 153-167.
8. **Ertl R, Korb M, Langbein-Detsch I, et al** (2015): *Prevalence and risk factors of gammaherpesvirus infection in domestic cats in Central Europe Herpes viruses.* Virol J, **12**, 146.
9. **Hartmann K** (2012): *Clinical aspects of feline retroviruses: A review*. Viruses, **4,** 2684-2710.
10. **Henzel A, Brum MCS, Lautert C, et al** (2012): *Isolation and identification of feline calicivirus and feline herpesvirus in Southern Brazil*. Brazilian J Microbiol, **43**, 560-568.
11. **Kumar S, Stecher G, Li M, et al** (2018): *MEGA X: Molecular evolutionary genetics analysis across computing platforms*. Mol Biol Evol, **35**, 1547-1549.
12. **Kurissio JK, Rodrigues MV, Taniwaki SA, et al** (2018): *Felis catus gammaherpesvirus 1 (FcaGHV1) and coinfections with feline viral pathogens in domestic cats in Brazil*. Ciência Rural, **48**, e20170480.
13. **Lewin AC, Kolb AW, McLellan GJ, et al** (2018): *Genomic, recombinational and phylogenetic characterization of global feline herpesvirus 1 isolates.* Virology, **518**, 385-397.
14. **Makundi I, Koshida Y, Endo Y, et al** (2018): *Identification of felis catus gammaherpesvirus 1 in*

Tsushima leopard cats (Prionailurus bengalensis euptilurus) on Tsushima Island, Japan. Viruses, **10**, 378.

15. **McLuckie AJ, Barrs VR, Smith AL, et al** (2016): *Detection of Felis catus gammaherpesvirus 1 (FcaGHV1) in peripheral blood B- and T-lymphocytes in asymptomatic, naturally-infected domestic cats.* Virology, **497**, 211-216.
16. **McLuckie AJ, Barrs VR, Lindsay S, et al** (2018): *Molecular diagnosis of Felis catus gammaherpesvirus 1 (FcaGHV1) infection in cats of known retrovirus status with and without lymphoma*. Viruses, **10**, 128.
17. **McLuckie AJ, Barrs VR, Wilson B, et al** (2017): *Felis catus gammaherpesvirus 1 DNAemia in whole blood from therapeutically immunosuppressed or retrovirus-infected cats.* Vet Sci, **4**, 16.
18. **Novacco M, Kohan NR, Stirn M, et al** (2019): *Prevalence, geographic distribution, risk factors and co-infections of feline gammaherpesvirus infections in domestic cats in Switzerland*. Viruses, **11**, 721.
19. **Ravi M, Wobeser GA, Taylor SM, et al** (2010): *Naturally acquired feline immunodeficiency virus (FIV) infection in cats from western Canada: Prevalence, disease associations, and survival analysis*. Can Vet J, **51**, 271-276.
20. **Roca AL, Nash WG, Menninger JC, et al** (2005): *Insertional polymorphisms of endogenous feline leukemia viruses.* J Virol, **79**, 3979-3986.
21. **Rushton JO, Kolodziejek J, Tichy A, et al** (2013): *Detection of equid herpesviruses 2 and 5 in a herd of 266 Lipizzaners in association with ocular findings*. Vet Microbiol, **164**, 139-144.
22. **Simons FA, Vennema H, Rofina JE, et al** (2005): *A mRNA PCR for the diagnosis of feline infectious peritonitis.* J Virol Methods, **124**, 111-116.
23. **Stiles J** (2014): *Ocular manifestations of feline viral diseases*. Vet J, **201**, 166-173.
24. **Tateno M, Takahashi M, Miyake E, et al** (2017): *Molecular epidemiological study of gammaherpesvirus in domestic cats in Japan*. J Vet Med Sci, 79, 1735-1740.
25. **Troyer RM, Lee JS, Vuyisich M, et al (2015):** *First complete genome sequence of Felis catus gammaherpesvirus 1*. Genome Announc, **3**, e01192-15.
26. **Vögtlin A, Fraefel C, Albini S, et al** (2002): *Quantification of feline herpesvirus 1 DNA in ocular fluid samples of clinically diseased cats by real-time TaqMan PCR*. J Clin Microbiol, **40**, 519-523.

Ankara Univ Vet Fak Derg, **68**, 61-68, 2021
DOI: 10.33988/auvfd.724846

Long-term prospective assessment of subconjunctival triamcinolone acetonide in addition to topical therapy in the management of chronic superficial keratitis

Florin BETEG[1,a], Cristina Alexa LELESCU[2,b✉], Andrada Elena URDĂ-CÎMPEAN[3,c], Marian Aurel TAULESCU[4,d], Cosmin MUREȘAN[1,e]

[1]University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Department of Surgery, Cluj-Napoca; [2]Modis Competence Center, Modis Life Sciences Belgium, Cluj-Napoca; [3]Iuliu Hațieganu University of Medicine and Pharmacy, Faculty of Medicine, Department of Medical Informatics and Biostatistics, Cluj-Napoca; [4]University of Agricultural Sciences and Veterinary Medicine, Faculty of Veterinary Medicine, Department of Pathology, Cluj-Napoca, Romania.
[a]ORCID: 0000-0001-6916-188X; [b]ORCID: 0000-0002-6726-6028; [c]ORCID: 0000-0001-7685-0085;
[d]ORCID: 0000-0002-8987-6038; [e]ORCID: 0000-0002-5064-6841.

✉Corresponding author: cristina.alexa.lelescu@gmail.com
Received date: 21.04.2020 - Accepted date: 21.07.2020

Abstract: Chronic superficial keratitis (CSK) is a progressive inflammatory disease in adult dogs, associated with corneal vascular proliferation, edema and melanic pigment deposition. In the absence of a curative therapy, the current approach for the management of CSK aims to control disease progression and eventually decrease the severity of corneal lesions. Subconjunctival injections of corticosteroids may be effective in severe cases, where topical therapy alone is inadequate. The aim of this study was to comprehensively evaluate the effectiveness of subconjunctival triamcinolone acetonide (TA) injection in addition to topical steroid treatment for the long-term management of CSK, in dogs unresponsive to conventional topical steroids. Treatment efficacy was assessed in 11 dogs with bilateral CSK by periodic evaluation of the cornea in terms of vascularization, edema and pigmentation for up to 120 weeks. Clinically significant reductions in corneal pigmentation ($P<0.0001$), vascularization ($P<0.0001$) and edema scores ($P<0.0001$) were achieved during therapy. The greatest reduction was exhibited by corneal edema, closely followed by vascularization. Corneal pigmentation was less responsive and complete regression has not been achieved in any of the dogs. Severely affected corneas exhibited greater improvements in terms of pigmentation, whereas moderately affected corneas showed a slight decrease. Corneal edema and vascularization were adequately controlled, whereas corneal pigmentation exhibited a lower decrease in severity and was more difficult to manage on a long-term basis. This study suggests that subconjunctival injection of TA in addition to topical steroids could represent an effective option for long-term management of CSK in dogs unresponsive to topical steroids alone.
Keywords: Chronic superficial keratitis, dogs, subconjunctival, triamcinolone acetonide.

Introduction

Chronic superficial keratitis (CSK), also known as *pannus*, is a chronic inflammatory disease of the cornea in adult dogs, characterized by local progressive vascular proliferation, edema, inflammatory mononuclear cell infiltration and melanic pigment deposition (4, 10). Etiopathogenesis of CSK is not fully understood, but generally, it is considered to be an autoimmune condition with a genetic component, commonly affecting German shepherds, Belgian shepherds and greyhounds (16, 18). In the early stage, a vascular conjunctival lesion is usually detected, followed by inflammatory cell infiltration of the corneal stroma, melanosis and corneal vascularization. The corneal changes progress gradually from the temporal limbal region towards the central area. As the disease progresses, opacification, edema and pigmentation may worsen and spread over the entire cornea (10, 18, 21).

Currently, no curable treatment for CSK is known (18). Therefore, the primary treatment goal is to control the disease progression and eventually decrease the severity of corneal lesions (9). Classical approach of slightly or moderately affected cases involves the administration of long-term corticosteroid eye drops or ointments, alone or in addition to topical cyclosporine (18, 31). Application of tacrolimus and dimethyl sulfoxide drops resulted in decreased inflammatory activity and vascularization; still, gradual progression of pigmentation could not be ceased (4). Soft X-ray therapy (1), UV-

blocking contact lenses (9), topical administration of pimecrolimus (24) and superficial keratectomy (18) were described as treatment options in order to control the disease progression and alleviate specific symptoms.

Subconjunctival injections of depot corticosteroids in addition to topical solutions (dexamethasone, prednisolone) are indicated in severe or non-responsive cases (21). Triamcinolone acetonide (TA) is a long-acting, moderate-potency synthetic corticosteroid with anti-inflammatory, anti-permeability and anti-fibrotic properties (2, 19). TA has a rapid onset of action, prolonged and enhanced anti-inflammatory effect (3–6 weeks or more), decreased mineralocorticoid activity and significant glucocorticoid activity (11, 14). However, there is a lack of knowledge regarding the effectiveness and safety of long-term subconjunctival administration of TA in CSK.

In this context, our aim was to describe a long-term subconjunctival and topical steroid-antibiotic combination therapy for the management of CSK in dogs unresponsive to topical steroids alone. Furthermore, a prospective assessment of its clinical effectiveness in terms of corneal vascularization, edema and pigmentation, was performed over 120 weeks.

Material and Methods

Animals: A total number of 22 corneas from 11 canine patients aged 3–9 years, *weighing between* 8 to 45 kg, with bilateral progressive corneal lesions of CSK of varying intensities (e.g. corneal vascularization, pigmentation, and edema) and with relevant cytological findings that were previously treated with conventional topical steroids and were unresponsive or relapsing, were enrolled in this study. Dogs were excluded if they had not been treated consistently due to poor owner compliance to treatment recommendations, or due to lack of follow-up. Severe corneal pigmentation that did not allow evaluation of vascularization was also considered as an exclusion criterion. The following patient's data were collected: breed, color, date of birth, gender, neuter status, country of origin and any concurrent diseases (if present). At the end of the study, all dogs remained under clinical observation.

***Study design*:** The present prospective analytical study was conducted over a period of 120 weeks and was approved by the Bioethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (No. 38/ 21.11.2016). The dogs were enrolled in the study consecutively as they arrived at the clinic for ophthalmologic evaluation and met the inclusion criteria. After informed consent was obtained from owners, the dogs were subjected to complete ophthalmologic examination in order to assess the severity of corneal lesions, including corneal edema, melanic pigmentation and vascularization, prior and during the treatment at 2, 4, 8, 12, 16, 20, 24, 48, 72, 96 and 120 weeks.

Ophthalmic examination and assessment of the corneal lesions: Each patient underwent direct (Heine® Beta 200) and indirect (Heine® Omega 500) ophthalmoscopy, fluorescein staining (Fluoro Touch), Schirmer tear test (Tear Touch Blu) and intraocular pressure measurement (Tono-Pen®, Reichert). The cornea was schematically divided into 24 sectors to assess corneal pigmentation (1, 12), and each sector was graded in terms of melanic pigmentation extent. Corneal vascularization and edema were quantified each, by using a specific four grade scale, as presented in Table 1.

Table 1. Grading of corneal alterations in dogs with CSK

Corneal alterations	Grading system	Reference
Corneal melanic pigmentation	**(a)** Schematic division of the cornea in 24 sectors	(1, 12)
	(b) Areas of each sector were graded according to the following grading system: 0= no pigmentation 1= pigmented area <30% of the sector area 2= pigmented area 30–60% of the sector area 3= pigmented area >60% of the sector area The resulting grades of each sector were re-added in order to obtain a final pigmentation grade.	(3, 4)
Corneal neovascularization	0= no vessels visible 1= mild superficial vascularization, thin vessels visible with magnification 2= profuse superficial vascularization 3= extensive vascularization with thick vessels present in all of the quadrants	(3)
Corneal edema	0= no signs 0.5–1= mild corneal haze 2= marked corneal opacity, but anterior chamber still visible 3= severe corneal opacity, anterior chamber not visible	(3)

Table 2. Administration protocol of topical and subconjunctival therapy

Dosing frequency	Day 1– week 4	Week 4– 8	Week 8– 24	Week 24–72
Neomycin sulfate–dexamethasone-polymyxin B sulfate ophthalmic suspension (1-2 drops)	6-8 times/ day		4 times/ day	2 times/ day
Triamcinolone acetonide injectable suspension (0.4 mg)	-	1 administration/ month		1 administration every 3 months

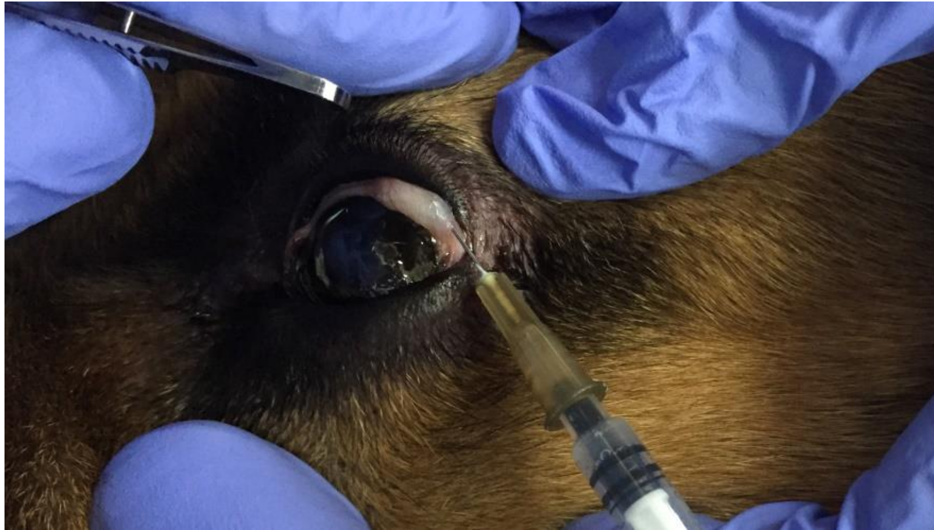

Figure 1. Site of subconjunctival injection

Treatment: Topical neomycin sulfate – dexamethasone – polymyxin B sulfate ophthalmic suspension (Maxitrol®, Alcon Couvreur, Belgium) was applied six to eight times a day, since day 1. Starting with the fourth week, 0.2% triamcinolone acetonide injectable suspension (Retardoesteroide®, Laboratorios Calier, Spain) was administered subconjunctivally once-monthly. Topical administration of the steroid-antibiotic combination was decreased gradually since the 8th week of treatment, to four times a day. Starting with the 24th week, TA was injected subconjunctivally once every 3 months, simultaneously decreasing the frequency of administration of the ophthalmic suspension to twice a day. After 72 weeks of treatment, TA was administered every 6 months, and the steroid-antibiotic combination was applied topically one-two times a day for the rest of the treatment period (Table 2).

The subconjunctival injection was performed following topical anesthesia with 0.4% oxibuprocaine chlorhydrate (Benoxi®, Unimed Pharma Ltd., Slovakia); in 4 patients, sedation was needed in order to complete the procedure. Using a fine needle (25G), 0.4 mg of TA injectable suspension (2mg/ml), was administered beneath the bulbar conjunctiva, at the superotemporal quadrant (Figure 1). All dogs received the topical treatment and the subconjunctival injections in both eyes.

Statistical analysis: Quantitative data without normal distribution were described using the median (Q1-Q3), where Q1-Q3 (interquartile range) stands for the range between 25th percentile (Q1) and 75th percentile (Q3). The Friedman test (for repeated measures, used with post hoc tests) was applied to check if there was a significant difference between all non-normally distributed variables. A P-value equal to or lower than 0.05 was considered statistically significant. In case of a significant difference between all measurements, separate Wilcoxon signed-rank tests (using the Bonferroni correction) were run to compare the measurements from week 0 with each of the other weeks. A P-value equal to or lower than 0.0045 was considered statistically significant for the Wilcoxon signed-rank tests.

Results

A total of 11 dogs (3 females, 8 males) diagnosed with bilateral CSK were enrolled in this study, including 6 German shepherds (54.5%), 2 German shepherd mix breeds (18.1%), 2 Belgian shepherds (18.1%) and one Pekingese (9.09%). The mean age at diagnosis was 6.45 ± 2.58 years. No other concurrent ophthalmic conditions were diagnosed in these dogs, besides CSK.

Cytologic examination of corneal scrapings revealed a moderate to large number of inflammatory cells,

including lymphocytes and plasma cells, admixed with fibroblasts, endothelial cells, melanin-laden macrophages and hyperplastic epithelial cells.

Assessment of corneal lesions: The evolution of corneal lesion scores measured during the study is presented in Table 3. Comparing all measurements performed during treatment, a statistically significant difference in corneal pigmentation (Friedman test: p<0.0001), neovascularization (Friedman test: p<0.0001) and edema (Friedman test: p<0.0001) scores was observed.

Corneal pigmentation assessment: The scores for corneal pigmentation decreased in time, from a median of 22.5 at week 0 to a median of 12.5 after 24 weeks of treatment (Table 3). A statistically significant reduction of corneal pigmentation scores was observed between the values measured at week 0 and those measured during the other weeks (Wilcoxon Signed Ranks Test: P≤0.0045), (Table 4).

Corneal vascularization assessment: The scores for corneal vascularization decreased in time (Figure 2), from a median of 3 at week 0 to a median of 1 after 20 weeks of treatment (Table 3). There was no significant difference detected between the measured scores at week 0 and week 2 (Wilcoxon Signed Ranks Test: P= 1), nor between the scores obtained at week 0 and week 4 (Wilcoxon Signed Ranks Test: P=0.00815). However, a statistically significant reduction was detected after 8 weeks of treatment, compared to baseline (week 0) (Table 4).

Table 3. Descriptive statistics for ophthalmic examination measurements

Week number	Corneal pigmentation (N=22) Median (Q1–Q3)	Corneal vascularization (N=22) Median (Q1–Q3)	Corneal edema (N=22) Median (Q1–Q3)
Week 0	22.50 (16.75–37.25)	3 (2–3)	2 (2–3)
Week 2	20.00 (15.75–34.00)	3 (2–3)	2 (1.75–3)
Week 4	19.00 (14.00–31.00)	2 (1.75–3)	1 (1–2)
Week 8	16.50 (13.75–28.55)	2 (1–2)	1 (1–2)
Week 12	14.50 (11.75–27.00)	2 (1–2)	1 (0–2)
Week 16	14.00 (9.75–24.25)	1.5 (1–2)	1 (0–1)
Week 20	14.50 (9.00–21.25)	1 (1–2)	0.5 (0–1)
Week 24	12.50 (8.75–20.25)	1 (0–1.25)	0 (0–1)
Week 48	11.00 (8.00–20.5)	1 (0.75–2)	0.5 (0–1)
Week 72	9.00 (7.00–18.25)	1 (1–1.25)	0.5 (0–1)
Week 96	9.00 (5.75–16.50)	1 (0 –1.25)	0 (0–1)
Week 120	8.50 (5.00–16.00)	1 (0–1.25)	0 (0–1)

Table 4. Comparison of corneal pigmentation, neovascularization and edema scores measured during treatment, with scores obtained at week 0.

