E-ISSN 1309 - 2251

KAFKAS ÜNİVERSİTESİ VETERİNER FAKÜLTESİ DERGİSİ

Journal of the Faculty of Veterinary Medicine, Kafkas University

Published Bi-monthly

Volume: 29
Issue: 3 (May - June)
Year: 2023

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 3, Year: 2023

E-ISSN: 1309-2251

This journal is published bi-monthly, by the Faculty of Veterinary Medicine, University of Kafkas, Kars - Turkey

This journal is indexed and abstracted in:

- Web of Science Core Collection: Science Citation Index Expanded (since 2007)
- Additional Web of Science Indexes: Essential Science Indicators - Zoological Record
- CABI - Veterinary Science Database
- DOAJ
- EBSCO - Academic Search Premier
- Elsevier - SCOPUS
- Elsevier - EMBASE
- SOBİAD Atıf Dizini
- TÜBİTAK/ULAKBİM TR-Dizin
- Türkiye Atıf Dizini

Address for Correspondence

Kafkas Üniversitesi Veteriner Fakültesi Dergisi Editörlüğü 36040, Kars - TÜRKİYE
Phone: +90 474 2426807-2426836/5228 Fax: +90 474 2426853 E-mail: vetdergi@kafkas.edu.tr

Electronic Edition http://vetdergikafkas.org

Online Submission http://submit.vetdergikafkas.org

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 3, Year: 2023

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 3, Year: 2023

EDITORIAL BOARD

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 3, Year: 2023

THE REFEREES LIST OF THIS ISSUE *(in alphabetical order)*

Aliye GÜLMEZ SAĞLAM	Kafkas Üniversitesi Veteriner Fakültesi
Alper ÇİFTCİ	Ondokuz Mayıs Üniversitesi Veteriner Fakültesi
Cenker Çağrı CINGI	Afyon Kocatepe Üniversitesi Veteriner Fakültesi
Ebru ERAVCI	İstanbul Üniversitesi- Cerrahpaşa Veteriner Fakültesi
Ekin Emre ERKILIÇ	Kafkas Üniversitesi Veteriner Fakültesi
Erdoğan UZLU	Balıkesir Üniversitesi Veteriner Fakültesi
Eren KUTER	Burdur Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
Eser ÖZGENCİL	Yakın Doğu Üniversitesi Veteriner Fakültesi
Fatih BÜYÜK	Kafkas Üniversitesi Veteriner Fakültesi
Fikret Özgür COSKUN	Kafkas Üniversitesi Diş Hekimliği Fakültesi
Fuat AYDIN	Erciyes Üniversitesi Veteriner Fakültesi
Hamid AKBAR	University of Veterinary and Animal Sciences, Pakistan
Hasan ÖNDER	Ondokuz Mayıs Üniversitesi Ziraat Fakültesi
Hasan Tarık ATMACA	Balıkesir Üniversitesi Veteriner Fakültesi
Hivda ÜLBEĞİ POLAT	TÜBİTAK MAM - Yaşam Bilimleri
İbrahim ÇAKIR	Bolu Abant İzzet Baysal Üniversitesi Mühendislik Mimarlık Fakültesi
Kristina MORKŪNIENĖ	Lithuanian University of Health Sciences, Lithuania
Levend COŞKUNTUNA	Tekirdağ Namık Kemal Üniversitesi Ziraat Fakültesi
Mehmet BAYRAKTAR	Harran Üniversitesi Tıp Fakültesi
Mehmet Nuri AÇIK	Bingöl Üniversitesi Veteriner Fakültesi
Memiş ÖZDEMİR	Atatürk Üniversitesi Ziraat Fakültesi
Mert SEZER	Kafkas Üniversitesi Veteriner Fakültesi
Miyase ÇINAR	Kırıkkale Üniversitesi Veteriner Fakültesi
Murat Can DEMİR	Kafkas Üniversitesi Veteriner Fakültesi
Mustafa SAATCI	Muğla Sıtkı Koçman Üniversitesi Veteriner Fakültesi
Mustafa SANDIKÇI	Aydın Adnan Menderes Üniversitesi Veteriner Fakültesi
Mükremin ÖLMEZ	Kafkas Üniversitesi Veteriner Fakültesi
Nilgün ÜNAL	Sağlık Bilimleri Üniversitesi Gülhane Eczacılık Fakültesi
Özgül GÜLAYDIN	Siirt Üniversitesi Veteriner Fakültesi
Özlem KARADAĞOĞLU	Kafkas Üniversitesi Veteriner Fakültesi
Ramazan ADANIR	Burdur Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi
Raziye IŞIK	Tekirdağ Namık Kemal Üniversitesi Ziraat Fakültesi
Rini Devijanti RIDWAN	Faculty of Dental Medicine, Universitas Airlangga, Indonesia
Ruhi KABAKÇI	Kırıkkale Üniversitesi Veteriner Fakültesi
Sadık YAYLA	Dicle Üniversitesi Veteriner Fakültesi
Salih OTLU	Kafkas Üniversitesi Veteriner Fakültesi
Semra KAYA	Kafkas Üniversitesi Veteriner Fakültesi
Seyda CENGİZ	Muğla Sıtkı Koçman Üniversitesi Veteriner Fakültesi
Veysel BAY	Ege Üniversitesi Ziraat Fakültesi
Zelal KARAKOÇ	Dicle Üniversitesi Veteriner Fakültesi
Zuhal ÖNDER	Erciyes Üniversitesi Veteriner Fakültesi

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 3, Year: 2023

CONTENTS

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 3, Year: 2023

CONTENTS

Kafkas Universitesi Veterinar Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 213-219, 2023
DOI: 10.9775/kvfd.2022.28781

RESEARCH ARTICLE

The Ability of Febuxostat on Periodontal Inflammation and Bone Loss in Rats with Periodontitis by Regulating Nuclear Factor-κB

Yanzhong YANG [1] Cuiqiong MO [2] Xiaohua TIAN [1] Ying ZHENG [2] Jie ZOU [2] Chen CHEN [3 (*)]

[1] Department of Integrated Medicine, Hangzhou Stomatology Hospital, 310000, Hangzhou, Zhejiang Province, P.R. CHINA
[2] The 903rd Hospital of the Joint Logistics Support Force of PLA, 310000, Hangzhou, Zhejiang Province, P.R. CHINA
[3] Zhuji Affiliated Hospital of Wenzhou Medical University, 311800, Zhuji, Zhejiang Province, P.R. CHINA

ORCIDs: Y.Y. 0000-0002-1426-0415; C.M. 0000-0003-0418-7960; X.T. 0000-0003-2282-4329; Y.Z. 0000-0002-7051-9188; J.Z. 0000-0001-6738-2064; C.C. 0000-0002-7914-3471

Article ID: KVFD-2022-28781 Received: 14.11.2022 Accepted: 27.05.2023 Published Online: 27.05.2023

Abstract: We aimed to evaluate the effects of febuxostat on periodontal inflammation and bone loss in rats with periodontitis by regulating nuclear factor-κB (NF-κB). Sixty rats were randomly divided into sham (Group S), periodontitis model (Group P), febuxostat (Group F), minocycline hydrochloride (Group M), NF-κB p65 inhibitor PDTC (Group C), and febuxostat + NF-κB p65 inhibitor PDTC (Group F+C) groups (n=10). The levels of serum tumor necrosis factor-α (TNF-α), interleukin-17 (IL-17), and IL-10 were detected by enzyme-linked immunosorbent assay. The pathological changes in periodontal tissues were detected by hematoxylin-eosin staining, and bone loss was detected using micro-CT. Western blotting was performed to measure the protein expression of NF-κB p65 in periodontal tissues. Group P had significantly higher levels of TNF-α and IL-17 and lower IL-10 levels than those of Group S (P<0.05). Group F had significantly lower levels of serum TNF-α and IL-17 and higher IL-10 levels than those of Group P (P<0.05). The protein expression of NF-κB p65 in the periodontal tissues of Group P was significantly higher than that of Group S, which was lower in Group F and M than that in Group P (P<0.05). Compared with Group P, Group C had significantly lower levels of TNF-α and IL-17, CEJ-AC and protein expression of NF-κB p65, but a higher level of IL-10 (P<0.05), and the trends were the same between Group F+C and Group F (P<0.05). Febuxostat can inhibit periodontal inflammation and reduce alveolar bone loss in rats with periodontitis, probably by inhibiting the activation of NF-κB.

Keywords: *Febuxostat, Alveolar bone loss, Periodontitis, NF-κB*

Febuksostat'ın Periodontitisli Sıçanlarda Nükleer Faktör-κB'yi Düzenleyerek Periodontal Enflamasyon ve Kemik Kaybı Üzerine Etkisi

Öz: Bu çalışmada, febuksostatın, periodontitisli sıçanlarda nükleer faktör-κB'yi (NF-κB) düzenleyerek periodontal enflamasyon ve kemik kaybı üzerine etkilerini değerlendirmeyi amaçladık. Altmış sıçan rastgele, sham (Grup S), periodontitis modeli (Grup P), febuksostat (Grup F), minosiklin hidroklorür (Grup M), NF-κB p65 inhibitörü PDTC (Grup C) ve febuksostat + NF-κB p65 inhibitörü PDTC (Grup F+C) gruplarına ayrıldı (n=10). Serum tümör nekroz faktörü-α (TNF-α), interlökin-17 (IL-17) ve IL-10 seviyeleri enzime bağlı immünosorbent yöntemi ile tespit edildi. Periodontal dokulardaki patolojik değişiklikler hematoksilen-eozin boyama ile ve kemik kaybı mikro-BT kullanılarak tespit edildi. Periodontal dokularda NF-κB p65 protein ekspresyonunu ölçmek için Western blotlama yapıldı. Grup P'nin TNF-α ve IL-17 düzeyleri, Grup S'ye göre anlamlı derecede yüksek ve IL-10 düzeyleri daha düşüktü (P<0.05). Grup F'nin serum TNF-α ve IL-17 düzeyleri, Grup P'ye göre anlamlı derecede düşük ve IL-10 düzeyleri daha yüksekti (P<0.05). Grup P'nin periodontal dokularında NF-κB p65 protein ekspresyonu, Grup S'den anlamlı derecede yüksek, Grup F ve Grup M'de ise Grup P'den daha düşüktü (P<0.05). Grup P ile karşılaştırıldığında, Grup C'de TNF-α ve IL-17 seviyeleri, CEJ-AC ve NF-κB p65 protein ekspresyonu anlamlı derecede düşükken, IL-10 seviyesi daha yüksekti (P<0.05) ve Grup F+C ile Grup F arasındaki eğilimler aynıydı (P<0.05).

Febuksostat, NF-κB aktivasyonunu inhibe ederek periodontitisli sıçanlarda periodontal enflamasyonu inhibe edebilir ve alveolar kemik kaybını azaltabilir.

Anahtar sözcükler: *Febuksostat, Alveolar kemik kaybı, Periodontitis, NF-κB*

How to cite this article?

Yang Y, Mo C, Tian X, Zheng Y, Zou J, Chen C: The ability of febuxostat on periodontal inflammation and bone loss in rats with periodontitis by regulating nuclear factor-κB. *Kafkas Univ Vet Fak Derg,* 29 (3): 213-219, 2023.
DOI: 10.9775/kvfd.2022.28781

(*) Corresponding author: C. Chen

Phone: +86-013867591616
E-mail: chenchenzahwmu@wl-asia.com

Introduction

Periodontitis is a chronic inflammatory disease mainly caused by microbial infection, which leads to the continuous destruction of periodontal tissues, such as gingival swelling, alveolar bone resorption, and tooth loosening, as one of the major causes of tooth loss in adults [1]. Periodontitis is closely linked to cardiovascular disease, diabetes mellitus, rheumatoid arthritis, osteoporosis, and other systemic diseases in addition to local periodontal damage [2]. Currently, periodontitis is primarily treated by removing pathogenic factors such as dental plaque and calculus on the tooth surface with instruments. However, such treatment causes discomfort to patients with dentin hypersensitivity [3]. Therefore, researchers have endeavored to overcome the problem. Local periodontal inflammation leads to metabolic syndromes such as hyperuricemia, which can raise the risk of the vascular inflammatory response [4]. Febuxostat is an oral selective inhibitor of xanthine oxidase (XO) that mediates uricogenesis as a catalytic enzyme for uric acid [5]. Currently, drugs that inhibit uricogenesis mainly function by inhibiting XO. Febuxostat can inhibit the aggregation of XO and substrates by binding the molybdopterin active site in XO [6]. However, the effect of febuxostat on serum inflammatory factors in rats with periodontitis has not been reported hitherto. Nuclear transcription factor-κB (NF-κB), as a pleiotropic eukaryotic transcription factor expressed in a variety of cells, is able to regulate cell growth, differentiation, apoptosis, and inflammation [7]. It has high expression in the case of local inflammation in various cells [8]. When gingival epithelial cells undergo pathogenic periodontal infection, NF-κB is activated, thereby inducing inflammation [9]. Furthermore, NF-κB can damage periodontal tissues by releasing multiple enzymes, aggravating the inflammatory response. Upon periodontitis, NF-κB is involved in the generation of inflammatory factors [10], but whether febuxostat can ameliorate periodontitis through NF-κB remains unclear. Therefore, the aim of this study was to explore the mechanism by which febuxostat affected periodontal inflammation and bone loss in rats with periodontitis by regulating NF-κB.

Material and Methods

Ethical Approval

This study has been approved by the animal ethics committee of Zhuji Affiliated Hospital of Wenzhou Medical University Medical University (approval No. ZAHWMU2020008).

Laboratory Animals

A total of 60 healthy SPF male SD rats (10 weeks old, 200-220 g) were purchased from Hunan SJA Laboratory Animal Co., Ltd. [certificate No. SYXK (Hunan) 2019-0004; China]. All rats were housed in an SPF animal room at 20-24°C and humidity of 40-70%, with a 12/12 h light/dark cycle.

Reagents and Apparatus

The following reagents and apparatus were used: febuxostat (2-[3-Cyano-4-(2-methylpropoxy)phenyl]-4-methyl-5-thiazolecarboxylic acid) *(Fig. 1)* (Jiangsu Wanbang Biochemical Pharmaceutical Co., Ltd., China), NF-κB p65 antibody (Cat. No. AN365, Wuhan Amyjet Scientific Inc., China), NF-κB p65 inhibitor pyrrolidine dithiocarbamate (PDTC) (Cat. No. S1809, Beijing Biolab Technology Co., Ltd., China), ST16 low-temperature high-speed centrifuge (Xi'an Kangrui Trading Co., Ltd., China), DM-1 dissecting microscope (Zeiss, Germany), Medesy periodontal probe (Shanghai Kangqiao Dental Instruments Factory, China), and enzyme-linked immunosorbent assay (ELISA) kit (Cat. No. RE52001, Wuhan Huamei Biological Engineering Co., Ltd., China).

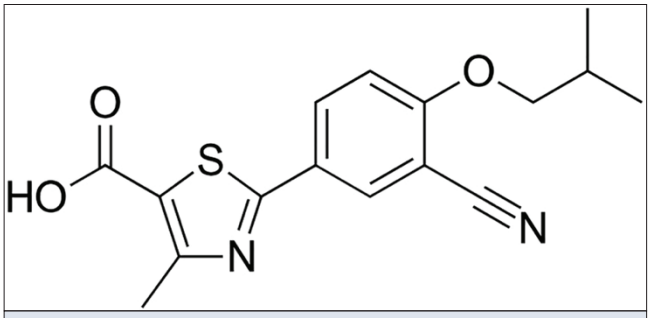

Fig 1. Chemical formula of febuxostat

Preparation of Periodontitis Model and Grouping

All rats were adaptively fed for one week, and given 2% co-trimoxazole suspension in the first week and normal drinking water in the second week. The rats were randomly divided into a sham group (Group S, n=10), a periodontitis model group (Group P, n=10), a febuxostat group (Group F, n=10), a minocycline hydrochloride group (Group M, n=10), an NF-κB p65 inhibitor PDTC group (Group C, n=10), and a febuxostat + NF-κB p65 inhibitor PDTC group (Group F+C, n=10) according to the principle of equal body weight.

After anesthesia by intraperitoneal injection using 10% chloral hydrate, the palatal gingival crevices of bilateral maxillary second molars were injected with 20 μL of sterile PBS only and applied with 100 μL of PBS for modeling in Group S. In the remaining five groups, 20 μL of *Porphyromonas gingivalis* W83 was injected into the palatal gingival crevices of bilateral maxillary second molars, the dental neck was ligated with silk ligature, and 100 μL of bacterial solution (1.5×10^9 CFU/mL) was applied at the site of ligation. The above treatment was repeated

every two days for a total of four times. Eight weeks after modeling, periodontal inflammation, attachment loss, tooth loosening and alveolar bone resorption shown in CT suggested successful modeling. The modeling failed in five rats, so five new ones were replenished. Local medication was given for each group after the ligature was removed. Febuxostat (8 mg/kg) was injected in the gingiva of Group F, 20 μL of 2% minocycline hydrochloride was injected in the gingiva of Group M, 20 μL of PDTC was injected in the gingiva of Group C, febuxostat (8 mg/kg) and 20 μL of PDTC were injected in the gingiva of Group F+C, and an equal amount of normal saline was injected in the gingiva of Group S and P. The rats were administered once daily for four consecutive weeks.

Detection of Levels of Serum Tumor Necrosis Factor-α (TNF-α), Interleukin-17 (IL-17), and IL-10 by ELISA

After drug intervention, the rats were anesthetized, and 4 mL of venous blood was drawn from the tail vein, left still, and centrifuged at 3.500 r/min for 15 min. The supernatant was harvested to detect the serum levels of TNF-α, IL-17, and IL-10.

Pathological Observation of Periodontal Tissues

After the last administration and 12-hour fasting, three rats from Group S, P, F, and M were randomly selected and sacrificed by cervical dislocation. The molar segment of the left mandible was fixed with 4% paraformaldehyde solution for 48 h. Then the tissue was decalcified with EDTA (100 g/L) for three weeks, embedded in paraffin, and sliced into 5 μm-thick sections along the median coronal section of the mandible. After hematoxylin-eosin (HE) staining, the sections were observed and photographed under a light microscope.

Detection of Bone Loss Using Micro-CT

At 12 h after the last administration, three rats randomly selected from each group were sacrificed by cervical dislocation. The maxilla, upper molar, dental tissue, and periodontal tissue were fixed with 4% paraformaldehyde for 24 h, followed by trimming. Jawbone and periodontal tissue in the molar section were preserved, and other tissues were removed. Then three-dimensional CT imaging was performed for Group S, P, F, and M, and the distances from the cemento-enamel junction to the alveolar crest (CEJ-AC) of all groups were measured. The alveolar bone resorption at the left mandibular molar was observed.

Detection of NF-κB p65 Protein Expression in Periodontal Tissues by Western Blotting

The periodontal tissues at the left mandibular molar were harvested and thoroughly ground. Then 100 mg of samples were placed into a pre-cooled 1.5 mL centrifuge tube, lysed with RIPA lysis buffer, and centrifuged in a centrifuge tube at 12.000 r/min for 10 min. The supernatant was harvested to measure the protein level, and 5% spacer gel and 8% separation gel were prepared. The protein samples were loaded, electrophoresed for 100 min, and transferred onto a PVDF membrane by wet transfer for 2 h. Then the membrane was blocked with 5% blocking buffer at room temperature for 1 h, and incubated with NF-κB p65 (1:800) and internal reference β-actin primary antibody solution at 4°C overnight. After the primary antibody was washed away, HRP-labeled secondary antibodies were added for 2-h incubation, and the membrane was washed again. Finally, the membrane was soaked in ECL solution for 2 min and imaged by a chemiluminescence system.

Statistical Analysis

GraphPad Prism 8.0 software (IBM, New York, USA) was used for statistical analysis. The measurement data were expressed as mean ± standard deviation (x ± s). The data were analyzed by one-way analysis of variance among groups and compared by the *t* test between two groups. P<0.05 was considered statistically significant.

Results

Group P had significantly higher levels of serum TNF-α and IL-17 and lower level of IL-10 than those of Group S (P<0.05). Group F had significantly lower levels of serum TNF-α and IL-17 and higher level of IL-10 than those of Group P (P<0.05). There was no significant difference in the serum levels of TNF-α, IL-17, and IL-10 between Group F and M (P>0.05) *(Table 1)*.

Table 1. *Serum levels of TNF-α, IL-17 and IL-10 (`x ± s, pg/mL)*

Group	n	TNF-α	IL-17	IL-10
S	10	100.16±5.42	312.45±15.32	286.33±10.25
P	10	312.73±14.68*	618.23±21.46*	113.28±5.31*
F	10	186.54±8.31*#	409.31±18.15*#	197.32±7.54*#
M	10	185.36±8.16*#	402.18±12.96*#	195.06±7.82*#
F		806.4	562.7	795.4
P		<0.0001	<0.0001	<0.0001

** P<0.05 vs. Group S, #P<0.05 vs. Group P*

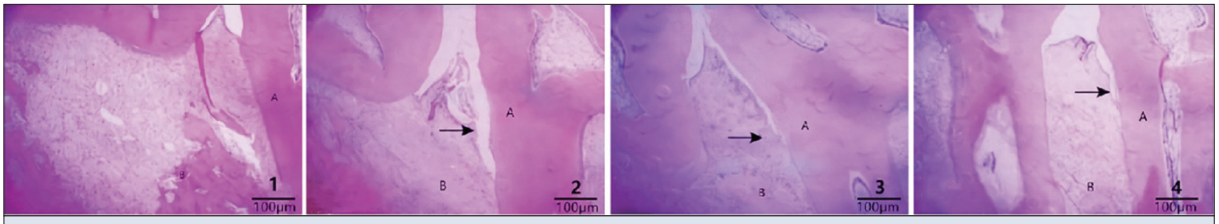

Fig 2. Pathological morphology of periodontal tissues (HE staining). 1: Group S, 2: Group P, 3: Group F, 4: Group M. A: Maxillary first molar; B: alveolar bone. Arrows indicate periodontal pockets

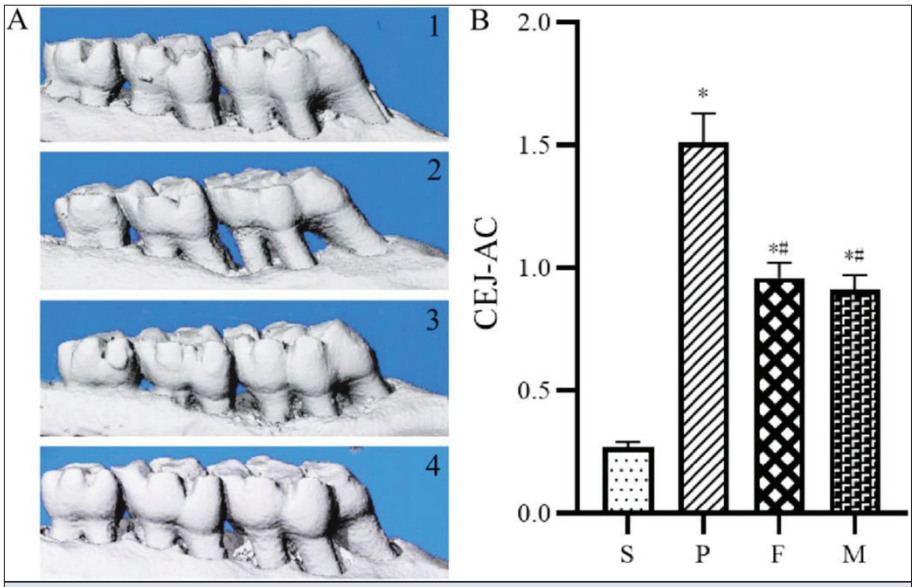

Fig 3. Bone loss. CEJ-AC significantly rose in Group P compared with that in Group S, declined in Group F and M compared with that in Group P, and had no significant difference between Group F and M. A: Micro-CT image reconstruction in each group, B: Comparison of CEJ-AC among groups. 1: Group S, 2: Group P, 3: Group F, 4: Group M. *P<0.05 *vs.* Group S, #P<0.05 *vs.* Group P. Unit for CEJ-AC: mm

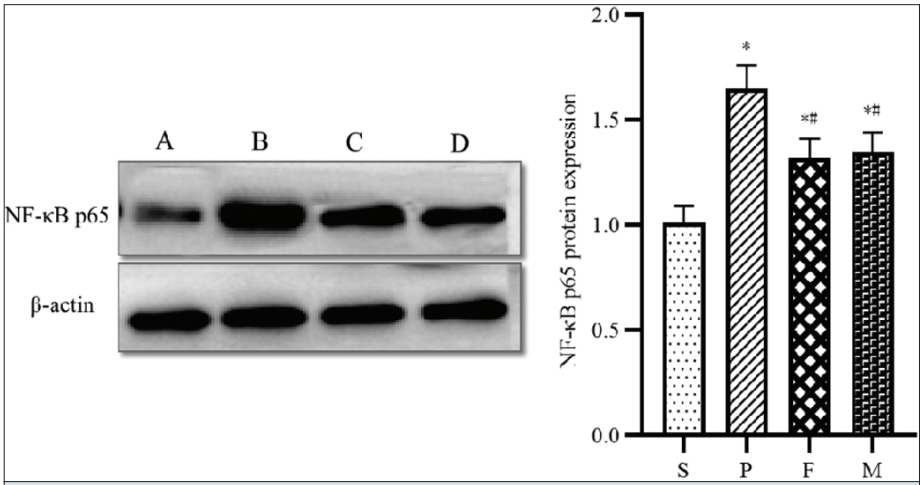

Fig 4. Protein expression of NF-κB p65 in periodontal tissues. The protein expression of NF-κB p65 in periodontal tissues was significantly higher in Group P than that in Group S, significantly lower in Group F and M than that in Group P, and had no significant difference between Group F and M. A: Group S, B: Group P, C: Group F, D: Group M. *P<0.05 *vs.* Group S, #P<0.05 *vs.* Group P

The HE staining results of Group S showed that the gingival epithelium was intact, without loss of attachment or abnormal changes in connective tissue. In Group P, the junctional epithelium underwent down-growth. The collagen fibers were degenerated, some of which were degraded. Meanwhile, there was significant alveolar bone

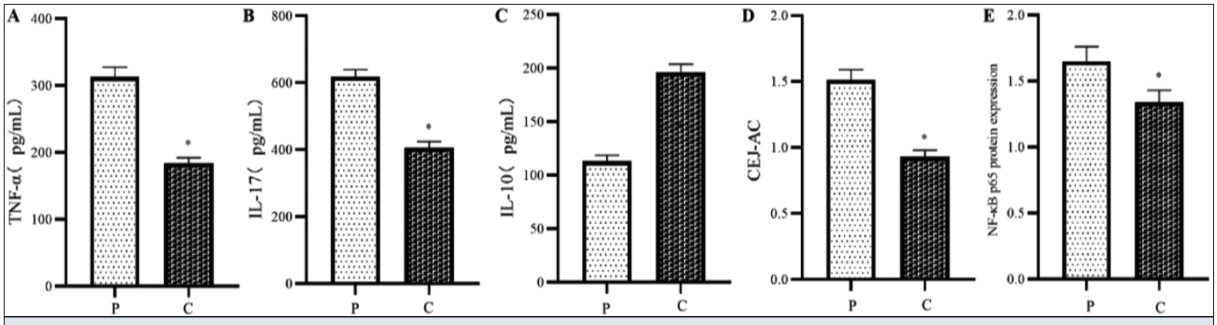

Fig 5. Effects of NF-κB p65 inhibitor PDTC on serum inflammatory factor levels, CEJ-AC and NF-κB p65 protein expression. Compared with Group P, Group C had significantly decreased levels of serum TNF-α and IL-17, CEJ-AC and protein expression of NF-κB p65, but increased level of IL-10. A: TNF-α level, B: IL-17 level, C: IL-10 level, D: CEJ-AC, E: NF-κB p65 protein expression. *P<0.05 *vs.* Group P

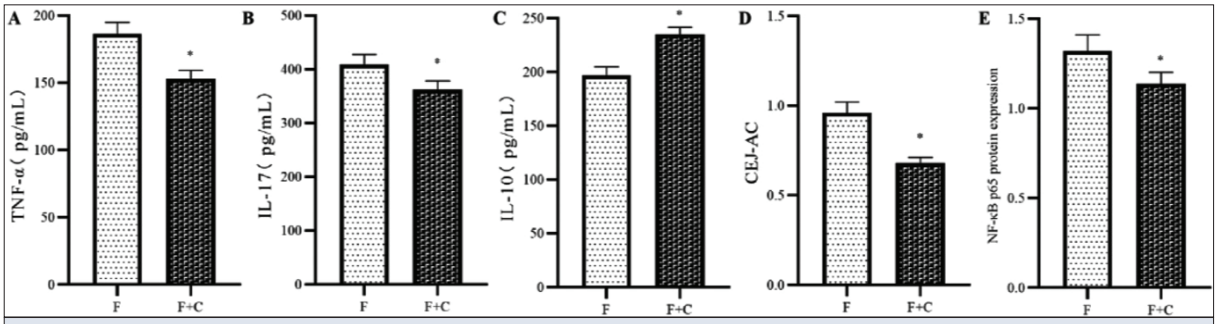

Fig 6. Effects of febuxostat combined with PDTC on serum inflammatory factor levels, CEJ-AC and NF-κB p65 protein expression. Compared with Group F, Group F+C had significantly decreased levels of serum TNF-α and IL-17, CEJ-AC and protein expression of NF-κB p65, but elevated level of IL-10. A: TNF-α level, B: IL-17 level, C: IL-10 level, D: CEJ-AC, E: NF-κB p65 protein expression. *P<0.05 *vs.* Group F

resorption. The periodontal tissues of Group F and M were markedly improved, and the periodontal pockets became shallow *(Fig. 2)*.

CEJ-AC was significantly greater in Group P than in Group S (P<0.05). Group F and M had lower CEJ-AC values than that of Group P (P<0.05), but there was no significant difference between the values of Group F and M (P>0.05) *(Fig. 3)*.

The protein expression of NF-κB p65 in periodontal tissues was significantly higher in Group P than that in Group S (P<0.05), significantly lower in Group F and M than that in Group P (P<0.05), and had no significant difference between Group F and M (P>0.05) *(Fig. 4)*.

Compared with Group P, Group C had significantly decreased levels of serum TNF-α and IL-17, CEJ-AC, and protein expression of NF-κB p65 but increased level of IL-10 (P<0.05) *(Fig. 5)*.

Compared with Group F, Group F+C had significantly decreased levels of serum TNF-α and IL-17, CEJ-AC, and protein expression of NF-κB p65 but elevated level of IL-10 (P<0.05) *(Fig. 6)*.

Discussion

Periodontitis is a destructive inflammatory disease, and its primary pathogenesis is the destruction of pathogenic bacteria to periodontal tissues and the local host response. Pathogenic bacteria, such as Gram-negative anaerobic bacteria, can induce infectious diseases in dental supporting tissues as a main cause for periodontitis [11]. Different methods have been proposed to establish periodontitis models. Currently, the rat model of periodontitis is usually established through molar ligation, inoculation of *P. gingivalis*, or the combination of them. A periodontitis model has been successfully induced in laboratory rabbits through silk-suture ligation, high-glucose water intake, and putative periodontal pathogens [12]. Until now, many kinds of animals, such as pigs, dogs, rats, and mice, have been used to successfully establish periodontitis models with the manifestations of induced periodontitis. Among them, the rat periodontitis model is most commonly used. Since rat and human molars have similar physiological structures and pathological mechanisms, their kinetics of inflammatory cytokines in the case of periodontitis also quite resemble each other [13,14]. In this study, the periodontitis model was established by gingival inoculation of *P. gingivalis* solution and silk suture ligation of the molar tooth cervix. The periodontal tissue destruction and disorderly distributed ligament fiber bundles, and considerable inflammatory cell infiltration suggested that *P. gingivalis* solution combined with silk suture ligation can cause inflammatory injury in periodontal tissue, verifying the successful establishment of the periodontitis model.