Comparison of measurements	Corneal pigmentation (N=22) Wilcoxon Signed Ranks Test: P-value*	Corneal neovascularization (N=22) Wilcoxon Signed Ranks Test: P-value*	Corneal edema (N=22) Wilcoxon Signed Ranks Test: P-value*
Week 0–Week 2	0.00005	1	0.1573
Week 0–Week 4	0.00003	0.00815	0.00001
Week 0–Week 8	0.00004	0.00018	0.00000
Week 0–Week 12	0.00004	0.00002	0.00001
Week 0–Week 16	0.00004	0.00002	0.00002
Week 0–Week 20	0.00004	0.00002	0.00002
Week 0–Week 24	0.00004	0.00002	0.00002
Week 0–Week 48	0.00004	0.00002	0.00004
Week 0–Week 72	0.00004	0.00002	0.00003
Week 0–Week 96	0.00004	0.00002	0.00002
Week 0–Week 120	0.00004	0.00002	0.00002

* Significance level: P≤0.0045

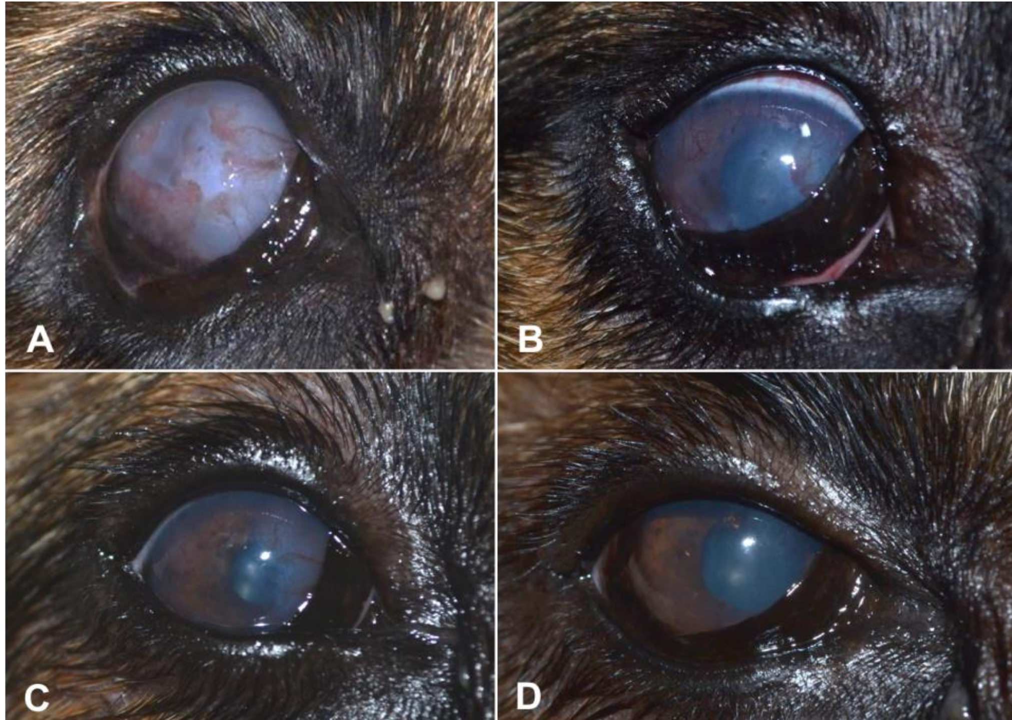

Figure 2. Evolution of corneal lesions during polytherapy (German Shepherd male, age at diagnosis: 4 years). **A.** 100% of the cornea is affected by severe edema, fibrovascular proliferation and pigmentation (at the lower temporal quadrant) before initiating the polytherapy (week 0). **B.** 6 weeks after starting the polytherapy, the corneal lesions are markedly drecreased, allowing visualization of the iris and pupil **C.** At week 20, edema and fibrovascular proliferation are significantly regressed, particularly in the temporal half of the cornea **D.** At week 120, regressed (ghost) vessels and partially regressed vessels with slight edema are still present at the nasal half of the cornea. Pigmentation is visible at the lower temporal quadrant.

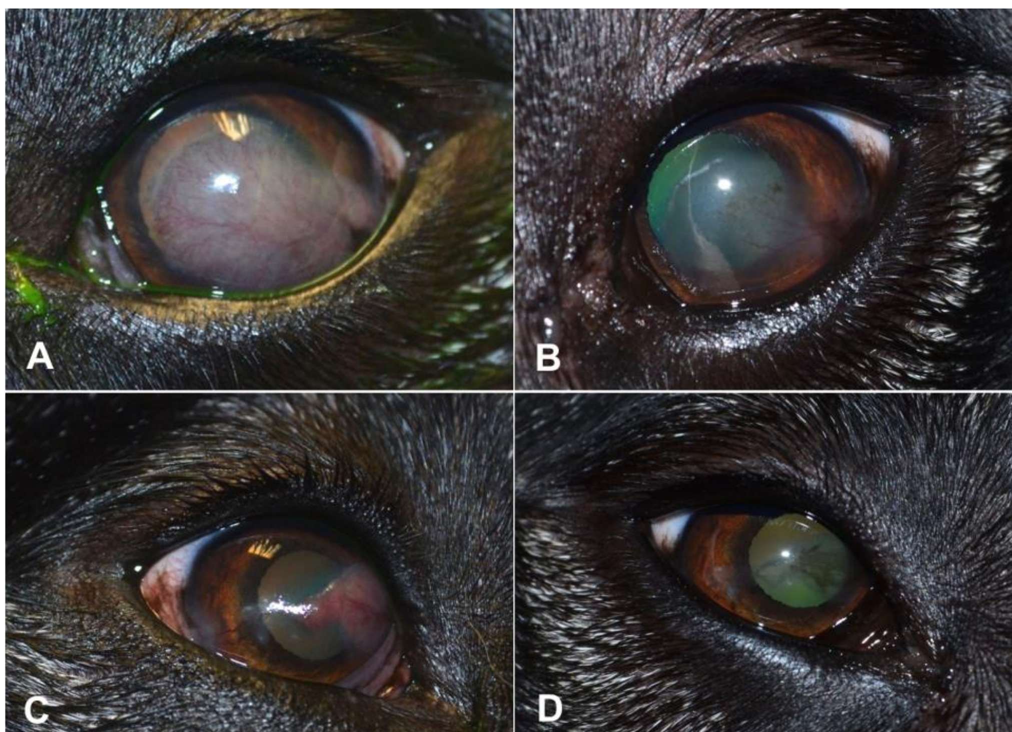

Figure 3. Evolution of corneal lesions during polytherapy (Mix breed female, age at diagnosis: 6 years). **A.** Left eye, week 0: more than 50% of the cornea is affected by severe fibrovascular proliferation and pigmentation (at the lower temporal quadrant) before initiating the polytherapy **B.** Left eye, week 72: the fibrovascular proliferation is markedly attenuated, allowing corneal transparency; mild vascularization with pigmentation are still present in the lower temporal quadrant **C.** Right eye, week 0: less than 50% of the cornea is affected by fibrovascular proliferation extending centrally from the nasal angle; pigmentation is present in the lower temporal quadrant **D.** Right eye, week 72: significant attenuation of the fibrovascular tissue, allowing corneal transparency.

Corneal edema assessment: The score for corneal edema decreased in time, from a median of 2 at week 0 to a median of 0.5 after 20 weeks of treatment. Subsequently, the mean score increased at week 48 and 72 and then decreased again, reaching a median value of 0 at week 120 (Table 3). There were no significant differences between the measurements at week 0 and week 2 in terms of corneal edema scores (Wilcoxon Signed Ranks Test: P=0.1573). However, there was a statistically significant reduction of the scores after 4 weeks of treatment, compared to week 0 (Table 4).

Epithelial and stromal damage of the cornea led to the development of subsequent corneal scarring in patients with severe lesions. However, all eyes remained visual during therapy and no signs of a decrease in visual acuity were observed.

***Side effects*:** There were no major side effects of using this therapeutic protocol that required its discontinuation. Mild to moderate discomfort, accompanied by blepharospasm, irritation and scratching of the eyeball were noted in 3 dogs, mainly after administration of the ophthalmic drops. These signs ceased after using an Elizabethan collar until the dogs were accustomed to the procedure.

Discussion and Conclusion

The results obtained in this study provide an accurate long-term assessment of an ophthalmic combination therapy, consisting of TA subconjunctival injections in addition to topical steroid-antibiotic administration, in dogs with CSK, by using a scoring system for each specific corneal lesion. To the author's knowledge, this is the first long-term follow-up study of such a polytherapy in the management of canine pannus. Because CSK is a progressive disease that requires life-long therapy (18), we strongly believe that long-term assessment of various treatment options provides a valuable contribution to the management plan of this condition.

Previous studies (5, 13, 17, 22) have mentioned the potential benefits of subconjunctival corticosteroid injections, including betamethasone and methylprednisolone acetate in the treatment of CSK. However, none of these researches performed a long-term follow-up of subconjunctival TA administration as part of a combination therapy. Although intravitreal TA has been evaluated in dogs in terms of safety and efficacy (23), much more is known about the subconjunctival administration of TA in human ophthalmology, particularly in the treatment of endothelial corneal allograft rejection (6) and non-necrotizing anterior scleritis (27). Therefore, difficulties were encountered in comparing the data obtained in this study with other previous studies carried out in a similar context; in spite of this, we compared our findings with studies that used a similar assessment model of corneal lesions treated with various adjuvant therapies.

Data periodically collected during this study on the independent evolution of CSK typical clinical signs showed a distinctive efficacy of the polytherapy on each type of lesion (pigmentation, neovascularization, edema). The main emerging result is that the scores measured in each type of lesion at 120 weeks of treatment were significantly decreased, compared to those obtained initially. Of the assessed lesions, corneal edema exhibited the greatest reduction of severity. In addition, total resolution of the edema was detected in 13 out of 22 corneas at the end of the study period. Immune-mediated keratitis may be associated with vascular damage of the corneal endothelium, leading to the absorption of aqueous humour and early-onset of edema (18). Even though the recurrence of edema was noticed during therapy, an overall decrease occurred in all of the cases. Adjunctive therapies such as cryotherapy and UV-blocking contact lenses showed a significant drawback from this point of view, by causing additional corneal edema due to hypoxic stress or epithelial injury (3, 9).

Any kind of corneal injury that causes inflammation may induce angiogenesis (20). Corneal vascularization was proved to be the second most responsive lesion to polytherapy. The initial average score of corneal vascularization decreased from 3 to 1 at 120 weeks after starting the polytherapy. Moreover, a considerable number of 6 out of 22 corneas showed a total resolution in terms of angiogenesis. Previous studies reported no effects of UV-blocking lens on vascularization (9), whilst topical application of pimecrolimus proved remarkable efficacy in half of the animals (24). Both polytherapy and topical pimecrolimus caused a similar effect on vascularization in the first 2 weeks of treatment, leading to a significant reduction in its severity. However, complete regression occurred after 11 weeks of topical pimecrolimus administration, while the present polytherapy indicated a similar effect after 16 weeks, in only one cornea. A significant decrease in corneal vascularization was also achieved with tacrolimus and dimethyl sulfoxide (DMSO) ophthalmic drops (4), but the long-term clinical evolution of vascular lesions is still unknown. Soft X-ray therapy caused a similar decrease in vascularization, that was maintained for 24 weeks after completion of the procedure in most eyes (1). Despite this, no data on the evolution of vascular lesions beyond this period is yet available.

The average corneal pigmentation scores obtained in periodical evaluations suggest a significant decrease of almost 50% after 120 weeks of treatment. Unfortunately,

complete resolution of this lesion has not been achieved in any of the treated corneas and relapses occurred during the treatment. The most prominent reduction in corneal pigmentation occurred in severely affected corneas, whereas mild to moderately affected corneas exhibited a slower and less pronounced improvement. This is in good agreement with the results obtained after topical administration of pimecrolimus (24), where regression of pigmentation in the least affected cornea took 24 weeks. While pigmentation increased in eyes wearing UV-blocking contact lenses (9), a very rapid improvement was observed 5 days following cryotherapy. Despite this rapid effect, after 30 days, recurrence of pigmentation was detected. Administration of DMSO and tacrolimus resulted in a reduction of pigmentation in 14 out of 32 corneas, over a period of 5 weeks (4). It is generally considered that corneal pigmentation is more difficult to manage for the long-term than other typical corneal changes, such as neoangiogenesis and granulation tissue proliferation (18, 28).

Various injectable preparations of corticosteroids are available, including betamethasone, dexamethasone, methylprednisolone acetate and triamcinolone (acetonide and diacetate). Subconjunctival depot injection of TA allows a slow release from the injection site, thus ensuring a prolonged duration of action which can last up to 3–6 weeks or more (9, 14, 15). Even if it was long believed that subconjunctival injections may have the potential to cause scleral thinning and even globe perforation (8, 30), subsequent studies performed on a representative number of patients indicated the opposite (7, 29). However, these studies have been conducted in human patients and there are no extensive reports on the safety of long-term subconjunctival administration in dogs, although it is a convenient and easy-to-perform therapeutic procedure in veterinary practice, as compared to certain therapeutic alternatives described above.

Suggested doses for subconjunctival injection in dogs are still contradictory. They may vary between 4–12 mg according to some authors (26), whereas others contraindicate intralesional administration of more than 0.6 mg at one site (25). Even if short-term administration of high doses does not appear to cause toxicity, long-term treatment with TA can lead to serious adverse effects (25). Since the present therapy included concomitant daily topical administration of dexamethasone, we considered using lower doses of subconjunctival TA than those typically recommended for this procedure. Furthermore, we aimed to gradually reduce the frequency of administration of the topical therapy, from six–eight times a day to only twice a day after 24 weeks, concomitantly increasing the intervals between subconjunctival administrations. This resulted in an inhibition of corneal pigmentation regression in most cases and slight recurrence in 10 out of 22 corneas. No significant effects on the other corneal scores were observed.

Our study has several limitations. First, it would have been beneficial to include a distinct control group, treated only with topical steroids. On the other hand, the study was performed on client-owned dogs diagnosed with a progressive corneal disease, that were previously treated with conventional topical therapy alone and were either unresponsive to treatment or had clinical signs of relapse after a period of time. Therefore, we consider that meaningful conclusions could be drawn despite this limitation. Secondly, the authors admit the relatively small number of dogs included in the study. Still, CSK is not frequently encountered in temperate climate countries and, moreover, the chosen inclusion criteria led to a significant reduction in the number of dogs enrolled.

The results of this study suggest that subconjunctival injection of triamcinolone acetonide in addition to topical steroid-antibiotic therapy could represent an effective option for the long-term management of CSK in dogs unresponsive to topical steroids. Among the corneal lesions evaluated, corneal edema and vascularization showed a superior response to treatment, while pigmentation was more difficult to manage on a long-term basis.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study was approved by Bioethics Committee of the University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca (No. 38/ 21.11.2016).

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Allgoever I, Hoecht S** (2010): *Radiotherapy for canine chronic superficial keratitis using soft X- rays*. Vet Ophthalmol, **13**, 20-25.
2. **Athanasiadis I, de Wit D, Patel AK, et al** (2012): *Subconjunctival injection of triamcinolone acetonide in the management of corneal graft rejection and new vessels*. J Clin Pharmacol, **52**, 607-612.
3. **Azoulay T** (2013): *Adjunctive cryotherapy for pigmentary keratitis in dogs: a study of 16 corneas*. Vet Ophthalmol, **17**, 241-249.
4. **Balicki I** (2012): *Clinical study on the application of tacrolimus and DMSO in the treatment of chronic superficial keratitis in dogs*. Polish J Vet Sci, **15**, 667-676.
5. **Bedford PGC** (1972): *The treatment of keratitis and corneal ulceration in the dog*. Vet Ann, **13**.

6. **Costa DC, de Castro RS, Kara-Jose N** (2009): *Case-control study of subconjunctival triamcinolone acetonide injection vs intravenous methylprednisolone pulse in the treatment of endothelial corneal allograft rejection*. Eye, **23**, 708-714.
7. **Croasdale CR, Brightbill MD** (1999): *Subconjunctival Corticosteroid Injections for Nonnecrotizing Anterior Scleritis.* Arch Ophthalmol, **117**, 978-979.
8. **de la Maza MS, Jabbur NS, Foster CS** (1993): *An analysis of therapeutic decision for scleritis*. Ophthalmology, **100**, 1372-1376.
9. **Denk N, Fritsche J, Reese S** (2011): *The effect of UV-blocking contact lenses as a therapy for canince chronic superficial keratitis*. Vet Ophthalmol, **14**, 186-194.
10. **Esson DW** (2015): Clinical atlas of canine and feline ophthalmic disease. JohnWiley & Sons Inc, Ames, Iowa.
11. **Grossmann C, Scholz T, Rochel M, et al** (2004): *Transactivation via the human glucocorticoid and mineralocorticoid receptor by therapeutically used steroids in CV-1 cells: a comparison of their glucocorticoid and mineralocorticoid properties*. Eur J Endocrinol, **151**, 397-406.
12. **Grüning G, Allgoewer I, Höcht S, et al** (2001): *Zur strahlentherapie der keratitis superficialis chronica mit strontium 90*. Kleintierpraxis, **46**.
13. Hallstrom M (1970): *Synspunkter ph behandling av keratitis chronica superficialis*. Svensk Veterinartidning, 22.
14. **Hardman JG, Limbird LE, Gilman AG** (2001): Goodman & Gilman's The Pharmacological Basis of Therapeutics. McGraw Hill, New York.
15. **Jermak CM, Dellacroce JT, Heffez J, et al** (2007): *Triamcinolone acetonide in ocular therapeutics*. Surv Ophthalmol, **52**, 503-522.
16. **Jokinen P, Rusanen EM, Kennedy LJ, et al** (2011): *MHC class II risk haplotype associated with canine chronic superficial keratitis in German Shepherd dogs*. Vet Immunol Immunopathol, **140**, 37-41.
17. **Krahenmann A** (1972): *Zur therapie der keratitis superficialis chronica (uberreiter) des deutschen schaferhundes*. Ophthalmologica, **165**.
18. **Ledbetter EC, Gilger BC** (2013): Diseases and Surgery of the Canine Cornea and Sclera. 976-1050. In: KN Gelatt (Ed), Veterinary Ophthalmology. Wiley-Blackwell, Ames, Iowa.
19. **Lewis G, Campbell W, Johnson A** (1986): *Inhibition of prostaglandin synthesis by glucocorticoids in human endothelial cells*. Endocrinology, **119**, 62-69.
20. **Maddula S, Davis DK, Maddula S, et al** (2011): *Horizons in therapy for corneal angiogenesis*. Ophthalmology, **118**, 591-599.
21. **Maggs DJ** (2008): Cornea and Sclera. 175-202. In: DJ Maggs, P Miller, R Ofri (Eds), Slatter's Fundamentals of Veterinary Ophthalmology. Elsevier Saunders, Philadelphia.
22. **Magrane WG** (1971): Canine Ophthalmology. Lea & Febiger, Philadelphia.
23. **Molleda JM, Tardón RH, Gallardo JM, et al** (2008): *The ocular effects of intravitreal triamcinolone acetonide in dogs*. Vet J, **176**, 326-332.
24. **Nell B, Walde I, Billich A, et al** (2005): *The effect of topical pimecrolimus on keratoconjunctivitis sicca and chronic superficial keratitis in dogs: results from an exploratory study*. Vet Ophthalmol, **8**, 39-46.
25. Plumb DC, Pharm D (2008): Plumb's Veterinary Drug Handbook. Blackwell Publishing, Ames, Iowa.
26. **Rankin A** (2013): Clinical Pharmacology and Therapeutics, Anti-Inflammatory and Immunosuppressant Drugs. 407-423. In: KN Gelatt (Ed), Veterinary Ophthalmology. Wiley-Blackwell, Ames, Iowa.
27. **Roufas A, Jalaludin B, Gaskin C, et al** (2010): *Subconjunctival triamcinolone treatment for non-necrotising anterior scleritis*. Br J Ophthalmol, **94**, 743-747.
28. **Sandmeyer LS, Bauer BS, Grahn BH** (2017): *Diagnostic Ophthalmology*. Can Vet J, **58**, 91–93.
29. **Tu EY, Culbertson WW, Pflugfelder SC, et al** (1995): *Therapy of nonnecrotizing anterior scleritis with subconjunctival corticosteroid injection*. Ophthalmology, **102**, 718-724.
30. **Watson PG** (1974): *Treatment of scleritis and episcleritis*. Trans Ophthalmol Soc UK, **94**, 773.
31. **Williams D, Hoey A, Smitherman P** (1995): *Comparison of topical cyclosporin and dexamethasone for the treatment of chronic superficial keratitis in dogs*. Vet Rec, **137**, 635-639.

Ankara Univ Vet Fak Derg, **68**, 69-75, 2021
DOI: 10.33988/auvfd.775884

Detection of SARS-CoV-2 using five primer sets

Alper KARAGÖZ[1,a], Hidayet TUTUN[2,b], Tutku ARSLANTAŞ[3,c], Özlem ALTINTAŞ [4,d,✉], Nadir KOÇAK[5,e], Levent ALTINTAŞ [6,f]

[1]Uşak University, Department of Molecular Biology and Genetics, Uşak; [2]Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Burdur; [3]Çankırı Public Hospital, Microbiology Laboratory, Çankırı; [4]Veterinary Control Central Research Institute, Ankara; [5]Selçuk University, Medical Genetics, Konya; [6]Ankara University, Faculty of Veterinary Medicine, Department of Pharmacology and Toxicology, Ankara, TURKEY
[a] ORCID: 0000-0002-8178-223X; [b] ORCID: 0000-0001-9512-8637; [c] ORCID: 0000-0002-4351-6600;
[d] ORCID: 0000-0001-6467-9647; [e] ORCID: 0000-0002-1727-1582; [f] ORCID: 0000-0002-5148-723X

✉Corresponding author: ozlembayram@hotmail.com
Received date: 30.07.2020 - Accepted date: 13.09.2020

Abstract: A novel coronavirus (SARS-CoV-2) outbreak, responsible for a pneumonia-associated respiratory disorder (COVID-19), has started in early December 2019 in Wuhan, China, and has rapidly spread around the world. Rapid and accurate diagnostic testing plays a crucial role in tackling the COVID-19 pandemic. In this study, it was aimed to compare 5 primer sets designed to amplify different regions for the detection of SARS-CoV-2 and to perform sequence analysis. Conventional RT-PCR was carried out using primers targeting different regions of the virus genome including ORF1ab, Envelope (E), RNA-dependent RNA polymerase (RdRp), Spike (S) and Nucleocapsid (N) genes for the diagnosis of COVID-19. DNA sequence of ORF1ab gene from each sample were compared with the DNA sequence data of SARS-CoV-2 stored in the GenBank and ORF1ab phylogenetic tree was constructed. The amplicon sizes of ORF1ab, S, E, N and RdRp genes were 588 bp, 440 bp, 145 bp, 323 bp and 196 bp, respectively. The SARS-CoV-2 RNA was detected from 74% of total samples from RdRp gene, 87% for N gene, 74% for S gene, 61% for E gene and 82% for ORF1ab region. The ORF1ab sequences of SARS-CoV-2 from 82 patients were had 100% identity to the sequence of Wuhan isolate and among themselves. The phylogenetic analysis revealed that all isolates formed a cluster. The results of this study suggest that the N region is the best for SARS-CoV-2 identification.
Keywords: Conventional RT-PCR, COVID-19, Diagnosis, E gene, N gene, ORF1ab.

SARS-CoV-2'nin beş primer seti kullanılarak belirlenmesi

Özet: Pnömoni ile ilişkili solunum bozukluğundan (COVID-19) sorumlu yeni bir koronavirüs (SARS-CoV-2) salgını Aralık 2019'un başında Çin'in Wuhan şehrinde başladı ve hızla dünyaya yayıldı. Hızlı ve doğru teşhis testleri COVID-19 salgını ile mücadelede çok önemli bir rol oynar. Bu çalışmada SARS-CoV-2'nin saptanması için farklı bölgelerinin amplifiye edilmesi amacıyla tasarlanmış 5 primer setin karşılaştırılması ve sekans analizinin yapılması amaçlanmıştır. Konvansiyonel RT-PCR, COVID-19 tanısı için ORF1ab, Zarf (E), RNA-bağlı RNA polimeraz (RdRp), Spike (S) ve Nükleokapsid (N) genleri içeren virüs genomunun farklı bölgelerini hedefleyen primerler kullanıldı ve ORF1ab geninin DNA dizisi, GenBank SARS-CoV-2 DNA dizisi verileri ile karşılaştırılarak, ORF1ab filogenetik ağacı oluşturuldu. ORF1ab, S, E, N ve RdRp genlerinin amplikon boyutları, sırasıyla 588 bp, 440 bp, 145 bp, 323 bp ve 196 bp idi. Toplam örneklerin %74'ünde RdRp geni, %87'sinde N geni, %74'ünde S geni, %61'inde E geni ve %82'sinde ORF1ab geni tespit edildi. 82 hastadan SARS-CoV-2'nin ORF1ab dizileri, Wuhan izolat dizisi ve kendi aralarında %100 özdeşliğe sahipti. Filogenetik analiz, tüm izolatların bir küme oluşturduğunu ortaya çıkarmıştır. Bu çalışmanın sonuçları, N bölgesinin SARS-CoV-2 tespiti için en iyisi olduğunu göstermektedir.
Anahtar Kelimeler: COVID-19, E geni, Konvansiyonel RT-PCR, N geni, ORF1ab, Tanı.

Introduction

Coronaviridae is a family of enveloped, positive sense and single-stranded ribonucleic acid (RNA) viruses, with a large genome between 27-31 kilobases in size (23). Coronaviruses have been identified in a wide range of hosts, including birds and mammals, and can cause respiratory, enteric, hepatic and neurological diseases of varying severity (12, 25, 26, 29). A novel human-infecting coronavirus (COVID-19), named Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2), emerged in December 2019, in Wuhan, China, and has spread rapidly throughout the world (32). Most of the

patients infected with the coronavirus develop clinical manifestations including fever, difficulty in breathing, cough, and chest radiographs showing invasive lesions of both lungs (5, 9). Multiple organ failures and death can be observed in severe cases (24).

Until today, no treatments have been found for treating SARS-CoV-2. However, several options including antiviral drugs such as Favipirarvir, Lopinavir, Ritonavir (3), anti-inflammatory agents such as Baricitinib, Ruxolitinib, Fedratinib (19) and other drugs such as chloroquine (18) have been used to improve clinical symptoms of the infection (30, 31).

In COVID-19 Guide of Turkey Ministry of Health (21), it is suggested to immediately start hydroxychloroquine (HCQ) treatment on patients with a probability of COVID-19, along with several other antiviral drugs. Oseltamivir was used with HCQ in March and April but was discontinued. Favipiravir or Remdesivir has been used in cases with progressing pneumonia or clinical signs becoming severe as an additional agent. Also, anticoagulants agents and monoclonal antibodies such as Tocilizumab are used in intensive-care units for patients with COVID-19 related pathologies such as thrombosis.

Coronavirus genome consists of 6-11 open reading frames (ORFs) encoding 27 proteins. ORF1ab constitutes about two-thirds of the whole genome length, encoding a total of 16 nonstructural proteins (nsps) and remaining one-third of the genome encodes four structural proteins and at least six accessory proteins (11, 28). The main structural proteins are Spike surface glycoprotein (S), Membrane (M), Nucleocapsid protein (N), Envelope (E) and accessory proteins (ORF3, ORF4a, ORF4b, ORF5, and ORF8b) encoded by ORFs (4). For conducting the PCR assays, these target proteins can be utilized for the SARS-CoV-2 diagnosis. Currently, real-time reverse transcription-polymerase chain reaction (RT-PCR) assay has been developed to rapidly detect the SARS-CoV-2 and used in clinics. RT-PCR assay, based on measuring the amount of amplified product with various primers and probe sets designed to the target in SARS-CoV-2 genome, is sensitive and suitable to detect the viruses in samples of sputum, throat swab and secretions from the upper respiratory tract (2, 7, 13).

In this study, it was aimed to confirm the diagnosis of COVID-19 in a hundred of samples from COVID-19 positive patients using sequence analysis and compare the five primer sets designed to amplify of different regions of SARS-CoV-2 for detecting the virus.

Materials and Methods

Permission to publish this study was obtained from the by Republic of Turkey Ministry of Health, General Directorate of Health Services (T13-27-31). The study was approved by the ethic committee of Burdur Mehmet Akif Ersoy University (Ethic approval Code: GO2020-238).

Samples: Patients who were admitted to Çankırı Public Hospital with complaining of symptoms including fever, cough, sore throat and trouble breathing were evaluated for SARS-CoV-2 using RT-qPCR detection kit (Biospeedy-USA Technologies Inc. COVID-19 RT-qPCR Detection Kit v2.0, Istanbul-Turkey). Oropharyngeal and nasopharyngeal swabs samples of one hundred of consecutive patients (55% male; 45% female) with confirmed SARS-CoV-2 from June 1 to July 20, 2020, were used for all analysis in this study.

One Step RT-PCR: One step RT-PCR kit (Qiagen, Germany) was used to amplify genes including ORF1ab, S, E, N and RNA depended on RNA Polymerase (RdRp) using specific primers (5’-CTA GGA CCT CTT TCT GCT CA-3’ and 5’-ACA CTC TCC TAG CAC CAT CA-3’ for ORF1ab gene, 5’-CCC TGT TGC TAT TCA TGC AG-3’ and 5’-CCC TAT TAA ACA GCC TGC AC-3’ for S gene, 5’-GGA AGA GAC AGG TAC GTT AA-3’ and 5’-AAG GTT TTA CAA GAC TCA CG-3’ for E gene, 5’-CCT CTT CTC GTT CCT CAT CA-3’ and 5’-CCT GGT CCC CAA AAT TTC CT-3’ for N gene, and 5’-CAT CTC ACT TGC TGG TTC CT-3’ and 5’-CCT TAA TAG TCC TCA CTT CTC TC-3’ for RdRp gene) designed by Mollaei et al. (14). Conventional RT-PCR was performed in a 20 µl reaction buffer contained 15 µl of 2 x Master Mix and 5 µl of nucleic acid extract. Amplification was carried out using the CFX Connect real-time system (Bio-Rad Laboratories, Hercules, CA, USA) with the following program: 5 min at 52°C for reverse transcription, 10 secs at 95°C for activation of the Taq DNA polymerase, and 40 cycles of 1 s at 95°C and 30 s in 55°C. PCR amplicons were analyzed by electrophoresis in 2% agarose gel (Bio-Rad, USA) with a molecular size marker 100bp.

Sequencing and phylogenetic analysis: Following the amplification of ORF1ab gene from each sample, two-way sequencing reactions were carried out with the primers used for PCR reactions. For DNA sequence analysis; ABI Prism 3700 Genetic Analyzer (Thermo Fisher Scientific, Massachusetts, USA) and BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) were used with the manufacturer's recommendation. The DNA sequence data of samples were compared with the DNA sequences data of SARS-CoV-2 stored in the GenBank using the Basic Local Alignment Search Tool (BLAST version 2.0) program. Multiple sequence alignments of ORF1ab genes were carried out using the 588 coding nucleotide sequences obtained from 100 SARS-CoV-2 isolates and the sequences of isolate Wuhan-12-1-1399-458/2020 (GenBank accession number MT544308.1) by the ClustalW2 program for determination of homology between the studied isolates and the references isolates from Wuhan. ORF1ab phylogenetic tree was constructed

using the neighbor-joining method within the Molecular Evolutionary Genetics Analysis software (MEGA; version 4.0).

Results

All SARS-CoV-2 positive patients exhibited several clinical manifestations including cough, fever, sore throat and difficulty in breathing. Among all patients with SARS-CoV-2, 20 patients were hospitalized due to severe pneumonia, and a total of 5 patients had an admission in the intensive-care unit and no death was observed among them. The reaction products generated by specific primers for each gene region were separated on 2% agarose gel and presented in Figure 2. The amplicon sizes of ORF1ab, S, E, N and RdRp genes were 588 bp, 440 bp, 145 bp, 323 bp and 196 bp, respectively. The SARS-CoV-2 RNA was detected from 74% of total samples from RdRp gene, 87% for N gene, 74% for S gene, 61% for E gene and 82% for ORF1ab region (Figure 1).

Sample No	ORF 1ab gene	RdRp gene	N gene	S gene	E gene
1	P	P	P	P	P
2	P	P	P	P	N
3	P	P	P	P	P
4	N	N	P	N	N
5	P	P	P	P	P
6	P	P	P	P	P
7	P	P	P	P	N
8	P	P	P	P	P
9	P	N	P	N	N
10	N	N	P	P	N
11	P	N	P	P	P
12	P	N	P	P	P
13	N	N	P	N	N
14	P	P	P	P	N
15	P	P	P	N	P
16	P	P	P	P	P
17	P	P	N	P	N
18	P	N	P	N	N
19	N	P	P	P	N
20	P	P	P	P	N
21	P	P	P	P	P
22	P	P	P	P	P
23	P	N	P	P	P
24	P	P	P	P	N
25	N	N	N	P	P
26	P	P	P	N	P
27	P	N	P	N	N
28	P	P	P	P	P
29	N	P	P	N	P
30	P	P	P	P	P
31	P	P	N	P	N
32	P	P	P	P	P
33	P	P	P	P	N
34	P	P	P	P	P
35	P	N	P	P	P
36	N	P	P	P	N
37	P	P	N	P	N
38	P	P	P	P	N
39	P	N	P	N	P
40	N	P	P	N	P
41	P	N	P	N	P
42	P	P	P	P	P
43	P	P	P	P	N
44	P	P	P	P	P
45	P	P	P	N	P
46	P	N	P	N	P
47	N	P	P	N	P
48	P	P	P	P	P
49	P	N	P	P	P
50	P	P	P	P	P
51	P	N	P	N	P
52	N	N	N	P	P
53	P	N	P	N	P
54	P	P	P	P	N
55	P	P	P	P	P
56	P	P	N	P	N
57	P	P	P	P	P
58	N	N	P	P	N
59	P	P	P	P	P
60	P	P	P	N	P
61	P	P	P	P	P
62	P	P	N	P	N
63	P	P	P	P	N
64	N	P	P	P	N
65	P	N	P	P	P
66	P	P	P	P	P
67	P	P	P	P	N
68	N	P	P	N	P
69	P	N	P	N	P
70	P	P	N	P	N
71	P	P	P	P	P
72	P	N	P	N	P
73	P	P	P	P	N
74	N	P	P	N	P
75	P	P	P	N	P
76	P	P	P	P	P
77	P	P	P	P	N
78	P	P	P	P	P
79	P	P	N	P	N
80	P	P	P	P	P
81	P	N	P	P	P
82	N	N	N	P	P
83	P	P	P	P	N
84	P	P	N	P	N
85	P	P	P	P	P
86	P	P	P	N	P
87	N	N	P	P	N
88	P	P	N	P	N
89	P	P	P	P	P
90	P	P	P	N	P
91	P	P	P	P	N
92	P	P	P	P	P
93	N	P	P	N	P
94	P	P	N	P	N
95	P	P	P	P	P
96	P	P	P	P	N
97	P	P	P	P	P
98	N	N	P	N	N
99	P	P	P	P	N
100	P	P	P	P	P
Total P/N	82/18	74/26	87/13	74/26	61/39

Conventional RT-PCR for N (Nucleocapsid), RdRp (RNA depended RNA Polymerase), E (Spike glycoprotein), S (Surface protein) gene, P: Positive, N: Negative.

Figure 1. Conventional RT-PCR results in different genes of SARS-CoV-2 samples.

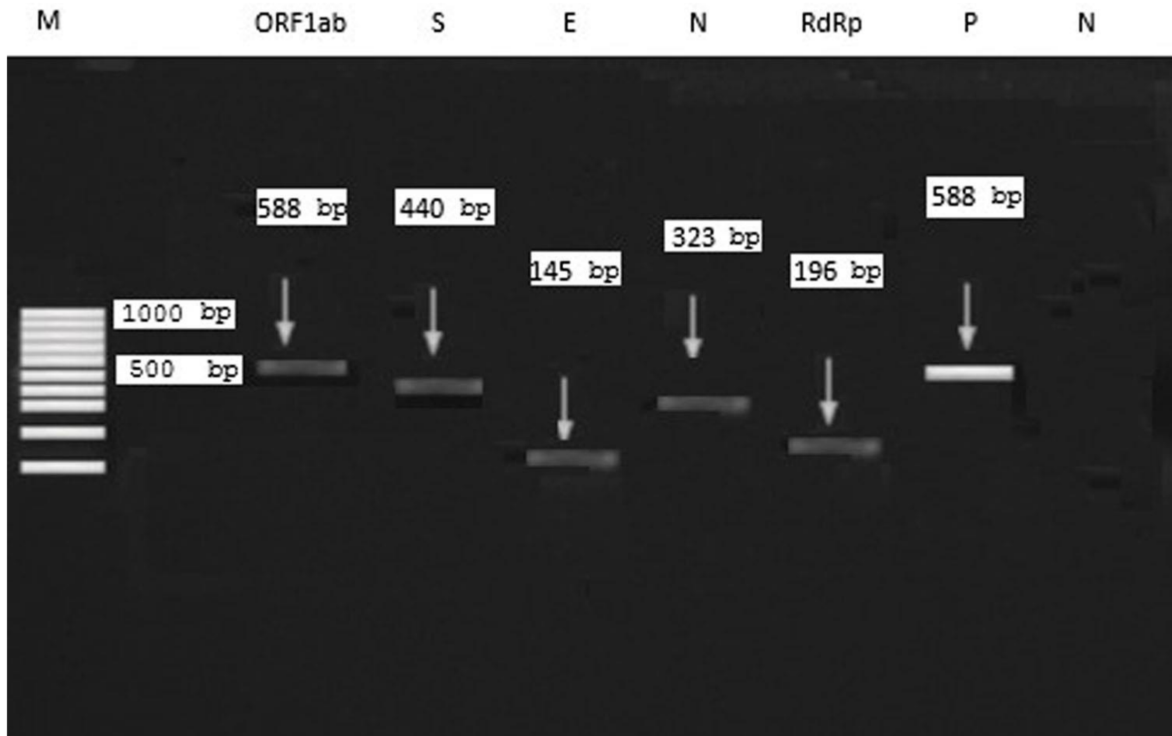

Figure 2. Agarose gel electrophoresis (2%) of 5 PCR products. Lane M: Size marker (100 bp); ORF1ab: 588 bp; S gene: 440 bp; E gene: 145 bp; N gene: 323 bp; RdRp: 196 bp, P: positive; N: Negative.