Both inflammatory response and host immunomodulatory response are of significance to remodeling after periodontal bone loss, and cytokines released by specific cell populations play crucial roles in the host response and alveolar bone resorption. TNF-α mediates various pathological processes, such as inflammatory response, alveolar bone resorption, and loss of connective tissue attachment [15]. Upon stimulus by IL-23, T cell subset Th17 can produce a variety of inflammatory chemokines and cytokines, including IL-17, which dominate the host immune response. The protein expression of IL-17 rises during the onset and progression of periodontitis [16]. The disorders of IL-17 enhance the generation of osteoclasts and promote local inflammatory response, accompanied by the increase in inflammatory mediators such as TNF-α. On the contrary, IL-10 produced by Treg cells is widely expressed in inflammatory periodontal tissues, which can alleviate bone loss by inhibiting osteoclast formation [17]. In this study, febuxostat reduced the serum levels of TNF-α and IL-17 and significantly raised the level of IL-10 in rats with periodontitis. Guo et al.[18] proved that febuxostat significantly reduced the levels of inflammatory mediators and ameliorated the endothelial function in patients with chronic periodontitis complicated by uricemia.

NF-κB regulates the inflammatory response by modulating inflammatory factors, as well as immune function- and inflammatory stimulus-related cytokines [19]. By regulating IL-6 and TNF-α, NF-κB controls the proliferation and differentiation of immune cells and mediates immune response. Suppressing its activation can inhibit the production of various inflammatory mediators [20]. Moreover, the enhanced activity of NF-κB corresponds to the enhanced synthesis of IL-6, IL-8, and TNF-α [21]. These cytokines are implicated in the regulation of immune response and osteoclasts, thereby inducing matrix degradation, bone resorption and loss of connective tissue attachment. Hiyari et al.[22] proved that the level of NF-κB in periodontal tissues significantly rose in mice with periodontitis. Minocycline hydrochloride is a semi-synthetic derivative of tetracycline which is effective in treating periodontitis [23]. In this study, febuxostat (Group F) worked similarly to minocycline hydrochloride (Group M) regarding all detected data, suggesting that febuxostat may also be effective for periodontitis treatment. Additionally, febuxostat had significantly decreased protein expression of NF-κB p65 in periodontal tissues and CEJ-AC, suggesting that it inhibited the activation of NF-κB, reduced CEJ-AC, and inhibited bone resorption by suppressing the expressions of inflammatory mediators. To further validate the postulation, the rats with periodontitis were subjected to PDTC intervention. PDTC significantly decreased the levels of serum inflammatory factors in rats with periodontitis and also CEJ-AC and NF-κB p65 protein expression. After the combined use of febuxostat and PDTC, the levels of serum inflammatory factors, CEJ-AC and NF-κB p65 protein expression all declined compared with those after the use of febuxostat alone. Collectively, febuxostat can, through regulating NF-κB, inhibit periodontal inflammation and alveolar bone loss in rats with periodontitis.

In conclusion, febuxostat can inhibit periodontal inflammation and reduce alveolar bone loss in rats with periodontitis, probably by suppressing the activation of NF-κB. Due to limited time and budget, HE staining and micro-CT image reconstruction were not performed for Group C and F+C in this study, so the results have limitations. Further experimental studies are ongoing in our group to provide a valuable experimental basis for the clinical treatment of periodontitis.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Financial Support

This study was not financially supported.

Ethical Approval

This study has been approved by the ethic committee of Zhuji Affiliated Hospital of Wenzhou Medical University, and great efforts have been made to minimize the animals' suffering.

Competing Interest

There is no conflict of interest.

Authors' Contributions

Y.Y., C.M. and X.T. designed this study and significantly revised the manuscript; Y.Z., J.Z. and C.C. performed this study and writing manuscript. The first two authors Y.Y. and C. M. contributed equally to this study. All authors have approved the submission and publication of this manuscript.

References

1. Pacheco CMF, Maltos KLM, Shehabeldin MS, Thomas LL, Zhuang Z, Yoshizawa S, Verdelis K, Gaffen SL, Garlet GP, Little SR, Sfeir C: Local sustained delivery of anti-IL-17A antibodies limits inflammatory bone loss in murine experimental periodontitis. *J Immunol,* 206, 2386-2392, 2021. DOI: 10.4049/jimmunol.2001432

2. Petain S, Kasnak G, Firatli E, Tervahartiala T, Gürsoy UK, Sorsa T: Periodontitis and peri-implantitis tissue levels of *Treponema denticola*-CTLP and its MMP-8 activating ability. *Acta Histochem,* 123:151767, 2021. DOI: 10.1016/j.acthis.2021.151767

3. Derman SH, Lantwin EM, Barbe AG, Noack MJ: Does a pretreatment with a dentine hypersensitivity mouth-rinse compensate the pain caused by professional mechanical plaque removal? A single-blind randomized controlled clinical trial. *Clin Oral Invest,* 25, 3151-3160, 2021. DOI: 10.1007/s00784-020-03643-4

4. Moro MG, Oliveira MDS, Santana MM, de Jesus FN, Feitosa K, Teixeira SA, Franco GCN, Spolidorio LC, Muscará MN, Holzhausen M: Leukotriene receptor antagonist reduces inflammation and alveolar bone loss in a rat model of experimental periodontitis. *J Periodontol,* 92, e84-e93, 2021. DOI: 10.1002/JPER.20-0718

5. Kim SC, Neogi T, Kim E, Lii J, Desai RJ: Trends in utilization of urate-lowering therapies following the US food and drug administration's boxed

warning on febuxostat. *Arthritis Rheumatol,* 73, 542-543, 2021. DOI: 10.1002/art.41550

6. Nessa N, Kobara M, Toba H, Adachi T, Yamamoto T, Kanamura N, Pezzotti G, Nakata T: Febuxostat attenuates the progression of periodontitis in rats. *Pharmacology,* 106, 294-304, 2021. DOI: 10.1159/000513034

7. Shi C, Shi R, Guo H, Shi Y, Liu X: β-amyloid-induced gonadotropin-releasing hormone decline involving Forkhead transcription factor FOXO3a and nuclear factor-κB. *Neuroreport,* 31, 923-927, 2020. DOI: 10.1097/WNR.0000000000001488

8. An L, Li Z, Shi L, Wang L, Wang Y, Jin L, Shuai X, Li J: Inflammation-targeted celastrol nanodrug attenuates collagen-induced arthritis through NF-κB and Notch1 pathways. *Nano Lett,* 20, 7728-7736, 2020. DOI: 10.1021/acs.nanolett.0c03279

9. Wang J, Wang B, Lv X, Wang L: NIK inhibitor impairs chronic periodontitis via suppressing non-canonical NF-κB and osteoclastogenesis. *Pathog Dis,* 78:ftaa045, 2020. DOI: 10.1093/femspd/ftaa045

10. Francis M, Gopinathan G, Salapatas A, Nares S, Gonzalez M, Diekwisch TGH, Luan X: SETD1 and NF-κB regulate periodontal inflammation through H3K4 trimethylation. *J Dent Res,* 99, 1486-1493, 2020. DOI: 10.1177/0022034520939029

11. Kalali N, Kadkhoda Z, Amid R, Ghourchian S, Douraghi M: Identification of oral anaerobic bacteria and the beta-lactamase resistance genes from Iranian patients with periodontitis. *Anaerobe,* 75:102515, 2022. DOI: 10.1016/j.anaerobe.2022.102515

12. Chen S, Lin G, You X, Lei L, Li Y, Lin M, Luo K, Yan F: Hyperlipidemia causes changes in inflammatory responses to periodontal pathogen challenge: implications in acute and chronic infections. *Arch Oral Biol,* 59, 1075-1084, 2014. DOI: 10.1016/j.archoralbio.2014.06.004

13. Gao J, Cai S, Wang Z, Li D, Ou M, Zhang X, Tian Z: The optimization of ligature/bone defect-induced periodontitis model in rats. *Odontology,* 110, 697-709, 2022. DOI: 10.1007/s10266-022-00715-7

14. Song JH, Yang JH, Kim MJ, Kim KY, Kim JK, Jin YB, Kim WH, Kim S, Kim KR, Park KI, Lee HJ: Anti-periodontitis effects of *Dendropanax morbiferus* H. Lév leaf extract on ligature-induced periodontitis in rats. *Molecules,* 28, 849, 2023. DOI: 10.3390/molecules28020849

15. Aleksandrowicz P, Brzezińska-Błaszczyk E, Kozłowska E, Żelechowska P, Borgonovo AE, Agier J: Analysis of IL-1β, CXCL8, and TNF-α levels in the crevicular fluid of patients with periodontitis or healthy implants. *BMC Oral Health,* 21:120, 2021. DOI: 10.1186/s12903-021-01478-3

16. Medara N, Lenzo JC, Walsh KA, Darby IB, O'Brien-Simpson NM, Reynolds EC: T helper 17 cell-related cytokines in serum and saliva during management of periodontitis. *Cytokine,* 134:155186, 2020. DOI: 10.1016/j.cyto.2020.155186

17. Cheng WC, Hughes FJ, Taams LS: The presence, function and regulation of IL-17 and Th17 cells in periodontitis. *J Clin Periodontol,* 41, 541-549, 2014. DOI: 10.1111/jcpe.12238

18. Guo ZS, Liu X, Cai YM: The efficacy of adjuvant febuxostat in the treatment of chronic periodontitis with hyperuricemia and its effect on serum inflammatory factors in patients. *China Pharmaceuti,* 27:4, 2018.

19. Bustami A, Lestari WP, Hayuningrum CF, Wuyung PE, Wibowo H, Natadisastra RM: The anti-inflammatory effect of octyl gallate through inhibition of nuclear factor-κB (NF-κB) pathway in rat endometriosis model. *J Reprod Infertil,* 21, 169-175, 2020.

20. He Y, Ruganzu JB, Zheng Q, Wu X, Jin H, Peng X, Ding B, Lin C, Ji S, Ma Y, Yang W: Silencing of LRP1 exacerbates inflammatory response via TLR4/NF-κB/MAPKs signaling pathways in APP/PS1 transgenic mice. *Mol Neurobiol,* 57, 3727-3743, 2020. DOI: 10.1007/s12035-020-01982-7

21. Phusuntornsakul P, Jitpukdeebodintra S, Pavasant P, Leethanakul C: Vibration activates the actin/NF-κB axis and upregulates IL-6 and IL-8 expression in human periodontal ligament cells. *Cell Biol Int,* 44, 661-670, 2020. DOI: 10.1002/cbin.11267

22. Hiyari S, Wong RL, Yaghsezian A, Naghibi A, Tetradis S, Camargo PM, Pirih FQ: Ligature-induced peri-implantitis and periodontitis in mice. *J Clin Periodontol,* 45, 89-99, 2018. DOI: 10.1111/jcpe.12817

23. Zhang T, Qiu Y, Song J, Zhou P, Liao H, Cheng Y, Wu X: Electrosprayed minocycline hydrochloride-loaded microsphere/SAIB hybrid depot for periodontitis treatment. *Drug Deliv,* 28, 620-633, 2021. DOI: 10.1080/10717544.2021.1902020

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 221-230, 2023
DOI: 10.9775/kvfd.2023.28970

RESEARCH ARTICLE

Whole-Genome Sequencing-Based Characterization of *Listeria monocytogenes* from Food and Animal Clinical Cases

Özkan ASLANTAŞ [1 (*)] Kaan BÜYÜKALTAY [2] Oktay KESKİN [3]
Ayfer GÜLLÜ YÜCETEPE [3] Adem ADIGÜZEL [4]

[1] Hatay Mustafa Kemal University, Faculty of Veterinary Medicine, Department of Microbiology, TR-31060 Hatay - TÜRKİYE

[2] Middle East Technical University, Enformatic Institute, TR-06800 Ankara - TÜRKİYE

[3] Harran University, Faculty of Veterinary Medicine, Department of Microbiology, TR-63290 Şanlıurfa - TÜRKİYE

[4] Food Control Laboratory Directorate, TR-63040 Şanlıurfa - TÜRKİYE

ORCIDs: Ö.A. 0000-0003-0407-8633; K.B. 0000-0001-6074-8940; O.K. 0000-0002-5977-7872; A.G.Y. 0000-0002-9842-3305; A.A. 0000-0002-7763-0882

Article ID: KVFD-2023-28970 Received: 05.01.2023 Accepted: 17.04.2023 Published Online: 19.04.2023

Abstract: Listeriosis is a rare but severe foodborne infection caused by *Listeria monocytogenes*. In this study, we performed comparative whole-genome sequencing (WGS) on 28 *Listeria monocytogenes* from seven invasive listeriosis cases in animals and 21 food samples in Türkiye for the first time. Food isolates were delineated into eleven clonal complexes (CCs), namely CC1, CC2, CC3, CC8, CC9, CC20, CC69, CC124, CC155, CC204, ST3002. The isolates from meningoencephalitis cases were associated with CC1, whereas CC9 and CC7 were associated with the isolates from sheep abortus cases. All the isolates carried the *fosX*, *lin*, *norB*, and *sul* genes. In addition, *emrC* (n=15), *bcrC* (n=4), *emrE* (n=2), *qacA* (n=1), *cadA* (n=5) and *cadC* (n=1) genes, conferring resistance to stress and disinfectants were detected. Listeria pathogenicity island (LIPI)-1 and LIPI-2 were distributed in all isolates, but LIPI-3 was closely related to CC1, CC3, and ST3002 isolates. LIPI-4 was not found in any of the *L. monocytogenes* isolates. The Inc18(rep25) and Inc18(rep26) plasmids were found in 16 (57.1%) isolates. A total of 15 different intact prophage genomes ranging from one to three were detected in the genomes of 24 isolates. The hypervirulent CC1 and CC2 clones that pose a significant threat to food safety and public health were detected among food isolates. These findings highlight the importance of continuous surveillance of hypervirulent *L. monocytogenes* strains in different settings.

Keywords: *Food, Genetic diversity, Invasive infection, Listeria monocytogenes, Whole Genome Sequencing*

Gıda ve Hayvan Klinik *Listeria monocytogenes* İzolatlarının Tam Genom Dizilimine Dayalı Karakterizasyonu

Öz: Listeriosis, *Listeria monocytogenes*'in neden olduğu nadir görülen fakat ciddi klinik seyre sahip gıda kaynaklı bir enfeksiyondur. Bu çalışmada, hayvanlardaki invaziv listeriozis vakalarından (n=7) ve farklı gıda örneklerinden (n=21) izole edilen 28 *Listeria monocytogenes* suşunun karşılaştırmalı tam genom dizileme (WGS) ile analizi yapıldı. Gıda izolatları CC1, CC2, CC3, CC8, CC9, CC20, CC69, CC124, CC155, CC204, ST3002 olmak üzere onbir klonal komplekse (CC) ayrıldı. Meningoensefalit vakalarına ait izolatlar CC1'e ait iken, koyun abortus vakalarına ait izolatlar CC9 ve CC7'e ait bulundu. Tüm izolatlarda *fosX*, *lin*, *norB* ve *sul* genleri belirlendi. Ayrıca, değişen oranlarda stres ve dezenfektan direncine aracılık eden *emrC* (n=15), *bcrC* (n=4), *emrE* (n=2), *qacA* (n=1), *cadA* (n=5) ve *cadC* (n=1) genleri tespit edildi. Listeria patojenite adası (LIPI)-1 ve LIPI-2 tüm izolatlarda tespit edilirken; LIPI-3 CC1, CC3 ve ST3002'e ait izolatlar ile yakın ilişkili bulundu. LIPI-4 *L. monocytogenes* izolatlarının hiçbirinde bulunmadı. Inc18(rep25) ve Inc18(rep26) plazmidleri 16 (%57.1) izolatta bulundu. Yirmidört izolatta bir - üç arasında değişen toplam 15 farklı intakt profaj genomu tespit edildi. Bu çalışmada gıda izolatları arasında gıda güvenliği ve halk sağlığı için önemli bir tehdit oluşturan hipervirülent CC1 ve CC2 klonları tespit edilmiştir. Bu bulgular, farklı ortamlarda hipervirülent *L. monocytogenes* suşlarının sürekli izlenmesinin önemini vurgulamaktadır.

Anahtar sözcükler: *Gıda, Genetik çeşitlilik, İnvaziv infeksiyon, Listeria monocytogenes, Tüm Genom Sekanslama*

How to cite this article?

Aslantaş Ö, Büyükaltay K, Keskin O, Güllü-Yücetepe A, Adıgüzel A: Whole-genome sequencing-based characterization of *Listeria monocytogenes* from food and animal clinical cases. *Kafkas Univ Vet Fak Derg,* 29 (3): 221-230, 2023.
DOI: 10.9775/ kvfd.2023.28970

(*) Corresponding author: Özkan ASLANTAŞ

Phone: +90 326 245 8545/11523 Cellular phone: +90 533 641 2236 Fax: +90 326 245 5704
E-mail: ozkanaslantas@yahoo.com, aslantas@mku.edu.tr

Introduction

Listeria monocytogenes is an opportunistic food-borne pathogen that may cause life-threatening infections in many mammalian species following ingestion [1]. In ruminants, listeriosis is associated with gastroenteritis, abortions, septicemia, central nervous system (CNS) infections (neurolisteriosis), as well as rarely anatomically localized infections such as mastitis, eye infections and keratitis [2]. Infected ruminants are frequent asymptomatic carriers of *L. monocytogenes* in their gastrointestinal tract that allows the pathogen to multiply and spread the environments [3]. Throughout the world, listeriosis most commonly occurs in a sporadic form affecting a single or a few animals or may occur as outbreaks within a farm [4]. In humans, the infection, is frequently linked to consumption of ready-to-eat (RTE) food, occurs mostly in immunocompromised individuals, the elderly people and pregnant women, which manifests itself symptoms similar to those seen in ruminants [5]. Although incidence of listeriosis is low in comparison with other food-borne infections, high fatality rate associated with this infection makes it a major public health concern [6]. *L. monocytogenes* is a ubiquitous microorganism that colonizes a variety of ecological niches, including soil, water, food processing facilities, mammalian intestinal tracts and faeces, making surveillance and control of environmental lifestyle of the pathogen very challenging [7]. In particular, persistence of *L. monocytogenes* in food processing environment for months even years despite sanitation measures applied often results in cross-contamination of the final product, which increases the risk of outbreaks [8]. Presence of same *L. monocytogenes* genotypes were also reported in farm environments and animals [9,10]. Multiple virulence factors (VFs) are key for the adaptation of *L. monocytogenes* to its host and different environmental niches [2].

Molecular epidemiological studies have revealed the variable distribution of genetic and serological subtypes of *L. monocytogenes* with respect to food products and processing environments as well as among human and animal clinical listeriosis cases. Lineage II or serotypes 1/2b, 1/2a, and 1/2c are more frequent in food isolates, and lineage I or serotype 4b is the predominant serotype among clinical isolates. Moreover, clones CC1, CC2, CC4, and CC6 are strongly associated with clinical strains, CC9 and CC121 are hypovirulent food-associated clones [11-14].

Whole genome sequencing (WGS) greatly improved the analysis of bacterial genome sequences and facilitated the evaluation of *L. monocytogenes* isolates from clinical, environmental, and food sources [13,15]. WGS also allows the detection of virulence genes responsible for the pathogenicity of different isolates. So far, four virulence gene clusters, known as Listeria pathogenicity islands (LIPIs) have been identified. LIPI-1 and LIPI-2 contain the key genes responsible for promoting adhesion or binding, invasion, polymerization, and cell-to-cell spread within the host organism [2]. LIPI-3 encodes listeriolysin S (LLS), shows hemolytic and cytotoxic activity associated with the destruction of gut microbiota and thus dysregulates host-microbiota homeostasis during infection [16]. More recently, LIPI-4 gene cluster considered as hypervirulent, strongly linked with neural and placental infections has been described [14].

The objectives of this study were to (i) assess the genomic diversity of *L. monocytogenes* isolates from food and animal invasive infections, (ii) determine the absence and presence of antimicrobial and disinfectant resistance and virulence genes.

Material and Methods

Bacterial Strains

The strains of *L. monocytogenes* tested were isolated from food (n = 21) and animal clinical cases of listeriosis (n = 7) [five from sheep abortus cases and two from CNS cases (one from sheep and one from cattle)] in four provinces of Türkiye over a period of three years (from January 2017 to July 2020). The characteristics and sources of the *L. monocytogenes* strains used in this study are reported in *Table 1*. Species identification was carried out both using MALDI-TOF MS (Bruker Daltonics, Billerica, MA, United States) and VIDAS system (Biomérieux, France).

DNA Isolation, Library Preparation and Sequencing

Genomic DNA was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. DNA concentration was evaluated using fluorometric (Qubit 3.0, ThermoFisher Scientific, Waltham, MA, USA) method. The sequencing libraries of genomic DNA were prepared according to the Illumina protocol and paired-end (2x150 bp) sequencing was performed on a NovaSeq 6000 platform (Illumina, San Diego, USA). After trimming low-quality reads and removing adapter sequences using Trimmomatic v0.36 [17], the quality of both raw reads and trimmed reads was assessed using FastQC (v 0.11.9). The *de novo* genome assembly was conducted using the Shovill pipeline (v 0.9.0) by applying the default parameters [18]. The quality of assemblies was evaluated using QUAST v4.5 [19]. The detailed *L. monocytogenes* sequence parameters used in the present study are listed in *Table 1*. The draft assemblies of all *L. monocytogenes* isolates were deposited in the BIGSdb-Lm database *(https://bigsdb.pasteur.fr/listeria)* under the accession number 86311-86338.

WGS Characteristics of *L. monocytogenes*

All *L. monocytogenes* sequences were analyzed using the publicly available web-based WGS tools on the BIGSdb-

Table 1. *L. monocytogenes sequence parameters*

No	BIGSdb id	Contigs	Total Length	Max. Contig Length	Min. Contig Length	Mean Contig Length	StdDev Contig Length	L50 Contig Number	N50 Contig Length (L50)	L90 Contig Number	N90 Contig Lenght	L95 Contig Number	N95 Contig Lenght	GC%
1	86311	17	3.022.337	745.156	238	177.785	232.446	3	513.002	7	224.989	8	97.911	37.79
2	86312	12	2.939.770	1.507.484	238	244.981	442.900	1	1.507.484	4	229.475	5	101.052	37.86
3	86313	16	3.022.564	745.156	238	188.911	236.106	3	518.154	7	97.911	8	97.911	37.79
4	86314	16	2.950.838	848.413	238	184.428	270.484	3	530.836	5	230.756	7	100.203	38.08
5	86315	16	2.950.938	848.173	238	184.434	270.430	3	530.836	5	230.756	7	100.203	38.08
6	86316	15	2.950.898	848.413	238	196.727	268.487	3	530.836	5	230.552	7	100.203	38.08
7	86317	16	2.950.975	848.173	238	184.436	261.841	3	530.836	5	230.756	7	100.203	38.08
8	86318	15	2.964.264	909.192	238	197.618	278.461	2	604.948	4	229.071	6	100.992	37.82
9	86319	15	3.010.367	816.421	221	200.692	289.618	2	774.368	5	228.558	6	101.088	37.83
10	86320	15	2.964.210	909.192	238	197.614	279.456	2	604.948	5	229.071	6	100.992	37.82
11	86321	16	2.964.383	909.192	238	185.274	274.770	2	604.948	5	228.923	6	100.992	37.82
12	86322	23	3.012.987	575.528	217	131.000	175.906	4	397.443	8	167.829	9	100.992	37.73
13	86323	18	2.996.628	1.486.655	238	166.480	335.230	2	430.186	6	99.054	8	75.397	37.86
14	86324	19	2.986.983	1.486.782	238	157.210	336.919	2	430.186	6	99.054	8	75.397	37.85
15	86325	16	2.964.377	909.192	238	185.274	274.769	2	604.942	5	228.923	6	100.992	37.82
16	86326	17	2.957.225	1.243.617	296	173.955	307.671	2	521.587	6	225.190	6	225.190	37.85
17	86327	28	2.877.061	632.635	201	102.753	178.162	3	476.856	7	225.046	8	99.748	37.93
18	86328	20	3.011.077	588.120	238	150.554	190.746	3	439.574	8	99.051	10	71.994	37.83
19	86329	16	2.964.363	909.186	238	185.273	274.773	2	604.942	5	229.071	6	100.992	37.82
20	86330	20	2.956.218	759.675	296	147.811	215.408	3	483.632	7	227.892	8	99.751	37.87
21	86331	16	2.964.368	909.192	238	185.273	274.774	2	604.942	5	229.071	6	100.992	37.82
22	86332	14	2.964.682	909.192	238	211.763	284.144	2	604.942	5	229.071	6	100.992	37.82
23	86333	16	2.964.376	909.192	238	185.274	274.772	2	604.942	5	228.923	6	100.992	37.82
24	86334	17	2.964.487	909.192	238	174.382	270.099	2	604.492	5	228.923	6	100.992	37.82
25	86335	14	2.882.660	1.502.479	238	205.479	386.440	1	1.502.479	5	167.055	6	99.228	37.89
26	86336	24	2.922.545	505.602	259	121.773	160.986	4	331.745	8	207.136	10	96.460	37.88
27	86337	20	3.026.339	745.155	238	151.317	212.219	3	478.320	7	225.128	8	151.808	37.91
28	86338	28	2.877.058	632.635	201	102.753	178.163	3	476.856	7	225.046	8	99.748	37.93

Lm platform *(https://bigsdb.pasteur.fr/listeria*, accessed on 12 July 2022). MLST profiles with the same alleles for seven loci were classified into sequence types (ST) and grouped into clonal complexes (CCs) if at least five out of seven loci were the same as previously described. cgMLST (1748 loci) profiles were grouped into cgMLST types (CTs) and sublineages (SLs), using the cut-offs of seven and 150 allelic mismatches, respectively, as previously described. Allele numbers, CTs, and SLs were determined according to the *Listeria* sequence typing database (BIGSdb-*Lm* platform) [13].

Identification of Virulence and Other Genetic Markers

Assemblies were also screened *in silico* for virulence, antimicrobial, metal, and biocide resistance genes, Listeria Stress Islands as well as the *sigB* and rhamnose operons using the BIGSdb-Lm platform [13]. The presence of premature stop codons (PMSCs) in the *inlA* gene was also investigated. When the BIGSdb-*Lm* database reported that a PMSC mutation was present, the mutation position and the length of the resulting truncated *inlA* protein were specified [13].

Detection of Prophage and Plasmid Sequences

The putative prophage determinants within the genomes of the *L. monocytogenes* were tested, the WGS sequences were analyzed with the PHASTER (PHAge Search Tool Enhanced Release) web server [20]. The criteria for scoring prophage regions (as intact, questionable or incomplete) have been described in PHASTER. If the region's total score was less than 70, it was marked as incomplete, between 70-90 as questionable, and when greater than 90 defined as intact. The presence of plasmid sequences was identified using the PlasmidFinder v2.1 for the specified Gram-positive scheme [21].

Results

WGS-based Typing of *L. monocytogenes*

Overall, all strains were grouped into five PCR-serogroups IIc (comprising serotypes 1/2c, 3c), IIa (1/2a, 3a), IIb (1/2b, 3b), IVb (4b, 4d, 4e) and L. The most frequent PCR-serogroup was IIc (n=9, 32.1%), followed by IIa (n=7, 25%), IVb (n=5, 17.9%), L (n=4, 14.3%), and IIb (n=3, 10.7%) *(Table 2)*. PCR-serogroup IVb was determined in two CNS related isolates and three food isolates. Of the five isolates related with aborted sheep fetuses, four were PCR-serogroup IIc, and one was IIa.

A total of three lineages was identified, Lineage II (17, 60.7%) was the most numerous, followed by Lineage I (7, 25%), and Lineage III (4, 14.3%), respectively. While CNS infection related isolates belonged to Lineage I, all isolates associated with sheep abortus cases belonged to Lineage II. Eleven different STs were identified, ST122 (9, 32.1%) was the most abundant, followed by ST202 (4, 14.3%), ST1 (4, 14.3%), ST20 (2, 7.2%), and ST3002 (2, 7.2%), respectively. Seven STs (25% of all STs) were represented by single isolates. ST3002 was submitted as new ST to the Listeria PasteurMLST database. Ten CCs and one singleton (ST3002) were identified. CC112 (9, 36%) was the most prevalent, followed by CC1 (4, 14.3%), CC69 (4, 14.3%),

Table 2. *Molecular characterization of L. monocytogenes strains tested*

BIGSdb-Lm ID	Source	Province	Phylogenetic Lineage	PCR-Serogroup	ST	CC	SL	cgMLST
86311	Butter	Diyarbakır	I	IIb	3002*	ST3002	SL3	CT11733
86312	Cheddar cheese	Diyarbakır	II	IIa	124	CC124	SL124	CT11738
86313	Cake	Diyarbakır	I	IIb	3002*	ST3002	SL3	CT11733
86314	Ice cream	Diyarbakır	III	L	202	CC69	SL69	CT996
86315	Cream	Diyarbakır	III	L	202	CC69	SL69	CT996
86316	Cream	Diyarbakır	III	L	202	CC69	SL69	CT996
86317	Cream cheese	Diyarbakır	III	L	202	CC69	SL69	CT996
86318	Sausage	Diyarbakır	II	IIc	122	CC9	SL9	CT630
86319	Roasted meat	Diyarbakır	II	IIa	155	CC155	SL155	CT11740**
86320	Meatball	Diyarbakır	II	IIc	122	CC9	SL9	CT630
86321	Lahmacun	Diyarbakır	II	IIc	122	CC9	SL9	CT630
86322	Hamburger	Hatay	II	IIa	204	CC204	SL204	CT11737
86323	Pastry	Hatay	II	IIa	20	CC20	SL20	CT11735
86324	Pizza	Hatay	II	IIa	20	CC20	SL20	CT11736
86325	Cake	Hatay	II	IIc	122	CC9	SL9	CT630
86326	Sausage	Adana	I	IVb	1	CC1	SL1	CT11732
86327	Ice cream	Adana	I	IVb	1	CC1	SL1	CT11730**
86328	Beans	Adana	II	IIa	8	CC8	SL8	CT11739
86329	Raw meat	Adana	II	IIc	122	CC9	SL9	CT630
86330	Cattle Meningoencephalitis	Şanlıurfa	I	IVb	1	CC1	SL1	CT11731
86331	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86332	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86333	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86334	Sheep aborted fetus	Şanlıurfa	II	IIc	122	CC9	SL9	CT630
86335	Sheep aborted fetus	Şanlıurfa	II	IIa	12	CC7	SL7	CT720
86336	Döner	Şanlıurfa	II	IVb	145	CC2	SL2	CT375
86337	Cake	Şanlıurfa	I	IIb	3	CC3	SL3	CT11734
86338	Sheep Meningoencephalitis	Şanlıurfa	I	IVb	1	CC1	SL1	CT11730**

**new ST type, **new cgMLST type*

CC20 (2, 14.3%) and ST3002 (2, 14.3%). The remaining seven (25%) strains, which were mainly recovered from food, were grouped into six CCs, with single isolates in each *(Table 2)*. Minimum spanning tree (MST) analysis based on the cgMLST profiles of 28 *L. monocytogenes* strains was generated using the publicly available web-based WGS tool on the BIGSdb-Lm platform.