/

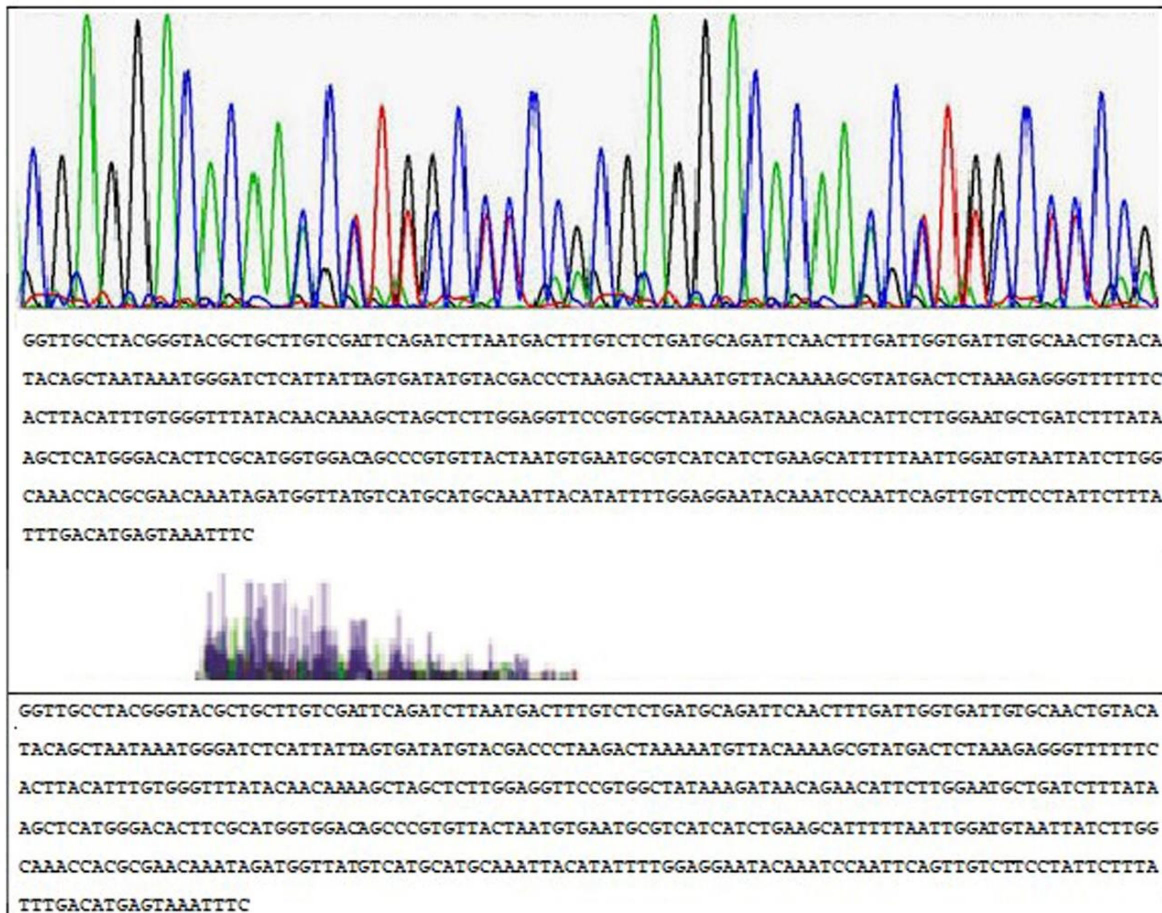

Figure 3. Sequence analysis peak of ORF1ab obtained from patients.

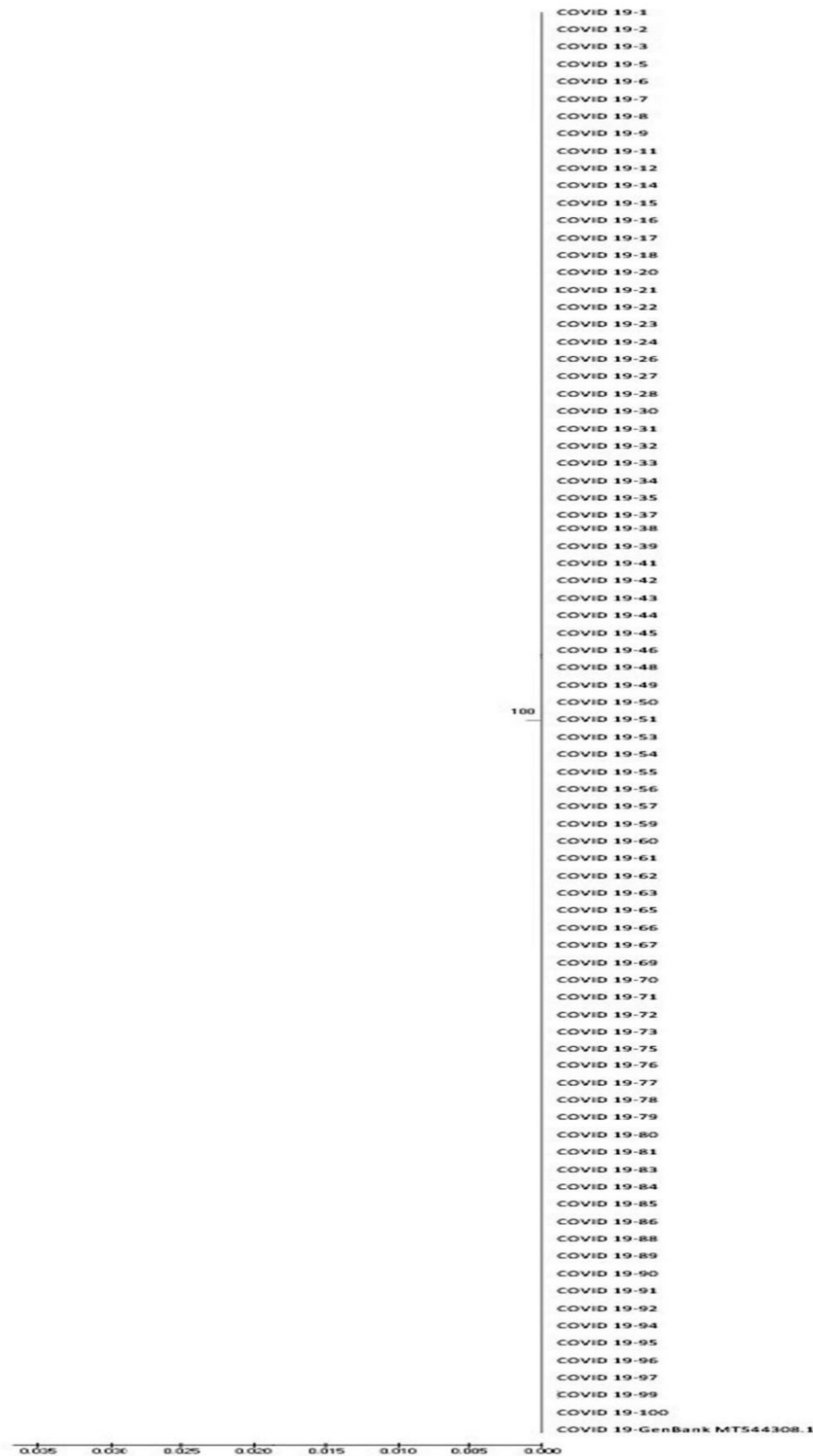

Figure 4. Phylogenetic relations among COVID-19 genotypes detected in patient with COVID-19. The phylogenetic tree was constructed by the neighbor-joining method.

After the sequencing of amplicons, the sequences were analyzed using the BLAST program. The sequence image related to ORF1ab gene was given as peaks in Figure 3. BLAST analysis of the ORF1ab gene sequences confirmed the identity of all 100 Turkish isolates as SARS-CoV-2. Wuhan-12-1-1399-458/2020 (GenBank accession number MT544308.1) was used as the reference genome for ORF1ab. The ORF1ab sequences of SARS-CoV-2 from all the patients had 100% identity to the sequence of the Wuhan isolate and among themselves. The phylogenetic analysis revealed that all isolates formed a cluster (Figure 4).

Discussion and Conclusion

The coronavirus COVID-19 has spread to 213 countries and territories around the world and two international conveyances with 28 989 073 confirmed cases, including 925 373 deaths, as of September 13, 2020 (27). As of 13 September 2020, the total number of confirmed cases of COVID-19 reached to 289 635 and the death toll has reached 6 999 in Turkey (17). The death rate (2.42%) in Turkey is lower than most of the developed countries in the world. Turkey has been quite effective in reducing the viral spread due to establishing a scientific committee, consisting of academics, at the earlier time of COVID-19 outbreak by the Turkish Government and effective and preventative measures taken in line with the recommendations of the committee. Also, Turkey successfully has kept the mortality rate very low with its a large amount of intensive-care unit (ICU) beds, well-qualified and trained staff in ICU, strong free-of-charge health service and a successful treatment strategy against COVID-19 outbreak (10).

Currently, no vaccines or specific medications is available for preventing or treating COVID-19. However, there are several drug options to have better treatment outcomes either as mono or combination therapy. The drugs being used to help manage the clinical manifestations of COVID-19 are chloroquine and its metabolite HCQ, azithromycin, monoclonal antibodies such as Tocilizumab, anticoagulants such as heparin, corticosteroids and several antiviral agents including Lopinavir, Ritonavir (15). Medical professionals in Turkey started using aggressive treatments and medication strategies for treating COVID-19 much earlier than Western counties. Briefly, each adult patient confirmed with SARS-CoV-2 is initially treated with HCQ and azithromycin. Also, Oseltamivir was used with HCQ and was discontinued after April 2020. Favipiravir or Remdesivir have been started to use in patients with severe pneumonia. Tocilizumab treatment has been used in ICU for patients with severe COVID-19 related cytokine release syndrome. Also, anticoagulant drugs were added to the treatment algorithm to treat the coagulopathy observed in patients hospitalized with COVID-19 and related to mortality as well (10, 21). The drugs or drug combinations used in the treatment of patients with COVID-19 in Turkey are generally recommended in COVID-19 treatment. Therefore, the causes of Turkey's success in COVID-19 may be its strong health care system and to take precautions much earlier than most of the affected countries by the COVID-19 outbreak.

The clinical signs caused by COVID-19 in patients are not specific and cannot be used for an accurate diagnosis. Nucleic Acid Amplification Tests (NAAT) such as RT-PCR and Chest-computed tomography (CT) have been used for diagnosis and screening COVID-19. NAAT for the detection of viral nucleic acids are more convenient, reliable and better than syndromic testing and CT scans (1). A large number of RT-PCR kits have been designed for detection SARS-CoV-2 genetically. Several sets of primer and probes were designed to amplify a number of molecular targets including RdRp gene, E gene, S gene, ORF1ab and N gene for real-time RT PCR assays (22). It has been reported that the RdRp and the E gene had high analytical sensitivity for the detection of SARS-CoV-2, whereas the N gene was slightly less sensitive (6). One study showed that the N gene was to be performing well for SARS-CoV-2 diagnosis (8). Another study showed that the ORF1ab, N and RdRp primers had sensitivity, specificity and positive predictive value higher than other primer (S gene, E gene) (14). In this study, the primers designed by Mollaei et al. (14) were used for diagnosis of SARS-CoV-2 RNA from the patients and the N gene (87%) had high analytical sensitivity for detection of SARS-CoV-2, whereas the ORF1ab gene was slightly less sensitive (82%). Therefore, the best gene for identifying cases of SARS-CoV-2 is the N gene, ORF1ab is the second-best option after this gene. A commercial kit, Bio-Speedy, used for detecting SARS-CoV-2, is carried out by RT-qPCR targeting the virus-specific RdRp gene region. In the present study, only 74 of the 100 samples confirmed with the commercial kit measuring using the RdRp gene region in the hospital could be verified by using primers for the RdRp gene region analysis. The reason for this difference in results may be the difference of the primers used for the detection of the gene region and the laboratory conditions.

Sequencing molecular methods such as next-generation sequencing are currently impractical for diagnosis of SARS-CoV-2 but may be used for determination mutations and epidemiological analysis of SARS-CoV-2 (16, 20). Epidemiologically, sequences alignments of ORF1ab gene showed that 82 patients in our study had 100% identity to the sequence of Wuhan isolate and among themselves, suggesting that they might have been infected with the virus from the infection source at the market in Wuhan, China.

In conclusion; the conventional RT-PCR assay with N and ORF1ab primers was the best option for detecting for SARS-CoV-2 in patients. The ORF1ab gene sequences of SARS-CoV-2 positive samples were 100% identical to the Wuhan genome reference.

Financial Support

This research received no grant from any funding agency/sector.

Conflict of Interest

The authors declared that there was no conflict of interest.

References

1. **Alsuliman T, Sulaiman R, Ismail S, et al** (2020): *COVID-19 paraclinical diagnostic tools: Updates and future trends*. Curr Res Transl Med. **68**, 83-91.
2. **Bustin SA, Nolan T** (2020): *RT-qPCR testing of SARS-CoV-2: a primer*. Int J Mol Sci, **21**, 3004.
3. **Cai Q, Yang M, Liu D, et al** (2020): *Experimental treatment with favipiravir for COVID-19: an open-label control study*. Engineering.
4. **Chen B, Tian EK., He B, et al** (2020): *Overview of lethal human coronaviruses*. Signal Transduct Tar, **5**, 1-16.
5. **Chen N, Zhou M, Dong X, et al** (2020): *Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study*. The Lancet, **395**, 507-513.
6. **Corman VM, Landt O, Kaiser M, et al** (2020): *Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR*. Eurosurveillance, **25**, 2000045.
7. **Emery SL, Erdman DD, Bowen MD, et al** (2004): *Real-time reverse transcription–polymerase chain reaction assay for SARS-associated coronavirus*. Emerg Infect Dis, **10**, 311-316.
8. **Holshue ML, DeBolt C, Lindquist S, et al** (2020): *First case of 2019 novel coronavirus in the United States*. N Engl J Med, **36**, 929-936.
9. **Kilic AU, Kara F, Alp E, et al** (2020): *New threat: 2019 novel Coronavirus infection and infection control perspective in Turkey*. North Clin Istanb, **7**, 95-98.
10. **Kodaz H** (2020): *Editorial: Successful Treatment Strategy of Turkey against Covid-19 Outbreak*. EJMO, **4**, 177-178.
11. **Kumar S, Nyodu R, Maurya VK, et al** (2020): Morphology, Genome Organization, Replication, and Pathogenesis of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2). 23-31. In: SK Saxena (Ed) Coronavirus Disease 2019 (COVID-19). Springer, Singapore.
12. **Luan J, Lu Y, Jin X, et al** (2020): *Spike protein recognition of mammalian ACE2 predicts the host range and an optimized ACE2 for SARS-CoV-2 infection*. Biochem Biophys Res Commun, **526**, 165-169.
13. **Mathuria JP, Yadav R** (2020): *Laboratory diagnosis of SARS-CoV-2- a review of current methods*. J Infect Public Heal, **13**, 901-905.
14. **Mollaei HR, Afshar AA, Kalantar-Neyestanaki D, et al** (2020): *Comparison five primer sets from different genome region of COVID-19 for detection of virus infection by conventional RT-PCR*. Iran J Microbiol, **12**, 185-193.
15. **Omolo CA, Soni N, Fasiku VO, et al** (2020): *Update on therapeutic approaches and emerging therapies for SARS-CoV-2 virus*. Eur J Pharmacol, **883**, 173348.
16. **Rodríguez-Morales AJ, Balbin-Ramon GJ, Rabaan AA, et al** (2020): *Genomic Epidemiology and its importance in the study of the COVID-19 pandemic*. InfezMed, **2**, 139-142.
17. **Sağlık Bakanlığı** (2020): Genel Koronavirus Tablosu. Available at https://covid19. saglik.gov.tr/TR-66122/genel-koronavirus-tablosu.html (Accessed September 13, 2020).
18. **Singh AK, Singh A, Shaikh A, et al** (2020): *Chloroquine and hydroxychloroquine in the treatment of COVID-19 with or without diabetes: A systematic search and a narrative review with a special reference to India and other developing countries*. Diabetes Metab Syndr, **14**, 241-246.
19. **Stebbing J, Phelan A, Griffin I, et al** (2020): *COVID-19: combining antiviral and anti-inflammatory treatments*. Lancet Infect Dis, **20**, 400-402.
20. **Tang YW, Schmitz JE, Persing DH, et al** (2020): *Laboratory diagnosis of COVID-19: current issues and challenges*. J Clin Microbiol, **58**, e00512-20.
21. **The Republic of Turkey Ministry of Health** (2020): COVID-19 (SARS-CoV-2 Infection) Guide. July 17, 2020. Available at https://hsgm.saglik.gov.tr/en/covid-19-i-ngilizce-dokumanlar/rehberler.html. (Accessed July 20, 2020).
22. **Udugama B, Kadhiresan P, Kozlowski HN, et al** (2020): *Diagnosing COVID-19: the disease and tools for detection*. ACS Nano, **14**, 3822-3835.
23. **Wei Q, Wang Y, Ma J, et al** (2020): *Description of the First Strain of 2019-nCoV, C-Tan-nCoV Wuhan Strain—National Pathogen Resource Center, China, 2020*. China CDC Weekly, **2**, 81-82.
24. **Weiss P, Murdoch DR** (2020): *Clinical course and mortality risk of severe COVID-19*. The Lancet, **395**, 1014-1015.
25. **Woo PC, Lau SK, Lam CS, et al** (2012): *Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus*. J Virol, **86**, 3995-4008.
26. **Woolhouse M, Scott F, Hudson Z, et al** (2012): *Human viruses: discovery and emergence*. Philos T R Soc B, **367**, 2864-2871.
27. **Wordometers** (2020): COVID-19 Coronavirus Pandemic. Available at https://www.worldometers.info/coronavirus/ (Accessed September 13, 2020).
28. **Yang D, Leibowitz JL** (2015): *The structure and functions of coronavirus genomic 3' and 5' ends*. Virus Res, **206**, 120-133.
29. **Yoldar ZA, Koç BT, Oğuzoğlu TÇ** (2020): *Phylogenetic analysis of partial transmembrane protein gene of canine coronaviruses detected in Turkey*. Ankara Univ Vet Fak Derg, **67**, 265-271.
30. **Yousefifard M, Zali A, Ali KM, et al** (2020): *Antiviral therapy in management of COVID-19: a systematic review on current evidence*. Arch Acad Emerg Med, **8**, e45.
31. **Zhang J, Zhou L, Yang Y, et al** (2020): *Therapeutic and triage strategies for 2019 novel coronavirus disease in fever clinics*. Lancet Respir Med, **8**, e11-e12.
32. **Zhu H, Wei L, Niu P** (2020): *The novel coronavirus outbreak in Wuhan, China*. Global Health Research and Policy, **5**, 1-3.

Ankara Univ Vet Fak Derg, **68**, 77-81, 2021
DOI: 10.33988/ auvfd.660568

Case Report / *Olgu Sunumu*

Clinicopathologic evaluation of oral squamous cell carcinoma in a young dog

Hazal ÖZTÜRK GÜRGEN[1,a,✉], Evrim EGEDEN[2,b], Gülbin ŞENNAZLI[1,c]

[1]İstanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Pathology, İstanbul; [2]Ada Veterinary Policlinic, İstanbul, Turkey.
[a]ORCID: 0000-0003-2748-6189; [b]ORCID: 0000-0002-1326-3200; [c]ORCID: 0000-0002-8419-2833.

✉Corresponding author: hazal.ozturk@istanbul.edu.tr
Received date: 17.12.2019- Accepted date: 18.04.2020

Abstract: Canine oral papilloma is a benign tumor of young dogs and caused by papillomavirus. The possible role of papillomavirus infection in the development of oral squamous cell carcinoma has recently been studied, but it has not been elucidated in veterinary medicine yet. A one-year-old, mixed, spayed, female dog was presented with severely disseminated oral lesions, lethargy, and weight loss. Physical examination of the patient revealed severely disseminated oral papillomatous lesions in the entire oral cavity and the complete blood test showed mild non-regenerative anemia and pancytopenia. In addition, the patient was found seropositive by the SNAP 4Dx Plus test for *Ehrlichia canis*. Histopathologic examination of oral lesions was performed using Hematoxylin and Eosin (HE) staining and immunohistochemistry for p16, antibody which increases in infections caused by papillomavirus. Histopathology revealed the histologic features of oral papilloma in association with squamous cell carcinoma. Cytoplasmic and nuclear positive reactions for p16 protein were observed within the neoplastic cells in the immunohistochemical examination. Thereafter, the dog was treated with combined therapy of vincristine, antibiotic, radiotherapy, and high doses of vitamin C. After long-term treatment, the dog completely recovered from the lesions. In this report, it was aimed to present a possible role of papilloma in the development of oral squamous cell carcinoma with the clinical, histopathological, immunohistochemical findings and treatment procedure.

Keywords: Canine, immunohistochemistry, oral papilloma, squamous cell carcinoma, treatment

Genç bir köpekte oral skuamöz hücreli karsinomun klinikopatolojik değerlendirilmesi

Özet: Oral papilloma, genç köpeklerin iyi huylu bir tümörüdür ve papillomavirüs enfeksiyonundan kaynaklanır. Papillomavirüsün oral skuamöz hücre karsinomu gelişimindeki olası rolü son yıllarda çalışılmıştır, fakat veteriner hekimlikte bu henüz ortaya konulmamıştır. Bir yaşında, melez, kısırlaştırılmış, dişi köpek şiddetli dağılım gösteren oral lezyonlar, letarji ve kilo kaybı şikâyeti ile getirildi. Hastanın fiziksel muayenesinde tüm ağız boşluğuna yayılmış şiddetli papillomatöz lezyonlar saptandı. Tam kan testinde rejeneratif olmayan hafif anemi ve pansitopeni tespit edildi. Ayrıca, yapılan SNAP 4Dx Plus testi ile hasta *Ehrlichia canis* seropozitif bulundu. Oral lezyonların histopatolojik incelemesi Hematoksilen ve Eosin (HE) boyama yöntemi ve papillomaviral enfeksiyonlarda artış gösteren p16 antikoru ile immünohistokimyasal olarak yapıldı. Mikroskobik bakıda, vakaya oral papilloma ve skuamöz hücreli karsinoma tanısı konuldu. İmmunohistokimyasal incelemede neoplastik hücrelerde p16 proteini için sitoplazmik ve nükleer pozitif reaksiyonlar gözlendi. Daha sonra vincristine, antibiyotik, radyoterapi ve yüksek C vitamini dozlarının kombine tedavisi uygulandı. Uzun süreli bir tedaviden sonra, hastada izlenen lezyonlar tamamen iyileşti. Bu olguda papillomanın oral skuamöz hücreli karsinom gelişimindeki potansiyel rolü ile birlikte klinik, histopatolojik, immünohistokimyasal bulgular ve tedavi prosedürünün sunulması amaçlanmıştır.

Anahtar sözcükler: İmmunohistokimya, köpek, oral papilloma, skuamöz hücreli karsinom, tedavi

Papillomaviruses (PVs) are non-enveloped, double-stranded DNA viruses with a circular genome of 8000 pairs. They are known to induce epithelial proliferation on the skin and mucous membranes in their natural hosts and related species (5). Oral papillomas are characterized by cauliflower-like exophytic warts, but can also be fringed or nodular, and arise in oral mucosa, including lips and mucocutaneous junctions (12). They are commonly seen

in young and immunosuppressed dogs (24). So, the diagnosis of PV infection in dogs is quite simple by clinical examination if the animal is young. But diagnostic methods are based on classical histopathology and polymerase chain reaction (PCR), as well as immunohistochemistry, in situ hybridization and electron microscopy (12).

Oral papilloma is accepted to be a benign tumor and usually regresses spontaneously (4). On the other hand, excessive proliferation of the epithelium can also result in malignant transformation of the lesion to squamous cell carcinoma (SCC) (3, 20, 26). Although there are several possible causes for the development of oral SCC in dogs, the actual role of PV infection remains uncertain (20).

Clinical approach to the oral SCC depends on the prognosis of the patient and includes surgical excision (19), chemotherapy (6), radiotherapy (8), photodynamic therapy (14), or combined therapy of these options (13, 22). Here, it was aimed to present the clinicopathologic evaluation of a young dog with severe oral papilloma progressing to oral SCC regarding increased attention on the possible role of PV infection in the development of oral SCC since the last decade in veterinary medicine, especially in dogs and cats.

A one-year-old, mixed breed spayed female dog was presented with the complaints of severely disseminated oral lesions, emaciation due to feeding difficulty, salivation, and mild anemia. In clinical examination, oral lesions were characterized by multiple, white to pinkish, pedunculated, cauliflower-like exophytic masses ranging from 1.5 to 3 cm in diameter and were present throughout the oral cavity including; the tongue, palatopharyngeal mucosa, superior and inferior labial mucosa, buccal mucosa, upper and lower lips and extending to the hairy skin (Figure 1). The masses located on the superior and inferior labial mucosa were also characterized by ulcerative changes. Complete blood test revealed mild non-regenerative anemia and pancytopenia. In addition, the dog was found seropositive by SNAP 4Dx Plus test for *Ehrlichia canis*. A single biopsy sample taken from the inferior labial mucosa was fixed in 10% formalin solution, embedded in paraffin, sectioned, and stained with HE. After preliminary histopathological evaluation, the biopsy sample was stained by streptavidin-biotin immunoperoxidase method, using anti- $p16^{INK4a}$ (E6H4), a mouse monoclonal primary antibody (REF 705-4713, ready-to-use, Ventana, Arizona) and Seconder antibody (REF 253-2188, ready-to-use, Ventana, Arizona). Immunoreaction was visualized by diaminobenzidine and the section was counterstained with Mayer’s hematoxylin.

Histopathological findings revealed characteristic features of papillomavirus infections with severe papillomatous hyperplasia within the mucosal epithelium and hyperkeratosis (Figure 2A), koilocytosis (Figure 2B), intra-nuclear eosinophilic inclusion bodies in the epithelial cells (Figure 2A, inset). Nonetheless, squamous neoplastic cells were observed to have arranged in nests or trabeculae-like structures, supported by fibrovascular stroma within the lamina propria and submucosa. The neoplastic cells, which were round-to-polygonal-shaped with eosinophilic cytoplasm, exhibited varying degrees of squamous differentiation, namely formation of keratin pearls, and also single-cell keratinization (Figure 3A). Neoplastic cells showed severe hyperchromasia, anisocytosis, anisokaryosis and atypical mitotic figures, and some neoplastic cells had multiple nuclei. In addition, infiltration of inflammatory cells and superficial ulceration were also observed (Figure 3B). Immunohistochemical staining against p16 antigen was visualized as cytoplasmic and nuclear positive reaction in a pattern of diffuse and homogenous brown coloring in the neoplastic cells (Figure 4). The case was diagnosed as severe oral papillomas progressing to oral SCC. A treatment protocol was concordantly established by the

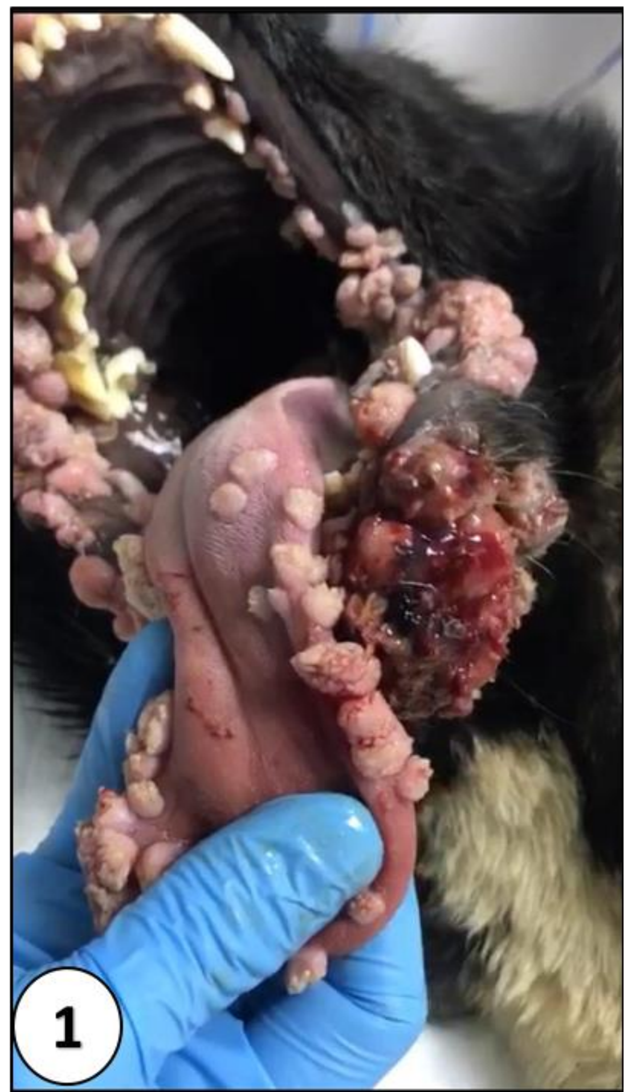

Figure 1. Pinkish, pedunculated, cauliflower-like exophytic masses on the lateral surface of the tongue and in the entire oral cavity.

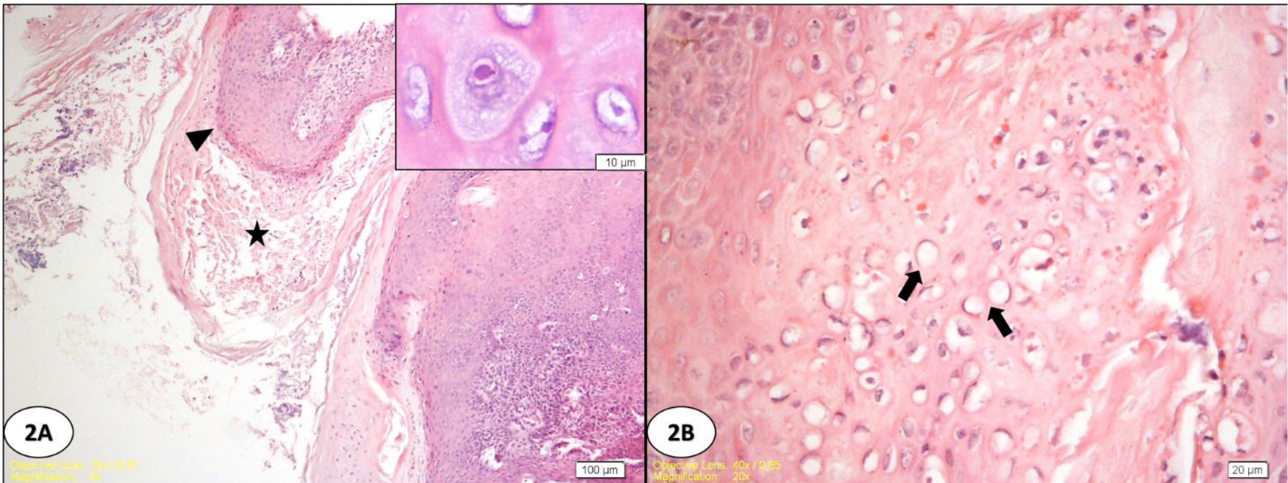

Figure 2 A. The appearance of papilloma with severe papillomatous hyperplasia (arrowhead) within the mucosal epithelium and hyperkeratosis (star). H&E. Bar = 100 µm. **B**. Numerous koilocytes with a swollen nucleus surrounded by a clear halo (arrows). H&E. Bar = 20 µm. Intra-nuclear eosinophilic inclusion bodies in the epithelial cell (2A, inset). H&E. Bar = 10 µm.

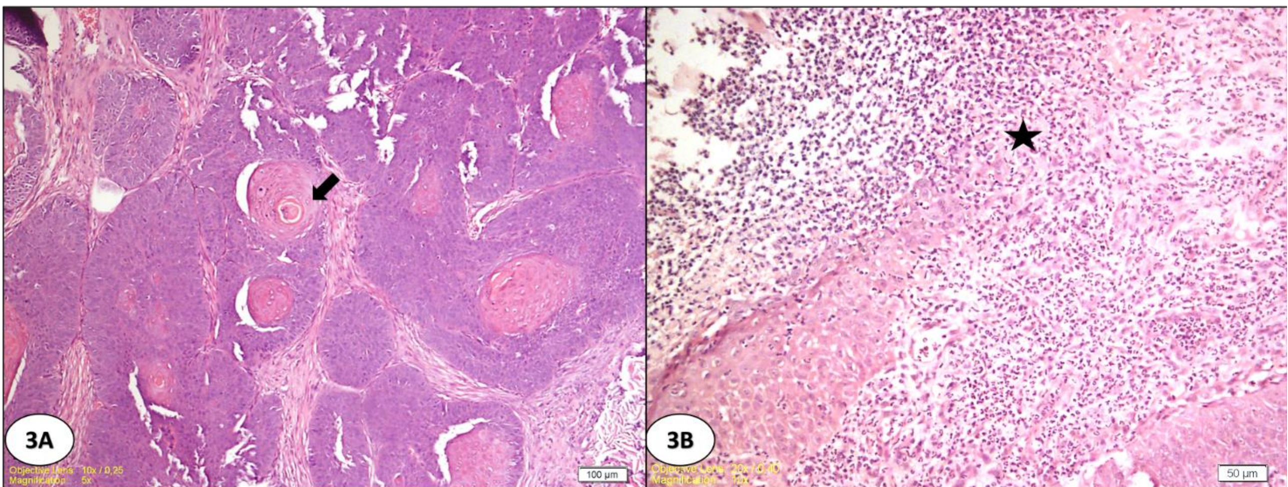

Figure 3 A. High-grade epithelial hyperplasia from the mucosa to the submucosa in the canine oral cavity. Neoplastic differentiation characterized by different degrees of keratinization (horn pearls /arrow) in squamous epithelial cells clustered as nests. H&E. Bar = 100 µm. **B.** Superficial ulceration on the mucosa with severe infiltration of polymorph neutrophil leukocytes (star). H&E. Bar = 50 µm.

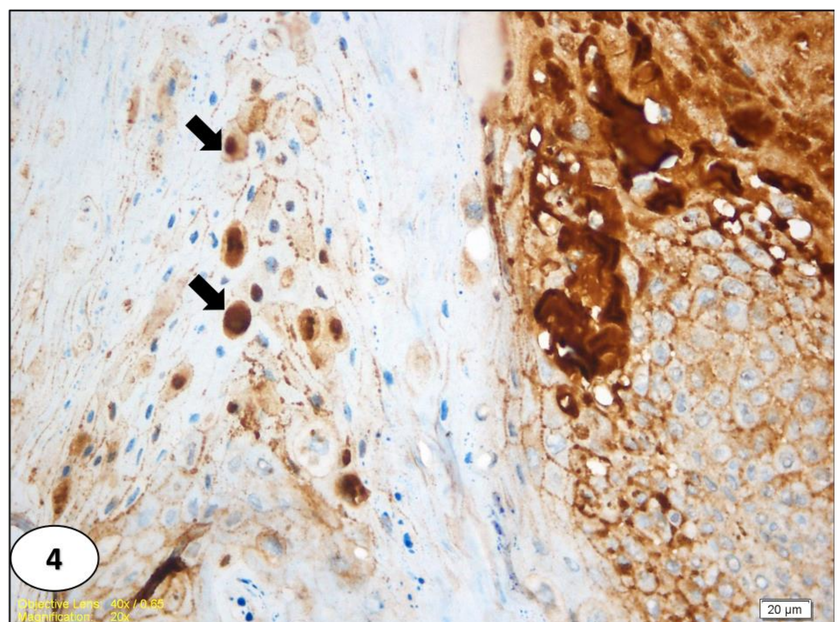

Figure 4. Positive immune reaction against P16 antigen in neoplastic canine oral SCC (arrows). IHC with Anti-p16^{INKA4a}(E6H4). Bar = 20 µm.

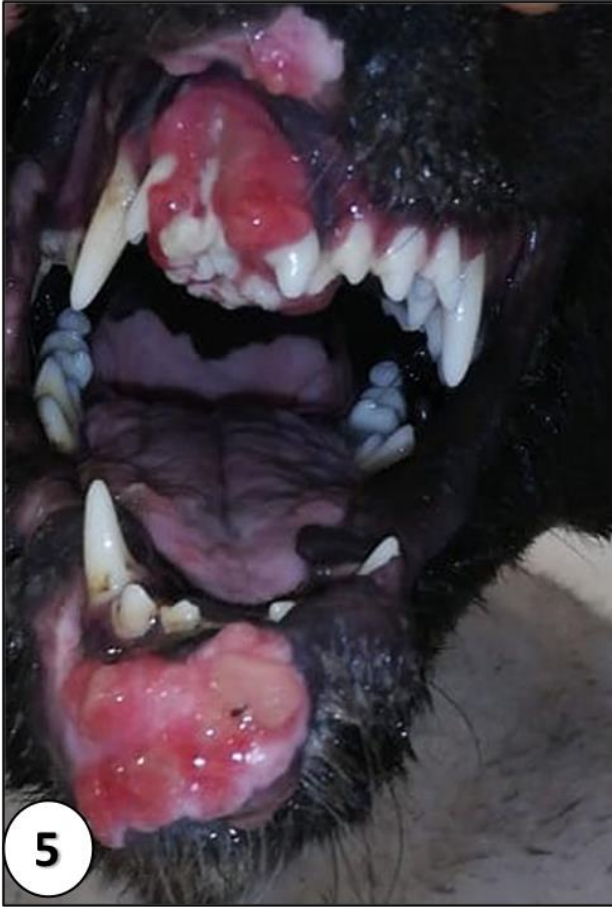

Figure 5. Decreased PV lesions after the treatment with vincristine and prominent ulcerative SCC in the oral cavity.

administration of vincristine (0.75 mg/kg IV, Vincristine®, Kocak Farma, Turkey) with 0.9% NaCl isotonic serum (10 ml/kg, Deva®, Turkey) once a week for 6 weeks and by doxycycline (10 mg/kg/day PO) for a month to improve the ehrlichiosis of the patient. Substantial improvement was achieved in oral PV lesions (Figure 5) by vincristine administration. Then, the patient also received 12 fractions of 48 Gy radiotherapy 3 times a week in 12 sessions. In addition, vitamin C (1 g/kg) was administered during the treatment. After the treatment, the lesions on the oral mucosa and the hairy skin completely healed.

The possible role of PV infection in the development of oral SCC has recently been studied in both human and veterinary medicine. While PV infection is considered to be one of the causes of head and neck tumors in human medicine (7), it is still questionable in veterinary medicine (17, 15, 26). In the presented case report, the gross and histopathological findings were found compatible with the oral SCC accompanied by severe oral papilloma. The young age of the dog was deemed to be the predisposing factor for PV infection, as previously indicated (25), and subsequently, the PV infection was assumed to have developed into SCC. Therefore, additional immunohistochemical staining was performed against p16, antigen which has been intended to underline a possible PV etiology in oral SCC (23). It has been shown that human PV infection has a breakdown effect on cell cycle regulation by affecting the function of retinoblastoma protein (2). It causes degradation of retinoblastoma protein, which results in the increased amount of cellular p16 protein (21). While immunodetection of p16 protein is currently in use as an indicator for the presence of PV in human medicine (11), the reports in veterinary medicine need to be improved (18, 16, 20). For instance, the role of PV in SCC has been investigated by both IHC for p16 and amplification of PV DNA via PCR in canine oral SCC, despite the positive results for p16 immunopositivity, DNA of the PV hasn't been detected in any case of that study (16). In the present study, due to the lack of PCR analysis the existence of PV infection and coexistence of oral SCC was diagnosed based on the typical macroscopic and histologic features of the animal. Although the immunohistochemical findings revealed the potential role of PV infection-induced development of SCC, the actual role of PV remains uncertain.