The isolates were further divided into 14 different types of cgMLST (CTs), with the most numerous CT630 (10, 35.7%) and CT996 (4, 14.3%). The isolates associated with sheep aborted fetuses belonged to CT630 and CT720, whereas CNS infection related isolates belonged to CT11731 and CT11730. CT11730 and CT11740 were new cgMLST types designated for the first time. cgMLST based analyses results revealed ten different sublineages (SLs). The predominant SLs identified were SL9 (10, 35.7%), SL69 (4, 14.3%), SL1 (4, 14.3%), SL3 (3, 10.7%) and SL20 (2, 3.6%). The remaining SLs (SL24, SL155, SL20, SL8, and SL2) were only determined in one isolate of each. Of the CNS infection related SLs, SL1 was also identified in two food isolates. SLs recovered sheep aborted fetus isolates were SL9 and SL7. Molecular relationship of 28 *L. monocytogenes* strains based on the cgMLST analysis are shown in *Fig. 1*.

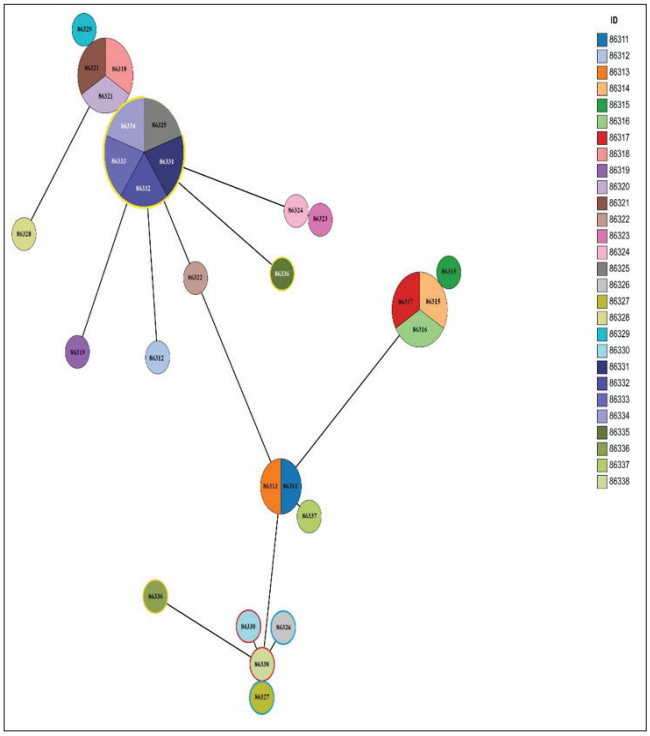

Fig 1. MST based on the cgMLST profiles of 28 *L. monocytogenes* strains studied. Circles with red rim represent *L. monocytogenes* strains from CNS infections. Circles with blue rim represent *L. monocytogenes* strains from foods belonged to CC1. Circles with yellow rim represent *L. monocytogenes* strains from sheep abortus cases

Antimicrobial Resistance and Virulence Gene Profiles

Half of the strains had the same resistance profile with the presence of the *fosX* (fosfomycin), *lin* (antibiotic ABC transporter ATP-binding protein), *norB* (multidrug efflux pump), and *sul* (dihydropteroate synthases) genes, according to the screening screening of *L. monocytogenes* WGS data for 17 antimicrobial resistance genes. Other half of the isolates also carried *mprF* (phosphatidyl glycerol lysyl transferase) gene together with the genes above-mentioned. Acquired antibiotic resistance traits were not detected.

Presence of LIPI-1 was observed in all strains. The LIPI-2 was present in all isolates. However, four strains belonged to ST202 (CC69) did not carry *inlC*, *inlE*, *inlF*, *inlG*, *inlH* and *inlJ* genes. In addition, *inlG* in six isolates and *inlJ* in one isolate were not present. The LIPI-3 virulence genes were identified in seven isolates. LIPI-4 was not found in any of the *L. monocytogenes* studied. The premature stop codon (PMSC) in the *inlA* gene was not present in all the isolates.

The *gltA* and *gltB* genes involved in teichoic acid biosynthesis were found in the only strains belonging to serogroup IVb (n=5) isolates. Similarly, *aut_IVb* gene (autolysin) was only present in the isolates belonging to serogroup IVb.

Benzalkonium Chloride (BC) and Other Tolerance Genes

Of the *bcrABC* gene cassette, encoding benzalkonium chloride resistance protein, *bcrC* was identified in four isolates. The *emrE* gene (encoding the putative small multidrug-resistant (SMR) efflux pump), the *qacA* gene (encoding the quaternary ammonium compound efflux major facilitator superfamily (MFS)), and the *Tn6188 qac* (*ermC*) sequence responsible for benzalkonium chloride tolerance were found in two, one and 15 isolates, respectively. The *cadA* and *cadC* genes, responsible for cadmium resistance, were present in five and one isolate, respectively. All five genes of the stress survival islet 1 (SSI-1) were observed in 21 isolates. The remaining isolates had only one of the SSI-1 islets.

Identification of Prophage Sequences

DNA sequence analysis of 28 *L. monocytogenes* isolates revealed the presence of 41 different intact, questionable or incomplete prophage sequences among the *L. monocytogenes* isolates. Intact prophage sequences were determined in 24 *L. monocytogenes* isolates *(Table 3)*. Phage_Lister_LP_HM00113468 (NC_049900) (n=10, 41.7%) were the most prevalent.

Detection of Plasmids

Plasmids were found in 57.1% (16/28) of the strains *(Table 4)*. pLM330006(pLM33) (rep25) and pLGUG1 (rep26) were detected in 13 (81.3%) and three (18.7%) isolates, respectively.

Table 3. *Prophages in L. monocytogenes strains tested*

No	BIGSdb id	cgMLST Type	Total Lenght (bp)	Total Number of Prophages Regions	No of Prophages Regions According to the Status			Names of Phages with Intact Status	Characteristics of Phages with Intact Status		
					Intact	Questionable	Incomplete		Size (Kb)	No of Proteins	Position
1	86311	CT11733	3.022.337	5	1	1	3	PHAGE_Lister_B054_NC_009813(44)	65.9	82	445354-511181
2	86312	CT11738	2.939.770	3	1	1	1	PHAGE_Lister_A118_NC_003216(12)	35.6	53	1042532-1078228
3	86313	CT11733	3.022.564	5	1	1	3	PHAGE_Lister_B054_NC_009813(44)	65.9	82	445354-511181
4	86314	CT996	2.950.838	5	2	1	2	PHAGE_Lister_LP_030_2_NC_021539(16)	41.3	62	140503-181867
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	6465-34603
5	86315	CT996	2.950.938	5	2	1	2	PHAGE_Lister_2389_NC_003291(16)	41.3	62	348970-390388
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	6465-34603
6	86316	CT996	2.950.898	5	2	1	2	PHAGE_Lister_LP_030_2_NC_021539(16)	41.3	62	140503-181867
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	44-28182
7	86317	CT996	2.950.975	5	2	1	2	PHAGE_Lister_2389_NC_003291(16)	41.3	62	140503-181867
								PHAGE_Lister_LP_030_3_NC_024384(28)	28.1	41	44-28182
8	86318	CT630	2.964.264	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
9	86319	CT11740	3.010.367	5	3	2	0	PHAGE_Strept_315.2_NC_004585(7)	35.3	48	359352-394680
								PHAGE_Lister_B054_NC_009813(65)	48.2	79	455703-503959
								PHAGE_Lister_A006_NC_009815(26)	53	60	34644-87714
10	86320	CT630	2.964.210	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
11	86321	CT630	2.964.383	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
12	86322	CT11737	3.012.987	4	1	2	1	PHAGE_Lister_vB_LmoS_188_NC_028871(23)	24.8	32	550716-575528
13	86323	CT11735	2.996.628	1	1	0	0	PHAGE_Lister_A006_NC_009815(39)	38.2	59	296189-334422
14	86324	CT11736	2.986.983	1	1	0	0	PHAGE_Lister_A006_NC_009815(39)	38.2	59	296189-334422
15	86325	CT630	2.964.377	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
16	86326	CT11732	2.957.225	3	1	1	1	PHAGE_Lister_vB_LmoS_188_NC_028871(31)	48.7	68	252224-300962
17	86327	CT11730	2.877.061	2	0	1	1	-	-	-	-
18	86328	CT11739	3.011.077	3	1	2	0	PHAGE_Lister_LP_HM00113468_NC_049900(38)	47.4	57	392173-439574
19	86329	CT630	2.964.363	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
20	86330	CT11731	2.956.218	4	2	1	1	PHAGE_Lister_2389_NC_003291(48)	37.9	56	153-38085
								PHAGE_Lister_vB_LmoS_293_NC_028929(34)	34.4	47	1223-35650
21	86331	CT630	2.964.368	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
22	86332	CT630	2.964.682	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
23	86333	CT630	2.964.376	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(40)	57.4	70	461710-519120
24	86334	CT630	2.964.487	3	1	1	1	PHAGE_Lister_LP_HM00113468_NC_049900(38)	42.9	69	389956-432915
25	86335	CT720	2.882.660	3	1	1	1	PHAGE_Lister_LP_101_NC_024387(47)	43.5	65	983003-1026580
26	86336	CT375	2.922.545	2	0	1	1	-	-	-	-
27	86337	CT11734	3.026.339	6	0	1	5	-	-	-	-
28	86338	CT11730	2.877.058	2	0	1	1	-	-	-	-

Discussion

This study describes the WGS-based characterization of *L. monocytogenes* strains isolated from food and animal clinical cases in Türkiye for the first time. MLST typing revealed that *L. monocytogenes* strains isolated from food were assigned to eleven clonal complexes. The detected CCs in the present study have been also shown to be globally distributed in foods and food-processing environments. As previously reported, CC1 and CC2 are highly associated with clinical human and animal listeriosis cases, but they have also been detected in food products and considered hypervirulent [22,23]. The higher gut colonization ability of hypervirulent *L. monocytogenes* clones, particularly CC1, may lead to prolonged fecal shedding, resulting in persistence dairy cattle farm environment and high frequency contamination of milk and dairy products and thus pose a potential health risk

Tablo 4. *Plasmids in L. monocytogenes strains tested*

No	BIGSdb id	cgMLST Type	Total lenght (bp)	No. of Plasmids	Locus Targeted	Note	Contig	Position	Lenght (bp)
1	86311	CT11733	3.022.337	1	rep25	pLM330006(pLM33)	NODE_9_length_57672_cov_44.684682	34694..36454	1761
2	86312	CT11738	2.939.770	1	rep25	pLM330006(pLM33)	NODE_6_length_48704_cov_35.285329	3761..5521	1761
3	86313	CT11733	3.022.564	1	rep25	pLM330006(pLM33)	NODE_9_length_57728_cov_40.328931	34750..36510	1761
4	86314	CT996	2.950.838	-	-	-	-	-	-
5	86315	CT996	2.950.938	-	-	-	-	-	-
6	86316	CT996	2.950.898	-	-	-	-	-	-
7	86317	CT996	2.950.975	-	-	-	-	-	-
8	86318	CT630	2.964.264	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_40.326559	43465..45225	1761
9	86319	CT11740	3.010.367	-	-	-	-	-	-
10	86320	CT630	2.964.210	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_35.097293	-	-
11	86321	CT630	2.964.383	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_36.925559	-	-
12	86322	CT11737	3.012.987	-	-	-	-	-	-
13	86323	CT11735	2.996.628	1	rep26	repA(pLGUG1)	NODE_8_length_75397_cov_15.714901	-	-
14	86324	CT11736	2.986.983	1	rep26	repA(pLGUG1)	NODE_8_length_75397_cov_16.905055	-	-
15	86325	CT630	2.964.377	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_42.370043	-	-
16	86326	CT11732	2.957.225	-	-	-	-	-	-
17	86327	CT11730	2.877.061	-	-	-	-	-	-
18	86328	CT11739	3.011.077	1	rep26	repA(pLGUG1)	NODE_9_length_79235_cov_25.598162	36670..38478	1809
19	86329	CT630	2.964.363	1	rep25	pLM330006(pLM33)	NODE_8_length_48469_cov_34.140432	43207..44967	1761
20	86330	CT11731	2.956.218	-	-	-	-	-	-
21	86331	CT630	2.964.368	1	rep25	pLM330006(pLM33)	NODE_8_length_48469_cov_34.332148	3503..5263	1761
22	86332	CT630	2.964.682	1	rep25	pLM330006(pLM33)	NODE_8_length_57720_cov_38.563263	34750..36510	1761
23	86333	CT630	2.964.376	1	rep25	pLM330006(pLM33)	NODE_8_length_48727_cov_38.600831	3503..5263	1761
24	86334	CT630	2.964.487	1	rep25	pLM330006(pLM33)	NODE_8_length_48469_cov_38.818954	43207..44967	1761
25	86335	CT720	2.882.660	-	-	-	-	-	-
26	86336	CT375	2.922.545	-	-	-	-	-	-
27	86337	CT11734	3.026.339	1	rep25	pLM330006(pLM33)	NODE_9_length_48469_cov_58.455540	3503..5263	1761
28	86338	CT11730	2.877.058	-	-	-	-	-	-

to humans [18]. Moura et al. [22] reported that CC1 was the most prevalent clonal group associated with human listeriosis cases in the world and was strongly associated with cattle and dairy products. In addition, the teichoic acid biosynthesis genes *gltA* and *gltB* and the invasion gene *aut_IVb* were only detected in isolates belonging to CC1 and CC2. Other CCs, despite, were defined as hypovirulent or intermediate clones with low clinical frequency [14], invasive infections caused by these clones were also reported in humans [24,25] and animals [26,27].

L. monocytogenes strains have many virulence factors that determine their pathogenic potential and all of which have an important role at various stages of the infection cycle. The current study looked for 69 potential virulence markers that could be used to predict the level of virulence in *L. monocytogenes* isolates. Based on virulence markers, it was suggested to classify *L. monocytogenes* isolates as putatively hypo-virulent, with unknown virulence potential, and putatively hypervirulent [8]. Markers were identified across the isolates suggesting that most virulence markers are ubiquitous across *L. monocytogenes* strains. Intact LIPI-1, which harbors Prf-A dependent virulence cluster genes that are critical in the infectious cycle of *L. monocytogenes*, was observed in all isolates. In the current study, the LIPI-3, which is associated with promoting the virulence capabilities of *L. monocytogenes*, was found in four isolates from ST1 from serogroup IVb belonging to lineage I, but was also present in two isolates from lineage I belonging to ST3002 which was identified as the new ST, and one isolate from lineage I belonging to ST3. The LIPI-3 carries a gene encoding the hemolytic and cytotoxic factor known as listeriolysin S, which contributes to the intracellular survival of *L. monocytogenes* in human polymorphonuclear neutrophils [8]. Painset et al. [28] and Chen et al. [29] reported similar findings and revealed that

LIPI-3 is ubiquitous to lineage I, which was also observed in the present study. The recently described pathogenicity island LIPI-4 that confers hypervirulence by enhancing the invasion of the CNS and placenta was not detected among the *L. monocytogenes* isolates. LIPI-4 was reported to be the most prevalent in CC4 strains in Western countries and in CC87 strains in Asia [2].

Except for *L. monocytogenes* strains belong to CC69 (ST202), the rest of the isolates generally carried the virulence genes examined. However, the known adhesion and invasion related genes, such as *inlC, inlE, inlF, inlG*, *inlH, inlJ*, *ami*, *vip*, and *aut* were not found in genomes of CC69 strains, which suggests a possible limitation of the invasiveness and virulence of these *L. monocytogenes* strains [30,31]. The *inlA* gene was found in all of the isolates in the current study. A recent study showed that the truncation of the gene *inlA* due to premature stop codon resulted in reduced invasiveness in *L. monocytogenes* strains [32]. Therefore, it has been noted that this mutation may serve as a marker of hypovirulence. None of the isolates showed mutations in the *inlA* gene leading to premature stop codon (PMSC).

Various resistance genes, *lin* (lincosamides), *mprF* (cationic antimicrobial peptides), *fosX* (fosfomycins), *norB* (quinolones) and *sul* (sulfonamides) were found in the WGS of *L. monocytogenes* isolates using the BIGSdb-Lm database (https://bigsdb.pasteur.fr/listeria/, accessed on 12 July 2022). Hanes and Huang [33] reported that the genes *fosX* and *lin* were present in nearly every *L. monocytogenes* isolate, presence of other resistance genes other than *fosX* and *lin* changed according to country or isolation sources (clinical, environmental or other).

In the current study, two isolates from central nervous system (CNS) infection belonged to CC1. It was shown that CC1 was the most prevalent CCs isolated from rhombencephalitis-associated cases in ruminants [1,23]. Dreyer et al.[1] reported that neurotropism of *L. monocytogenes* strains belonged to this clone was associated with their hyperinvasiveness and increased intracellular replication. Furthermore, cgMLST analyses of *L. monocytogenes* ST1 strains revealed two different cgMLST types (CT11730 and CT11731), were not previously reported among ruminant rhomboencephalitis isolates. This could be explained by the fact that such strains have not been previously isolated or such sequences have not been submitted to the PasteurMLST BIGSdb-*Lm* database.

CC9 (ST122) and CC7 (ST12) were detected an abortion-associated CCs in the current study. CC7 was reported in Latvia [27] and in Slovenia [23] from abortus cases, despite not being amongst the three most common clones. In contrast, CC7 (7/46) were reported as the most prevalent CCs among *L. monocytogenes* isolates from different animal the clinical cases (abortus, bacteremia, CNS infection) from the USA [26]. CC9 is considered as food-associated hypovirulent MLST clones which were rarely implicated in clinical listeriosis cases. The genes of LIPI-1 and internalin family are the main virulence factors involved in the pathogenesis of *L. monocytogenes*. These genes are required for the intestinal infection stage, the entry into the host cells, and the adaptation to an intracellular lifestyle [17]. These genes were observed in all the isolates from abortus cases. Indeed, even if prevalence rate is low, invasive infections caused by CC9 have been reported both in humans [24,25] and animals [27]. It could be argued that the emergence of infections caused by CC9 could be related with the predisposing factors as well as virulence factors.

Like many bacterial species, *L. monocytogenes* strains are known to harbor plasmids with frequencies reaching as high as 92%. To date, plasmids obtained from *L. monocytogenes* strains have been shown to contain genes that confer resistance to disinfectants, heavy metals, antimicrobials, biotics. In addition, plasmids have been reported to carry the genes related with oxidative, osmotic, and heat stress [34]. The most common plasmid was Inc18(rep25), and only three strains carried Inc18(rep26). These plasmids contain genetic determinant for cadmium resistance [35]. Environmental and foodborne isolates have been reported to harbor plasmid in higher rates than clinical isolates [36,37]. Similarly, plasmids were detected with food-related isolates, none of the isolates belonged to CC1, CC2 and CC3 strains did not harbor plasmids.

WGS analysis revealed 15 different intact prophages across the *L. monocytogenes* isolates. Multiple prophages were observed in some isolates, supporting the previous studies that prophages were highly prevalent in the genomes of *L. monocytogenes* [38,39]. The prophages have been regarded as an important contributor to the evolution and virulence of *L. monocytogenes* [40,41], conferring an ecological advantage for persistence and survival over time [38]. This demonstrates that prophage diversification is a driving force for the adaptation to specific environmental niches and genetic evolution of *L. monocytogenes* strains [34].

Quaternary ammonium compounds (QACs), such as benzalkonium chloride (BC) are the factors that could contribute to the persistence and survival of *L. monocytogenes* in food processing environments, through the activity of various efflux pumps encoded by *brcABC* cassette, *qacA*, *qacC*, *qacH*, *emrE* and *emrC* [42]. QACs, like the factors that could contribute to the persistence and survival of *L. monocytogenes* in food processing environments, through the activity of various efflux pumps encoded by *brcABC* cassette, *qacH*, *qacA*, *qacC*, *emrE* and *emrC* [7]. In the present study, *emrC* gene (53.6%), was first described on plasmid pLMST6 [43], was most frequently detected in *L. monocytogenes* isolates, followed by *bcrC* (14.3%), *emrE* (7.1%), and *qacA* (3.6%). Similarly, *emrC*

(40%) was the most frequently detected gene responsible for BC resistance in *L. monocytogenes* strains originating from food chain in South Africa [39]. In contrast, a higher prevalence for *qacH* were reported in France (18.8%) [44], Norway (22%) [45], and Czechia [42]. In the present study, two chromosomal major efflux pump genes, *mdrL* and *lde*, were detected in all *L. monocytogenes* isolates. The high prevalence of QAC efflux genes may give *L. monocytogenes* an advantage for survival and persistence in their specific environments [46].

The WGS analysis performed in the current study revealed the genetic diversity of *L. monocytogenes* strains from different food and clinical cases of animals in Türkiye. Novel ST and cgMLST types were also identified, which were previously not present in the *Listeria* database. Several hypervirulent strains were detected among the food-associated isolates belonging to CC1 and CC2, which could present a major public health threat. *L. monocytogenes* strains from CNS infections belonged to CC1, whereas strains from maternal-neonatal infections belonged to CC9 and CC7. According to our knowledge, this is the first study based on WGS analysis of *L. monocytogenes* from different food and animal invasive infections in Türkiye. The findings of this study also highlight the importance of WGS to provide more detailed genetic information on *L. monocytogenes* obtained from different sources. The present study also shows the necessity of implementation of effective hygienic procedures to prevent contamination of food with *L. monocytogenes*.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (Ö. Aslantaş) on reasonable request.

Ethical Statement

The study does not require ethical approval from Animal Experiments Local Ethics Committee Funding Support

Funding support

This study was supported by the Scientific Research Fund of Hatay Mustafa Kemal University with a grant number of 21.GAP.051.

Conflict of Interest

The authors declared that there is no conflict of interest related to this study.

Author Contributions

ÖA and OK planned, designed, and supervised the research procedure, AGY and AA performed all microbiological experiments, ÖA and KB performed bioinformatic analyses, and ÖA wrote the manuscript. All authors have read and approved the manuscript.

References

1. Dreyer M, Aguilar-Bultet L, Rupp S, Guldimann C, Stephan R, Schock A, Otter A, Schüpbach G, Brisse S, Lecuit M, Frey J, Oevermann A: *Listeria monocytogenes* sequence type 1 is predominant in ruminant rhombencephalitis. *Sci Rep*, 6:36419, 2016. DOI: 10.1038/srep36419

2. Disson O, Moura A, Lecuit M: Making sense of the biodiversity and virulence of *Listeria monocytogenes*. *Trends Microbiol,* 29, 811-822, 2021. DOI: 10.1016/j.tim.2021.01.008

3. Nightingale KK, Schukken YH, Nightingale CR, Fortes ED, Ho AJ, Her Z, Grohn YT, McDonough PL, Wiedmann M: Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm environment. *Appl Environ Microbiol*, 70, 4458-4467, 2004. DOI: 10.3389/fmicb.2022.866462

4. Dhama K, Karhik K, Tiwari R, Shabbir MZ, Barbuddhe S, Malik SVS, Singh RK: Listeriosis in animals, its public health significance (food-borne zoonosis) and advances in diagnosis and control: A comprehensive review. *Vet Q*, 35, 211-235, 2015. DOI: 10.1080/01652176.2015.1063023

5. Charlier C, Perrodeau É, Leclercq A, Czenave B, Pilmis B, Henry B, Lopes A, Maury MM, Moura A, Goffinet F, Fieye HB, Thouvenot P, Ungeheuer MN, Tourdjman M, Goulet V, de Valk H, Lortholary O, Ravaid P, Lecuit M: Clinical features and prognostic factors of listeriosis: The MONALISA national prospective cohort study. *Lancet Infect Dis*, 17, 510-519, 2017. DOI: 10.1016/S1473-3099(16)30521-7

6. European Centre for Disease Prevention and Control (ECDC): Annual Epidemiology Report 2017-Listeriosis, https://www.ecdc.europa.eu/sites/default/files/documents/listeriosis-annual-epidemiological- report-2017.pdf; *Accessed:* 12 July 2022.

7. Osek J, Lachtara B, Wieczorek K: *Listeria monocytogenes* - How this pathogen survives in food-production environments? *Front Microbiol*, 13:866462, 2022. DOI: 10.3389/fmicb.2022.866462

8. Hurley D, Luque-Sastre L, Parker CT, Huynh S, Eshwar AK, Nguyen SV, Andrews N, Moua A, Fox EM, Jordan K, Lehner A, Stephan R, Fanning S: Whole-genome sequencing-based characterization of 100 *Listeria monocytogenes* isolates collected from food processing environments over a four-year period. *mSphere*, 4:e00252-e00319, 2019. DOI: 10.1128/mSphere.00252-19

9. Terentjeva M, Šteingolde Z, Meistere I, Elferts D, Avsenjenko J, Streikiša M, Gradovska S, Alksne L, Ķibilds J. Bērziņš A: Prevalence, genetic diversity and factors associated with distribution of *Listeria monocytogenes* and other *Listeria* spp. in cattle farms in Latvia. *Pathogens*, 10:851, 2021. DOI: 10.3390/pathogens10070851

10. Elsayed MM, Elkenany RE, Zakaria AI, Badawy BM: Epidemiological study on *Listeria monocytogenes* in Egyptian dairy cattle farms' insights into genetic diversity of multi-antibiotic-resistant strains by ERIC-PCR. *Environ Sci Pollut Res,* 29, 54359-54377, 2022. DOI: 10.1007/s11356-022-19495-2

11. Doumith M, Cazalet C, Simoes N, Frangeul L, Jacquet C, Kunst F, Martin P, Cossart P, Glaser P, Buchrieser C: New aspects regarding evolution and virulence of *Listeria monocytogenes* revealed by comparative genomics and DNA arrays. *Infect Immun*, 72, 1072-1083, 2004. DOI: 10.1128/IAI.72.2.1072-1083.2004

12. Ragon M, Wirth T, Hollandt F, Lavenir R, Lecuit M, Le Monnier A, Brisse S: A new perspective on *Listeria monocytogenes* evolution. *PLoS Pathog*, 4:e1000146, 2008. DOI: 10.1371/journal.ppat.1000146

13. Moura A, Criscuolo A, Pouseele H, Maury MM, Leclercq A, Tarr C, Bjorkman JT, Dallman T, Reimer A, Enouf V, Larsonneur E, Carleton H, Bracq-Dieye H, Katz LS, Jones L, Touchon M, Tourdjman M, Walker M, Stroika S, Cantinelli T, Chenal-Francisque V, Kucerova Z, Rocha EP, Nadon C, Grant K, Nielsen EM, Pot B, Gerner-Smidt P, Lecuit M, Brisse S: Whole genome-based population biology and epidemiological surveillance of *Listeria monocytogenes*. *Nat Microbiol*, 2:16185, 2016. DOI: 10.1038/nmicrobiol.2016.185

14. Maury MM, Tsai YH, Charlier C, Touchon M, Chenal-Francisque V, Leclercq A, Criscuolo A, Gaultier C, Roussel S, Brisabois A, Disson O, Rocha EPC, Brisse S, Lecuit M: Uncovering *Listeria monocytogenes* hypervirulence by harnessing its biodiversity. *Nat Genet*, 48, 308-313, 2016. DOI: 10.1038/ng.3501

15. Kwong JC, Mercoulia K, Tomita T, Easton M, Li HY, Bulach DM, Stinear TP, Seeman T, Howden BP: Prospective whole-genome sequencing enchances national surveillance of *Listeria monocytogenes*. *J Clin Microbiol*, 54, 584-640, 2016. DOI: 10.1128/JCM.02344-15

16. Cotter PD, Draper LA, Lawton EM, Daly KM, Groeger DS, Casey PG,

Ross RP, Hill C: Listeriolysin S, a novel peptide haemolysin associated with a subset of lineage I *Listeria monocytogenes*. *PLoS Pathog*, 4:e1000144, 2008. DOI: 10.1371/journal.ppat.1000144

17. Bolger AM, Lohse M, Usadel B: Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*, 30, 2114-2120, 2016. DOI: 10.1093/bioinformatics/btu170

18. Seemann T: Shovill, 2019. https://github.com/tseemann/shovill; *Accessed:* 12 July 2022.

19. Gurevich A, Saveliev V, Vyahhi N, Tesler G: QUAST: Quality assessment tool for genome assemblies. *Bioinformatics*, 29, 1072-1075, 2013. DOI: 10.1093/bioinformatics/btt086

20. Arndt D, Grant J, Marcu A, Sajed T, Pon A, Liang Y, Wishart DS: PHASTER: A better, faster version of the PHAST phage search tool. *Nucleic Acids Res*, 44, 16-21, 2016. DOI: 10.1093/nar/gkw387

21. Carattoli A, Zankari E, García-Fernández A, Larsen MV, Lund O, Villa L, Aarestrup FM, Hasman H: *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother,* 58, 3895-3903, 2014. DOI: 10.1128/AAC.02412-14

22. Moura A, Lefrancq N, Wirth T, Leclercq A, Borges V, Gilpin B, Dallman TJ, Frey J, Franz E, Nielsen EM, Thomas J, Pightling A, Howden BP, Tarr CL, Gerner-Smidt P, Cauchemez S, Salje H, Brisse S, Lecuit M: Emergence and global spread of *Listeria monocytogenes* main clinical clonal complex. *Sci Adv*, 7:eabj9805, 2021. DOI: 10.1126/sciadv.abj9805

23. Papić B, Pate M, Félix B, Kušar D: Genetic diversity of *Listeria monocytogenes* strains in ruminant abortion and rhombencephalitis cases in comparison with the natural environment. *BMC Microbiol,* 19, 1-13, 2019. DOI: 10.1186/s12866-019-1676-3

24. Yin Y, Doijad S, Wang W, Lian K, Pan X, Koryciński I, Hu Y, Tan W, Ye S, Wang Z, Pan Z, Chakraborty T, Jiao X: Genetic diversity of *Listeria monocytogenes* isolates from invasive Listeriosis in China. *Foodborne Pathog Dis*, 17, 215-227, 2020. DOI: 10.1089/fpd.2019.2693

25. Kuch A, Goc A, Belkiewicz K, Filipello V, Ronkiewicz P, Gołębiewska A, Wróbel I, Kiedrowska M, Waśko I, Hryniewicz W, Lomonaco S, Skoczyńska A: Molecular diversity and antimicrobial susceptibility of *Listeria monocytogenes* isolates from invasive infections in Poland (1997-2013). *Sci Rep*, 8:14562, 2018. DOI: 10.1038/s41598-018-32574-0

26. Steckler AJ, Cardenas-Alvarez MX, Ramsett MKT, Dyer N, Bergholz TM: Genetic characterization of *Listeria monocytogenes* from ruminant listeriosis from different geographical regions in the U.S. *Vet Microbiol*, 215, 93-97, 2018. DOI: 10.1016/j.vetmic.2017.12.021