The dog was initially treated with vincristine for PV infection. Concurrent therapy was administered by vitamin C and doxycycline, which is a tetracycline group of antibiotics used with adequate doses for ehrlichiosis (9). Following the regression of the viral papillomas and the improvement of the immunity, the dog received radiotherapy for oral SCC. Radiotherapy has already been recommended as a sole treatment method or an adjuvant treatment for incompletely excised oropharyngeal SCC in dogs (19). In humans, concurrent chemotherapy and radiotherapy have widely been accepted for the management of locally advanced epithelial tumors (10) and have been established as standard non-surgical therapy for patients with head and neck carcinomas (1).

In conclusion, this case can provide additional support for the possible role of papillomavirus in the development of oral SCC, which was supported by macroscopic, histopathologic, and immunohistochemical findings. In addition, the treatment of choice was proved quite effective and the dog completely recovered after the treatment. Combination of vincristine, antibiotics, and vitamin C exhibited a favorable outcome in the regression of oral papilloma. The treatment protocol was completed with radiotherapy for oral SCC. The condition of the dog is known to have remained stable since the termination of the treatment. Therefore, this case was considered to be a contribution to veterinary literature.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Argiris A** (2002): *Update on chemoradiotherapy for head and neck cancer.* Curr Opin Oncol **14**, 323-329.
2. **Boyer SN, Wazer DE, Band V** (1996): *E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway.* Cancer Res, **56**, 4620-4624.
3. **Bregman CL, Hirth RS, Sundberg JP, et al** (1987): *Cutaneous neoplasms in dogs associated with canine oral papilloma virus vaccine*. Vet Pathol **24**, 477-487.
4. **Chambers VC, Evans CA** (1959): *Canine oral papillomatosis I. Virus assay and observations on the various stages of the experimental infection.* Cancer Res, **19**, 1188-1195.
5. **De Villiers EM, Fauquet C, Broker TR, et al** (2004): *Classification of papillomaviruses.* Virology **324**, 17–27.
6. **De Vos JP, Burm AGD, Focker AF, et al** (2005): *Piroxicam and carboplatin as a combination treatment of canine oral non-tonsillar squamous cell carcinoma: a pilot study and a literature review of a canine model of human head and neck squamous cell carcinoma.* Vet Comp Oncol, **3**,16-24.
7. **Gillison ML** (2007): *Current topics in the epidemiology of oral cavity and oropharyngeal cancers.* Head Neck, **29**, 779-792.
8. **Grier CK, Mayer MN** (2007). *Radiation therapy of canine nontonsillar squamous cell carcinoma.* Can Vet J, **48**,1189-1191.
9. **Harrus S, Kenny M, Miara L, et al** (2004). *Comparison of simultaneous splenic sample pcr with blood sample pcr for diagnosis and treatment of experimental ehrlichia canis infection.* Antimicrob Agents Chemother, **48**, 4488–4490.
10. **Hennequin C, Favaudon V** (2002): *Biological basis for chemo-radiotherapy interactions.* Eur J Cancer, **38**, 230-233.
11. **Konig F, Krekeler G, Hönig JF, et al** (2007). *Relation between human papillomavirus positivity and p16 expression in head and neck carcinomas – A tissue microarray study.* Anticancer Res, **27**, 283-288.
12. **Lange CE, Favrot C** (2011). *Canine papillomaviruses.* Vet Clin Small Anim, **41**, 1183-1195.
13. **Mas A, Blackwood L, Cripps P, et al** (2011). *Canine tonsillar squamous cell carcinoma – a multi-centre retrospective review of 44 clinical cases.* J Small Anim Pract, **52**, 359-365.
14. **McCaw DL, Pope ER, Payne JT, et al** (2000): *Treatment of canine oral squamous cell carcinomas with photodynamic therapy.* Br J Cancer, **82**, 1297-1299.
15. **Munday JS, Dunowska M, De Grey S** (2009): *Detection of two different papillomaviruses within a feline cutaneous squamous cell carcinoma.* N Z Vet J, **57**, 248-51.
16. **Munday JS, French A, Harvey CJ** (2015a): *Molecular and immuohistochemical studies do not support a role for papillomaviruses in canine oral squamous cell carcinoma development.* Vet J, **204**, 223-225.
17. **Munday JS, Kiupel M, French AF, et al** (2008): *Amplification of papillomaviral DNA sequences from a high proportion of feline cutaneous in situ and invasive squamous cell carcinomas using a nested polymerase chain reaction.* Vet Dermatol, **19**, 259-263.
18. **Munday JS, Knight CG, French AF** (2011). *Evaluation of feline oral squamous cell carcinomas for p16CDKN2A protein immunoreactivity and the presence of papillomaviral DNA.* Res Vet Sci, **90**, 280-283.
19. **Munday JS, Löhr CV, Kiupel M** (2017): Tumors of the alimentary tract. 500-507. In: Meuten DJ (ed.): Tumors in Domestic Animals. John Wiley & Sons, Inc. Ames, Iowa.
20. **Munday JS, Tucker RS, Kiupel M, et al** (2015b): *Multiple oral carcinomas associated with novel papillomavirus in a dog.* J Vet Diagn Invest, **27**, 221-225.
21. **Parry D, Bates S, Mann DJ, et al** (1995): *Lack of cyclin D-Cdk complexes in Rb-negative cells correlates with high levels of p16INK4IMTS1 tumor suppressor gene product.* EMBO J, **14**, 503-511.
22. **Rejec A, Benoit J, Tutt C, et al** (2015): *Evaluation of an accelerated chemoradiotherapy protocol for oropharyngeal squamous cell carcinoma in 5 cats and 3 dogs.* J Vet Dent, **4**, 212-221.
23. **Smeets SJ, Hesselink AT, Spell EJM, et al** (2007): *A novel algorithm for reliable detection of human papillomavirus in paraffin embedded head and neck cancer specimen.* Int J Cancer, **121**, 2465–2472.
24. **Sunberg JP, Smith EK, Herron AJ, et al** (1994): *Involvement of canine oral papillomavirus in generalized oral and cutaneous verrucosis in a chinese shar pei dog.* Vet Pathol, **31**, 183-187.
25. **Sykes J, Luff JA** (2014): Viral papillomatosis. 261-268. In: Skyes JE (ed.), Canine and Feline Infectious Diseases. Saunders, Elsevier, Missouri.
26. **Teifke JP, Löhr CV, Shirasawa H** (1998): *Detection of canine oral papillomavirus-DNA in canine oral squamous cell carcinomas and p53 overexpressing skin papillomas of the dog using the polymerase chain reaction and non-radioactive in situ hybridization*. Vet Microbiol, **60**, 119-130.

Ankara Univ Vet Fak Derg, **68**, 83-85, 2021
DOI: 10.33988/ auvfd.679530

Case Report / *Olgu Sunumu*

Uterine papillary adenocarcinoma in a Pit-bull dog

Yanad ABOU MONSEF[1,a], Tuncer KUTLU[2,b,✉], Osman KUTSAL[1,c]

[1]Ankara University, Faculty of Veterinary Medicine, Department of Pathology, Ankara; [2]Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Pathology, Hatay, Turkey.
[a]ORCID: 0000-0002-4929-9395; [b]ORCID: 0000-0002-8771-1256; [c]ORCID: 0000-0003-3599-6867

✉Corresponding author: tuncerkutlu83@gmail.com
Received date: 24.01.2020 - Accepted date: 25.03.2020

Abstract: The uterus of an 18 month old Pit-bull removed during ovariohysterectomy formed the material of this study. Macroscopically, the uterus was whitish in color and elastic in consistency, the uterine horns were 15 cm long with a 1 cm of diameter. On cut section uterine mucosa showed variable sized cysts and was filled with a yellowish white mucus. Microscopical evaluation demonstrated a proliferation of the glandular component of the endometrium and a migration of these glands into the myometrium. Glands located in both the endometrium and the myometrium formed large cystic structures. The atypical glandular epithelium showed papillary projections into the cystic spaces. The tumor was diagnosed as uterine adenocarcinoma, an extremely rare canine tumor, especially in young dogs.
Keywords: Dog, papillary adenocarcinoma, uterus

Pitbull ırkı bir köpekte uterusta papiller adenokarsinom

Özet: Bu çalışmada, Pitbull ırkı, 18 aylık bir köpekten ovariohisterektomi ile alınan uterusta rastlanan papiller adenokarsinom tanımlandı. Makroskopik incelemede, kesit yüzünde sarımsı beyaz renkte mukus bulunan, kistik yapılar içeren, 15 cm uzunluğunda ve 1cm çapında kornu uterileri bulunan uterusun sarımsı renkte ve elastik kıvamda olduğu gözlendi. Histopatolojik incelemede ise, endometriyumda bezlerin sayıca arttığı ve miyometriyumda da bu bezlerin bulunduğu dikkat çekti. Hem endometriyum hem de miyometriyumda bulunan bezlerin geniş kistik yapılar şekillendirdiği ve atipik bez epitellerinin papiler uzantılar yaptığı gözlendi. Bu çalışmayla köpeklerde oldukça az sayıda rapor edilen uterus adenokarsinomunu 18 aylık bir köpekte tanımlandı.
Anahtar sözcükler: Köpek, papiller adenokarsinom, uterus

Compared to other genital tract tumors, uterine tumors are less reported. According to a study on canine and feline urogenital tumors, among 36 tumors noted in the female genital system, 14 were found within the ovary, 19 in the vagina and only 3 in the uterus (10). Canine uterine and ovarian tumors are extremely rare and this is thought to be due to surgical castration at early ages (10). Contrary to its prevalence in human, uterine carcinomas are considered truly rare in all domestic animals except cows and rabbits (3). Canine uterine endometrial adenocarcinoma is a very rare malignant tumor arising from the endometrium and specially seen in geriatric dogs (8). It was previously reported in two young dogs; a 2 years old mixed breed dog and a 10 months old Golden Retriever (1, 6). Clinical signs of uterine tumors depend on the size of the tumor and the same clinical signs are also reported in pyometra and mucometra cases according to some reports (7, 8). The aim of the present study is to investigate the pathomorphological findings of a papillary adenocarcinoma case encountered in the uterus of an 18 month old Pit-bull.

An 18 month old Pit-bull dog formed the material of this study. On the history, the owner reported a drug intervention done by the veterinarian to prevent gestation of the bitch at 6 months old. The owner also stated that until 18 months old, the animal presented no heat sign or any other clinical sign. In order to completely prevent gestation, the owner elected surgical ovariohysterectomy. The uterus removed during surgery was noted to be larger than normal, so it was sent to pathology department for histopathological examination. Grossly, the uterus was whitish in color and elastic in consistency, the uterine horns were 15 cm long with a 1 cm of diameter (Figure 1a). On cut section uterine mucosa showed variable sized

Figure 1. Macroscopical view of uterus **a:** The uterus fixed in formalin, whitish in color and elastic in consistency. **b:** On cut section uterine mucosa filled with a yellowish white mucus.

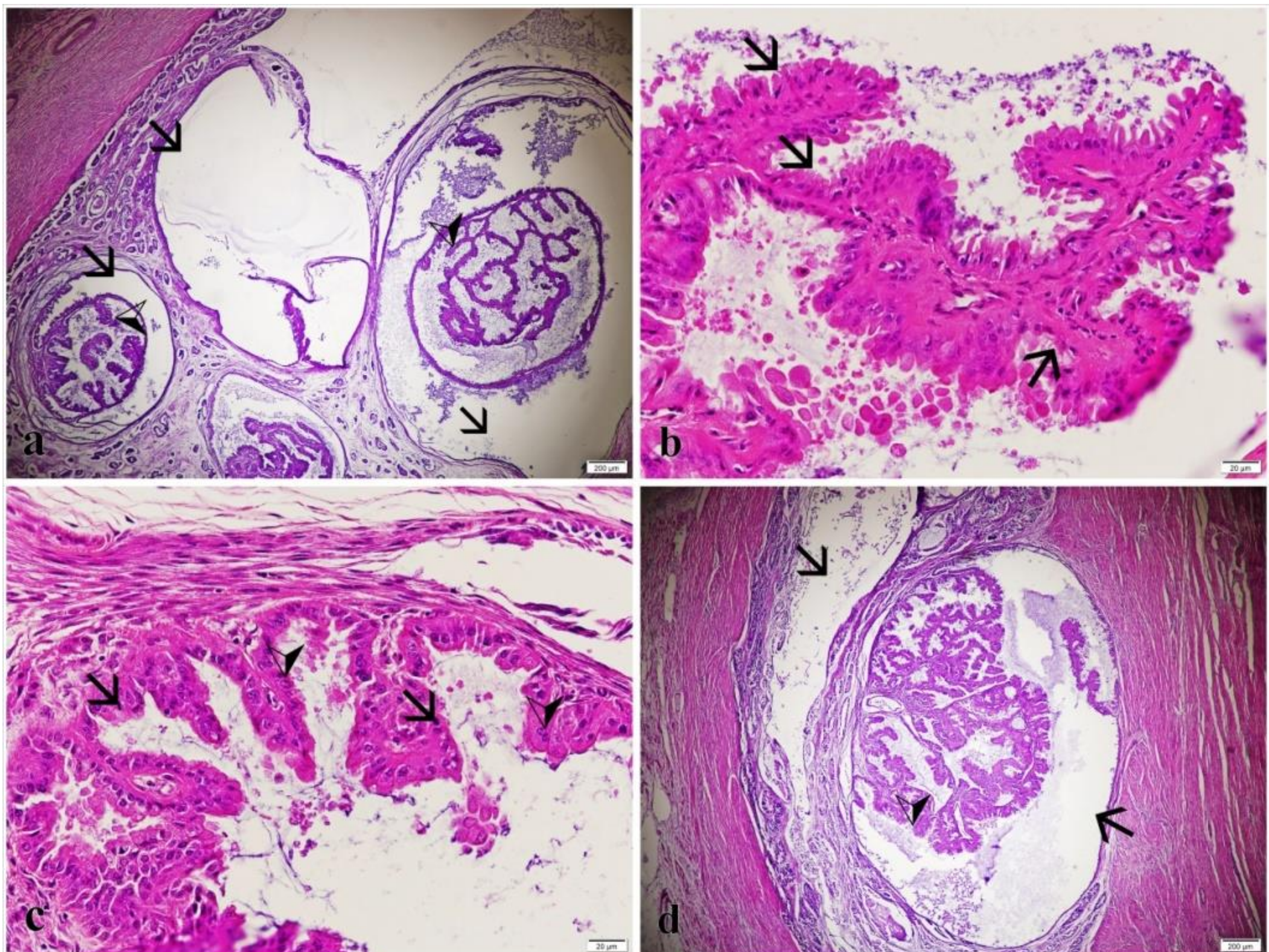

Figure 2. Histopathological findings **a**: Several endometrial large cystic structures, varying in size (arrows) and papillary projections (arrowheads), Hematoxylin-Eosin, X200. **b:** Glandular epithelium showing long papillary projections (arrows), Hematoxylin-Eosin, X400. **c:** Epithelial cells with anisonucleosis, anisocytosis (arrowheads) and some mitotic figures (arrows), Hematoxylin-Eosin, X400. **d:** Cystic structures (arrows) and papillary projections (arrowhead) in the myometrium, Hematoxylin-Eosin, X200.

cysts and was filled with a yellowish white mucus (Figure 1b). Tissue samples were fixed in 10 % buffered formalin, embedded in paraffin then the sections were cut at 4 µm and stained with hematoxylin and eosin. On microscopic examination, several large cystic structures, varying in size were revealed in the endometrium (Figure 2a). Glandular epithelium showed long papillary projections resembling tree branches into the cystic spaces (Figure 2b). Epithelial cells forming papillary projections showed anisonucleosis, anisocytosis and some mitotic figures (Figure 2c). The nuclei of tumor cells were pleomorphic, ranging from vesicular to hyperchromatic and presented multiple nucleoli. The cytoplasm of these cells marked variable forms, some were acidophilic, and some were vacuolar in structure. Besides, the endometrial cystic glands and the anaplastic epithelial cells showing papillary projections invaded the myometrium and presented infiltration between the muscle bundles (Figure d).

Uterine tumors are not frequent tumors in domestic animals but are apparently more frequent in cattle when compared to other species (2). Mammary tumors are one of the most common neoplasms in bitches (5). As opposed to mammary tumors, uterine tumors account for only 0.4 % of all canine tumors (1). Of those tumors reported, leiomyomas are the most predominant type accounting for 85–90% and the most encountered malignant uterine tumor is leiomyosarcoma (4). Carcinomas are very rare and only few related reports are found in veterinary literature.

In previously reported cases of uterine adenocarcinoma, an exposition to exogenous pregnancy prevent drug was present but a direct relationship could not be established between the use of pregnancy prevent drugs and the pathogenesis of uterine adenocarcinoma (7, 10). In the present report a chronic use of pregnancy prevent drug was reported until the owner elected ovariohysterectomy as a final choice. We can deduce that, despite the absence of a reasonable contribution of this drug to the carcinogenesis of adenocarcinoma, a possible predisposition can be concluded.

Surgery is still the treatment of choice in canine endometrial carcinomas cases specially if metastases are not present (1, 9). After removal of the uterus, the abdomen should be investigated for any metastatic foci. No studies reported efficacy of radiotherapy and chemotherapy in veterinary cases. However adjunctive chemotherapy should be considered related to the histopathological diagnosis (1). In the case reported here, no metastasis or complications were reported after ovariohysterectomy was performed.

Specially reported in geriatric dogs, uterine adenocarcinoma is extremely rare in young dogs. Only 2 cases of adenocarcinoma in young dogs were reported in the literature, the first was a 2 years old mixed breed dog reported by Payne-Johnson et al (6) and a 10 months old dog reported by Cave et al (1).

In conclusion, within this study; uterine adenocarcinoma reported in an 18 month old young dog showed that uterine adenocarcinomas, though rare, should be considered in the differential diagnosis of canine uterine lesions of young dogs. The dog presented no oestrus cycle and showed no clinical signs before ovariohysterectomy was performed. The diagnosis was totally based on histopathological findings.

Acknowledgements

This case was presented as a poster at 50th European Veterinary Conference Woorjaarsdagen 2017 held in Den Haag, between 19-21 April 2017.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Cave TA, Hine R, Howie F, et al** (2002): *Uterine carcinoma in a 10-month-old Golden Retriever.* J Small Anim Pract, **43**, 133-135.
2. **Foster RA** (2017): Female Reproductive System and Mammae. 1178. In: Zachary JF (Ed), Pathologic Basis of Veterinary Disease, 6th ed. Elsevier Health Sciences, Missouri.
3. **MacLachlan NJ, Kennedy PC** (2002): Tumors of the Genital Systems. 547-573. In: Meuten DJ (Ed), Tumors in Domestic Animals, 4th ed. Iowa State Press, Iowa.
4. **Murphy ST, Kruger JM, Watson GL** (1994): *Uterine adenocarcinoma in the dog: a case report and review.* J Am Anim Hosp Assoc, **30**, 440-444.
5. **Mülazimoğlu SB, Beceriklisoy HB, Schäfer-somi S, et al** (2016): *B-mode echotexture analysis and color doppler sonography in canine mammary tumors.* Kafkas Univ Vet Fak Derg, **22**, 961-969.
6. **Payne-Johnson CE, Kelly DF, Davies PT** (1986): *Endometrial carcinoma in a young dog*. J Comp Pathol, **96**, 463-467.
7. **Pena FJ, Gines JA, Duque J, et al** (2006): *Endometrial adenocarcinoma and mucometra in a 6-year-old Alaska Malamute dog*. Reprod Domest Anim, **41**, 189-190.
8. **Pires MA, Seixas F, Palmeira C, et al** (2010): *Histopathologic and immunohistochemical exam in one case of canine endometrial adenocarcinoma.* Reprod Domest Anim, **45**, 545-549.
9. **Plaxe SC, Mundt MJ, MD** (2019): Overview of endometrial carcinoma. Available at https://www.uptodate.com/contents/overview-of-endometrial-carcinoma. (Accessed Dec 12, 2019)
10. **Sapierzyński R, Malicka E, Bielecki W, et al** (2007): *Tumors of the urogenital system in dogs and cats. Retrospective review of 138 cases.* Pol J Vet Sc, **10**, 97-103.

Ankara Univ Vet Fak Derg, **68**, 87-90, 2021
DOI: 10.33988/ auvfd. 683190

Case Report / *Olgu Sunumu*

Priapism and its surgical treatment in a cat

Zülfükar Kadir SARITAŞ[1,a,✉], Musa KORKMAZ[1,b], Fatma GÖRÜCÜ[1,c], Sefa ÇELİK[2,d]

[1]Afyon Kocatepe University, Faculty of Veterinary Mecicine, Department of Surgery, Afyonkarahisar; [2]Afyonkarahisar Health Sciences University, Faculty of Medicine, Department of Biochemistry, Afyonkarahisar, Turkey.
[a]ORCID: 0000-0002-7659-6635; [b]ORCID: 0000-0002-7646-0009; [c]ORCID: 0000-0001-7630-0788;
[d]ORCID: 0000-0002-5187-378X

✉Corresponding author: zksaritas@hotmail.com
Received date: 02.02.2020 - Accepted date: 09.05.2020

Abstract: This study details a case of priapism in a 3-year-old, male, crossbreed cat weighing 5,4 kg treated successfully with surgery. The cat was brought to the Afyon Kocatepe University Veterinary Health Application and Research Centre for general examination and castration. Clinical examination revealed a fully erect and hard/solid penis. Serum biochemistry, complete blood count, and testosterone results of the animal were within the reference ranges except for urea. When the conservative treatment was unsuccessful, it was decided to perform a perineal urethrostomy operation. Postoperative examination on day 10 revealed healing and comfortable urination; thus, the stitches and urinary catheter were removed. In conclusion, this report of successful treatment of priapism in a cat through perineal urethrostomy can contribute to veterinary literature.
Keywords: Cat, perineal urethrostomy, priapism.

Bir kedide priapizm ve cerrahi sağaltımı

Özet: Bu çalışmada, 3 yaşında, erkek, 5,4 kg ağırlığındaki melez kedide görülen priapizm olgusu ve cerrahi sağaltımı tanımlanmaktadır. Kedi, genel kontrol ve kastrasyon isteği ile Afyon Kocatepe Üniversitesi, Veteriner Sağlık Uygulama ve Araştırma Merkezine getirildi. Klinik muayenede penisin tamamen erekte ve katı-sert kıvamda olduğu görüldü. Hayvanın üre dışındaki serum biyokimya, tam kan ve testosteron sonuçları referans değerler içindeydi. Konservatif tedavi başarısız olduğunda, perineal üretrostomi operasyonu yapılmasına karar verildi. Hayvanın postoperatif on gün sonra yapılan kontrolünde iyileşmenin şekillendiği ve idrarını rahatça yapabildiği gözlendi, dikişler alınarak idrar sondası uzaklaştırıldı. Sonuç olarak bir kedide perineal üretrostomi ile başarılı bir şekilde sağaltılan priapizm olgusunun, veteriner literatüre katkı sağlayacağı kanısına varılmıştır.
Anahtar sözcükler: Kedi, perineal üretrostomi, priapizm.

Priapism is defined as a painful erection of the penis that persists for more than an hour without any sexual stimulation (1, 5, 8). This erectile dysfunction, while being rare in pets, has been reported in dogs, horses, cats, rats, and sea lions (3-5, 7, 8). Priapism is due to the engorgement of corpus cavernosum. Corpus spongiosum is less swollen compared to a normal erection (3, 7, 8). When normal erection mechanism is interrupted, partial pressure of carbon dioxide of blood collected in corpus cavernosum increases and increased viscosity of blood disturbs the venous return. Obstruction of venous return and permanent erection cause oedema in the corpus cavernosum. If priapism occurs once, an erection may continue even though its underlying cause is eliminated (5). Permanent damage to the erectile tissue is a potential result of priapism. Corporal fibrosis occurs following thrombosis and destruction of endothelial and smooth muscle cells in cavernous sinus, leading to eventual vascular stasis and corpus ischemia (8).

The causes of priapism include administration of phenothiazine derivative drugs (such as acepromazine), nematodiasis, trauma-induced spinal cord injuries, cauda equina lesions, constipation, purpura hemorrhagica, severe inflammation, and septic or aseptic secondary infections (7). Priapism occurs as a complication of distemper-related spinal cord lesions and lower urinary tract diseases (8). It can also develop secondarily after castration or due to trauma during mating (3). Majority of

mechanisms of the factors causing priapism are unknown, but phenothiazine-derivative tranquilizers inhibit sympathetic transmission that induces paralysis of retractor penis muscles resulting in priapism (7). This study details a case of priapism in a cat and its surgical treatment.

A 3-year-old, male, crossbreed, uncastrated cat weighing 5.4 kg was brought to the Afyon Kocatepe University Veterinary Health Application and Research Center for general examination and castration. According to the information provided by its owner, the cat had been routinely vaccinated. It had a normal appetite; however, its water intake had doubled. The cat had a hard penis for the last 20 days, which it was constantly licking. It had mated a few days before being brought to the clinic and did not have any history of trauma or surgery. Clinical examination revealed a body temperature of 38.3°C, respiratory rate per minute of 40 and pulse rate per minute of 130. Skin turgor and lymph nodes were normal. Inspection revealed that the penis was completely outside the preputium (Figure 1) and swollen, its tip was red and blue, and palpation revealed that it was hard and solid. Serum biochemistry, complete blood count, and testosterone results were within the reference ranges except for urea (Table 1 and 2). A rapid test kit (FIV Ab/FeLV Ag Test Kit, Bionote, Korea) confirmed that the cat was negative for FeLV and FIV. In light of these findings, the cat was diagnosed with priapism and treated accordingly. As a conservative treatment, the penis was massaged with emollients in addition to cold compressions, and blood was aspirated by puncturing the corpus cavernosum. Finally, adrenalin-containing 0.9% NaCl solution was injected intracavernously. Interviews with the owner revealed that the symptoms were slightly reduced, eating and drinking were normal, and the cat was able to urinate comfortably; however, priapism did not fully resolve, leading to the decision to perform a perineal urethrostomy.

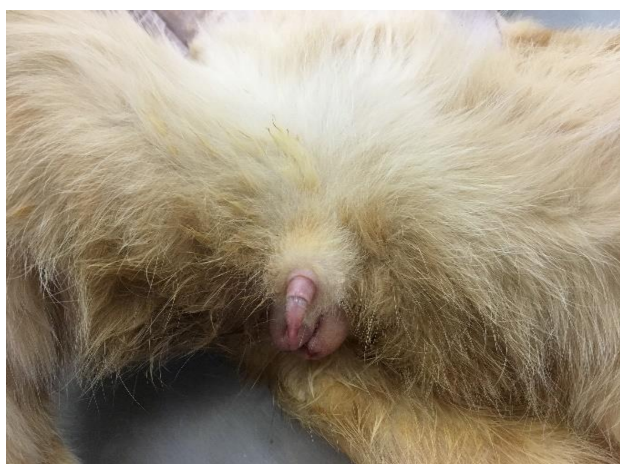

Figure 1. Preoperative clinical presentation

Table 1. Complete blood count results

Parameters	Value	Reference ranges
WBC (10^9/L)	12.5	5.5-19.5
Lymph# (10^9/L)	4.2	0.8-7.0
Mon# (10^9/L)	0.7	0.0-1.9
Gran# (10^9/L)	7.6	2.1-15.0
Lymph (%)	33.5	12.0-45.0
Mon (%)	5.5	2.0-9.0
Gran (%)	61	35.0-85.0
RBC (10^{12}/L)	7.69	4.60-10.00
HGB (g/dL)	12	9.3-15.3
HCT (%)	34.2	28.0-49.0
MCV (fL)	44.6	39.0-52.0
MCH (pg)	15.6	13.0-21.0
MCHC (g/dL)	35	30.0-38.0
RDW (%)	12.7	14.0-18.0
PLT (10^9/L)	357	100-514
MPV (fL)	11.4	5.0-11.8
PDW	15.9	
PCT (%)	0.406	
Eos (%)	1.2	

Table 2. Serum biochemistry results

Parameters	Value	Reference ranges
Chol (mg/dL)	100.3	71-156
Glucose (mg/dL)	115.5	60-120
Crea (mg/dL)	1.04	0.9-2.2
Uric Acid (mg/dL)	0.1	0-0.5
Na (mmol/L)	151	146-156
K (mmol/L)	4.57	3.7-6.1
Cl (mmol/L)	117.7	115-130
P (mg/dL)	4.61	3.0-6.1
Total Protein (g/dL)	6.77	6.0-7.9
Albumin (g/dL)	3.71	2.8-3.9
AST (U/L)	31.5	7-38
ALT (U/L)	41.1	25-97
Bild2 (mg/dL)	0.045	0-1.7
CA (mg/dL)	9.43	8.7-11.7
LDLC3 (mg/dL)	9.5	
Urea (mg/dL)	40.6	19-34
Trigl (mg/dL)	30.3	25-160
Total Bilirubin (mg/dL)	0.036	0-0.1
Testo (ng/dL)	0.4398	<0.5

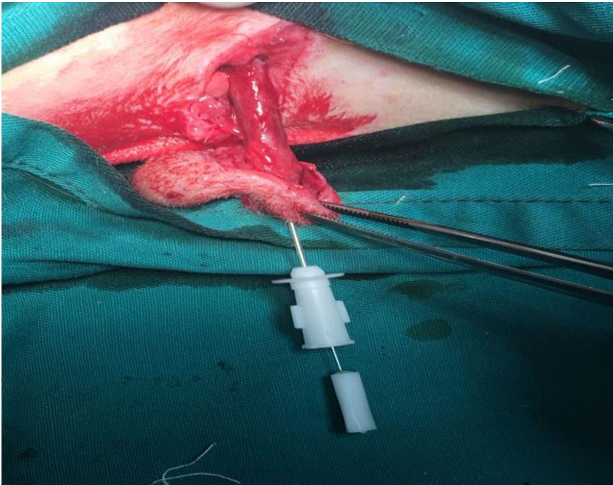

Figure 2. Preoperative view of the case.

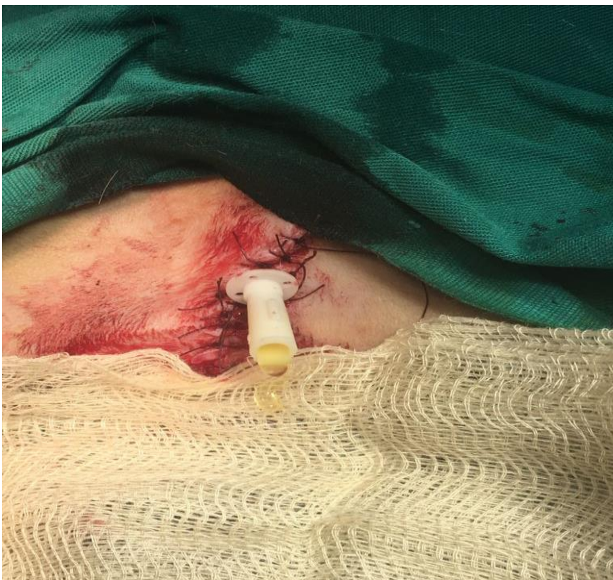

Figure 3. Postoperative view of the case.

Routine examination was performed prior to the operation. For premedication, atropine sulphate at a dose of 0.045 mg/kg was injected subcutaneously. The cat was sedated with intramuscular injection of 2 mg/kg Xylazine HCl and general anaesthesia was induced with an IM injection of 10 mg/kg Ketamine HCl.

Perineal region, including 4-5 cm at the ventral base of the tail, was shaved and prepared for surgery. The animal was laid ventrally and its hind legs were tied slightly tight and its tail was secured to its back. Paying attention to the anal sacs, the anus was closed with a 3-0 nylon stitch using the circular suture technique. First, castration was performed. Urethra was catheterized to determine its location. An elliptical incision was made to skin from the side of the penis and preputium. Penis and ischiocavernosus and ischiourethral muscles were dissected bluntly with subcutaneous tissues and penis was freed from its surrounding attachments (Figure 2). Penile urethra was incised from the dorsal end of penis to the bulbourethral gland using thin-tipped scissors. Approximately two-thirds of the incised pelvic urethra and penile urethra were stitched to the skin with simple interrupted suturing using a 3-0 monofilament polypropylene suture material. Penis and urethra remaining outside urethrostomy region were removed. A permanent urinary catheter was secured to the skin (Figure 3) and the circular stitching of the anus was removed. An Elizabethan collar was put on the cat to protect the region from licking for 7-10 days after the operation and until the stitches were removed. Amoxicillin/clavulanic acid at a dose of 8.75 mg/kg was administered IM for post-operative 5 days. Post-operative check up on day 10 revealed that the cat had fully recovered and was able to urinate comfortably; thus, stitches and urinary catheter were removed.

Although it can be observed at any age, priapism has usually been reported in animals older than one year (1, 5, 6, 8). While most of the priapism cases in cats have been observed in Siamese cats (1) priapism has also been observed in crossbreeds (1, 5, 6, 8). The three-year-old crossbreed cat described in this case is in line with the data found in the literature.

It has been reported that paraphimosis or priapism can develop both in uncastrated and castrated cats and both cases are urgent and should be treated immediately (2). Literature typically includes mating (1), castration (5), FIP (1, 8) hCG application (6) and unknown causes (1) related to the etiology of priapism. In the case presented here, the cat was brought to the clinic for castration. Moreover, the owner reported that the cat mated a few days prior to their visit to the clinic and penis was protruded before mating. Accordingly, we concluded that priapism, in this case, was not due to castration or mating. In addition, the cat was negative for FIP, which is listed among the possible causes of priapism.

Priapism can be mistaken for paraphimosis or penile paralysis (4, 7). In priapism, the penis is erect and hard, whereas it is soft in paraphimosis and penile paralysis (3, 4). In paraphimosis, preputial orifice is too narrow and, thus, blocks the entrance penis to preputium, while in penile paralysis, soft penis cannot enter preputium (7). In priapism, preputium can easily be moved to tip of the penis, which helps distinguishing priapism from paraphimosis (5). In line with the literature, in the presented case, the penis was hard and remained erect and a manual inspection revealed that preputium could easily cover penis and penis easily moved inside preputium. These types of clinical findings should be considered during diagnosis of priapism.

In priapism, penile mucosa changes color to varying degrees from red to purple due to congestion, and becomes dry, inflamed, and excoriated (3, 7). Male cats frequently

lick the area because of the pain, and by doing so, damage the penis. If the condition is neglected, it can lead to necrosis. In the case of urethral congestion, strangury may occur, and if untreated, secondary uraemia may emerge due to rupture of the urinary bladder (3). In this study, the congestion and change of color in the penis and the continuous licking of the penis were in line with the data found in the literature. In addition, the cat was able to urinate comfortably.

The main purposes of the treatment of priapism are to allow normal blood circulation in the penile erectile tissues and protect the penis from severe injuries, drying, ischemia, necrosis, and urethral obstruction (3). Conservative treatments include various combinations of cold-water compresses, topical hypertonic solutions, massaging, hydrotherapy, anti-inflammatory drugs, broad-spectrum antibiotics, and diuretics. A fully-erect penis is too large and too long to be pulled back into the preputium and, thus, becomes congested, dry, and eventually necrotic (3, 10). Venous return can be increased with a combination of intracavernous phenylephrine infusion and rinsing and drainage with heparin-containing salt (0.9% NaCl) solution (3). If intracavernous drainage and injections fail, or serious tissue damage occurs, amputation of the penis or a perineal urethrostomy might be necessary (4, 9). Conservative methods are usually unsuccessful in treatment of priapism (3, 7). In this case, penis was massaged with emollients along with cold compressions as reported in the literature. Corpus cavernosum was punctured, a fair amount of blood was aspirated, and adrenalin-containing 0.9% NaCl was applied intracavernously; however, these applications did not yield successful results. Thus, the case was eventually treated with a perineal urethrostomy and no complications were observed during postoperative period.

In conclusion, documentation of our successful treatment of a case of priapism in a cat via perineal urethrostomy can contribute to veterinary literature and help veterinary practitioners.

Financial Support

This research received no grant from any funding agency/sector.

Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Gunn-Moore DA, Brown PJ, Gruffydd-Jones TJ** (1995): *Priapism in seven cats.* J Small Anim Pract, **36**, 262-266.
2. **Jordan LA, Brainard BM** (2011): Triage *in the veterinary emergency room: part 2.* Vet Nurs J, **2**, 560-566.
3. **Kutzler MA** (2012): *Paraphimosis.* 686-690. In: E Monnet (Ed), Small Animal Soft Tissue Surgery. John Wiley & Sons, United Kingdom.
4. **Lavely JA** (2009): *Priapism in dogs*. Top Companion Anim Med, **24**, 49-54.
5. **Orima H** (1989): Surgical *treatment of priapism observed in a dog and a cat.* Jpn J Vet Sci, **51**, 1227-1229.
6. **Quaranta G, Rota A, Dogliero A, et al** (2015): Priapism *following hCG administration in a cat.* Acta Vet-Beograd, **65**, 568-571.
7. **Rochat MC** (2001): *Priapism: a review.* Theriogenology, **56**, 713-722.
8. **Rota A, Paltrinieri S, Jussich S, et al** (2008): *Priapism in a castrated cat associated with feline infectious peritonitis.* J Feline Med Surg, **10**, 181-184.
9. **Smith CW** (2002): *Perineal urethrostomy.* Vet Clin Small Anim Prac, **32**, 917-925.
10. **Taylor AH, Bolt DM** (2011): *Persistent penile erection (priapism) after acepromazine premedication in a gelding.* Vet Anaesth Analg, **38**, 523-525.

Ankara Univ Vet Fak Derg, **68**, 91-95, 2021
DOI: 10.33988/auvfd.651527

Short Communication / *Kısa Bilimsel Çalışma*

Slaughter and carcass characteristics of Kivircik lambs in different rearing seasons

Hülya YALÇINTAN[a, ✉], Nurşen ÖZTÜRK, Pembe Dilara KEÇİCİ[c], Bülent EKİZ[d], Ömür KOÇAK[e], Alper YILMAZ[f]

İstanbul University-Cerrahpaşa, Faculty of Veterinary Medicine, Department of Animal Breeding and Husbandry, İstanbul, Turkey.
[a]ORCID: 0000-0001-7062-1521; [b]ORCID: 0000-0002-0091-5812; [c]ORCID: 0000-0003-1151-179X;
[d]ORCID: 0000-0001-6458-5747; [e]ORCID: 0000-0002-2827-4471; [f]ORCID: 0000-0003-0223-4444.

✉Corresponding author: hyalcint@istanbul.edu.tr
Received date: 28.11.2019 - Accepted date: 15.06.2020

Abstract: The aim of this study was to determine the slaughter and carcass characteristics of Kivircik lambs reared in different seasons. A Total of 36 Kivircik male lambs, which were reared in different seasons, were used in the study; autumn rearing (AR, n= 12 lambs), spring-summer rearing (SSR, n= 12 lambs), winter rearing (WR, n= 12 lambs). All the lambs in a specific rearing season were slaughtered at approximately 134 days old. Pre-slaughter live weight, hot carcass weight and real dressing percentage for WR, SSR and AR groups were 27.97, 20.65, and 21.70 kg; 12.94, 7.83, and 8.87 kg, and 55.15, 50.25, and 50.05%, respectively ($P < 0.001$). WR lambs had significantly higher carcass and hind limb compactness score as well as ribs percentage than SSR and AR lambs ($P < 0.001$). The results indicated that lambs from WR group had better carcass characteristics than lambs from AR and SSR groups.
Keywords: Indoor lambs, lamb finishing, pasture lambs, seasonal effect.