27. Šteingolde Ž, Meistere I, Avsejenko J, Ķibilds J, Bergšpica I, Streikiša M, Gradovska S, Alksne L, Roussel S, Terentjeva M, Bērziņš A: Characterization and genetic diversity of *Listeria monocytogenes* isolated from cattle abortions in Latvia, 2013-2018. *Vet Sci,* 8:195, 2021. DOI: 10.3390/vetsci8090195

28. Painset A, Björkman JT, Kiil K, Guillier L, Mariet JF, Félix B, Amar C, Rotariu O, Perez-Reche F, Brisse S, Moura A, Lecuit M, Forbes K, Strachan N, Grant K, Møller-Nielsen E, Dallman TJ: LiSEQ-whole-genome sequencing of a cross-sectional survey of *Listeria monocytogenes* in ready-to-eat foods and human clinical cases in Europe. *Microb Genom,* 5:e000257, 2019. DOI: 10.1099/mgen.0.000257

29. Chen Y, Chen Y, Pouillot R, Dennis S, Xian Z, Luchansky JB, Porto-Fett ACS, Lindsay JA, Hammack TS, Allard M, Van Doren JM, Broen EW: Genetic diversity and profiles of genes associated with virulence and stress resistance among isolates from the 2010-2013 interagency *Listeria monocytogenes* market basket survey. *PLoS One*, 15:e0231393, 2020. DOI: 10.1371/journal.pone.0231393

30. Lindén SK, Bierne H, Sabet C, Png CW, Florin TH, McGuckin MA, Cossart P: *Listeria monocytogenes* internalins bind to the human intestinal mucin MUC2. *Arch Microbiol*, 190 (1): 101-114, 2008. DOI: 10.1007/s00203-008-0358-6

31. Martins M, Custódio R, Camejo A, Almeida MT, Cabanes D, Sousa S: *Listeria monocytogenes* triggers the cell surface expression of Gp96 protein and interacts with its N terminus to support cellular infection. *J Biol Chem*, 287 (51): 43083-43093, 2012. DOI: 10.1074/jbc.M112.422568

32. Matle I, Mafuna T, Madoroba E, Mbatha KR, Magwedere K, Pierneef R: Population structure of Non-ST6 *Listeria monocytogenes* isolated in the red meat and poultry value chain in South Africa. *Microorganisms*, 8 (8):1152, 2020. DOI: 10.3390/microorganisms8081152

33. Hanes RM, Huang Z: Investigation of antimicrobial resistance genes in *Listeria monocytogenes* from 2010 through to 2021. *Int J Environ Res Public Health*, 19:5506, 2022. DOI: 10.3390/ijerph19095506

34. Schmitz-Esser S, Anast JM, Cortes BW: A large-scale sequencing-based survey of plasmids in *Listeria monocytogenes* reveals global dissemination of plasmids. *Front Microbiol*, 12:653155, 2021. DOI: 10.3389/fmicb.2021.653155

35. Kuenne C, Voget S, Pischimarov J, Oehm S, Goesmann A, Daniel R, Hain T, Chakraborty T: Comparative analysis of plasmids in the genus *Listeria. PLoS One*, 5:e12511, 2010. DOI: 10.1371/journal.pone.0012511

36. McLauchlin J, Hampton MDD, Shah S, Threlfall EJJ, Wieneke AAA, Curtis GD: Subtyping of *Listeria monocytogenes* on the basis of plasmid profiles and arsenic and cadmium susceptibility. *J Appl Microbiol*, 83, 381-388, 1997. DOI: 10.1046/j.1365-2672.1997.00238.x

37. Lebrun M, Loulergue J, Chaslus-Dancla E, Audurier A: Plasmids in *Listeria monocytogenes* in relation to cadmium resistance. *Appl Environ Microbiol,* 58, 3183-3186, 1992. DOI: 10.1128/aem.58.9.3183-3186.1992

38. Castro H, Douillard F, Korkeala H, Lindström M: Mobile elements harboring heavy metal and bacitracin resistance genes are common among *Listeria monocytogenes* strains persisting on dairy farms. *mSsphere,* 6 (4):e0038321, 2021. DOI: 10.1128/mSphere.00383-21

39. Mafuna T, Matle I, Magwedere K, Pierneef RE, Reva ON: Whole genome-based characterization of *Listeria monocytogenes isolates* recovered from the food chain in South Africa. *Front Microbiol*, 12:669287, 2021. DOI: 10.3389/fmicb.2021.669287

40. Fortier LC, Sekulovic O: Importance of prophages to evolution and virulence of bacterial pathogens. *Virulence*, 4, 354-365, 2013. DOI: 10.4161/viru.24498

41. Brüssow H, Canchaya C, Hardt W, Bru H: Phages and the evolution of bacterial pathogens: From genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev,* 68, 560-602, 2004. DOI: 10.1128/MMBR.68.3.560-602.2004

42. Gelbicova T, Florianova M, Hluchanova, L, Kalova A, Korena K, Strakova N, Karpiskova R: Comparative analysis of genetic determinants encoding cadmium, arsenic, and benzalkonium chloride resistance in *Listeria monocytogenes* of human, food, and environmental origin. *Front Microbiol,* 11:599882, 2021. DOI: 10.3389/fmicb.2020.599882

43. Kremer PH, Lees JA, Koopmans MM, Ferwerda B, Arends AW, Feller MM, Schipper K, Valls Seron M, van der Ende A, Brouwer MC, van de Beek D, Bentley SD: Benzalkonium tolerance genes and outcome in *Listeria monocytogenes* meningitis. *Clin Microbiol Infect*, 23 (4):265, 2017. DOI: 10.1016/j.cmi.2016.12.008

44. Maury MM, Bracq-Dieye H, Huang L, Vales G, Lavina M, Thouvenot P, Disson O, Leclercq A, Brisse S, Lecuit M: Hypervirulent *Listeria monocytogenes* clones' adaption to mammalian gut accounts for their association with dairy products. *Nat Commun*, 10:2488, 2019. DOI: 10.1038/s41467-019-10380-0

45. Møretrø T, Schirmer BCT, Heir E, Fagerlund A, Hjemli P, Langsrud S: Tolerance to quaternary ammonium compound disinfectants may enhance growth of *Listeria monocytogenes* in the food industry. *Int Food Microbiol*, 241, 215-224, 2017. DOI: 10.1016/j.ijfoodmicro.2016.10.025

46. Daeschel D, Pettengill JB, Wang Y, Chen Y, Allard M, Snyder AB: Genomic analysis of *Listeria monocytogenes* from US food processing environments reveals a high prevalence of QAC efflux genes but limited evidence of their contribution to environmental persistence. *BMC Genomics,* 23:488, 2022. DOI: 10.1186/s12864-022-08695-2

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 231-238, 2023
DOI: 10.9775/kvfd.2023.29090

RESEARCH ARTICLE

Intra-Breed Genetic Diversity and Genetic Bottleneck Tests in a Karacabey Merino Sheep Breeding Farm Using Microsatellite Markers

Adem KABASAKAL [1(*)]

[1] Bandırma OnyediEylül University, Susurluk Vocational School, Department of Food Processing Division, TR-10600 Balıkesir - TÜRKİYE

ORCIDs: 0000-0002-6857-4380

Article ID: KVFD-2023-29241 Received: 24.01.2023 Accepted: 19.04.2023 Published Online: 23.04.2023

Abstract: In the present study, it was aimed to reveal the genetic diversity and bottleneck status of Karacabey Merino sheep with the help of 14 microsatellite markers recommended by the FAO. The study was carried out in a sheep breeding farm in Bandırma and 103 unrelated Karacabey merino sheep. The microsatellites used in this study showed high levels of polymorphism. A total of 290 alleles were detected in this study. The mean values of polymorphic information content (PIC=0.90), observed heterozygosity (Ho=0.89) and expected heterozygosity (He=0.91) were high, suggesting that the total analysed population is characterized by noticeable genetic variability. Ten out of the fourteen microsatellite markers studied had a positive F_{IS} value. The mean value of F_{IS} was 0.032. The infinite allele model (IAM), two-phase mutation model (TPM) and stepwise mutation model (SMM) in the Bottleneck software were used to check genetic bottlenecks. The L-shaped curve obtained from the analysis indicates the absence of a bottleneck in the Karacabey Merino sheep population studied.

Keywords: *Genetic bottleneck, Genetic diversity, Karacabey merino sheep, Microsatellite*

Karacabey Merinos Koyunu Yetiştirme Çiftliğinde Mikro Uydu İşaretleyiciler Kullanılarak Irk İçi Genetik Çeşitlilik ve Genetik Darboğaz Testleri

Öz: Sunulan çalışmada bir Karacabey Merinosu koyun çiftliğinde populasyon içi genetik çeşitlilik ve darboğaz durumunun FAO tarafından önerilen 14 mikrosatellit belirteç yardımıyla ortaya konması amaçlanmıştır. Bu çalışma, Bandırma'da bir koyun yetiştirme çiftliğinde akraba olmayan 103 Karacabey merinos koyunu ile yürütülmüştür. Kullanılan mikrosatellitler yüksek düzeyde polimorfizm göstermiştir. Çalışmada toplam 290 allel gözlemlenmiştir. Polimorfik bilgi içeriği (PIC=0.90), gözlemlenen heterezigotluk (Ho=0.89) ve beklenen heterozigotluk (He=0.91) değerlerine ait genel ortalamalar çalışılan populasyonların dikkat çekici düzeyde genetic çeşitliliğe sahip olduğunu göstermektedir. İncelenen on dört mikrosatellit işaretleyicinin onunda pozitif F_{IS} değerleri gözlemlenmiştir. F_{IS} değerlerinin ortalaması 0.032 olmuştur. Çalışılan ırktaki genetik darboğaz durumunun kontrolünü sağlamak Bottleneck programındaki sonsuz alel modeli (IAM), iki fazlı mutasyon modeli (TPM) ve aşamalı mutasyon modeli (SMM) kullanılmıştır. Analizden elde edilen mode-shift grafiğindeki L şeklindeki eğri, çalışılan Karacabey Merinosu koyun populasyonunun yakın zamanlarda herhangi bir genetik darboğaza girmediğini göstermiştir.

Anahtar sözcükler: *Genetik çeşitlilik, Genetik darboğaz, Karacabey merinos koyunu, Mikrosatellit*

Introduction

Türkiye has rich genetic diversity in terms of sheep breeding. Nevertheless, it is known that there have been losses in terms of farm animal genetic resources in the past 50 years. When evaluated on a world scale, 14% of the sheep breeds in the world, especially in Europe, have disappeared [1]. Despite these aforementioned adversities, issues such as the fact that Türkiye is in a suitable geography for different animal production models with its ecological and genetic richness, the role of animal production in rural development, and the protection of animal genetic resources, which have been developing in recent years, increase their importance [2,3].

How to cite this article?

Kabasakal A: Intra-breed genetic diversity and genetic bottleneck tests in a Karacabey Merino Sheep breeding farm using microsatellite markers. *Kafkas Univ Vet Fak Derg,* 29 (3): 231-238, 2023.
DOI: 10.9775/kvfd.2023.29090

(*) Corresponding author: Adem KABASAKAL

Phone: +90 266 606 8401 Celluler phone: +90 542 343 8156 Fax: +90 266 717 0030
E-mail: akabasakal@bandirma.edu.tr

Genetic diversity is variation in the genetic material possessed by individuals in a population. Genetic diversity, calculated by characterizing statistically, includes allelic diversity, allele richness, observed and expected heterozygosity at the population level [4]. As the number of individuals in the population decreases, genetic diversity decreases, as it increases, genetic diversity increases and this is associated with high evolutionary resistance [5]. To maintain genetic diversity and variability, the number of individuals capable of effective reproduction within the population is important. Genetic bottlenecks occur when the effective population size is subject to serious reductions due to human influence, environmental effects, diseases and inbreeding. Genetic bottlenecks resulting from a founding event cause loss of genetic diversity in the population [6-8]. On the other hand, high inbreeding in small populations is one of the important causes of loss of genetic diversity [9].

Genetic drift as a result of the decrease due to the genetic bottleneck causes a decrease in the number of alleles in the population and especially the loss of rare alleles. For this reason, the occurrence of genetic bottlenecks that cause a decrease in genetic diversity should be genetically monitored [10-12]. The study of genetic diversity is especially important for the conservation and continuation of genetic resources [13]. All over the world, studies have been carried out using various molecular techniques based on DNA to reveal genetic variation in sheep breeds and to define intra and interbreed diversity. Molecular definitions for populations that are valuable genetic resources play a guiding role in conservation programs and the effectiveness of conservation activities can be tested with these studies. Genetic diversity can only be revealed in a healthy way with definitions made at the DNA level [14]. Through the molecular techniques, local gene resources, evaluation of conservation studies in public and institutes at the molecular level, genetic similarity, intrabreed difference, genetic diversity, possible bottleneck and genetic drift in populations can be determined. For this purpose, it was reported by FAO [15] that microsatellite markers can be used safely to reveal intra and interbreed genetic diversity in genetic resources.

FAO [15] has recommended over 30 microsatellite loci that can be used for genetic diversity. However, the use of such a large number of microsatellites causes high costs in genetic diversity studies [16]. Yılmaz et al.[17] tested the reliability of microsatellite panels with different loci numbers, and the reliability of panels with 8 or fewer loci was found to be low. They found that panels with 12 or more loci can be used with high reliability.

Molecular markers are genetic markers used to evaluate genetic differences between two or more individuals. They are capable of detecting polymorphism that exists in a genetically related population [16]. Microsatellites are found in large numbers in the genome, represent noncoding intron regions of DNA, and have multiple and codominant inheritance. For this reason, it is widely used in studies of detecting intra and interbreed genetic diversity and detecting bottlenecks in farm animals [6,14,18-21]. Particularly in small populations, genetic bottlenecks may cause a decrease in genetic diversity due to genetic drift and inbreeding. Models used to understand the processes that lead to reduced genetic diversity must yield consistent results. For this reason, it has been reported that the two-phase mutation model (TPM) is the most useful model to test the excess heterozygosity in bottleneck tests with microsatellites [22].

In this study, we aimed to reveal the parameters of the genetic diversity, similarity and inbreeding levels and the bottleneck status with the help of microsatellite markers of Karacabey Merino sheep bred on a farm in Bandırma, Türkiye.

Material and Methods

Ethical Statement

The study was conducted with the permission of the Balikesir University Animal Experiments Local Ethics Committee dated 27.10.2021 and numbered 2019/9-5.

Animals

The animal material of the study consisted of a total of 103 unrelated Karacabey Merino sheep in a farm operating in Bandırma, Balıkesir.

Blood Sample Collection

After taking the necessary precautions to prevent direct contact with blood, the animal to be bled was sedated, and the vein was slowly entered with a vacuum needle placed in the needle holder by slowly pressing the vena jugularis from the lateral side. Then, the blood was filled into a vacuum tube containing K3-EDTA in a controlled manner. Animal number, gender and date were written on the tubes from which approximately 10 mL of blood was collected and stored at -20°C until use.

DNA Isolation from Blood

DNA from blood samples was isolated using a commercial isolation kit (Applied Biological Materials Column-Pure Blood Genomic DNA Kit, Canada). The quantity and quality of the obtained DNA samples were checked with a NanoDrop 2000 (ThermoScientific, USA).

Microsatellite Markers Used in the Study

Fourteen microsatellite markers recommended by FAO were used in the study [15]. For use in capillary electrophoresis and fragment analysis, the forward primer of

Table 1. *Some information about the microsatellites used in thestudy*

Multiplex	Marker	Microsatellite	Primary Base Sequence		Allel Size Ranges
M1	D2	OarFCB20	F	AAATGTGTTTAAGATTCCATACAGTG	92-118
			R	GGAAAACCCCCATATATACCTATAC	
	D2	OarAE0129	F	AATCCAGTGTGTGAAAGACTAATCCAG	135-165
			R	GTAGATCAAGATATAGAATATTTTTCAACACC	
	D3	INRA0023	F	GAGTAGAGCTACAAGATAAACTTC	195-225
			R	TAACTACAGGGTGTTAGATGAACTC	
	D3	OARFCB193	F	TTCATCTCAGACTGGGATTCAGAAAGGC	96-136
			R	GCTTGGAAATAACCCTCCTGCATCCC	
	D4	INRA0132	F	AACATTTCAGCTGATGGTGGC	152-172
			R	TTCTGTTTTGAGTGGTAAGCTG	
	D4	D5S2	F	TACTCGTAGGGCAGGCTGCCTG	190-210
			R	GAGACCTCAGGGTTGGTGATCAG	
	D4	BM1818	F	AGCTGGGAATATAACCAAAGG	258-270
			R	AGTGCTTTCAAGGTCCATGC	
M2	D2	OARJMP29	F	GTATACACGTGGACACCGCTTTGTAC	96-150
			R	GAAGTGGCAAGATTCAGAGGGGAAG	
	D3	BM8125	F	CTCTATCTGTGGAAAAGGTGGG	110-130
			R	GGGGGTTAGACTTCAACATACG	
	D3	McM0527	F	GTCCATTGCCTCAAATCAATTC	165-179
			R	AAACCACTTGACTACTCCCCAA	
	D3	CSRD0247	F	GGACTTGCCAGAACTCTGCAAT	209-261
			R	CACTGTGGTTTGTATTAGTCAGG	
	D4	OARFCB128	F	ATTAAAGCATCTTCTCTTTATTTCCTCGC	96-130
			R	CAGCTGAGCAACTAAGACATACATGCG	
	D3	BM8125	F	AGTGCTTTCAAGGTCCATGC	110-130
			R	CTCTATCTGTGGAAAAGGTGGG	
	D4	HSC	F	CTGCCAATGCAGAGACACAAGA	267-301
			R	GTCTGTCTCCTGTCTTGTCATC	

each locus is labeled with a WELL-RED (D4, D3 or D2) fluorescentdye suitable for the Beckman CoulterGe XP Genetic Analysis System. Detailed information about the microsatellites used and the fluorescent dyes used in marking are given in *Table 1*.

DNA Amplificationby PCR

In the PCR stage, 0.2 mL thin-walled Eppendorf tubes were used to amplify the primer-specific regions. 10X PCR Buffer, $MgCl_2$, dNTP mix (dATP, dTTP, dGTP, dCTP), 18 fluorescently labeled microsatellite markers used in the study (Sigma, Interlab), Taq DNA Polymerase Enzyme, ~100 ng Genomic DNA and sterile PCR mix containing ddH_2O was created. In this study, the touch-down (TD) PCR technique was applied to perform DNA replication more effectively and quickly. Optimization of this PCR method is accomplished by focusing on annealing (heat of adhesion) rather than buffers used and cycling conditions. TD-PCR is widely used in studies with markers with unknown annealing temperatures. In this method, annealing degrees are arranged to change sequentially during the course of a single cycle program. Since the ratio of the target sequence that begins to be amplified in the template population increases, only the target sequence increases at decreasing temperature [23].

The PCR programs specific to the multiplex groups used for amplification of the DNA regions specific to the primers in the thermal converter are summarized in *Table 2*.

Statistical Analysis

Allele counts (Na), mean allele count (MNa), effective allele number (Ne), polymorphic information content (PIC),

Table 2. *Thermalcycler conditions according to the touchdown PCR method*

Multiplex Group	I. Denaturation	II. Denaturation	Annealing	Extension	Cycle	Final Extention
1	95°C (5 min)	95°C (40sec)	63-54°C (40 sec)	72°C (60 sec)	40	72°C (10 min)
2	95°C (5 min)	95°C (40sec)	60-50°C (40 sec)	72°C (60 sec)	34	72°C (10 min)

observed heterozygosity (Ho), expected heterozygosity (He), compliance with Hardy-Weinberg equilibrium, Wright's F- statistics (F_{IS}) [24,25] and null allele frequencies GenAlEx [26,27], POPGENE [28] and CERVUS 3.0.3 [29,30] were calculated using the programs.

The population structure was tested in the STRUCTURE program [31,32] using the clustering technique based on the Bayesian approach. In STRUCTURE analyses using independent allele frequencies and admixture model, the length value was taken as 20.000 and the Markov Chain Monte Carlo iteration number as 100.000, and the analysis was performed with 20 replications at different K values (K=2-5). The CLUMPAK [33,34] program was used to generate alignment charts from the obtained STRUCTURE results. The most appropriate cluster (cluster-K) value from the findings obtained as a result of the analysis was determined by considering the method (ΔK= m|L''(K)|/s[L(K)]) reported by Evanno et al.[35]. The STRUCTURE HARVESTER program was used to determine the cluster-K value [36]. To reveal the status of the populations in terms of genetic bottlenecks, the data set was tested using IAM (InfiniteAllel Model), SMM (Stepwise Mutation Model) and TPM (TwoPhase Mutation Model) in the Bottleneck 1.2.0.2 program [37], using Sign, Standardized and Wilcoxon tests and 1000 simulations.

Results

In this study, 290 alleles belonging to 14 microsatellite loci from the Karacabey Merino sheep breed were determined. Molecular genetic polymorphism statistics are presented in *Table 3*.

The highest number of alleles was obtained from HSC(28), and the lowest number of alleles was obtained from INRA0132(14). The overall mean of the observed heterozygosity value (0.89) was lower than the expected heterozygosity value (0.91).

The PIC values of microsatellite markers greater than 0.5 and the number of alleles greater than 4 in genetic diversity studies are an indication that these markers can be used in population genetic analyses [38,39]. The lowest PIC value obtained from the markers used in the present study was obtained from INRA0132 (0.86), and the highest was obtained from McM0527 and HSC (0.93). According to the results obtained, the average allele numbers and PIC values show that the studied breeds have high genetic diversity.

Table 3. *Polymorphism statistics of microsatellite loci*

Locus	N	Na	Ne	Ho	He	PIC	F_{IS}	HWE	F(Null)
OarFCB193	108	22	12.01	0.89	0.92	0.91	0.035	ns	0.0148
INRA0023	106	17	10.09	0.87	0.90	0.89	0.041	ns	0.0188
OarFCB20	106	21	13.03	0.93	0.92	0.92	-0.007	*	-0.0057
BM1818	106	19	10.91	0.82	0.91	0.90	0.101	ns	0.0508
INRA0132	107	14	8.00	0.84	0.87	0.86	0.043	ns	0.0171
OARAE129	108	18	10.37	0.94	0.90	0.90	-0.030	ns	-0.0158
D5S2	108	15	10.31	0.93	0.90	0.90	-0.021	***	-0.0136
CSRD0247	107	25	13.38	0.85	0.93	0.92	0.086	ns	0.0423
McM0527	106	22	14.11	0.85	0.93	0.93	0.091	ns	0.0457
HSC	106	28	14.85	0.88	0.93	0.93	0.064	ns	0.0306
OarFCB128	105	20	11.82	0.88	0.92	0.91	0.048	ns	0.0216
OarJMP29	107	26	11.82	0.88	0.92	0.91	0.045	ns	0.0214
MAF214	107	25	10.76	0.89	0.91	0.90	0.026	ns	0.0107
BM8125	108	18	10.33	0.98	0.90	0.90	-0.082	***	-0.0433
Mean		20.71	11.56	0.89	0.91	0.90	0.032		

Na:*Number of alleles,* ***Ne:****Effective number of alleles,* ***PIC:****Polymorphic information content,* ***Ho:****Observed heterozygosity,* ***He:****Expected heterozygosity,* F_{IS}*:Wright's F-statistics (According to Wright's statistics to Weir and Cockerham*[25]*,* ***HWE:****Hardy-Weinberg equilibrium,* ***F (Null):*** *Null allele frequency* P<0.05, ** P<0.01, *** P<0.001*

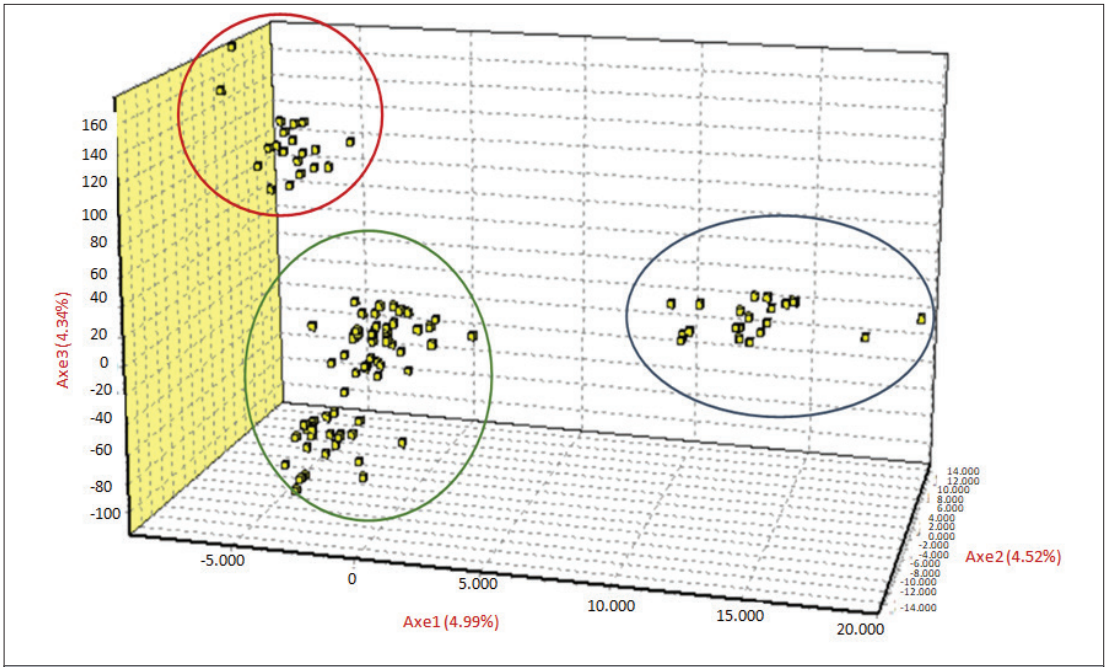

Fig 1. Graph of factorial relationship analysis (FCA) of individuals in the study

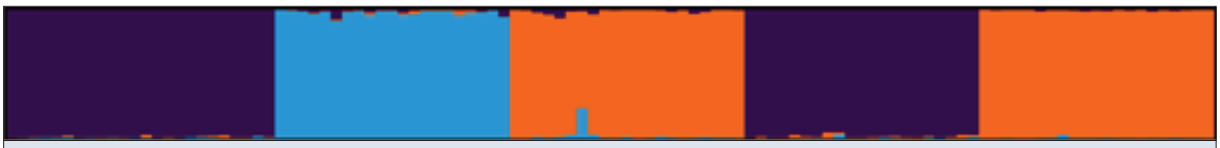

Fig 2. Structure test analysisresults in the Karacabey Merinopopulation (K=4)

F_{IS} values, which are a measure of the deviations of genotypic frequencies from Panmixia in populations, are a parameter used to determine heterozygous deficiency or excess. The overall F_{IS} value calculated for all populations in the study was 0.032. F_{IS} values, which are of great importance in terms of defining the population structure and determining heterozygosity losses in the study, varied between 0.082 and 0.101. When the general average of the F_{IS} value, which is defined as the inbreeding coefficient, is examined, it can be said that there is no loss of heterozygosity in the population. In the chi-square test, it was determined that allele distributions of 11 loci, excluding 3 microsatellite loci, did not statistically deviate from Hardy-Weinberg equilibrium.

The graph of the factorial relationship analysis (FCA) of the individuals included in the study is given in *Fig. 1*, and the STRUCTURE analysis results containing different clustering numbers (K=2-4) are given in *Fig. 2*.

The results of the factorial relationship analysis (FCA) indicate that there are 3 different groups in the studied population. Similarly, the results obtained for the STRUCTURE analysis are in agreement with the FCA results, as expected.

Findings including the estimation of posterior probabilities ([Ln Pr(X|K)]) for clustering numbers (K) and ΔK values are presented in *Table 4*.

Table 4. *Estimated posterior probabilities [Ln Pr(X|K)] and ΔK statistics*

K	[Ln Pr(X|K)]	ΔK
2	-7436.1250	-
3	-6976.8750	3.1499
4	-6616.4150	-

Particularly in the STRUCTURE analysis, it was observed that the studied populations were partially intertwined [36]. It is noteworthy that the most appropriate number of groups was 3 *(Table 4)*.

Genetic bottlenecks were investigated using the Infinite Allele Model (IAM), Stepwise Mutation Model (SMM), and Two Phase Model of Mutation (TPM) [37,40,41]. These three distinct mutation models were examined using the obtained data set *(Table 5)*.

To identify potential bottlenecks in the studied population, Mod-shift plots were obtained using allele frequency classes of 14 microsatellite loci *(Fig. 3)*. An L-shaped graph consistent with the distribution ranges of the normal frequency class is obtained from the mod-shift plot.

Table 5. *Test results according to three different mutation models forbottleneck analysis*

Mutation Model	Sign Test			Standardized Differences Test		Wilcoxon Rank Test (One Tail for H Excess)
	Hee	He	P	T2	P	P
IAM	16.44	19	0.20937	2.267	0.01168	0.00480
TPM	16.01	9	0.00577	-4.577	0.00000	0.99131
SMM	15.80	3	0.00000	-17.356	0.00000	1.00000

IAM: *The infinite allele model,* ***TPM:*** *Two-phase model,* ***SMM:*** *The stepwise mutation model,* ***Hee:*** *Expected number of loci with heterozygosity excess,* ***He:*** *heterozygosity excess*

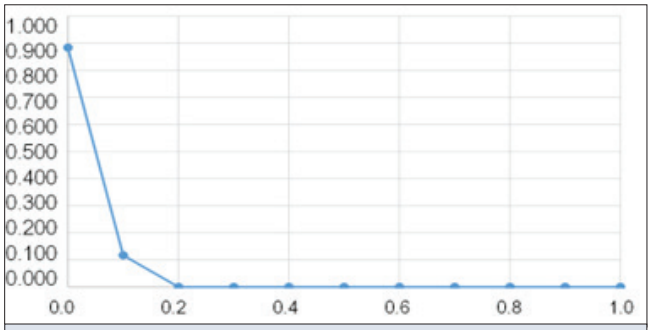

Fig 3. Mode-shift plot for bottleneck analysis in the studied population

Discussion

The variation between changes in allele number and heterozygosity has been used as a basis for statistical testing in identifying recent genetic bottlenecks in the population [37]. The PIC value indicates the probability of the presence or absence of that marker in two randomly selected individuals in a population [16]. Accordingly, the PIC value ranges from 0 to 1 and should be 0.50 for genetic diversity. Bostein et al.[42] classified the PIC value as highly informative (PIC >0.5), reasonably informative (0.5> PIC >0.25), and slightly informative (PIC <0.25). The PIC value close to 1 is desirable for high genetic diversity in that population [42]. Considering that the PIC values of microsatellite markers must be greater than 0.5 and the number of alleles must be greater than 4 to discuss genetic diversity, the results obtained in the study are quite high. As a matter of fact, as a result of the findings in the study, it is seen that the PIC value varies between 0.86-0.93 and its average is 0.90, and the average number of alleles varies between 14-28 and the average is 20.71. Considering the average allele numbers and PIC values, the population has high genetic diversity. The Na, PIC, Ho, and He values obtained in the aforementioned population were observed to be higher than the values obtained in previous similar studies on Turkish native breeds and foreign breeds [12,43-45]. High PIC value and allele number indicate that the studied population has high genetic diversity and no genetic bottleneck. It can be said that genetic diversity will continue in the future unless there are situations that will reduce genetic diversity, such as inbreeding.