Farklı büyüme mevsimlerinde Kıvırcık ırkı kuzuların kesim ve karkas özellikleri

Özet: Bu projede, mevsime bağlı kuzu üretim sistemlerinde büyütülen Kıvırcık ırkı kasaplık kuzuların kesim ve karkas özelliklerinin belirlenmesi amaçlanmıştır. Çalışmada farklı mevsimlerde büyütülen toplam 36 baş Kıvırcık ırkı erkek kuzu [sonbahar (AR, n=12); ilkbahar-yaz (SSR, n=12); kış (WR, n=12)] kullanılmıştır. WR, SSR ve AR grubu kuzulara ait kesim öncesi canlı ağırlık, sıcak karkas ağırlığı ve gerçek karkas randımanı ortalamaları sırasıyla 27,97; 20,65 ve 21,70 kg; 12,94; 7,83 ve 8,87 kg; %55,15; 50,25 ve 50,05 olarak tespit edilmiştir ($P < 0,001$). WR grubu kuzuların karkas kompaktlığı, but kompaktlığı ve kaburga oranı bakımından SSR ve AR grubu kuzulara kıyasla daha yüksek ortalamalara sahip oldukları belirlenmiştir ($P < 0,001$). Çalışma bulguları WR grubu kuzuların SSR ve AR grubu kuzulara kıyasla daha iyi karkas özelliklerine sahip olduğu tespit edilmiştir.
Anahtar sözcükler: Ağıl besisi, kuzu besisi, mera kuzusu, mevsim etkisi.

Consumers' growing interest in naturally reared lambs, which grazed on pasture, was observed in recent years (10). In traditional sheep breeding program in Marmara region, matings usually take place between June-August months, and therefore lambing occurs during winter. Because of the unsuitability of pasture areas for lambs' grazing, feeding lambs with concentrate feeds and their mothers' milk on sheepfold during winter is the most common lamb production system. However, conditions of pasture may vary due to season. Some farmers intend to supply quality lambs to market throughout the year, producing lambs out of the breeding season by using the advantage of the pasture (2). They use pastures to provide lambs' nutritional needs.

The feeding systems of animals may change according to the rearing season. Also, we know that husbandry conditions and feeding systems influence the carcass quality characteristics of lambs (10). Carcass weight (14), fatness level and dressing percentage of the lambs (8, 10) fed with concentrate feeds were reported to be higher than grazed lambs. This study was produced from a project, which investigated the effect of rearing season (spring-summer, winter and autumn) on slaughter characteristics, carcass and meat quality properties in male Kivircik lambs. This part includes certain slaughter and carcass characteristics results of the main project.

Animal handling procedures of the study were approved by the Ethics Committee of İstanbul University

(Approval no: 2015/05). The material was formed by 36 male Kivircik lambs which were born in winter (n = 12 lambs), spring-summer (n = 12 lambs) and autumn (n = 12 lambs) in the research farm of İstanbul University. At the beginning of the natural breeding season, the herd were separated into three groups to organize the synchronizations of ewes in each mating period (May, October, and February). Mean values of daily minimum ambient temperature, maximum ambient temperature and rainfall, which calculated by averaging daily data over the study, were 7.2 °C, 12.5 °C and 1.9 mm in the winter season, 15.3 °C, 23.4 °C and 1.1 mm in spring-summer season, 18.2 °C, 24.9 °C and 1.1 mm in the autumn season, respectively. Winter rearing (WR) lambs received concentrate feed (87.7% dry matter, 17.15% crude protein, 11.10 MJ/kg ME; 500 g / per animal / day) in the sheepfold after the age of 15 days until slaughter age. These lambs were also allowed to suckle their dams throughout the study. Lambs reared in spring-summer (SSR) and autumn (AR) seasons received the same nutrition programme as WR lambs until the age of two months, after this age these lambs grazed at the natural pasture in the day time with their dams. While SSR lambs grazed summer pasture, AR lambs were taken to autumn pasture. Lambs in both groups received concentrate feed when they returned from the pasture and after being separated from their dams. Alfalfa hay, which contained 87.8% dry matter, 12.88% crude protein, 7.72 MJ/kg ME, was given to all sub-groups with ad libitum access after two weeks of age in the sheepfold. Detailed descriptions of the feeds used in the present study were given in Yalcintan et al. (15). The pasture characteristics were widely described in Ekiz et al. (8).

All the lambs in a specific rearing season were slaughtered at approximately 134 days old in the same day at the experimental slaughterhouse of İstanbul University Faculty of Veterinary Medicine. Hot carcasses were weighted after the elimination of the non-carcass components (skin, head, feet, trachea and lungs, spleen, heart, liver, and gastro-intestinal tract) and then chilled for 24 h at 4 ºC. Hot carcasses included kidney and kidney knob and channel fat (KKCF). We estimated the commercial dressing percentage using pre-slaughter live weight and real dressing percentage with empty body weight. After kept for 24 h at 4 ºC, firstly the cold carcasses were divided into two halves, then left halves were divided into five parts including neck, shoulder, ribs, flank, and hind limb (6).

Lengths of carcass and leg, widths of buttock and carcass, circumferences of buttock and chest were measured on whole carcasses, while lengths of internal carcass and hind limb, and thoracic depth were measured from half carcasses (3, 9, 11). Hind limb compactness, carcass compactness and chest roundness index were calculated according to Ekiz et al. (9). One-way ANOVA and Duncan's multiple range tests in SPSS 13 programme were used to evaluate the differences among rearing seasons.

Differences among the season groups in terms of pre-slaughter weight and hot carcass weight were found significant for the lambs slaughtered at 134 days of age (Table 1). A higher pre-slaughter weight of the lambs from

Table 1. Means ± standard errors for slaughtering characteristics of lambs reared in different seasons

Characteristics	WR	SSR	AR	*P*-value
Pre-slaughter live weight, kg	27.97^{a}±1.38	20.65^{b}±0.58	21.70^{b}±1.00	<0.001
Hot carcass weight, kg	12.94^{a}±0.65	7.83^{b}±0.29	8.87^{b}±0.56	<0.001
Commercial dressing [1], %	46.29^{a}±0.80	37.83^{c}±0.63	40.56^{b}±0.80	<0.001
Real dressing [2], %	55.15^{a}±0.55	50.25^{b}±0.44	50.05^{b}±0.72	<0.001
Head, %	6.96^{b}±0.11	8.32^{a}±0.30	7.40^{b}±0.14	<0.001
Feet, %	3.16^{b}±0.08	3.87^{a}±0.05	3.31^{b}±0.09	<0.001
Skin, %	11.45±0.47	11.07±0.62	12.07±0.18	0.313
Lungs and trachea, %	1.74^{b}±0.05	2.23^{a}±0.09	2.12^{a}±0.10	<0.001
Liver, %	1.94^{b}±0.05	2.37^{a}±0.07	2.23^{a}±0.08	<0.001
Heart, %	0.53±0.02	0.61±0.02	0.57±0.05	0.220
Spleen, %	0.24^{b}±0.01	0.38^{a}±0.05	0.27^{b}±0.01	0.004
Omental and mesenteric fat, %	0.82^{a}±0.09	0.07^{b}±0.01	0.73^{a}±0.10	<0.001
Stomachs, %	19.42^{b}±1.48	30.15^{a}±2.31	22.28^{b}±0.87	<0.001
Empty stomachs, %	3.75^{b}±0.15	4.97^{a}±0.24	4.58^{a}±0.18	<0.001
Intestine, %	9.48^{b}±0.33	14.03^{a}±1.63	12.44^{a}±0.31	0.008
Empty intestine, %	5.79^{b}±0.22	6.10^{b}±0.11	6.59^{a}±0.15	0.007
Gastro-intestinal tract, kg	4.59±0.47	5.10±0.24	4.09±0.13	0.095

$^{a, b, c}$ Means in the same line with different superscripts are significantly different.
[1] Commercial dressing: hot carcass dressing based on pre-slaughter live weight;
[2] Real dressing: hot carcass dressing based on empty body weight.
WR: Winter rearing, SSR: Spring-summer rearing, AR: Autumn rearing.

the WR group caused higher hot carcass weights for these lambs compared to lambs from the SSR and AR groups. Previously, some researchers have reported an increase in the hot carcass weight in parallel to an increase in the pre-slaughter weight (1, 13). AR and WR groups had higher mean values regarding the omental and mesenteric fat percentages than the SSR group. This difference was probably due to the high energy intake of lambs fed with concentrated feed (4). Ekiz et al. (8), who were investigating the effect of rearing systems on carcass quality, determined that lambs fed with concentrates at sheepfold had higher omental and mesenteric fat percentages than weaned pasture lambs. SSR lambs had lower omental and mesenteric fat percentages than AR and WR groups, which could be linked by the heat stress for the SSR lambs due to grazing on the summer pasture and decreased feed intake. In our previous study (15), which was conducted similar to the current study, we also found lower carcass fatness score for spring-summer rearing lambs than winter and autumn rearing lambs, while winter rearing lambs had the highest backfat thickness in that study.

Winter rearing lambs which were kept with their dams during the rearing period presented lower lung and trachea, liver, intestine, and empty stomach percentages. Supporting the findings of this study Karim et al. (12) determined a higher liver percentage for the lambs fed on pasture than the lambs fed in sheepfold. Authors explained this difference with possible consumption of harmful vegetation which leads to liver growth caused by detoxification. On the other hand, Majdoub-Mathlouthi et al. (13) reported decreased liver percentage in parallel to the increased pre-slaughter weight. Percentage of the gastrointestinal tract might be associated with several factors, such as type of feed (pasture, concentrate), pre-slaughter age/weight of animals (8, 12, 13, 14). It has been suggested that digestive tract development is usually faster in animals grazed on pasture (8, 13). In the current study, there were no significant differences between season groups in terms of animal age. As expected, SSR and AR lambs had higher empty stomach percentage than WR lambs. This could be related to the higher development of digestive tract in pasture lambs. Supporting our results Ekiz et al. (8) reported a higher empty stomach percentage for the pasture-based lambs than lambs fed with concentrate. Moreover, lower pre-slaughter weights of SSR and AR lambs could be another explanation for higher percentages of empty stomachs.

Dressing percentage is describing the ratio between carcass weight and pre-slaughter live weight of the animal and depends on many factors such as age/weight at slaughter, husbandry system, breed and gender, feeding system, and whether it is calculated on the bases of empty or full body weight, which can be influenced by gastrointestinal content (7). As expected, WR lambs which were fed concentrate and reared sheepfold had higher commercial and real dressing percentage compared to SSR and AR lambs. However, these results cannot be explained by the only higher energy content of the diet. In addition, it should be considered that SSR and AR lambs which were grazed on pasture had higher lungs and trachea, liver and empty stomach percentages. Similarly, Priolo et al. (14) also determined a higher dressing percentage for lambs fed with concentrates in the sheepfold, even though slaughtering the pasture and sheepfold lambs at similar weights. The researchers explained this result by the lower intestinal tract content of sheepfold based lambs. Ekiz et al. (8) explained a lower dressing percentage of pasture lambs compared to concentrate fed lambs by a higher percentage of non-carcass components in the pasture lambs.

SSR lambs showed the highest values for the shoulder, flank and neck proportion, while WR lambs had the highest values for the ribs percentage (Table 2). SSR lambs presented lower KKCF percentage than AR and WR lambs ($P < 0.001$). Ekiz et al. (8) also found higher shoulder proportion for lambs grazed at pasture than lambs fed with concentrates at sheepfold. Lambs from WR group showed higher mean values than SSR and AR lambs in terms of internal carcass length, buttock width and circumference, carcass width, thoracic depth, and chest circumference (Table 2). In contrast to this study, Carrasco et al. (5) for Churra Tensina lambs and Ekiz et al. (10) for Kivircik lambs reported higher mean values for longitudinal carcass measurements (internal carcass length and hind limb length) for the grazed lambs than lambs fed with concentrates. The variation between researches might be explained by slaughtering the lambs from pasture and concentrate groups at different ages in those studies. Ekiz et al. (10) reported that pasture lambs had higher lengths of hind limb and internal carcass among the four different rearing groups, where lambs were slaughtered at similar weights due to fact that pasture lambs reached the target weight at older ages. Supporting our findings, Abdullah and Qudsieh (1) reported an increase in hind limb compactness and carcass measurements in parallel to the increase in the carcass weight. WR lambs had higher values for the carcass and hind limb compactness indices than lambs from AR and SSR groups which could be attributed to the high pre-slaughter weight for WR lambs. It has been determined that characteristics which have considerable economic importance, such as pre-slaughter live weight, dressing percentage, carcass compactness and hind limb compactness were higher in WR lambs compared to AR and SRR lambs. The results indicated that lambs from WR group had better slaughter and carcass characteristics than lambs from AR and SSR groups.

Table 2. Means ± standard errors for proportion of carcass cuts and certain carcass measurements of lambs reared in different seasons

Characteristics	WR	SSR	AR	*P*-value
Shoulder, %	18.90^{b}±0.36	20.30^{a}±0.21	19.29^{b}±0.31	0.007
Flank, %	9.14^{c}±0.30	11.64^{a}±0.38	10.62^{b}±0.19	<0.001
Neck, %	8.25^{b}±0.21	9.53^{a}±0.29	8.21^{b}±0.19	<0.001
Ribs, %	27.86^{a}±0.50	23.91^{c}±0.39	25.78^{b}±0.43	<0.001
Thoracic region, %	18.64^{a}±0.41	16.68^{b}±0.27	17.07^{b}±0.32	0.001
Lumbar region, %	9.25^{a}±0.22	7.22^{b}±0.27	8.71^{a}±0.27	<0.001
Hind limb, %	33.27±0.49	33.00±0.23	33.69±0.34	0.410
Tail, %	0.99^{a}±0.08	0.47^{c}±0.05	0.77^{b}±0.07	<0.001
Kidney, %	0.65^{b}±0.02	0.92^{a}±0.03	0.89^{a}±0.05	<0.001
KKCF ^{g}, %	0.95^{a}±0.12	0.24^{b}±0.03	0.75^{a}±0.09	<0.001
Carcass measurements				
Carcass length, cm	63.55±2.60	62.13±0.81	60.88±0.88	0.526
Internal carcass length, cm	56.78^{a}±0.87	49.18^{c}±0.65	52.48^{b}±0.65	<0.001
Leg length, cm	19.93^{a}±0.26	19.72^{a}±0.47	18.57^{b}±0.17	0.012
Hind limb length, cm	26.15±0.46	26.88±0.41	25.65±0.30	0.104
Buttock width, cm	16.25^{a}±0.33	13.90^{b}±0.30	14.34^{b}±0.41	<0.001
Buttock circumference, cm	51.88^{a}±0.99	41.87^{b}±1.05	43.76^{b}±0.94	<0.001
Carcass width, cm	19.43^{a}±0.43	15.14^{c}±0.40	16.99^{b}±0.54	<0.001
Thoracic depth, cm	23.47^{a}±0.47	20.76^{b}±0.30	21.30^{b}±0.44	<0.001
Chest circumference, cm	67.53^{a}±1.07	58.48^{b}±0.57	58.16^{b}±1.08	<0.001
Carcass compactness, g/cm	221.44^{a}±9.60	152.17^{b}±4.17	163.34^{b}±8.75	<0.001
Hind limb compactness, g/cm	80.03^{a}±3.72	47.58^{b}±1.88	54.55^{b}±5.96	<0.001
Chest roundness index	0.83^{a}±0.02	0.73^{b}±0.02	0.80^{a}±0.02	0.001

$^{a, b, c}$ Means in the same line with different superscripts are significantly different.
WR: Winter rearing, SSR: Spring-summer rearing, AR: Autumn rearing.

Financial Support

This study was supported by The Scientific and Technological Research Council of Turkey (Project Number: 115 O 840) and Scientific Research Projects Coordination Unit of İstanbul University-Cerrahpasa (Project Number: BYP-2019-32193).

Ethical Statement

This study was approved by the İstanbul University Animal Research Ethics Committee of (Approval no: 2015/05)

Conflict of Interest

The authors declared that there is no conflict of interest.

References

1. **Abdullah AY, Qudsieh RI** (2008): *Carcass characteristics of Awassi ram lambs slaughtered at different weights*. Livest Sci, **117**, 165-175.
2. **Akçapınar H** (1994): Koyun Yetiştiriciliği. Medisan Yayınevi, Ankara.
3. **Cañeque V, Pèrez C, Velasco S, et al** (2004). *Carcass and meat quality of light lambs using principal component analysis*. Meat Sci, **67**, 595-605.
4. **Cañeque V, Velasco S, Diaz MT, et al** (2003): *Use of whole barley with a protein supplement to fatten lambs under different management systems and its effect on meat and carcass quality*. Anim Res, **52,** 271-285.
5. **Carrasco S, Ripoll G, Sanz A, et al** (2009*): Effect of feeding system on growth and carcass characteristics of Churra Tensina light lambs*. Lives Sci, **121**, 56-63.
6. **Colomer-Rocher F, Morand-Fehr P, Kirton AH** (1987): *Standard methods and procedures for goat carcass evaluation, jointing and tissue separation*. Livest Prod Sci, **17**, 149-159.
7. **Corazzin M, Bianco SD, Bovolenta S, et al** (2019): Carcass Characteristics and Meat Quality of Sheep and Goat. 119-166. In: JM Lorenzo, PES Munekata, FJ Barba, F Toldra (Eds), More than Beef, Pork and Chicken – The Production, Processing, and Quality Traits of Other Sources of Meat for Human Diet. Springer Nature, Switzerland.
8. **Ekiz B, Demirel G, Yilmaz A, et al (2013):** *Slaughter characteristics, carcass quality and fatty acid composition of lambs under four different production systems*. Small Rum Res, **114**, 26-34.
9. **Ekiz B, Ozcan M, Yilmaz A, et al (2010):** *Carcass measurements and meat quality characteristics of dairy suckling kids compared to an indigenous genotype*. Meat Sci, **85**, 245-249.
10. **Ekiz E, Yilmaz A, Ozcan M, et al** (2012): *Effect of production system on carcass measurement and meat quality of Kivircik lambs*. Meat Sci, **90**, 465-471.

11. **Fisher AV, De Boer H** (1994): *The EAAP standart method of sheep carcass assessment. Carcas measurement and dissection procedures*. Report of the EAAP working group on carcass evaluation, in cooperation with the CIHEAM Instituto Agronomico Mediterraneo of Zaragoza and the CEC Directorate General for Agriculture in Brussels. Livest Prod Sci, **38**, 149-159.
12. **Karim SA, Porwal K, Kumar S, et al** (2007): *Carcass traits of Kheri lambs maintained on different system of feeding management*. Meat Sci, **76**, 395-401.
13. **Majdoub-Mathlouthi L, Saïd B, Say A, et al** (2013): *Effect of concentrate level and slaughter body weight on growth performances, carcass traits and meat quality of Barbarine lambs fed oat hay based diet*. Meat Sci, **93**, 557-563.
14. **Priolo A, Micol D, Agabriel J, et al** (2002): *Effect of grass or concentrate feeding systems on lamb carcass and meat quality*. Meat Sci, **62**, 179-185.
15. **Yalcintan H, Ekiz B, Kocak O, et al** (2017): *Carcass and meat quality characteristics of lambs reared in different seasons*. Arch Anim Breed, **60**, 225-233.

Veterinary Journal of Ankara University

E-ISSN 1308-2817 Cilt/Volume 68 ● Sayı/Number 1 ● 2021

Ankara Univ Vet Fak Derg - vetjournal.ankara.edu.tr - Open Acsess