While the overall F_{IS} value obtained in the study [44] was observed to be low, some studies [16,46-50] reported higher values. Although it is considered that these results are due to the difference in the number of microsatellites used in the relevant studies and the ampling methodology, attention should be paid to the loss of heterozygosity that may occur in existing populations. It was reported by Dakin and Avise [49] that null allele frequencies below 0.20 do not have a significant effect on paternity tests and determination of genetic diversity. When an allele is not oxidized by polymerase chain reaction (PCR) in heterozygous individuals, but only one allele gives a peak such as homozygous and thus causes erroneous reading, it is noteworthy that all studied loci are lower than the specified value. This indicates that the loci used in the study can be used safely.

Infinite allele models (IAMs) and stepwise mutation models (SMMs) are known to cause inconsistent results in studies using microsatellites. Therefore, the two-phase mutation model (TPM) has been reported to be the most useful model for testing heterozygous excess in bottleneck tests with microsatellites [37,41,50]. On the otherhand, it has been reported that the Wilcoxon test can be used with high confidence even in studies using a limited number of loci (<20) for bottleneck analysis [45] In the sheep population that is the subject of the research, Wilcoxon test results, which were carried out considering the TPM model, indicate that serious demographic bottlenecks do not occur.

First, Luikart et al.[41], the L-shaped graph obtained from the mode-shift plot, which graphically shows the allele frequency distribution, which is widely used in the detection of bottlenecks, shows that no genetic bottlenecks have occurred in the studied population in the recent past.

One of the limitations of this study may be the determination of the number of sheep used within the scope of the Project possibilities. However, this situation can be ignored as the situation in a single sheep farm is tried to be determined in the study.

In conclusion molecular genetic studies to identify the variation within and between populations of sheep breeds have been ongoing for a long time. Determining the relationships of individuals with each other in revealing the kinship within the same herd is very important for a healthy selection practice. In this context, this study makes important contributions to the literature. The findings obtained in the study show that the microsatellite markers used are polymorphic and can be used successfully in genetic diversity studies. The bottleneck test was used

to determine whether there was any genetic bottleneck danger as a result of increased inbreeding levels in the studied population. The findings indicated that the population did not enter any bottleneck in the recent past. As a result, the findings obtained from this study clearly revealed that the microsatellites used can be used safely in the identification of genetic diversity and detection of genetic bottlenecks in the studied Karacabey Merino population. At the same time, it can be said that the microsatellite markers used in the study can be used safely in future studies.

Availability of Data andMaterials

Data sets are not deposited in different repositories, and data from a third party were not used. The data are original, and users can get it from corresponding author (A. Kabasakal).

Acknowledgments

I would like to thank the Adnan Menderes University Agricultural Biotechnology and Food Safety Application and Research Center (ADUTARBİYOMER) for providing laboratory facilities for molecular genetic analysis. I would like to thank Assoc. Prof. Dr. Onur YILMAZ for performing the statistic alanalysis.

Funding Support

This work was supported by the Scientific Research Projects Coordination Unit of Bandırma Onyedi Eylül University University. Project Number: BAP-21-1003-010.

CompetingInterest

The author reports no declarations of interest.

Ethical Statement

The study was conducted with the permission of the Balikesir University Animal Experiments Local Ethics Committee dated 27.10.2021 and numbered 2019/9-5.

References

1. Ertugrul M, Dellal G, Elmacı C, Soysal İ, Akın O, Arat S, Barıtçı İ, Pehlivan E, Yılmaz O: Türkiye yerli ırklarının korunması. *J Agric Fac Uludag Univ,* 23 (2): 97-119, 2009.

2. Anonymous: Onuncu Kalkınma Planı (2014-2018) Hayvancılık Özel İhtisas Komisyon Raporu. Yayın No: KB: 2873 - ÖİK: 723, s.46-67, Ankara, 2014.

3. Parmaksız A, Oymak A, Yüncü E, Demirci S, Koban Boştanlar E, Özkan Ünal E, Togan İ, Özer F: Y-chromosome polymorphisms in 12 native, Karagül, Karacabey Merino breeds from Turkey and Anatolian mouflon *(Ovis gmelinii anatolica), Kafkas UnivVet Fak Derg,* 24 (6): 821-828, 2018. DOI: 10.9775/kvfd.2018.19962

4. Banks SC, Cary GJ, Smith AL, Davies ID, Driscoll DA, Gill AM, Lindenmayer D B, Peakall R: How does ecological disturbance influence genetic diversity? *Trends Ecol Evol,* 28 (11): 670-679, 2013.DOI: 10.1016/j.tree.2013.08.005

5. Barbosa S, Andrews KR, Harris RB, Gour DS, Adams JR, Cassirer EF, Miyasaki HM, Schwantje HM, Waıts LP: Genetic diversity and divergence among Bighorn sheep from reintroduced herds in Washington and Idaho. *J Wildl Manag,* 85 (6): 1214-1231, 2021. DOI: 10.1002/jwmg.22065

6. Gürler Ş, Bozkaya F: Geneticdiversity of threenativegoatpopulationsraised in the South-Easternregion of Turkey. *Kafkas UnivVet Fak Derg,* 19 (2): 207-213, 2013. DOI: 10.9775/kvfd.2012.7475

7. Rafter P, McHugh N, Pabiou T, BerryI: Inbreeding trends and genetic diversity in purebred sheep populations. *Anim*, 16 (8):1000604, 2022. DOI: 10.1016/j.animal.2022.100604

8. Flesch E, Graves T, Thomson J, Proffitt K, Garrott R: Average kinship within bighorn sheep populations is associated with connectivity, augmentation, and bottlenecks. *Ecosphere,* 13 (3):e3972, 2022. DOI: 10.1002/ecs2.3972

9. AddoI S, Klingel S, Thaller G, Hinrichs D: Genetic diversity and the application of runs of homozygosity-based methods for inbreeding estimation in German White-headed Mutton sheep. *PLoSOne,* 16 (5):e0250608, 2021. DOI: 10.1371/journal.pone.0250608

10. Machova K, Milerski M, Rycht J, Hofmanov B, Vostra-VydrovH, MoravcíkovN, Kasarda R, Vostrý L: Assessment of the genetic diversity of two Czech autochthonous sheep breeds. *Small Ruminant Res,* 195:106301, 2021. DOI: 10.1016/j.smallrumres.2020.106301

11. SajidAlia S, Kuralkara SV, Dasb R, Rainab V, Katariac RS, Vohrab V: Assessmentof genetic diversity and bottleneck in Purnathadi buffaloes using short tandem repeat markers. *Anim Biotechnol,* 32 (4): 495-506, 2021. DOI: 10.1080/10495398.2020.1724126

12. Selvam R andKathiravan P: Genetic diversity and bottleneck analysis of sheep based on microsatellite markers. *Indian J Small Rumin,* 25 (1): 13-18, 2019. DOI: 10.5958/0973-9718.2019.00012.6

13. Alnajm H, Alijani S, Javanmard A, Rafat SA, Hasanpur K: Genetic diversity analysis of four sheep breeds of Iran: Towards genetic maintenance and conservation decision. *Iran J Appl Anim Sci,* 11 (3): 527-538, 2021.

14. Cemal İ, Yilmaz O, Karaca O, Binbaş P, Ata N: Analysis of genetic diversity in indigenous Çine Çaparı sheep under conservation by microsatellite markers. *Kafkas UnivVet Fak Derg,* 19 (3): 383-390. 2013. DOI: 10.9775/kvfd.2012.7857

15. FAO: Molecular Genetic Characterization of Animal Genetic Resources. Food and Agricultural Organization of the United Nations; Rome, Italy, 2011.

16. Serrote CML, Reiniger LRS, Silva KB, Rabaiolli SMS, Stefanel CM: Determining the polymorphism ınformation content of a molecular marker. *Gene,* 726:144175, 2020, DOI: 10.1016/j.gene.2019.144175

17. Yılmaz O, Cemal İ, Coşkun B, Oğrak YZ, Ata N: Comparison of different paternity test panels in sheep. *Turk J Vet Anim Sci,* 42 (6): 633-641, 2018. DOI: 10.3906/vet-1805-80

18. Togan İ, Soysal İ, Berkman CC, Koban E: Irkların korunmasında moleküler işaretler. *Tekirdağ Ziraat Fak Derg*, 2 (1): 44-49, 2005.

19. Schlötterer C: The evolution of molecular markers - Just a matter of fashion? *Nat Rev Genet,* 5 (1): 63-69, 2004. DOI: 10.1038/nrg1249

20. Lasagna E, Bianchi M, Ceccobelli S, Landi V, Martínezm AM, Pla, JLV, Panella F, Bermejo JVD, Sarti FM: Genetic relationships and population structure in three Italian Merino-derived sheep breeds. *Small Rumantin Res,* 96 (2-3): 111-119, 2011. DOI: 10.1016/j.smallrumres.2010.11.014

21. Yilmaz O, Karaca O: Paternity analysis with microsatellite markers in Karya sheep. *Kafkas Univ Vet Fak Derg,* 18 (5): 807-813, 2012. DOI: 10.9775/kvfd.2012.6512

22. Akay N, Canatan T, Yılmaz O, Ata N, Karaca O, Cemal İ: Genetic diversity and bottleneck analysis of endangered Güney Karaman sheep. *J Anim Sci Prod,* 3 (2): 143-154, 2020.

23. Hecker KH, Roux KH: High and low annealing temperatures increase both specifity and yield in touchdown and stepdown PCR. *Biotechniques,* 20 (3): 478-485, 1996. DOI: 10.2144/19962003478

24. Wright S: Evolution in Mendelian populations. *Genetics,* 16 (2): 97-159, 1931. DOI: 10.1093/genetics/16.2.97

25. Weir BS, Cockerham CC: Estimating F-statistics for the analysis of population-structure. *Evol*, 38 (6): 1358-1370, 1984. DOI: 10.2307/2408641

26. Peakall R, Smouse PE: GENALEX 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Mol Ecol Notes,* 6, 288-295, 2006. DOI: 10.1111/j.1471-8286.2005.01155.x

27. Peakall R, Smouse PE: GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research -An update. *Bioinformatics,* 28, 2537-2539, 2012. DOI: 10.1111/j.1471-8286.2005.01155.x

28. Yeh FC, Yang RC, Boyle TBJ, Ye ZH, Mao JX: POPGENE: The User-

Friendly Shareware for Population Genetic Analysis. Edmonton, AB, Canada: University of Alberta; 1997.

29. Marshall TC: Cervus, 3.0, Cervus is a computer program for assignment of parents to their offspring using genetic markers. Cervus, a Windows package for parentage analysis using likelihood approach. CERVUS was written by Tristan Marshall (1998/2006). http://www.fieldgenetics.com; *Accessed:* 02.07.2008.

30. Kalinowski ST, Taper ML, Marshall TC: Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol Ecol,* 16, 1099-1106, 2007. DOI: 10.1111/j.1365-294X.2007.03089.x

31. Pritchard JK, Stephens M, Donnelly P: Inference of population structure using multilocus genotype data. *Genetics,* 155 (2): 945-959, 2000. DOI: 10.1093/genetics/155.2.945

32. Falush D, Stephens M, Pritchard JK: Inference of population structure using multi locus genotype data: Dominant markers and null alleles. *Mol Ecol Notes,* 7, 574-578, 2007. DOI: 10.1111/j.1471-8286.2007.01758.x

33. Hubisz MJ, Falush D, Stephens M, Pritchard JK: Inferring weak population structure with the assistance of sample group information. *Mol Ecol Resour,* 9, 1322-1332, 2009. DOI: 10.1111/j.1755-0998.2009.02591.x

34. Kopelman NM, Mayzel J, Jakobsson M, Rosenberg NA, Mayrose I: CLUMPAK: A program for identifying clustering modes and packaging population structure inferences across K. *Mol Ecol Res,* 15 (5): 1179-1191, 2015. DOI: 10.1111/1755-0998.12387

35. Evanno G, Regnaut S, Goudet J: Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study. *Mol Ecol,* 14, 2611-2620, 2005. DOI: 10.1111/j.1365-294X.2005.02553.x

36. Earl D A, vonHoldt BM: STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv Genet Resour,* 4 (2): 359-361, 2012. DOI: 10.1007/s12686-011-9548-7

37. Piry S, Luikart G, Cornuet JM: BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. *J Hered,* 90 (4): 502-503, 1999. DOI: 10.1093/jhered/90.4.502

38. Fatima S, Bhong CD, Rank DN, Joshi CG: Genetic variability and bottleneck studies in Zalawadi, Gohilwadi and Surti goat breeds of Gujarat (India) using microsatellites. *Small Ruminant Res,* 77 (1): 58-64,2008. DOI: 10.1016/j.smallrumres.2008.01.009

39. Korkmaz Ağaoğlu Ö, Ertuğrul O: Mikrosatellitbelirteçleri ile darboğaz (Bottleneck) testi. *Erciyes ÜnivVet Fak Derg,* 8 (3): 187-192, 2011.

40. Cornuet JM, Luikart G: Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics,* 144 (4): 2001-2014, 1996. DOI: 10.1093/genetics/144.4.2001

41. Luikart G, Cornuet JM: Empiricale valuation of a test for identifying recently bottlenecked populations from allele frequency data. *Conserv Biol,* 12 (1): 228-237, 1998. DOI : 10.1111/j.1523-1739.1998.96388.x

42. Botstein D, White RL, Skolnick M, Davis RW: Construction of a genetic linkage map in manusing restriction fragment length polymorphisms. *Am J Hum Genet,* 32, 314-331, 1980.

43. Peter C, Bruford M, Perez T, Dalamitra S, Hewitt G, Erhardt G: Genetic diversity and subdivision of 57 European and Middle-Eastern sheep breeds. *Anim Genet,* 38 (1): 37-44, 2007. DOI: 10.1111/j.1365-2052.2007.01561.x

44. Kırıkcı K, Cam MA, Mercan L: Genetic diversity and relationship among indigenous Turkish Karayaka sheep subpopulations. *Arch Anim Breed,* 63 (2): 269-275, 2020. DOI: 10.5194/aab-63-269-2020

45. Kurar E, Bulut Z, Çağlayan T, Garip M, Yılmaz A, Nizamlıoğlu M: Kangal Akkaraman koçlarında genetik çeşitlilik ve ebeveyn testinin uygulanabilirliğinin mikrosatellit belirteçler kullanılarak araştırılması. *Kafkas Univ Vet Fak Derg,* 18 (6): 973-977, 2012. DOI: 10.9775/kvfd.2012.6879

46. Yılmaz O, Cemal İ, Ata N, Karaca O: Genetic diversity and bottleneck analysis of three different sheep breeds in Turkey. *International Conference on Scienceand Technology ICONST 2018,* 5-9 September, Prizren, KOSOVO, 2018.

47. Loukovitis DA, SiasiouI, Mitsopoulos AG, Lymberopoulos Laga V, Chatziplis D: Genetic diversity of Greek sheep breeds and trans humant populations utilizing microsatellite markers. *Small Ruminant Res,* 136, 238-242, 2016. DOI: 10.1016/j.smallrumres.2016.02.008

48. Öner Y, Üstüner H, Orman A, Yılmaz O, Yılmaz A: Genetic diversity of Kıvırcık sheep breed reared in different regions and its relationship with other sheep breeds in Turkey. *Ital J Anim Sci,* 13 (3): 588-593, 2014. DOI: 10.4081/ijas.2014.3382

49. Dakin EE, Avise JC: Microsatellite null alleles in parentage analysis. *Heredity,* 93, 504-509, 2004. DOI: 10.1038/sj.hdy.6800545

50. Dirienzo A, Peterson AC, Garza JC, Valdes AM, Slatkin M, Freimer NB: Mutational processes of simple-sequence repeat loci in human-populations. *Proc Natl Acad Sci,* 91 (8): 3166-3170, 1994. DOI: 10.1073/pnas.91.8.3166

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 239-246, 2023
DOI: 10.9775/kvfd.2023.29132

RESEARCH ARTICLE

Evaluation of Antimicrobial and Antibiofilm Efficacy of Bee Venom and Exosome Against *Escherichia coli* K99 Strain

Demet CELEBI [1 (*)] Ozgur CELEBI [2] Sumeyye BASER [2] Ali TAGHIZADEHGHALEHJOUGHI [3]

[1] Ataturk University, Faculty of Veterinary Medicine, Department of Microbiology, TR-25240 Erzurum - TÜRKİYE
[2] Ataturk University, Faculty of Medicine, Department of Medical Microbiology, TR-25240 Erzurum - TÜRKİYE
[3] University of Seyh Edebali, Faculty of Medicine, Department of Pharmacology, TR-11000 Bilecik - TÜRKİYE

ORCIDs: D.C. 0000-0002-2355-0561; O.C. 0000-0003-4578-9474; S.B. 0000-0003-2391-8191; A.T. 0000-0002-3506-0324

Article ID: KVFD-2023-29132 Received: 01.02.2023 Accepted: 28.04.2023 Published Online: 03.05.2023

Abstract: *Escherichia coli* K99 (F5) strain is one of the bacterial agents that cause calf deaths. F5 is an adhesin that allows pathogenic *E. coli* attach to the small intestine cells and colonize there. The presence of F5 in *E. coli* strains in isolated bacteria is classified as Enterotoxigenic. Bee venom and bee venom-derived exosomes are bioactive compounds that exhibit antimicrobial and antibiofilm activity. The aim of this study is to demonstrate the antimicrobial and antibiofilm activity of bee venom and bee venom-derived exosomes against *E. coli*, which cause calf diarrhea. Bee venom-derived exosomes and bee venom effects against *E. coli* strains were determined by using Minimal inhibition concentration (MIC), antibiofilm activity, fractional inhibition concentrations (FIC), and measurement of L929 cells viability ratio. Cell damage was examined under a fluorescent microscope by an immunohistochemical method. In our study, the MIC value of the bee venom-derived exosome was determined as 1.95 µg/mL. A synergistic effect was detected with a value of 0.44 in combinations of amoxicillin with clavulanic acid. Antibiofilm activity was determined at the rate of 48.8% in bee venom, while bee venom-derived exosomes inhibited the biofilm layer by 60.4%. In L929 cell lines, combination groups have been reported to reduce viability. Bee venom-derived exosomes are more effective on bacteria than pure bee venom. In conclusion; It is important that the bee venom-derived exosome, which is a biocompatible molecule and acts as a cargo element, exhibits antimicrobial and especially antibiofilm activity and is an alternative approach against increasing antibiotic resistance.

Keywords: *Antibacterial activity, Antibiofilm activity, Escherichia coli, Exosome, Fractional inhibition concentration, Synergistic effect*

Escherichia coli K99 Suşuna Karşı Arı Zehiri ve Arı Zehrinden İzole Edilen Eksozomun Antimikrobiyal ve Antibiyofilm Etkinliğinin Değerlendirilmesi

Öz: *Escherichia coli* K99 (F5) suşu buzağı ölümlerine sebep olan bakteriyal etkenlerden biridir. F5, patojenik *E. coli* suşlarının bağırsak hücrelerine yapışmasını ve ince bağırsağı kolonize etmesini sağlayan bir adezindir. F5'in varlığı, bakteri izolatının Enterotoksijenik *E. coli* olarak sınıflandırılmasını sağlar. Arı zehiri ve arı zehrinden izole edilen eksozomlar, antimikrobiyal ve antibiyofilm aktivite sergileyen bioaktif bileşiklerdir. Bu çalışmanın amacı buzağı ishaline sebep olan *E. coli*,'ye karşı arı zehiri ve arı zehrinden izole edilen eksozomların antimikrobiyal ve antibiyofilm aktivitesini ortaya koymaktır. *E. coli* suşlarına karşı hem eksozom hem de arı zehirinin minimum inhibisyon konsantrasyonu (MIC), antibiyofilm aktivitesi ve fraksiyonel inhibisyon konsantrasyonları (FIC) ve L929 hücrelerinde canlılık oranları belirlendi. İmmünohistokimyasal olarak hücre hasarı floresan mikroskop altında incelendi. Çalışmamızda arı zehirinden izole edilen eksozomların MIC değeri 1.95 µg/mL olarak tespit edildi. Amoksisilin klavulonik asit ile yapılan kombinasyonlarda 0.44 değer ile sinerjik etki tespit edildi. Antibiyofilm aktivitesi arı zehrinde %48.8 oranında belirlenirken arı zehri eksozomu % 60.4 oranında biyofilm tabakasını inhibe ettiği tespit edildi. L929 hücre hatlarında kombinasyon grupların canlılık oranını düşürdüğü rapor edildi. Arı zehri eksozomları arı zehrinden daha fazla bakteriler üzerinde etkili olmaktadır. Sonuç olarak; biyouyumlu molekül olan ve kargo elemanı olarak görev yapan arı zehiri eksozomunun antimikrobiyal ve özellikle antibiyofilm aktivite sergilemesi artan antibiyotik direncine karşı alternatif bir yaklaşım olması önem arz etmektedir.

Anahtar sözcükler: *Antibakteriyel aktivite, Antibiyofilm aktivitesi, Eksozom, Escherichia coli, Fraksiyonel inhibisyon konsantrasyonu, Sinerjistik etki*

How to cite this article?

Celebi D, Celebi O, Baser S, Taghizadehghalehjoughi A: Evaluation of antimicrobial and antibiofilm efficacy of bee venom and exosome against *Escherichia coli* K99 strain. *Kafkas Univ Vet Fak Derg,* 29 (3): 239-246, 2023.
DOI: 10.9775/kvfd.2023.29132

(*) Corresponding author: Demet CELEBI
Phone: +90 532 666 6938
E-mail: celebiidil@atauni.edu.tr

Introduction

Resistance to antimicrobials has reached the level of red alert all over the world [1]. Although humanity won a great victory against microorganisms in the early days of the struggle that started with the discovery of antibiotics, this victory gained a great momentum towards defeat with factors such as resistance developed by microorganisms and internal mutations [2]. Unconscious, and excessive use of antibiotics, leads to appear resistance of microorganisms. Biofilm prevented antimicrobial drugs from penetrating at an effective dose [1,2]. This is how they managed to stay alive. In addition, they allowed the spread of mutant strains with their biofilm properties, which are a silent communication community [1,2]. Multi-drug resistance struggle, which threatens human health, has recently started to be seen as a major threat in terms of animal health. Among these factors, *Eschericia coli* strains are among the effective pathogens in the transfer of antimicrobial resistance genes in cattle and milk *E. coli* strains, which cause calf deaths in particular [3,4]. Resistance to β-lactams and fluoroquinolones, which are widely used in human and veterinary fields, causes alternative searches. The resistance to the carbapenem antibiotics in human medicine reveals the importance of its precautions once again. In addition, reasons such as increased resistance with mutant strains and the inadequacy of available antimicrobials led to the search for new antimicrobial candidates with the same mode of action. Among the candidates in these searches, many bee products rich in bioactive compounds were also of interest. Data showing that bee venom (BV) and other natural products exhibit remarkable activity against various diseases have taken their place in the literature [3-6]. Bee venom, called apitoxin, has been used in the treatment of arthritis, rheumatism, pain, cancer, skin diseases and in the field of traditional medicine. Studies have shown that it has anti-inflammatory, antimicrobial and antioxidant activities [7-11]. The peptides determined in the bee venom have antimicrobial activity against some gram-negative and gram-positive bacteria. It has been determined that the bee venom has a synergistic effect, especially in studies conducted with combinations with antimicrobials. The synergy that exists in poison combinations with antibiotics such as vancomycin and amikacin is promising [5,12,13]. Bee venom contains biologically active amines, enzymes, peptides, and non-peptide components. 50% of the dry weight of bee venom is a peptide component called melittin. Melittin is a characteristic component, especially with its strong cytotoxic properties and antimicrobial activity [14]. Exosomes with a double lipid layer and nanoscale membrane vesicles are involved in intercellular signal trafficking involving protein regulation mechanisms. They are secreted from almost all cells and have the characteristics of the cell of origin [15,16]. They were detected in biological fluids, isolated from cell culture media, and have cell-specific cargo properties [17-19]. The cargo molecules in them are composed of lipids, protein, DNA, mRNA, miRNA, and sRNA (small RNA) [19]. In addition to all these cargo elements, exosomes also contain tetraspanins, which play an important role in cell penetration and fusion [20]. Thanks to all these cargo elements that mediate signalling to recipient cells or tissues, exosomes are promising to become a biomarker and therapeutic tool in the treatment of cancer and pathogens with their role in intercellular signalling, cell-cell communication, immune responses, cellular homeostasis and autophagy. Recently, in addition to mortality rates due to various cancer types, the high mortality rates caused by microorganisms with multidrug resistance make it necessary to develop new treatment methods urgently. At this point, more research is needed on the mechanisms of action of exosomes in order to use them as biomarkers in the diagnosis, prognosis, and surveillance of multidrug pathogens. In addition, the antimicrobial properties and carrier capacities of the vesicles need to be determined in order to use drug-delivery vesicles without undesirable side effects. Based on all these concerns and information, we aimed to determine the antimicrobial activity of bee venom and exosome and to examine its antibiofilm ability. We planned to examine the synergistic effects due to the Fractional Inhibitory Concentration Index-Combination FIC indices with antibiotics approved by EUCAST. We designed to investigate the MTT values formed in the cells according to the synergy concentrations and cell damage immunohistochemically.

Material and Methods

Ethical Approval

Since the *E. coli* F82 (O101:K-:F5(K99)+) bacterial strain used in our study is the reference strain, ethical approval is not required.

Bacterial Strain Production

Bacteria to small intestinal epithelial cells K99 (F5) fimbrial antigen in classical Enterotoxigenic *E. coli* isolates isolated from calves is the most commonly detected antigenic structure. *E. coli* F82 (O101:K-:F5(K99)$^{+}$) strain was stored in trypticase soy agar at room temperature. Standard bacteriological methods were used to isolate and identify the *E.coli* strain. *E. coli* strain was inoculated into Eosin Methylene Blue (EMB) medium and incubated at 37°C for 24 h. Then, 10^{8} CFU/mL suspension was prepared from the growing colonies according to McFarland 0.5 chart.

MIC Values

MIC values of bee venom and bee venom-derived

exosome compounds against *E. coli* were determined using microdilution method. Bee venom and bee venom-derived exosome were traditionally determined in triplicate by the microdilution broth method. Serial dilutions of both bee venom and bee venom-derived exosome were prepared in microdilution at concentrations ranging from 1028-32 µg/mL. Bacterial colonies prepared according to McFarland 0.5 scale (10^8 CFU/mL) with serial dilutions were inoculated into all wells as 100 µL. Then, 100 µL of Mueller Hinton Broth (MHB) (MilliporeSigma) medium and a bee venom and exosome were added to the wells by dilution. The sample was incubated at 37°C for 24 h. MIC values were determined depending on the formation of agglutination [21].

Biofilm Analysis

The bacteria strain was incubated in MHB medium at 37°C for 18-24 h. Bacterial suspensions were prepared by standardizing them according to the McFarland 0.5 chart; 100 µL were added to the flat-bottomed wells and incubated at 37°C for 24 h. At the end of the incubation period, the wells were washed with distilled water and the cell residues associated with the biofilm were stained with 1% crystal violet (MilliporeSigma) for 37°C for 15 min. Biofilms observed in bacteria were photographed after the excess dye was washed off with water. To quantitatively determine biofilm formation, optical densities were measured on an ELISA reader (Biotek ELX800; BioTek Instruments, Inc.) at OD 570 - OD 630 nm. During the test, sterile TSB was used as a negative control [22]. After these procedures, bee venom and bee venom-derived exosome were added to each well and the antibiofilm activity was determined according to the formula below.

$$SBF = (AB-CW)/G$$

SBF: Specific biofilm formation; AB: Absorbance of 570 nm the attached end stained bacteria; CW: Absorbance of 570 nm of stained control wells containing only bacteria-free medium; G: Absorbance of 630 nm of cell growth in broth

Fractional Inhibitor Concentration Index-Combination (FIC)

When the in-vitro effectiveness of antibiotic combinations based on the Clinical and Laboratory Standards Institute (CLSI) and European Committee for Antimicrobial Susceptibility Test (EUCAST) standards are performed, if the effect is higher than the sum of the effect obtained when the same drugs are used alone, it is synergistic interaction, if it is equal to the sum, additive interaction. It is the test principle in which it is defined as indifference if the result obtained with one drug is equal, and as antagonism if it is lower than the effect of both drugs. Bee venom and bee venom-derived exosome with amoxicillin-clavulanic acid on bacterial strain checkerboard to test the effect of the combination (checkerboard) method was applied. This test is one of the synergy tests based on microdilution. Combination activity of antimicrobial agent has been tested on 96-well plate. 4xMIC and 1/32xMIC dilution was determined. First, each test tube was containing cation-regulated MHB. Graded dilutions from specified concentrations of the agent were prepared. Solutions of amoxicillin clavulanic acid plaque vertically, bee venom and bee venom-derived exosome were placed in the horizontal plane from right to left. Bacterial inoculum 0.5 McFarland ($1x10^8$ CFU/mL) in sterile 0.9% NaCl solution by standard density prepared. The final bacterial concentration in the wells 10 µL was added to each well at a rate of 5 $x10^5$ CFU/mL. Microdilution plates was incubate at 37°C for 24 h.

It was applied according to the FIC index formula used to determine the effectiveness of the combinations. And the results were determined according to the formula.

Calculation of the FIC index:

$$FIC\ A = \frac{\text{MIC numerical value of A in the presence of B}}{\text{MIC numerical value of A alone}}$$

$$FIC\ B = \frac{\text{MIC numerical value of B in the presence of A}}{\text{MIC numerical value of B alone}}$$

A: Antimicrobial 1 used in combination; B: Antimicrobial 2 used in combination

Σ FIC index = FIC A + FIC B

Σ FIC index ≤ 0.5: synergy

Σ FIC index >0.5 and <1: additive

Σ FIC index ≥1 and 4 ≤: ineffective (indifference)

Σ FIC index >4: antagonism

Bee Venom and Bee Venom-Derived Exosome Isolation

Bee venom New Techniques Laboratory Ltd. (Certificate No: 1543, Batch#1-5, Mtskheta Str. Tbilisi, 0149. Georgia). It was first centrifuged at 1000 g to remove debris. After the exosome isolation kit procedure (Total Exosome Isolation Reagent; Thermo Fisher; Massachusetts, USA) was applied, isolation was performed by centrifugation at 10.000 g for 30 min.

Scanning Electron Microscopes Analysis

The dimensions of the exosomes were evaluated by scanning electron microscopy (SEM) and images were taken under high vacuum and 20 kV EHT with the Carl ZeissEvo 40 SEM device (Jena, Germany).

Cell Culture

The L929 (CCL-1, ATCC) cell line was obtained from the medical pharmacology department of Bilecik Seyh Edebali University (Bilecik, Turkey). Briefly, the cell suspension was centrifuged at 1200 rpm for 5 min. Cells were resuspended in fresh medium (% Dulbeco-modified eagle medium (DMEM-F12), Fetal bovine serum (FBS) 10%, and antibiotic 1% (penicillin, streptomycin, and amphotericin B) and seeded in 25 cm^2 flask (Corning, USA) planted [21].

MTT Test

Control (cell medium only), *E. coli*, Amoxicilin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom-derived exosome) 1.95 µg/mL, Amoxicilin 4 mg/mL + *E. coli*, BV 62.5 µg For the determination of cytotoxicity of /mL + Amoxicilin 4 mg/mL + *E.coli*, BVE 1.95 µg/mL + Amoxicilin 4 mg/mL + *E. coli* groups, 'direct contact test method' will be applied, 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltatrazilium bromide containing MTT material (Sigma Aldrich inc, St.Louis, USA) will be evaluated. In order to determine the cytotoxicity with the MTT test, the mixture to be prepared with 5 mg of MTT powder in 1 mL of PBS will be passed through a sterile 0.20 µm filter (Corning, Wiessbaden, Germany) and kept at +4°C until the time of use, after its outer surface is covered with aluminium foil. After the medium liquids of the incubated cells are withdrawn, the previously prepared samples will be placed in each well and left to incubate again for 24 h at 37°C in an environment containing 5% CO_2. Thus, the cytotoxic effects of the groups at the end of the 24th h will be evaluated. In order to solubilize the formazan crystals formed as a result of the application of MTT, 99.4 mL dimethylsulfoxide (DMSO), 0.6 mL (HCl) and 10 g sodium laurylsulfate (SDS) will be added to the mixture as 100 µL/well and allowed to incubate again for 4 h. After this, the absorbance (optical density) will be measured in a spectrophotometer (µQuant, Bad Friedrichshall, Biotek, CA, United States) at a wavelength of 570 nm.

Immunofluorescence Analysis

Cells cultivated in cell culture were incubated for 30 min in paraformaldehyde solution for 30 min. The cells were then incubated in 3% H_2O_2 for 5 min. 0.1% Triton-X solution was dripped onto the cells washed with PBS and left for 15 min. After the incubation period, protein blocks were dripped onto the cells and kept in the dark for 5 min. Then, the primary antibody (8-OHdG cat no: sc-66036, Dilution Ratio: 1/100 US) was dropped and incubated in accordance with the instructions for use. Immunofluorescence secondary antibody was used as a secondary marker (FITC Cat No: ab6785 Diluent Ratio: 1/500. UK) and kept in the dark for 45 min. Then, DAPI with mounting medium (Cat no: D1306 Dilution Ratio: 1/200 UK) was dripped onto the sections and kept in the dark for 5 min, then the sections were closed with a coverslip. The stained sections were examined under a fluorescent microscope (Zeiss AXIO GERMANY) [21].

Statistical Analysis

In order to determine the intensity of positive staining from the pictures obtained as a result of the dyeing; 5 random areas were selected from each image and evaluated in the ZEISS Zen Imaging Software program. Data were statistically defined as mean and standard deviation (mean±SD) for % area. Mann-Whitney U test was performed to compare positive immunoreactive cells and immunopositively stained areas with healthy controls. As a result of the test, an AP value of <0.05 was considered significant and the data were presented as mean ± SD.

Results

Microbiological Results

In our study, minimal inhibition concentrations of bee venom and the obtained bee venom-derived exosome were determined against *E. coli* K99 (F5) strain. Amoxicillin clavulonic acid, one of the β-lactam antibiotics, was included in the study as a positive control in the MIC range determined by EUCAST. Minimal inhibition concentration value of bee venom, bee venom-derived exosome and amoxicillin-clavulanic acid against *E.coli* respectively, it was determined as 62.5 µg/mL 1.95 µg/mL and 4000 µg/mL. The MIC concentration of bee venom and exosome against *E. coli* is shown in *Table 1*.

Bee venom, bee venom-derived exosome and antibiotic concentrations prepared according to MIC values were determined by the checkerboard method to determine the FIC index. According to the FIC index formula, the synergistic effect of bee venom and exosome with amoxicillin clavulonic acid was observed. All these values are shown in *Table 2*.

Antibiofilm activity against biofilm ability was measured at a wavelength of 570 nm. And the results are summarized in *Table 3* and *Table 4*. In the results obtained, it was determined that the exosome structure inhibited the formation of biofilm. In our study results, while the antibiofilm activity was determined at the rate of 48.8% in bee venom, it was determined that the bee venom-

Table 1. *MIC values of bee venom, bee venom exosome and antibiotics against reference bacteria strains*

Bacteria Strains ATCC No	Bee Venom MIC µg/mL	Bee Venom Exosome MIC µg/mL	Antibiotics MIC mg/L
E. coli K99 (F5)	62.5 µg/mL	1.95 µg/mL	4 mg/L[a]

[a] *Amoxicillin-clavulanic acid*

Table 2. *Results of the checkerboard assay with fractional inhibitory concentration and FIC indices of two-drug combinations*

Bacteria Strains ATCC No	Agent	FIC	Interpretation
E. coli K99 (F5)	Bee venom Amoxicillin-clavulanic acid	0.33	Synergy
E. coli K99 (F5)	Bee venom exosome Amoxicillin-clavulanic acid	0.44	Synergy

Table 3. *Biofilm OD values for bee venom at 570 nm wavelength*

Bacteria Strains ATCC No	Positive Control	Negative Control	Highest OD Value
E. coli K99 (F5)	0.795	0.426	2.700
E. coli K99 (F5) + Bee venom			1.381

Table 4. *Biofilm OD values for bee venom exosome at 570 nm wavelength*

Bacteria Strains ATCC No	Positive Control	Negative Control	Highest OD Value
E. coli K99 (F5)	0.149	0.079	0.500
E. coli K99 (F5)+Bee venom exosome			0.198

derived exosome inhibited the biofilm layer by 60.4%. *E. coli* ATCC 25922 strain was used as positive control in our study.

Scanning Electron Microscopes Results

The obtained data are shown in *Fig.1*. Looking at the data obtained, it was determined that the particle sizes were between 67.47 nm and 105.9 nm.

MTT Results

Control (cell medium only), *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom-derived exosome) 1.95 µg/mL, Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg. The cytotoxic effects of BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli and* BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups were determined after 24 h using the MTT method *(Fig. 2)*. *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE data were compared with the control group. The cell viability rate of the control group was 100%. Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups were compared with the *E. coli* group. The *E. coli* group was compared with the control group (## P<0.001). The lowest viability was observed at Amoxicillin 4 mg/mL + *E. coli* (viability rate was 158%) (P<0.05). BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups showed more toxicity (P<0.001) *(Fig. 2)*.

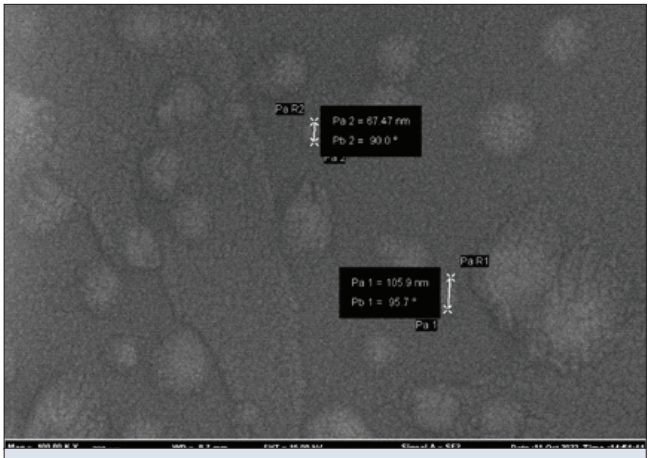

Fig 1. SEM evaluation of bee venom exosomes

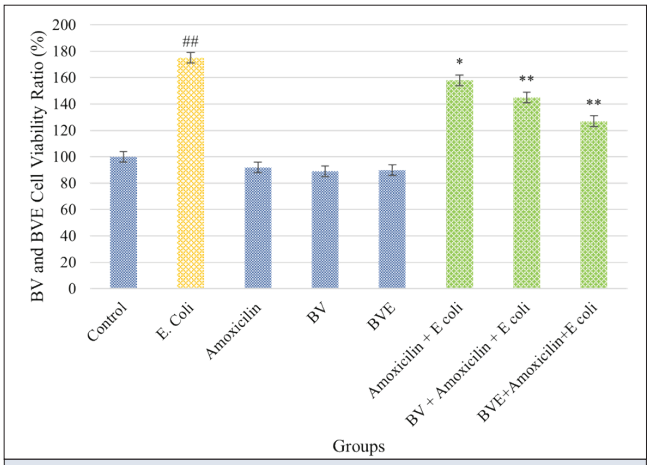

Fig 2. Cell viability rate of L929 cell after 24 hours. Control (cell medium only), *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom exosome) 1.95 µg/mL, Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg Viability rates of /mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups are shown. Control (cell medium only), *E. coli*, Amoxicillin 4 mg/mL, BV (bee venom) 62.5 µg/mL, BVE (Bee venom exosome) 1.95 µg/mL compared with the control group (## P<0.001). The viability rates of Amoxicillin 4 mg/mL + *E. coli*, BV 62.5 µg/mL + Amoxicillin 4 mg/mL + *E. coli*, BVE 1.95 µg/mL + Amoxicillin 4 mg/mL + *E. coli* groups were compared with the *E. coli* group (*P<0.05, ** P<0.001)

Immunofluorescence Results

Data of immunofluorescent staining results and statistical analysis results are also presented in *Table 5* and *Fig. 3*. Our results were in line with cell culture results.

Discussion

Bee venom (BV) antimicrobial and antibiofilm activity may be due to the presence of various peptides such as melittin, melectin, apamin, adolapin, mast cell degranulating peptides, enzymes, biologically active amines, and non-peptide components [23-25]. There are many studies on the antimicrobial and antibiofilm activity of bee products [26-30]. In this study, we tried to determine the antibacterial and antibiofilm activity of bee venom and exosome, which is a

Table 5. *Data and statistical analysis results of immunofluorescent staining results*

Groups	8-OHdG
Control	18.19±2.79[a]
E. coli	71.73±4.5[c]
Amoxi (Amoxicillin)	26.44±1.96[a]
B (BV)	28.55±3[a]
Bexo (BVE)	27.56±2.08[a]
Amoxi (Amoxicillin) + *E. coli*	58.13±2.76[d]
BV (B) + Amoxi (Amoxicilin) + *E. coli*	41.12±4.94[b]
Bexo (BVE) + Amoxi (Amoxicilin) + *E. coli*	29.18±4.46[b]

[a,b,c,d] *different letters in the same column are considered statistically significant difference (P<0.05)*

bee product, against *E. coli* K99 (F5) strain. Keles et al.[31], in their study investigated the etiology and predisposing factors of diarrheal calves from Kayseri province and surrounding provinces between January 2016 and September 2019. 270 newborn calves from diarrhea included in this study. It was determined that 15.6% (42) caused by *E. coli* K99 strain. Alternative treatments are important in diarrhea cases due to the antibiotic resistance of *E. coli* strains. Increasing antibiotic resistance has led to an increase in the search for bee products and alternative treatments [32-36]. Studies have shown that *E. coli* strains show high resistance to antibiotics. Karacan Sever et al.[37] in their study, 99 *E. coli* strains were isolated from poultry. High antibiotic resistance in isolated *E. coli* strains and serotyped *E. coli*. It was determined that O78 was the dominant serotype in strains. Cujova et al.[17] reported that honey BV contains melittin, which is more active against gram-positive bacteria than gram-negative bacteria. In our results, antimicrobial and antibiofilm effects of bee venom and bee venom-derived exosome were determined. The antimicrobial activity of the exosome was 1.95 µg/mL, and the FIC concentration created by the antibiotic showed a synergistic effect of 0.44. In a study, the MIC values of bee venom against *S. salyarius*, *S. sobrinus*, *S. mutans, S. mitis, S. sanguinis, L. casei* and *E. faecalis* were found to be between 20 and 40 µg/mL. Melittin, one of the main components of this poison, showed MIC values ranging from 4 to 40 µg/mL, while the MIC value of PLA2 was found to be over 400 µg/mL [26]. FIC values of bee venom combined with traditionally administered drugs yielded fractional inhibitory concentration (FIC) indices ranging from 0.24 to 0.5. [27] BV and melittin are a potent antimicrobial against Methicillin-resistant *Staphylococcus aureus* (MRSA) at MIC values of 6-800 µg/mL showed activity [28]. In another study, it was determined that both melittin and bee venom had a bactericidal effect on MRSA ATCC 33591 strain [29]. Previously, honey and honey-derived defensin-1 have reported antibiofilm activity on

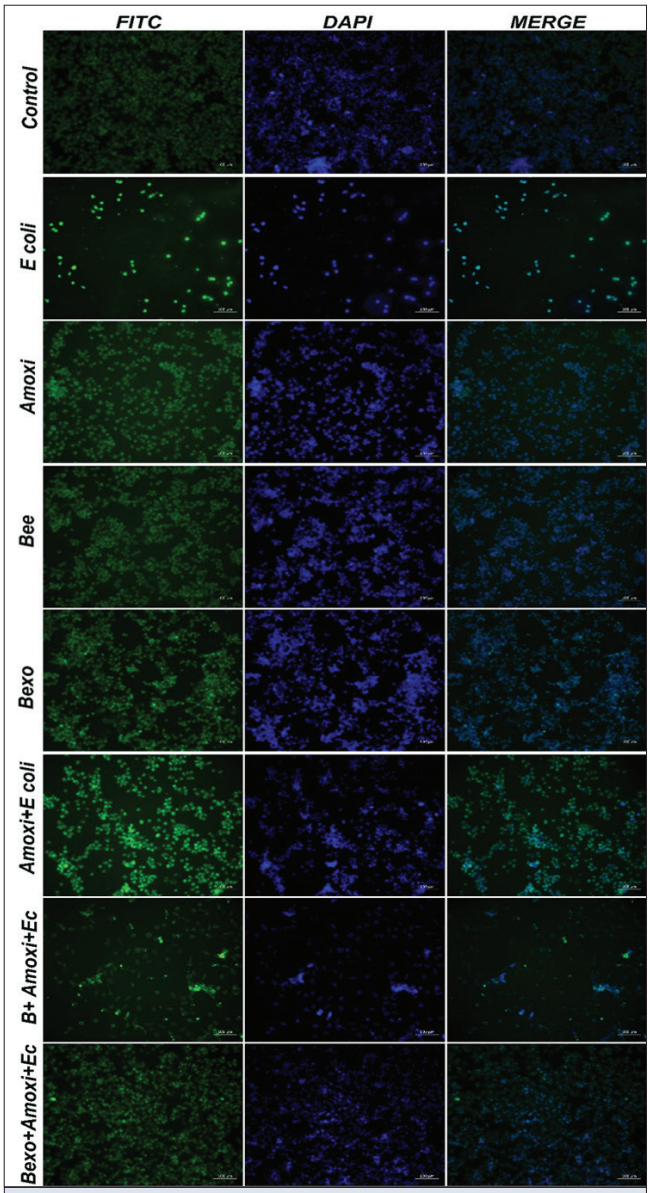

Fig 3. Control, *E. coli*, Amoxi (Amoxicillin) 4 mg/mL, B (BV) 62.5 µg/mL, Bexo (BVE) 1.95 µg/mL, Amoxi (Amoxicillin) 4 mg/mL + *E. coli*, B (BV) 62.5 µg/mL + Amoxi (Amoxicillin) 4 mg/mL + *E. coli*, Bexo (BVE) 1.95 µg/mL + Amoxi (Amoxicillin) 4 mg/mL + *E. coli* groups, 8-OHdG expressions in L929 cells are shown. (FITC), IF Bar:100 µm

wound pathogens [30,32]. In a study by Arteaga et al.[33], a MIC value of 512 µg/mL was determined against *S. enterica* isolated from poultry, and their potential to inhibit biofilm formation was found to be up to 68%. In a study by Elsayed et al.[34], when the antimicrobial activities of *Apis mellifera* venom were examined, it was reported that a MIC value of 15.65 µg/mL was detected in *E. coli* ATCC 8739 strain. In our study, the MIC value of bee venom was determined as 62.5 µg/mL. The MIC value obtained from the exosome of bee venom was determined as 1.95 µg/mL. Considering the damage of *E. coli*, which is the causative agent of calf diarrhea. In addition, the synergistic effect of antibiotic and bee venom-derived exosome adds originality to our

study. It is understood that exosomes, which act as a cargo element, which is a bioactive molecule, are effective especially at low concentrations and will be considered as an alternative in the search for new antimicrobials. In the study of Lima et al.[35], *in vitro* and *in vivo* antibacterial and anti-biofilm activities of melittin, a peptide derived from honey bee venom, against uropathogenic *E. coli* were examined and the MIC values were found to be 0.5 to 8 µM. It has also been reported that it degrades the biofilm layer by 39.58%. In a study by Picoli et al.[36], melittin had 40-42.5 µg/mL (~13 µM) MIC and 64-128 µg/mL (~20-40 µM) MBC's against *E. coli* ATCC 8739. In a study by Han et al.[38], it was reported that the MIC of melittin purified from honey bee venom against *E. coli* ATCC 25922 was 0.125 µg/mL (~0.04 µM). The cytotoxicity test with melectin, a component of bee venom, was evaluated using normal human fibroblast cells and it was determined that melectin at 32 µM showed low cytotoxicity, such as 10%, at concentrations below 16 µM. In our study, cytotoxicity was very evident in the toxicity model made with fibroblast cells [39]. Although not, it has been shown to significantly and significantly reduce the bacterial population in co-cultures. Similar results are shown in immunohistochemistry analyses.

Although studies on bee venom and its peptides are presented in the literature, there are no studies on the antibacterial and antibiofilm activity of exosomes obtained from bee venom. It has been determined that bee venom exosome has a synergistic effect when used in combination with antibiotics. It is important that more studies should be done on the cytotoxic effect, which is not seen in studies on cells. We see that bee venom and exosome will shed light on further studies and as a bioactive antimicrobial candidate against increasing antibiotic resistance.

Availability of Data and Materials

The data that support the findings of this study are available from the corresponding author (D. Celebi) on reasonable request.

Funding Support

The authors declared that this study has received no financial support.

Competing Interests

The authors have no conflicts of interest to declare.

Author Contributions

D.C., O.C., S.B. and A.T.: Concept, Design, Supervision, Resources, Materials Data, Collection and/or Processing, Analysis and/or Interpretation, Literature Search, Writing and Critical Reviews

References

1. Rehman K, Fiayyaz F, Khurshid M, Sabir S: Antibiotics and antimicrobial resistance: Temporal and global trends in the environment. **In,** Antibiotics and Antimicrobial Resistance Genes in the Environment. Vol. 1. 7-27, Elsevier, 2020. DOI: 10.1016/B978-0-12-818882-8.00002-4

2. Memariani H, Memariani M, Shahidi-Dadras M, Nasiri S, Akhavan MM, Moravvej H: Melittin: From honeybees to superbugs. *Appl Microbiol Biotechnol,* 103, 3265-3276, 2019. DOI: 10.1007/s00253-019-09698-y

3. Elmonir W, Shalaan S, Tahoun A, Mahmoud SF, Remela EMA, Eissa R, El-Sharkawy H, Shukry M, Zahran RN: Prevalence, antimicrobial resistance, and genotyping of shiga toxin-producing *Escherichia coli* in foods of cattle origin, diarrheic cattle, and diarrheic humans in Egypt. *Gut Pathog,* 13:8, 2021. DOI: 10.1186/s13099-021-00402-y

4. de Verdier K, Nyman A, Greko C, Bengtsson B: Antimicrobial resistance and virulence factors in *Escherichia coli* from Swedish dairy calves. *Acta Vet Scand,* 54 (1):2, 2012. DOI: 10.1186/1751-0147-54-2

5. Han SM, Kim JM, Hong IP, Woo SO, Kim SG, Jang HR, Pak SC: Antibacterial activity and antibiotic-enhancing effects of honeybee venom against methicillin-resistant *Staphylococcus aureus*. *Molecules,* 21 (1):79, 2016. DOI: 10.3390/molecules21010079

6. Shin S, Ye M, Choi S, Park K: The effects of melittin and apamin on airborne fungi-induced chemical mediator and extracellular matrix production from nasal polyp fibroblasts. *Toxins*, 9 (11):384, 2017. DOI: 10.3390/toxins9110348

7. Sobral F, Sampaio A, Falcão S, Queiroz MJR, Calhelha RC, Vilas-Boas M, Ferreira ICFR: Chemical characterization, antioxidant, anti-inflammatory and cytotoxic properties of bee venom collected in Northeast Portugal. *Food Chem Toxicol,* 94, 172-177, 2016. DOI: 10.1016/j.fct.2016.06.008

8. Son DJ, Lee JW, Lee YH, Song HS, Lee CK, Hong JT: Therapeutic application of anti-arthritis, pain-releasing, and anti-cancer effects of bee venom and its constituent compounds. *Pharmacol Ther,* 115, 246-270, 2007. DOI: 10.1016/j.pharmthera.2007.04.004

9. El-Seedi H, El-Wahed A, Yosri N, Musharraf SG, Chen L, Moustafa M, Zou X, Al-Mousawi S, Guo Z, Khatib A: Antimicrobial properties of *Apis mellifera*'s bee venom. *Toxins,* 12 (7):451, 2020. DOI: 10.3390/toxins12070451

10. Mata ÉCGd, Mourão CBF, Rangel M, Schwartz EFJJoVA, Diseases TiT: Antiviral activity of animal venom peptides and related compounds. *J Venom Anim Toxins Incl Trop Dis,* 23:3, 2017. DOI: 10.3390/molecules25102402

11. Somwongin S, Chantawannakul P, Chaiyana W: Antioxidant activity and irritation property of venoms from Apis species. *Toxicon,* 145, 32-39, 2018. DOI: 10.1016/j.toxicon.2018.02.049

12. Al-Ani I, Zimmermann S, Reichling J, Wink M: Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens. *Phytomedicine,* 22, 245-255, 2015. DOI: 10.1016/j.phymed.2014.11.019

13. Han S, Yeo J, Baek H, Lin SM, Meyer S, Molan P: Postantibiotic effect of purified melittin from honeybee (*Apis mellifera*) venom against *Escherichia coli* and *Staphylococcus aureus*. *J Asian Nat Prod Res,* 11, 796-804, 2009. DOI: 10.1080/10286020903164277

14. Jamasbi E, Batinovic S, Sharples RA, Sani MA, Robins-Browne RM, Wade JD, Separovic F, Hossain MA: Melittin peptides exhibit different activity on different cells and model membranes. *Amino Acids,* 46, 2759-2766, 2014. DOI: 10.1007/s00726-014-1833-9

15. Raposo G, Stoorvogel W: Extracellular vesicles: Exosomes, micro-vesicles, and friends. *J Cell Biol,* 200 (4): 373-383, 2013. DOI: 10.1083/jcb.201211138

16. Cocucci E, Meldolesi J: Ectosomes and exosomes: Shedding the confusion between extracellular vesicles. *Trends Cell Biol,* 25 (6): 364-372, 2015. DOI: 10.1016/j.tcb.2015.01.004

17. Takahashi A, Okada R, Nagao K, Kawamata Y, Hanyu A, Yoshimoto S, Takasugi M, Watanabe S, Kanemaki MT, Obuse C, Hara E: Exosomes maintain cellular homeostasis by excreting harmful DNA from cells. *Nat Commun*, 8:15287, 2017. DOI: 10.1038/s41467-018-06613-3

18. Maia J, Caja S, Strano Moraes MC, Couto N, Costa-Silva B: Exosome-based cell-cell communication in the tumor microenvironment. *Front Cell Dev Biol,* 6:18, 2018. DOI: 10.3389/fcell.2018.00018

19. Zhang Q, Higginbotham JN, Jeppesen DK, Yang YP, Li W, McKinley ET, Graves-Deal R, Ping J, Britain CM, Dorsett KA, Hartman CL, Ford DA, Allen RM, Vickers KC, Liu Q, Franklin JL, Bellis SL, Coffey RJ:

Transfer of functional cargo in exomeres. *Cell Rep,* 27 (3): 940-954, 2019. DOI: 10.1016/j.celrep.2019.01.009

20. Kalluri R: The biology and function of exosomes in cancer. *J Clin Invest*, 126 (4): 1208-1215, 2016. DOI: 10.1172/JCI81135

21. Celebi D, Taghizadehghalehjoughi A, Baser S, Genc S, Yilmaz A, Yeni Y, Yesilyurt F, Yildirim S, Bolat I, Kordali S, Yilmaz F, Hacimuftuoglu A, Celebi O, Margina D, Nitulescu GM, Spandidos DA, Tsatsakis A: Effects of boric acid and potassium metaborate on cytokine levels and redox stress parameters in a wound model infected with methicillin-resistant *Staphylococcus aureus*. *Mol Med Rep*, 26 (3):294, 2022. DOI: 10.3892/mmr.2022.12809

22. Niu C, Gilbert ES: Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. *Appl Environ Microbiol,* 70 (12): 6951-6956, 2004. DOI: 10.1128/AEM.70.12.6951-6956.2004

23. Park JW, Jeon JH, Yoon J, Jung TY, Kwon KR, Cho CK, Lee YW, Sagar S, Wong R, Yoo HS: Effects of sweet bee venom pharmacopuncture treatment for chemotherapy-induced peripheral neuropathy: A case series. *Integr Cancer Ther*, 11 (2): 166-171, 2012. DOI: 10.1177/1534735411413265

24. Kim HW, Kwon YB, Ham TW, Roh DH, Yoon SY, Lee HJ, Han HJ, Yang IS, Beitz AJ, Lee JH: Acupoint stimulation using bee venom attenuates formalin-induced pain behavior and spinal cord fos expression in rats. *J Vet Med Sci,* 65 (3): 349-355, 2003. DOI: 10.1292/jvms.65.349

25. Zolfagharian H, Mohajeri M, Babaie M: Bee venom *(Apis Mellifera)* an effective potential alternative to gentamicin for specific bacteria strains: Bee venom an effective potential for bacteria. *J Pharmacopuncture*, 19 (3): 225-230, 2016. DOI: 10.3831/KPI.2016.19.023

26. Han SM, Lee KG, Yeo JH, Baek HJ, Park K: Antibacterial and anti-inflammatory effects of honeybee *(Apis mellifera)* venom against acne-inducing bacteria. *J Med Plants Res,* 4, 459-464, 2010. DOI: 10.5487/TR.2012.28.1.001

27. Leandro LF, Mendes CA, Casemiro LA: Antimicrobial activity of apitoxin, melittin and phospholipase A2 of honey bee *(Apis mellifera)* venom against oral pathogens. *An Acad Bras Cienc,* 87, 147-155, 2015. DOI: 10.1590/0001-3765201520130511

28. AL-Ani I, Zimmermann S, Reichling J, Wink M: Pharmacological synergism of bee venom and melittin with antibiotics and plant secondary metabolites against multi-drug resistant microbial pathogens. *Phytomedicine,* 22, 245-255, 2015. DOI: 10.1016/j.phymed.2014.11.019

29. Flávia A, Pereira M, Albano M, Cristina F, Alves B, Fernanda B, Teles M, Furlanetto A, Mores VL: Influence of apitoxin and melittin from *Apis mellifera* bee on *Staphylococcus aureus* strains. *Microb Pathog*, 141:104011, 2020. DOI: 10.1016/j.micpath.2020.104011

30. Sojka M, Valachova I, Bucekova M, Majtan J: Antibiofilm efficacy of honey and bee- derived defensin-1 on multispecies wound biofilm. *J Med Microbiol,* 65, 337-344, 2016. DOI: 10.1099/jmm.0.000227

31. Keleş İ, Ekinci G, Tüfekçi E, Çitil M, Güneş V, Aslan Ö, Onmaz AC, Karaca Bekdik İ, Varol K, Deniz: Etiological and predisposing factors in calves with neonatal diarrhea: A clinical study in 270 case series. *Kafkas Univ Vet Fak Derg*, 28 (3): 315-326, 2022. DOI: 10.9775/kvfd.2021.26981

32. Majtan J, Bohova J, Horniackova M, Klaudiny J, Majtan V: Anti-biofilm effects of honey against wound pathogens *Proteus mirabilis* and *Enterobacter cloacae*. *Phytother Res*, 28, 69-75, 2014. DOI: 10.1002/ptr.4957

33. Arteaga V, Lamas A, Regal P, Vázquez B, Miranda JM, Cepeda A, Franco CM: Antimicrobial activity of apitoxin from *Apis mellifera* in *Salmonella enterica* strains isolated from poultry and its effects on motility, biofilm formation and gene expression. *Microb Pathog,* 137:103771, 2019. DOI: 10.1016/j.micpath.2019.103771

34. Elsayed K, Bakhiet Hosni AM, Elshehaby HM: *Apis mellifera* venom inhibits bacterial and fungal pathogens *in vitro*. *Pak J Biol Sci*, 25, 875-884, 2022. DOI: 10.3923/pjbs.2022.875.884

35. Lima WG, Batista Filho FL, Lima IP, Simião DC, Brito JCM, da Cruz Nizer WS, Cardoso VN, Fernandes SOA: Antibacterial, anti-biofilm, and anti-adhesive activities of melittin, a honeybee venom-derived peptide, against quinolone-resistant uropathogenic *Escherichia coli* (UPEC). *Nat Prod Res,* 36 (24): 6381-6388, 2022. DOI: 10.1080/14786419.2022.2032047

36. Picoli T, Peter CM, Zani JL, Waller SB, Lopes MG, Boesche KN, Vargas GDÁ, Hübner SO, Fischer G: Melittin and its potential in the destruction and inhibition of the biofilm formation by *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* isolated from bovine milk. *Microb Pathog,* 112, 57-62, 2017. DOI: 10.1016/j.micpath.2017.09.046

37. Karacan Sever N, Şahan Yapıcıer Ö, Akan M: Distribution of serotypes and antibiotic resistance of avian pathogenic *Escherichia coli* strains isolated from chickens. *Kafkas Univ Vet Fak Derg*, 28 (6): 767-772, 2022. DOI: 10.9775/kvfd.2022.28271

38. Han S, Yeo J, Baek H, Lin SM, Meyer S, Molan P: Postantibiotic effect of purified melittin from honeybee *(Apis mellifera)* venom against *Escherichia coli* and *Staphylococcus aureus*. *J Asian Nat Prod Res*, 11 (9): 796-804, 2009. DOI: 10.1080/10286020903164277

39. Ko SJ, Park E, Asandei A. Choi JY, Lee SC, Seo CH, Luchian T, Park Y: Bee venom-derived antimicrobial peptide melectin has broad-spectrum potency, cell selectivity, and salt-resistant properties. *Sci Rep,* 10:10145, 2020. DOI: 10.1038/s41598-020-66995-7

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 247-254, 2023
DOI: 10.9775/kvfd.2023.29134

Research Article

The Effect of *Bacillus subtilis* and Fructooligosaccharide as Antibiotic Substituent on Goose Performance Parameters, Serum Biochemical Indicators and Intestinal Morphology

Guang-quan LI [1 (‡)] Xian-ze WANG [1 (‡)] Yi LIU [1] Yun-zhou YANG [1] Cui WANG [1] Shao-ming GONG [1] Da-qian HE [1 (*)] Hui-ying WANG [1 (*)]

(‡) These authors contributed equally to this study

[1] Institute of Animal Husbandry and Veterinary Science, Shanghai Academy of Agricultural Science, Shanghai, 201106, P. R. CHINA

ORCIDs: G.Q.L. 0000-0003-1050-634X; X.Z.W. 0000-0002-7507-3437; Y.L. 0000-0002-7237-6010; Y.Z.Y. 0000-0003-1036-8047; C.W. 0000-0003-2723-5929; S.M.G. 0000-0002-9354-1804; D.Q.H. 0000-0002-8714-4781; H.Y.W. 0000-0002-8312-7028

Article ID: KVFD-2023-29134 Received: 05.02.2023 Accepted: 02.05.2023 Published Online: 10.05.2023

Abstract: As antibiotics are now prohibited or heavily regulated in farming in most countries, nutritionists and veterinarians are increasingly turning to probiotic supplements, prebiotics, and synbiotics to promote animal health and improve production performance. The study aimed to evaluate the effects of *Bacillus subtilis*, fructooligosaccharides (FOS), and their combined use on geese, compared to antibiotics, and evaluate their suitability as an alternative to antibiotics. 240 14-day-old geese were randomly divided into five groups and were fed a basal diet or supplemented with 100 mg/kg chlortetracycline, 400 mg/kg FOS, 2x10^{5}cfu g/kg *B. subtilis*, or a combination of 200 mg/kg FOS and 1x10^{5}cfu g/kg *B. subtilis* for 56 days. The study measured the body weight, feed consumption, serum parameters, and intestinal morphology of the geese. The results showed that the combination of *B. subtilis* and FOS had a positive impact on the body weight, feed consumption, and serum parameters of the geese, while improving their intestinal morphology. The results suggest that the combination of *B. subtilis* and FOS may provide multiple benefits to animal health and performance and could be used as a suitable alternative to antibiotics.

Keywords: *Bacillus subtilis, Fructooligosaccharide, Goose, Intestinal morphology, Performance parameters, Serum*

Antibiyotik İkamesi Olarak *Bacillus subtilis* ve Fruktooligosakkaritin Kazlarda Performans Parametreleri, Serum Biyokimyasal Göstergeleri ve Bağırsak Morfolojisi Üzerine Etkisi

Öz: Antibiyotikler artık çoğu ülkede çiftçilikte yasaklandığından veya ağır düzenlemelere tabi tutulduğundan, beslenme uzmanları ve veterinerler hayvan sağlığını desteklemek ve üretim performansını artırmak için probiyotik takviyelere, prebiyotiklere ve sinbiyotiklere giderek daha fazla yönelmektedir. Bu çalışmanın amacı, antibiyotiklere kıyasla *Bacillus subtilis*, fruktooligosakkaritler (FOS) ve bunların birlikte kullanımının kazlar üzerindeki etkilerini ve antibiyotiklere alternatif olarak uygunluğunu değerlendirmektir. İkiyüz kırk adet 14 günlük kaz rastgele beş gruba ayrılmış ve 56 gün boyunca bazal diyet veya 100 mg/kg klortetrasiklin, 400 mg/kg FOS, 2x10^{5}cfu g/kg *B. subtilis* ve 200 mg/kg FOS ve 1x10^{5}cfu g/kg *B. subtilis* kombinasyonu ile beslenmiştir. Çalışmada kazların vücut ağırlığı, yem tüketimi, serum parametreleri ve bağırsak morfolojisi ölçülmüştür. Sonuçlar, *B. subtilis* ve FOS kombinasyonunun kazların vücut ağırlığı, yem tüketimi ve serum parametreleri üzerinde olumlu bir etkiye sahip olduğunu ve bağırsak morfolojilerini iyileştirdiğini göstermiştir. Sonuçlar, *B. subtilis* ve FOS kombinasyonunun hayvan sağlığı ve performansına birçok fayda sağlayabileceğini ve antibiyotiklere uygun bir alternatif olarak kullanılabileceğini göstermektedir.

Anahtar sözcükler: *Bacillus subtilis, Bağırsak morfolojisi, Fruktooligosakkarit, Kaz, Performans parametreleri*

How to cite this article?

Li GQ, Wang XZ, Liu Y, Yang YZ, Wang C, Gong SM, He DQ, Wang HY: The effect of *Bacillus subtilis* and fructooligosaccharide as antibiotic substituent on goose performance parameters, serum biochemical indicators and intestinal morphology. *Kafkas Univ Vet Fak Derg,* 29 (3): 247-254, 2023.
DOI: 10.9775/kvfd.2023.29134

(*) Corresponding author: Da-qian HE & Hui-ying WANG

Phone: +86-18918162216 (D.Q. He); +86-18964198956 (H.Y. Wang)
E-mail: daqianhe@aliyun.com (D.Q. He); yjshywang@sina.com (H.Y. Wang)

Introduction

Antibiotics have a long history of being added to animal feed as growth promoters [1], It is widely used because of its ability to treat diseases infected by microorganisms such as bacteria, chlamydia, and mycoplasma [2]. However, with the continuous understanding of antibiotics, many bacteria have developed resistance to antibiotics [3], and the abuse of antibiotics will remain in animals or products [4,5], which will damage human health [6].

Probiotics widely exist in nature, animals and plants, can secrete a variety of metabolites, have a wide range of beneficial effects on the host [7]. As a typical probiotic, *B. subtilis* can secrete protease and cellulase to promote the digestion and utilization of nutrients [8]. At the same time, *B. subtilis* can produce spores during the dormant period [9], and has tolerance to high temperature and pressure during feed processing and the acidic environment of the animal gastrointestinal tract [10]. FOS have been widely used in animal husbandry as a prebiotic supplement in animal feed [11,12]. FOS are short chains of fructose molecules that act as a substrate for beneficial bacteria in the gut, promoting the growth of probiotics and maintaining gut health [13]. The application of FOS in animal husbandry has been found to be effective in improving gut microbiota balance, increasing feed efficiency, promoting growth, and reducing the incidence of digestive disorders. FOS have been particularly useful in poultry and swine production, where they have been shown to enhance gut health and improve feed utilization [14,15]. Overall, FOS have been a valuable addition to animal nutrition, providing multiple benefits to animal health and performance.

The use of probiotics or prebiotics alone can have limited benefits when compared to combining them [16,17]. There are few studies on *B. subtilis* and FOS in geese [18]. This experiment aims to study the effects of *B. subtilis*, FOS and their combined use on geese, compare its effect with that of antibiotics, and evaluate whether it can be used as a suitable alternative to antibiotics.

Material and Methods

Animal Ethics

Animal experimentation was approved by the Laboratory Animal Ethics Committee of the Shanghai Academy of Agricultural Sciences (SAASPZ0522046).

Material

The *B. subtilis* used in the experiment (the number of viable bacteria ≥1.0x10^{8}cfu/g) was purchased from Shanghai Shenya Animal Health Products Fuyang Co., Ltd, Anhui, China. FOS (content ≥97.15%) was purchased from Dongguan Zhenshang Industrial Co., Ltd, Guangdong, China. Zhedong white geese were purchased from Xiangshan County Zhejiang White Goose Research Institute, Zhejiang, China.

Experimental Design

Total 240 14-day-old Zhedong white geese were randomly divided into five groups, six replicates in each group, each replicate has eight geese (half male and half female). The five groups were basal diet group, 100mg/kg chlortetracycline (CTC) group, 400 mg/kg FOS group, 2x10^{5}cfu g/kg *B. subtilis* group and 200mg/kg FOS+ 1x10^{5}cfu g/kg *B. subtilis* group, the experiment lasts for 56 d [19]. The basal diet refers to the nutritional level recommended by NRC-1994 *(Table 1)* [20]. During the experiment period, Geese in each replicated was housed in an 80 cm x 80 cm cage with access to natural light during the day and low-level artificial light at night. They had free access to food and water, and routine feeding management and immunization procedures were carried out.

Performance Parameters

Body weight (BW) was recorded before the start and end of the experiment (8 h fasting, 2 h water deprivation), then weigh the amount of feed consumed for each repetition, and the average daily gain (ADG), average daily feed intake (ADFI) and feed/gain (F/G) were calculated at last.

Table 1. *Feed ingredients and analyzed chemical composition of geese diets (air-dry basis %)*

Ingredients	Content %	
	1-28 d	28-70 d
Corn	60.30	58.80
Soybean meal (43% CP)	32.60	25.60
Fish meal (60.3% CP)	2.00	10.10
Soybean oil	2.00	1.50
Lys + Met	0.10	0.00
Limestone	0.00	1.00
Premix [a]	3.00	3.00
Total	100	100
Nutritional Level		
ME/(MJ/kg)	12.13	12.55
Crude protein	20.23	16.00
Crude fiber	3.07	7.00
Ca	0.55	0.68
P	0.45	0.43

[a] *Per kilogram of diets including: Vit-A: 1500 IU, Vit-B_1: 2.3 mg, Vit-B_2: 5.0 mg, Vit-B_6: 5 mg, Vit-B_{12}: 2 mg, Vit-D_3: 200 IU, Vit-E: 12.5 IU, Vit-K: 1.5 mg, Trace elements: 50 g, Garlicin: 30 g, Lysine: 100 g, Methionine: 50 g, Salt: 100 g, Stone powder: 100 g, Myco-Ad: 100 g, Zeolite powder: 420 g;* [b] *Nutrient levels were all calculated values*

Table 2. Effects of B. subtilis and FOS on growth performance in geese

Items	Treatments				
	Basal Diet	100 mg/kg CTC	400 mg/kg FOS	2×10^5cfu g/kg *B. subtilis*	200 mg/kg FOS+1×10^5cfu g/kg *B. subtilis*
Initial BW, g	677.08±14.44	656.47±13.76	659.57±11.23	662.78±13.54	655.93±13.15
Final BW, g	3904.92±103.72^b	4111.11±123.98^a	3919.33±117.51^b	4055.82±85.16^a	4076.97±107.88^a
ADFI, g/d	304.41±1.31	304.96±1.05	301.87±1.06	309.66±1.22	310.03±1.62
ADG, g/d	57.64±8.59^b	61.69±10.26^a	58.21±7.93^{ab}	60.59±8.65^{ab}	61.90±7.86^a
F/G	5.21±0.56^a	4.92±0.86^b	5.13±0.53^{ab}	5.07±0.57^{ab}	5.03±0.93^{ab}

*The data in the table are compared in the same row, and different lowercase letters indicate that the difference has reached a significant level (P<0.05); **CTC:** chlortetracycline, **FOS**: Fructo oligosaccharide*

Serum Biochemical Indicators

At the end of the experiment, one male and female geese with close to average body weight were selected for each repetition, and blood was collected from the wing vein to prepare serum and stored at -20°C for later use. All serum were sent to Shanghai Pinyi Biological Co., Ltd. for testing total protein (TP), albumin (ALB), globulin (GLOB), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (GLU), blood urea nitrogen (BUN), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C).

Intestinal Morphology

The geese were slaughtered through exsanguination of the jugular vein.In reference to the methods used by Xue et al.[21], the duodenum, jejunum, and ileum were collected and analyzed. In summary, the contents of their intestines were carefully emptied, rinsed with saline solution, and dried with filter paper. The first quarter of the duodenum, jejunum, and anterior ileum were then cut into 2 cm sections for paraffin embedding. From each sample, five representative intestinal villus crypts were selected, and measurements were taken of the villus height (VH) and crypt depth (CD). The ratio of VH to CD (VH/CD) was calculated.

Statistical Analysis

The data were preliminarily processed using Excel 2019, followed by a one-way ANOVA using SPSS 26.0 software. Multiple comparisons were conducted using Duncan's post hoc test, and the results were presented as mean ± standard deviation. Statistical significance was defined as P<0.05, indicating significant differences.

Results

Performance Parameters

It can be seen from *Table 2* that adding 100 mg/kg CTC to the goose diet was the best for improving the growth performance, compared to the basic diet group, it

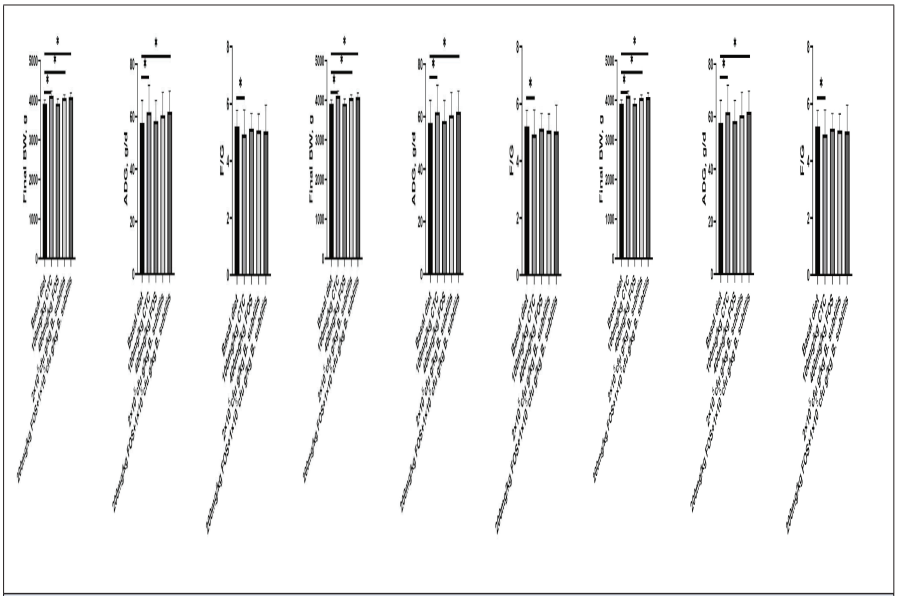

Fig 1. The effect of different treatments on goose performance parameters. **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide, **BW:** body weight, **ADG:** average daily gain, **F/G:** feed/gain

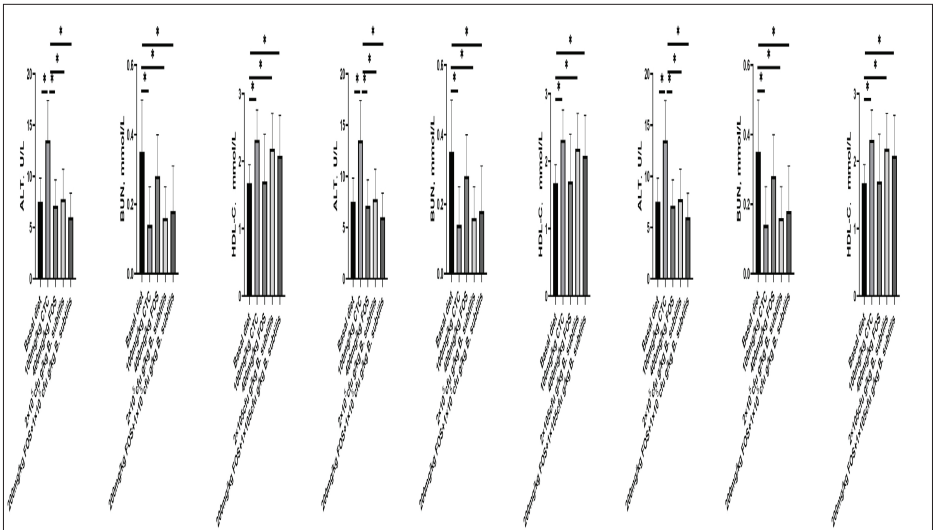

Fig 2. The effect of different treatments on goose serum biochemical indicators. **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide, **ALT:** alanine aminotransferase, **BUN:** blood urea nitrogen, **HDL-C:** high-density lipoprotein cholesterol

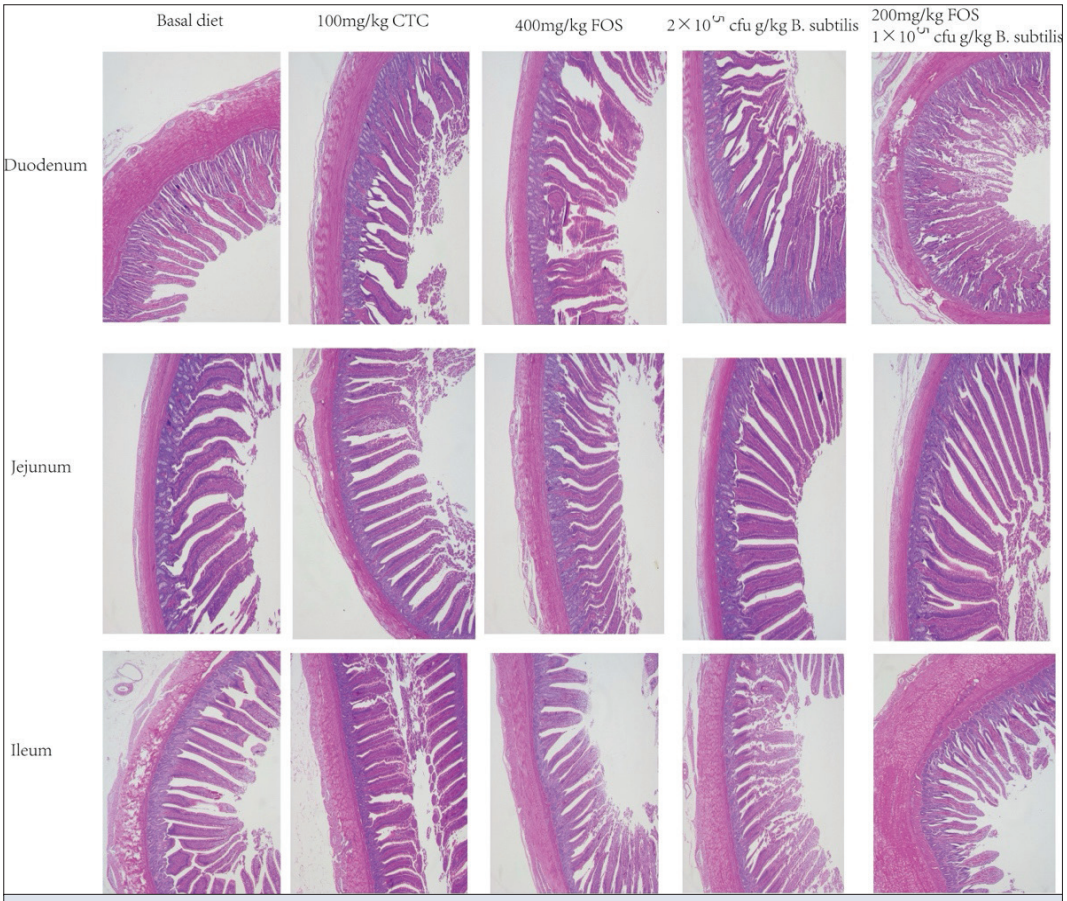

Fig 3. The effect of different treatments on goose intestinal morphology. **CTC:** chlortetracycline, **FOS:** Fructo oligosaccharide

significantly increased the final BW, ADG and F/G ($P<0.05$). The ADG and Final BW of the 200mg/kg FOS+1x10^{5}cfu g/kg *B. subtilis* group improved significantly compared to the basic diet group ($P<0.05$). Adding 2x10^{5}cfu g/kg *B. subtilis* can also improve the Final BW ($P<0.05$), but there is no significant difference in other parameters. There was no significant difference between 400 mg/kg FOS and basal diet group. Significant differences in performance parameters between groups are shown in *Fig. 1*.

Table 3. *Effects of B. subtilis and FOS on serum biochemical indicators in geese*

Items	Treatments				
	Basal Diet	100 mg/kg CTC	400 mg/kg FOS	2×10^{5}cfu g/kg *B. subtilis*	200 mg/kg FOS+1×10^{5}cfu g/kg *B. subtilis*
TP, g/L	31.43±6.48	39.89±3.42	32.90±8.77	37.06±8.83	39.02±7.00
ALB, g/L	12.06±1.97	15.19±1.13	13.74±2.86	14.56±2.67	14.68±2.34
GLOB, g/L	19.36±4.61	24.70±2.50	19.16±5.96	23.50±6.24	24.34±4.67
ALT, U/L	7.50±2.33^b	13.50±3.89^a	7.13±2.53^b	7.75±2.96^b	6.00±2.35^b
AST, U/L	23.25±10.28	21.63±4.47	19.88±7.36	21.13±7.16	20.2±5.12
BUN, mmol/L	0.35±0.15^a	0.14±0.11^b	0.28±0.12^{ab}	0.16±0.09^b	0.18±0.13^b
GLU, mmol/L	6.10±1.45	9.46±1.25	5.50±1.70	6.70±1.26	5.04±2.25
TC, mmol/L	2.74±0.63	3.39±0.69	2.89±1.14	3.75±1.22	2.83±1.08
HDL-C, mmol/L	1.67±0.28^b	2.32±0.44^a	1.70±0.70^b	2.18±0.53^a	2.08±0.60^a
LDL-C, mmol/L	0.98±0.26	0.78±0.17	1.00±0.25	0.76±0.29	0.88±0.50

The data in the table are compared in the same row, and different lowercase letters indicate that the difference has reached a significant level (P<0.05); ***CTC:*** *chlortetracycline,* ***FOS:*** *Fructo oligosac charide,* ***TP:*** *total protein,* ***ALB:*** *albumin,* ***GLOB:*** *globulin,* ***ALT:*** *alanine aminotransferase,* ***AST:*** *aspartate aminotransferase,* ***GLU:*** *glucose,* ***BUN:*** *blood urea nitrogen,* ***TC:*** *total cholesterol,* ***HDL-C:*** *high-density lipoprotein cholesterol,* ***LDL-C:*** *low-density lipoprotein cholesterol*

Table 4. *Effects of B. subtilis and FOS on intestinal morphology in geese*

Intestine	Items	Treatments				
		Basal Diet	100 mg/kg CTC	400 mg/kg FOS	2×10^{5}cfu g/kg *B. subtilis*	200 mg/kg FOS+1×10^{5}cfu g/kg *B. subtilis*
Duodenum	VH, um	504.54±134.18	511.64±66.11	516.74±82.99	504.02±37.84	501.24±66.70
	CD, um	124.03±16.89^a	107.27±37.72^b	115.86±23.58^{ab}	109.85±17.27^{ab}	111.30±40.63^{ab}
	VH/CD	4.16±0.94^b	4.54±1.82^a	4.25±1.07^{ab}	4.38±1.20^{ab}	4.30±2.95^{ab}
Jejunum	VH, um	504.98±50.81	506.92±55.92	507.72±51.80	511.14±54.23	506.89±68.33
	CD, um	100.73±21.13^a	92.21±22.21^b	96.34±30.43^{ab}	95.24±21.78^{ab}	93.18±21.67^b
	VH/CD	4.80±1.59^b	5.29±0.90^a	5.00±1.79^{ab}	5.13±1.28^{ab}	5.23±1.43^a
Ileum	VH, um	518.49±36.85	534.50±62.81	531.09±75.33	537.14±97.02	540.45±49.71
	CD, um	129.72±21.91	135.84±42.42	135.49±17.47	134.77±14.53	136.16±18.01
	VH/CD	4.10±0.66	4.15±1.46	4.12±0.98	4.14±1.23	4.15±0.77

The data in the table are compared in the same row, and different lowercase letters indicate that the difference has reached a significant level (P<0.05); ***CTC:*** *chlortetracycline,* ***FOS:*** *Fructo oligosaccharide,* ***VH:*** *villus height,* ***CD:*** *crypt depth*

Serum Biochemical Indicators

As shown in *Table 3*, There was no significant difference in serum TP, ALB, GLOB, AST, GLU, TC and LDL-C levels among the groups. The ALT level in 100 mg/kg CTC group was significantly higher than in other groups (P<0.05). Adding 100 mg/kg CTC, 2x10^{5}cfu g/kg *B. subtilis* and 200 mg/kg FOS+1x10^{5}cfu g/kg *B. subtilis* can improve the BUN and HDL-C (P<0.05). Significant differences in serum biochemical indicators between groups are shown in *Fig. 2*.

Intestinal Morphology

The intestinal morphology is shown in *Fig. 3*. It can be seen from *Table 4* that in duodenum, the CD of the CTC group was lower than that of the BD group (P<0.05), and the VH/CD were higher (P<0.05), but there is no difference between VH/CD of 100 mg/kg CTC, 2x10^{5}cfu g/kg *B. subtilis* and 200 mg/kg FOS+1x10^{5}cfu g/kg *B. subtilis* groups. In jejunum, the CD of the 100 mg/kg CTC and 200 mg/kg FOS+1x10^{5}cfu g/kg *B. subtilis* groups was lower than that of the BD group (P<0.05), and the VH/CD

were higher (P<0.05). There is no difference between VH, CD and VH/CD among the groups in ileum and there is no difference in the VH of each intestinal tract.

Discussion

Measuring animal growth performance is important because it provides information on the efficiency of feed utilization and overall health of the animal [22]. The ADG and F/G are critical metrics for assessing the growth performance of animals, a high ADG is indicative of strong digestive and absorption capabilities, while a low F/G signifies efficient feed conversion. The better an animal's ability to transform feed into weight gain, the more successful it will be in its growth performance [23]. Antibiotics have a direct impact on the gut microbiome by reducing the population of harmful bacteria and promoting the growth of beneficial bacteria [24]. This can improve gut health and enhance the absorption of nutrients from feed, leading to increased weight gain [25]. Alternatively, using probiotics, prebiotics, and synbiotics can achieve similar results without leaving any residue of antibiotics [26,27]. FOS is non-digestible compounds that stimulate the growth of beneficial bacteria in the gut [28]. Many studies have shown that FOS can improve the growth performance of animals [29]. However, an excessive amount of FOS can also put a strain on the intestines. As a result, FOS is typically used in conjunction with probiotics in animals [30,31]. *B. subtilis* is known for its ability to improve digestive health, increase nutrient absorption, and boost the immune system in animals. This bacterium also has antimicrobial properties, which can help reduce the risk of infection. In studies, the application of *B. subtilis* in livestock has been shown to improve feed efficiency, increase weight gain, and enhance overall performance in chickens, pigs, and ruminants [32-34]. In this experiment, adding 0.2% *B. subtilis* or 0.1% *B. subtilis* + 0.2% FOS increased the final body weight and adding 0.1% *B. subtilis*+0.2% FOS average daily gain, so we think it improved the growth performance of geese.

A higher villus height or shallower crypt depth can contribute to higher digestibility [35,36]. At the same time a higher VH/CD ratio is generally associated with a more efficient small intestine, as there is a greater surface area for nutrient absorption [37]. Conversely, a lower VH/CD ratio is associated with a less efficient small intestine and can be a sign of damage to the intestinal lining [38]. In the experiment, no significant differences were observed in the Ileum indicators among the groups. This is due to the fact that the Ileum is the final segment of the small intestine, where nutrient absorption capacity is limited. Antibiotics can reduce the proliferation of harmful bacteria and promote the differentiation of intestinal epithelial cells, leading to a decrease in the crypt depth and an increase in VH/CD [39]. In our experiment, we observed similar outcomes, however, it is noteworthy that the simultaneous addition of FOS and *B. subtilis* also yielded comparable results, particularly in the jejunum, which has the highest digestion capacity. FOS have been found to enhance the synthesis of polyamines and play a positive role in regulating the growth and development of the small intestine and colonic mucosa. Studies have shown that adding 0.25% FOS to pig feed can increase the villus height and the VH/CD in the proximal small intestine [40]. Additionally, adding 0.4% or 0.6% FOS to pig feed resulted in an increase in villus height and the VH/CD in the jejunum [41]. A study by Howard et al.[42] found that feeding neonatal piglets with FOS led to an increase in the density of cecal epithelial cells and the depth of mucosal crypts in the proximal and distal epithelial cells of the colon. This was attributed to the short-chain fatty acids produced by bacteria metabolizing FOS, which provide energy for intestinal cell proliferation, thus effectively promoting intestinal development. *B. subtilis* has been found to reduce the proliferation of harmful bacteria in the gut, which is likely a contributing factor to the decrease in crypt depth. Several studies have demonstrated that the presence of *B. subtilis* can positively impact the morphological structure of the intestine [43,44]. In this experiment, the combination of *B. subtilis* and FOS was found to have an improved effect on the intestinal morphology of geese.

Serum ALB, GLOB, and TP are important indicators of overall health and nutrition [45]. Despite the absence of significant differences between the groups, we observed an improvement compared with basal diet group. ALT, and AST are commonly used as markers for liver and heart damage or disease [46,47]. Antibiotic-induced liver injury is a known adverse effect of certain antibiotics, and can cause an increase in ALT levels [48]. This increase can be a sign of liver damage, and may be seen in animals receiving certain antibiotics, such as tetracyclines, aminoglycosides, and sulfonamides [49]. The findings of this experiment align with our expectations. The highest levels of both ALT and AST were observed in the group treated with 0.1% CTC. It is worth noting that the level of ALT in the 0.1% CTC group was significantly higher than those seen in any of the other groups. HDL-C, a type of cholesterol commonly referred to as "good" cholesterol. Some antibiotics, such as statins, can lower serum HDL-C level [50], while others may have no significant effect. While the 0.1% CTC group showed elevated levels of HDL-C, it is important to note that this did not diminish the observed liver damage in this group. BUN is a non-protein nitrogenous compound in the blood and is one of the primary products of protein metabolism in the body. Impaired renal function and excessive protein intake can lead to an elevated level of BUN in the blood [51,52]. In this experiment, we found that both CTC and *B. subtilis* can reduce the level of BUN, which indicates that there is more protein or amino acids

degraded into urea in the basal diet group. Both antibiotics and probiotics can reduce the abundance of harmful bacteria, improve intestinal villi, and enhance the ability to absorb protein [19]. Therefore, we believe that adding CTC and *B. subtilis* can both improve the absorption level of protein in geese, and adding 0.1% B. subtilis + 0.2% FOS and 0.1% CTC has a similar effect.

In summary, adding 100 mg/kg *B. subtilis* and $2x10^5$cfu g/kg FOS to the diet as an alternative to antibiotics can significantly improve the growth performance of geese in 14-70 d, improve the intestinal morphology of the jejunum, and prevent liver damage associated with antibiotics.

Availability of Data and Materials

The full dataset and supporting materials are provided within the study for reference. If additional information is desired, please direct inquiries to the corresponding author (H-Y Wang).

Acknowledgements

We would like to express our heartfelt gratitude to the staff at the Zhuanghang Experimental Station, Shanghai, China for their invaluable support and assistance in carrying out this research.

Ethical Approval

Animal experimentation was approved by the Laboratory Animal Ethics Committee of the Shanghai Academy of Agricultural Sciences (SAASPZ0522046).

Funding Support

This study was generously supported by China Agriculture Research System (CARS-42-35), the Climbing Plan of Shanghai Academy of Agricultural Sciences (PG21171), and the Excellent Research Team Program of SAAS (2022-021).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

The conception and design of the experiment were contributed by H-Y Wang and D-Q He. G-Q Li and X-Z Wang conducted the data analysis and drafted the manuscript. Yi L, Y-Z Yang, S-M Gong and C Wang were instrumental in data collection. All authors reviewed and approved the final manuscript for publication.

References

1. Castanon JIR: History of the use of antibiotic as growth promoters in European poultry feeds. *Poult Sci*, 86 (11): 2466-2471, 2007. DOI: 10.3382/ps.2007-00249

2. Ahrens P, Andersen LOB, Lilje B, Johannesen TB, Dahl EG, Baig S, Falk L: Changes in the vaginal microbiota following antibiotic treatment for *Mycoplasma genitalium, Chlamydia trachomatis* and bacterial vaginosis. *PloS One*, 15 (7):e0236036, 2020. DOI: 10.1371/journal.pone.0236036

3. Pokhrel A: Antimicrobial (antibiotic) resistance: An evolving threat to medicine. *Ann Med Surg*, 81:104357, 2022. DOI: 10.1016/j.amsu.2022.104357

4. Lavrukhina OI, Amelin VG, Kish, LK, Tretyakov AV, Pen'kov TD: Determination of residual amounts of antibiotics in environmental samples and food products. *J Anal Chem*, 77 (11): 1349-1385, 2022. DOI: 10.1134/S1061934822110077

5. Wang F, Lu Y, Cao J: Dynamics impacts of oxytetracycline on growth performance, intestinal health and antibiotic residue of grouper in exposure and withdrawal treatment. *Ecotox Environ Safe*, 247:114203, 2022. DOI: 10.1016/j.ecoenv.2022.114203

6. Hua Y, Yao Q, Lin J, Li X, Yang Y: Comprehensive survey and health risk assessment of antibiotic residues in freshwater fish in southeast China. *J Food Compos Anal*, 114:104821, 2022. DOI: 10.1016/j.jfca.2022.104821

7. Komura T, Takemoto A, Kosaka H, Suzuki T, Nishikawa Y: Prolonged lifespan, improved perception, and enhanced host defense of *Caenorhabditis elegans* by *Lactococcus cremoris* subsp. *cremoris*. *Microbiol Spectr*, 10 (3):e00454-21, 2022. DOI: 10.1128/spectrum.00454-21

8. Özbek YS, Özmen I: Optimization for coproduction of protease and cellulase from *Bacillus subtilis* M-11 by the Box-Behnken design and their detergentcompatibility. *Braz J Chem Eng*, 37, 49-59, 2020. DOI: 10.1007/s43153-020-00025-x

9. Mutlu A, Trauth S, Ziesack M, Nagler K, Bergeest JP, Rohr K, Bischofs IB: Phenotypic memory in *Bacillus subtilis* links dormancy entry and exit by a spore quantity-quality tradeoff. *Nat Commun*, 9 (1):69, 2018. DOI: 10.1038/s41467-017-02477-1

10. Elegbeleye JA, Buys EM: Molecular characterization and biofilm formation potential of *Bacillus subtilis* and *Bacillus velezensis* in extended shelf-life milk processing line. *J Dairy Sci*, 103 (6): 4991-5002, 2020. DOI: 10.3168/jds.2019-17919

11. Muniyappan M, Baek DH, Kim IH: Effects of dietary supplementation of quillaja saponin or fructooligosaccharide and a mixture of both on the growth performance, nutrient utilisation, faecal microbial and faecal noxious gas emissions in growing pigs. *Ital J Anim Sci*, 21 (1): 1489-1497, 2022. DOI: 10.1080/1828051X.2022.2093656

12. Abd El-Aziz AH, Abo Ghanima MM, Alsanie WF, Gaber A, Alsenosy AE, Easa AA, Moawed SA, Raza SHA, Elfadadny A, Yossef HA, Ghoneem WM, Shukry M, Hendawy AO, Mahrose K: Fructooligosaccharide supplementation boosts growth performance, antioxidant status, and cecal microbiota differently in two rabbit breeds. *Animals*, 12 (12):1528, 2022. DOI: 10.3390/ani12121528

13. Guerreiro I, Serra CR, Oliva-Teles A, Enes P: Gut microbiota of European sea bass *(Dicentrarchus labrax)* is modulated by short-chain fructooligosaccharides and xylooligosaccharides. *Aquacul Int*, 26, 279-288, 2018. DOI: 10.1007/s10499-017-0220-4

14. Bird AR, Vuaran M, Crittenden R, Hayakawa T, Playne MJ, Brown IL, Topping DL: Comparative effects of a high-amylose starch and a fructooligosaccharide on fecal bifidobacteria numbers and short-chain fatty acids in pigs fed Bifidobacterium animalis. *Digest Dis Sci,* 54, 947-954, 2009. DOI: 10.1007/s10620-008-0451-3

15. Porcheron G, Chanteloup NK, Trotereau A, Brée A, Schouler C: Effect of fructooligosaccharide metabolism on chicken colonization by an extra-intestinal pathogenic *Escherichia coli* strain. *PLoS One*, 7 (4):e35475, 2012. DOI: 10.1371/journal.pone.0035475

16. Mirghaed AT, Yarahmadi P, Hosseinifar SH, Tahmasebi D, Gheisvandi N, Ghaedi A: The effects singular or combined administration of fermentable fiber and probiotic on mucosal immune parameters, digestive enzyme activity, gut microbiota and growth performance of Caspian white fish *(Rutilus frisii kutum)* fingerlings. *Fish Shellfish Immun*, 77, 194-199, 2018. DOI: 10.1016/j.fsi.2018.02.007

17. Li WJ: Effects of probiotics, acidifiers and enzymes on growth performance, meat quality, serum biochemistry and antioxidant capacity of Dagu chickens. *Feed Res,* 5, 36-41, 2023.

18. Zhang B, Sui F, Wang B, Wang Y, Li W: Dietary combined supplementation of iron and *Bacillus subtilis* enhances reproductive performance, eggshell quality, nutrient digestibility, antioxidant capacity, and hematopoietic function in breeder geese. *Poult Sci*, 99 (11): 6119-6127, 2020. DOI: 10.1016/j.psj.2020.06.077

19. He T, Long S, Mahfuz S, Wu D, Wang X, Wei X, Piao X: Effects of probiotics as antibiotics substitutes on growth performance, serum biochemical parameters, intestinal morphology, and barrier function of broilers. *Animals*, 9 (11):985, 2019. DOI: 10.3390/ani9110985

20. Dale N: National Research Council (1994) Nutrient Requirements of Poultry. 9th revised ed., National Academy Press, Washington DC, 1994).

21. Xue JJ, Huang XF, Liu ZL, Chen Y, Zhang, YK, Luo Y, Wang C: Effects of citric acid supplementation on growth performance, intestinal

morphology and microbiota, and blood parameters of geese from 1 to 28 days of age. *Poult Sci,* 102 (2):102343, 2023. DOI: 10.1016/j.psj.2022.102343

22. Marx FO, Massuquetto A, Bassi LS, Krabbe E L, Rocha C, Oliveira SG, Maiorka A: Different soybean meal particle sizes on growth performance, nutrient ileal digestibility, digestible energy, and carcass yield of broiler chickens. *Lives Sci,* 247, 104467, 2021. DOI: 10.1016/j.livsci.2021.104467

23. Zhang J, He H, Yuan Y, Wan K, Li L, Liu A: Effects of yeast culture supplementation on growth performance, nutrient digestibility, blood metabolites, and immune response in geese. *Animals*, 12 (10):1270, 2022. DOI: 10.3390/ani12101270

24. Cremonesi P, Biscarini F, Castiglioni B, Sgoifo CA, Compiani R, Moroni P: Gut microbiome modifications over time when removing in-feed antibiotics from the prophylaxis of post-weaning diarrhea in piglets. *PloS One*, 17 (3):e0262199, 2022. DOI: 10.1371/journal.pone.0262199

25. Wang Y, Wang Y, Lin X, Gou Z, Fan Q, Ye J, Jiang S: Potential effects of acidifier and amylase as substitutes for antibiotic on the growth performance, nutrient digestion and gut microbiota in yellow-feathered broilers. *Animals*, 10 (10):1858, 2020. DOI: 10.3390/ani10101858

26. Wang B, Zhou Y, Tang L, Zeng Z, Gong L, Wu Y, Li WF: Effects of Bacillus amyloliquefaciens instead of antibiotics on growth performance, intestinal health, and intestinal microbiota of broilers. *Front Vet Sci*, 8:679368, 2021. DOI: 10.3389/fvets.2021.679368

27. Wang LM, Mandell IB, Bohrer BM: Effects of feeding essential oils and benzoic acid to replace antibiotics on finishing beef cattle growth, carcass characteristics, and sensory attributes. *Appl Anim Sci*, 36 (2): 145-156, 2020. DOI: 10.15232/aas.2019-01908

28. Yan H, Zhou P, Zhang Y, Zhang Z, Liu J, Zhang H: Short-chain fructo-oligosaccharides alleviates oxidized oil-induced intestinal dysfunction in piglets associated with the modulation of gut microbiota. *J Funct Foods,* 64:103661, 2020. DOI: 10.1016/j.jff.2019.103661

28. Liu ZY, Yang HL, Yan YY, Seerengaraj V, Zhang CX, Ye JD, Sun YZ: Supplementation of tributyrin, alone and in combination with fructo-oligosaccharide in high soybean meal diets for shrimp *(Litopenaeus vannamei)*: Effects on growth, innate immunity and intestinal morphology. *Aquacul Nutr*, 27 (2): 592-603, 2021. DOI: 10.1111/anu.13209

29. Zhang CN, Li XF, Xu WN, Zhang DD, Lu KL, Wang LN, Liu WB: Combined effects of dietary fructooligosaccharide and *Bacillus licheniformis* on growth performance, body composition, intestinal enzymes activities and gut histology of triangular bream *(Megalobrama terminalis). Aquacul Nutr*, 21 (5): 755-766, 2015. DOI: 10.1111/anu.12200

30. Bomhof MR, Saha DC, Reid DT, Paul HA, Reimer RA: Combined effects of oligofructose and Bifidobacterium animalis on gut microbiota and glycemia in obese rats. *Obesity*, 22 (3): 763-771, 2014. DOI: 10.1002/oby.20632

31. Sen S, Ingale SL, Kim JS, Kim KH, Kim YW, Khong C, Chae BJ: Effect of supplementation of *Bacillus subtilis* LS 1-2 grown on citrus-juice waste and corn-soybean meal substrate on growth performance, nutrient retention, caecal microbiology and small intestinal morphology of broilers. *Asian-Australas J Anim Sci*, 24 (8): 1120-1127, 2011. DOI: 10.5713/ajas.2011.10443

32. Hossain MM, Begum M, Kim IH: Effect of *Bacillus subtilis, Clostridium butyricum* and Lactobacillus acidophilus endospores on growth performance, nutrient digestibility, meat quality, relative organ weight, microbial shedding and excreta noxious gas emission in broilers. *Vet Med*, 60 (2): 77-86, 2015. DOI: 10.17221/7981-VETMED

33. Lee SH, Ingale SL, Kim JS, Kim KH, Lokhande A, Kim EK, Chae BJ: Effects of dietary supplementation with *Bacillus subtilis* LS 1-2 fermentation biomass on growth performance, nutrient digestibility, cecal microbiota and intestinal morphology of weanling pig. *Anim Feed Sci Technol*, 188, 102-110, 2014. DOI: 10.1016/j.anifeedsci.2013.12.001

34. Fukumori R, DOI K, Mochizuki T, Oikawa S, Gondaira S, Iwasaki T, Izumi K: Sodium butyrate administration modulates the ruminal villus height, inflammation-related gene expression, and plasma hormones concentration in dry cows fed a high-fiber diet. *Anim Sci J*, 93 (1):e13791, 2022. DOI: 10.1111/asj.13791

35. Chwen LT, Foo HL, Thanh NT, Choe DW: Growth performance, plasma Fatty acids, villous height and crypt depth of preweaning piglets fed with medium chain triacylglycerol. *Asian-Australas J Anim Sci,* 26 (5): 700-704, 2013. DOI: 10.5713/ajas.2012.12561

36. Gurung M, Rosa F, Yelvington B, Terry N, Read QD, Piccolo BD, Yeruva L: Evaluation of a plant-based infant formula containing almonds and buckwheat on gut microbiota composition, intestine morphology, metabolic and immune markers in a neonatal piglet model. *Nutrients*, 15 (2):383, 2023. DOI: 10.3390/nu15020383

37. Xu Y, Zhang B, Zhao R, Gao K, Liang S, Wu Y, Tang J: Effect of riboflavin deficiency on intestinal morphology, jejunum mucosa proteomics, and cecal microbiota of Pekin ducks. *Anim Nutr*, 12, 215-226, 2023. DOI: 10.1016/j.aninu.2022.09.013

38. Zhang Q, Li J, Yi X, Li Z, Liang S, Fang Z, Che L: Rhodotorula benthica culture as an alternative to antibiotics improves growth performance by improving nutrients digestibility and intestinal morphology, and modulating gut microbiota of weaned piglets. *Front Microbiol*, 13:964651, 2022. DOI: 10.3389/fmicb.2022.964531

40. Shim S: Effects of prebiotics, probiotics and synbiotics in the diet of young pigs. *PhD Thesis,* Wageningen Univ Res, 28238877, 2005.

41. Xu ZR, Zou XT, Hu CH, Xia MS, Zhan XA, Wang MQ: Effects of dietary fructooligosaccharide on digestive enzyme activities, intestinal microflora and morphology of growing pigs. *Asian-Australas J Anim Sci*, 15 (12): 1784-1789, 2022. DOI: 10.5713/ajas.2002.1784

42. Howard MD, Gordon DT, Pace LW, Garleb KA, Kerley MS: Effects of dietary supplementation with fructooligosaccharides on colonic microbiota populations and epithelial cell proliferation in neonatal pigs. *J Pediatr Gastr Nutr*, 21 (3): 297-303, 1995. DOI: 10.1097/00005176-199510000-00007

43. Xing Y, Wang S, Fan J, Oso AO, Kim SW, Xiao D, Zhang B: Effects of dietary supplementation with lysine-yielding Bacillus subtilis on gut morphology, cecal microflora, and intestinal immune response of Linwu ducks. *J Anim Sci*, 93 (7): 3449-3457, 2015. DOI: 10.2527/jas.2014-8090

44. Al-Fataftah AR, Abdelqader A: Effects of dietary Bacillus subtilis on heat-stressed broilers performance, intestinal morphology and microflora composition. *Anim Feed Sci Technol*, 198, 279-285, 2014. DOI: 10.1016/j.anifeedsci.2014.10.012

45. Ölmez M, Şahin T, Özlem Ö, Öğün M, Yörük MA, Dalğa S: Effect of probiotic mixture supplementation to drinking water on the growth performance, carcass parameters and serum biochemical parameters in native Turkish geese. *Kafkas Univ Vet Fak Derg*, 28 (1): 131-138, 2022. DOI: 10.9775/kvfd.2021.26633

46. Zhu CW, Liu JC, Wang J, Wang H, Liu YL, Li YG, Zhu L, Ding WM, Qiu YW, Yang YF, Li J, Huang R, Wu C: Liver inflammation activity in autoimmune hepatitis patients with normal ALT and IgG levels. *J Hepatol*, 77, S326-S327, 2022. DOI: 10.1016/S0168-8278(22)01017-0

47. Kogan M, Klempfner R, Lotan D, Wasserstrum Y, Goldenberg I, Segal G: Low ALT blood levels are associated with lower baseline fitness amongst participants of a cardiac rehabilitation program. *J Exerc Sci Fit*, 16 (1): 1-4, 2018. DOI: 10.1016/j.jesf.2017.11.002

48. Liu P, Yang M, Jia CX, Zhang QJ, Qu JQ: Effect of enrofloxacin on transaminase activity in serum and liver of Siberian sturgeon. *Fish Sci*, 2, 273-279, 2022. DOI: 10.16378/j.cnki.1003-1111.20090

49. Haque MI, Ahmad N, Miah MA: Comparative analysis of body weight and serum biochemistry in broilers supplemented with some selected probiotics and antibiotic growth promoters. *J Adv Vet Anim Res*, 4 (3): 288-294, 2017. DOI: 10.5455/javar.2017.d226

50. Mori T, Tanno Y, Kasakura S, Yoshioka K, Nakai N: Influence of statins on LDL-and HDL-cholesterol and plasma fatty acids in elderly Japanese ischemic stroke patients. *Austin J Cerebrovasc Dis Stroke*, 4 (3): 1062-1064, 2017. DOI: 10.26420/AUSTINJCEREBROVASCDISSTROKE.2017.1062

51. Hou H, Li L: Effects of continuous renal replacement therapy on Apache-II score, creatinine, and urea nitrogen levels in patients with acute kidney injury. *Pak J Med Sci*, 39 (1):6591, 2023. DOI: 10.12669/pjms.39.1.6591

52. Yu D, Zhu W, Hang S: Effects of low-protein diet on the intestinal morphology, digestive enzyme activity, blood urea nitrogen, and gut microbiota and metabolites in weaned pigs. *Arch Anim Nutr*, 73 (4): 287-305, 2019. DOI: 10.1080/1745039X.2019.1614849

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 255-263, 2023
DOI: 10.9775/kvfd.2023.29148

Research Article

Histomorphometric and Immunohistochemistry Studies of the Corpus Luteum of Bedouin Goats Reared in Arid Environment

Sara KASSOURI-MAOUCHE [1,2 (*)] Nouria BOUKENAOUI-FERROUK [1,2,3]
Lynda AINOUZ-AMMAR AOUCHICHE [4,5] Elara MOUDILOU [6,d] Jean-Marie EXBRAYAT [6]
Farida KHAMMAR [1,2]

[1] Houari BOUMEDIENE University of Sciences and Technology, Faculty of Biology, Laboratory of Research on Arid Lands, Bab Ezzouar 16111, Algiers, ALGERIA

[2] Benyoucef BENKHEDDA University of Algiers 1, Department of Biology, 16000, Algiers, ALGERIA

[3] Saad DAHLEB University Blida 1, Institute of Veterinary Sciences, 09100, Blida, ALGERIA

[4] Rabie BOUCHAMA National Higher Veterinary School, Oued Smar, 16059, Algiers, ALGERIA

[5] Mohamed Bachir EL IBRAHIMI Normal Higher School of Kouba, Department of Biology, Laboratory of Biology and Animal Physiology, 16308, Algiers, ALGERIA

[6] Science and Humanities Confluence Research Center - (EA 1598), 69002, Lyon, FRANCE

ORCIDs: S.K.M. 0000-0002-7468-1190; N.B.F. 0000-0002-8688-4614; L.A.A.A. 0000-0002-9986-9085; E.M. 0000-0002-4186-2948; J.M.E. 0000-0003-4280-2688; F.K. 0000-0001-7499-1431

Article ID: KVFD-2023-29148 Received: 13.02.2023 Accepted: 28.05.2023 Published Online: 29.05.2023

Abstract: The aim of this work was to study the histomorphometry and the immunohistochemistry of the Corpus luteum (CL) of Bedouin goat living in arid zones. Pregnant and non-pregnant females ovaries were collected from slaughterhouses, weighed, measured and then fixed in buffered formalin for histological, histochemical and immunolocalization of Ki-67, active caspase-3, aromatase and progesterone receptor. Our results showed that CL affected significantly the ovarian weight (P<0.001). The CL (P<0.05) and the large luteal cells (P<0.001) diameters were higher in pregnant compared to the non-pregnant CL. In the non-pregnant CL, the immunostaining of Ki-67 was only observed in the small luteal cell's nuclei whereas the active caspase-3 was detected in the large and small luteal cells cytoplasm. The aromatase was also detected in the CL and capsule cells cytoplasm, in contrast the progesterone receptor was observed in all luteal cell's cytoplasm and in some luteal cell nuclei. We conclude that in the Bedouin goat, the CL affects the ovarian biometric parameters. The development and luteolysis of non-pregnant CL seem to be under the balance between luteotropic and luteolytic markers, with luteolysis occurring by apoptosis via the caspase-3 pathway.

Keywords: *Active caspase-3, Aromatase, Corpus luteum, Goat, Histomorphometry, Immunohistochemistry, Progesterone receptor*

Kurak Ortamda Yetiştirilen Göçebe Keçilerin Corpus Luteum'u Üzerine Histomorfometrik ve İmmünohistokimyasal Çalışmalar

Öz: Bu çalışmanın amacı, kurak bölgelerde yaşayan göçebe keçilerin Corpus luteum'unun (CL) histomorfometrisini ve immünohistokimyasını incelemektir. Gebe ve gebe olmayan keçilerin ovaryumları mezbahalardan toplanmış, tartılmış, ölçülmüş ve daha sonra Ki-67, aktif kaspaz-3, aromataz ve progesteron reseptörünün histolojik, histokimyasal ve immünolokalizasyonu için tamponlu formalin içinde sabitlenmiştir. Bulgular, CL'nin ovaryum ağırlığını önemli ölçüde etkilediğini göstermiştir (P<0.001). CL (P<0.05) ve büyük luteal hücrelerin (P<0.001) çapları gebe CL'de gebe olmayan CL'ye kıyasla daha yüksekti. Gebe olmayan CL'de, Ki-67'nin immün boyanması sadece küçük luteal hücre çekirdeklerinde gözlenirken, aktif kaspaz-3, büyük ve küçük luteal hücre sitoplazmalarında tespit edilmiştir. Aromataz, CL ve kapsül hücrelerinin sitoplazmasında tespit edilmiş, buna karşın progesteron reseptörü tüm luteal hücrelerin sitoplazmasında ve bazı luteal hücre çekirdeklerinde gözlenmiştir. Göçebe keçilerinde, CL'nin ovaryum biyometrik parametrelerini etkilediği sonucuna vardık. Gebe olmayan CL'nin gelişimi ve luteolizi, luteotropik ve luteolitik belirteçler arasındaki denge altında görünmektedir ve luteoliz, kaspaz-3 yolu üzerinden apoptoz ile gerçekleşmektedir.

Anahtar sözcükler: *Aktif kaspaz-3, Aromataz, Korpus luteum, Keçi, Histomorfometri, İmmünohistokimya, Progesteron reseptörü*

How to cite this article?

Kassouri-Maouche S, Boukenaoui-Ferrouk N, Ainouz-Ammar Aouchiche L, Moudilou E, Exbrayat JM, Khammar F: Histomorphometric and immunohistochemistry studies of the corpus luteum of Bedouin goats reared in Arid environment. *Kafkas Univ Vet Fak Derg,* 29 (3): 255-263, 2023. DOI: 10.9775/kvfd.2023.29148

(*) Corresponding author: S. Kassouri-Maouche

Phone: +213 540 064 159
E-mail: kassourisara@gmail.com

Introduction

The Algerian Sahara is reputed to be the hottest and most deserted region in the world. Despite this hostile conditions, Bedouin goat, a small ruminant perfectly adapted to its environment [1], manages to maintain a good reproductive performance. This testifies to their great capacity for adaptation, the mechanism of which remains poorly understood. These species play an important economic role for the population living in arid regions. The Bedouin goat was known for its high fertility, fecundity, prolificacy rates and seasonal breeding activity [2-5], to ensure the survival of the offspring by coinciding parturition with the adequate period of nutrition and climatic conditions [6].

The corpus luteum (CL), called "temporary endocrine gland" is an important ovarian structure resulting from the differentiation of follicular cells from the ovulatory follicle after the expulsion of the oocyte [7]. The presence of CL characterizes the luteal phase in the non-pregnant female and determines the appropriate course of the oestrous cycle; it also ensures the subsequent maintenance of pregnancy [8]. Pregnancy in the goat has been shown to be dependent on the presence of CL even after mid-gestation due to the low amount of progesterone produced by the goat's uterus. Indeed, the goat placenta of the goat does not produce progesterone in sufficient amounts to support pregnancy [9]. The lifespan of CL is a function of luteotropic and luteolytic factors [10]. Many studies have reported that the CL regulation is under hypothalamic and pituitary-gonadal hormones, progesterone, aromatase, and oestrogen cited as local regulatory hormones that acting as paracrine and/or autocrine factors and angiogenic factor like Ki-67, their activity is a sign of maintained luteal activity and CL progression [11]. Luteolysis is classified into two forms; functional and morphological luteolysis. Functional luteolysis is the underlying deterioration of progesterone discharge while morphological luteolysis is the consequent change in the CL [12]. It was reported that apoptotic cell death are mediated by locally produced factors such as caspase family molecules [13]. However, the process of CL growth and regression remains poorly understood in the goat due to lack of evidence. Some aspects of ovarian structure in the Bedouin goat have been carried out in previous studies [3,14]. The CL homeostasis must be maintained in order to avoid any reproductive issues. Indeed, luteolysis is involved in the maintenance of pregnancy if this later took place, otherwise the CL must regress in order to give the female the opportunity to start another ovarian cycle and thus become pregnant.

The aim of the current study was to provide more information on the histomorphometric analysis of non-pregnant and pregnant CL and also the immunolocalization of Ki-67, active caspase-3, aromatase (P450-Arom) and progesterone receptor (PR) in the non-pregnant CL of the Bedouin goat which will allow us to understand the molecular mechanisms that regulate the function of the non-pregnant LC

Material and Methods

Ethical Statement

This study was approved by the Algerian Ministry of Higher Education and Scientific Research (Executive Decree 10-90 supplementing the Algerian government decree 04-82) and the AASEA (45/DGLPAG/DVA. SDA.14).

Animal Protocol and Sample Preparation

A total of 29 adult goat, pregnant (n=12) and non-pregnant (n=17) aged from 2 to 5 years were used in this study. The diagnosis of pregnancy was carried out in post-mortem. After the slaughtering of goats at Bechar slaughterhouses (South West of Algeria, 31°62'N, 2°22'W), the genital tract was removed, examined and ovaries were excised immediately, washed with phosphate-buffered saline (PBS), classified according to the presence or absence of CL and then measured by estimating the weight, length, width and thickness. The diameter of non-pregnant and pregnant CL was measured after longitudinal sectioning, along the axis of the helium, using the caliper *(Fig. 1)*. Then, the ovaries were fixed in buffered formalin (10%) to perform histological, histochemical and the immunohistochemical technics.

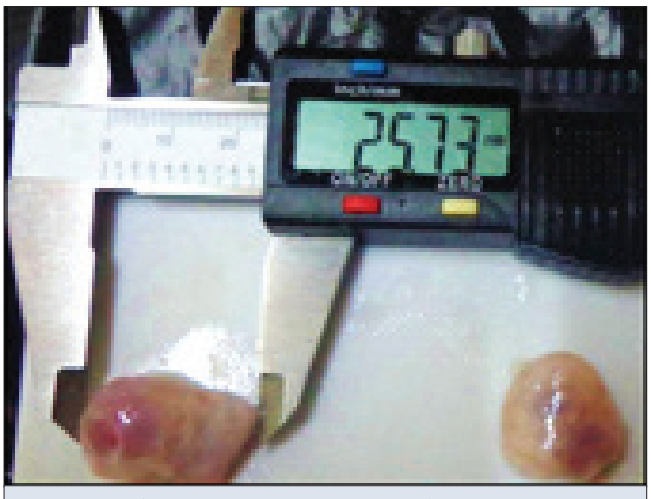

Fig 1. Ovarian biometric measurements using caliper

Histological Preparation

The fixed and sectioned ovaries were dehydrated in a graded series of ethanol (70, 80, 90 and 100°), clarified in xylene, embedded in paraffin and sectioned at 4 µm. Sections were used for the histological technique, mounted

on slides, deparaffinised and hydrated then stained with modified azan to demonstrate collagen fibres. For histochemical studies, the sections were stained by alcian blue at pH 2.5 to visualize carbohydrates [15].

Immunohistochemistry

Immunohistochemical detection of Ki-67, active caspase-3, P450-Arom and PR was performed on paraffin-embedded sections of ovaries using avidin-biotin complex method (ABC) (vectastain Elite ABC kit, Vector Laboratories, Burlingame). Before proceeding with the immunodetection of active caspace-3, sections were deparaffinised, hydrated and put in PBS (0.1 M; pH=7.2) then permeabilized at room temperature in 0.2 mg/mL saponin (Fischer Scientific UK) and proteinase K (Eurobio) mixture [3]. For the immunolocalisation of P450-Arom, PR and Ki-67 [15], the antigen retrieval step was realised by immersing of the section in 10 mM of sodium citrate solution (pH=6.0) in a water bath during 40 min at 95°C. The endogenous peroxidases are blocked by H_2O_2 (3% in PBS) during 5 min then rinsed in PBS. The nonspecific background was blocked by a normal horse serum for 30 min at room temperature. Sections were incubated with different primary antibodies at room temperature: rabbit monoclonal active caspase-3 antibody (1:50, ab32042 Abcam, Cambridge, UK Cambridge, UK) for 1h, mouse monoclonal Ki-67 antibody (1:50, RM-9106-S, Thermo Fisher Scientific, USA) for 1h, rabbit polyclonal CYP19A1 antibody (1:100, H-300, SC-30086 Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse monoclonal PR antibody (1:50, AB-52, sc-810, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. After rinsing for PBS, all sections were incubated with a biotinylated anti-mouse/rabbit IgG secondary antibody (CA 94010, Vectastain Elite ABC Kit, Vector Laboratories, Burlingame) for 1 h at room temperature. They were incubated for 1h at room temperature with Avidin-Horse Radish Peroxidase complex and then rinsed in PBS. For the visualisation of the immunolabeling, the DAB (3,3′-diaminobenzidine) chromogen (Dako) was added as a chromogen staining substrate. Sections were counterstained in Harris haematoxylin (Hematoxylin QS, H-3404; Vector lab, Burlingame, A, USA). After rinsing in water, the sections were dehydrated and cover slipped with Eukitt. For negative controls, a similar protocol was performed for each antibody, except for incubation of the primary antibody which is replaced by the normal horse serum.

Morphometric Analysis

The histological slides were stained with modified azan coloration and observed with light microscope (Optika B-350) using a computer program Ts View connected to a digital eye-camera (Hirocam MA88-500). The parameters evaluated for CL were: CL, large luteal cell (LC), small luteal cell (SC), large luteal cell nuclei (LCN), small luteal cell nuclei (SCN) diameters and cytoplasm-to-nucleus ratio (CNR) of LCN and SCN. The area (A) of these parameters was measured using Axiovision software. The shape of the CL cells and nuclei was assumed to be spherical; their diameter (D) was deduced by the mathematical relationship applied for the calculation of the area.

$$D = \sqrt{\left(\frac{4A}{\pi}\right)}$$

with $A = \pi \times r^2$ and $r = D/2$

Statistical Analysis

The results were represented by means ± SEM. Statistical analyses were performed using the SPSS for Windows v.26.0 (IBM Corp., NY, USA). Data which were not normally distributed were submitted to logarithmic transformations. The data were analysed with independent *t*-test or with Mann-Whitney test as appropriate. These tests were used to compare the differences in parameters between the ovaries with and without CL also between pregnant and non-pregnant CL groups. We considered that $P<0.05$ was statistically significant.

Results

Morphological Study

The morphological study of the ovaries *(Fig. 2-A)* revealed an ovoid shape with an irregular surface *(Fig. 2-B)* containing some follicles and CL *(Fig. 2-C)*. Both of ovaries from pregnant and non-pregnant females were exhibited the same colour and did not show apparent differences in their morphology.

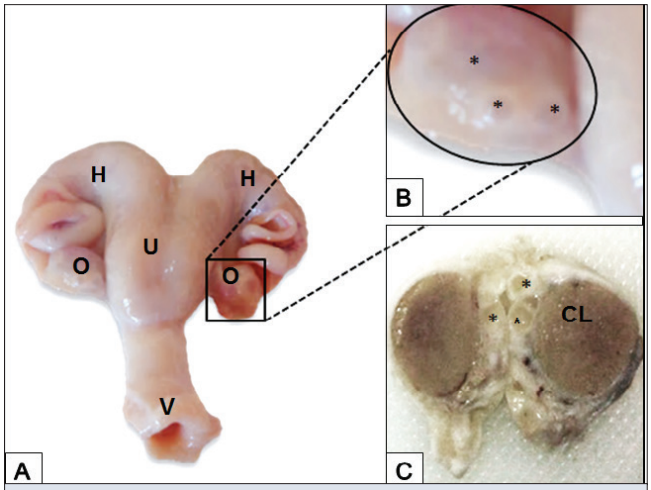

Fig 2. Genital tract of Bedouin goats reared in arid environment. A- Localisation of the ovary in the genital tract. B- External view of ovary. C- Sectioned ovary. CL: corpus luteum, H: horn, O: ovaries, U: uterus, V: vulva, *: Ovarian follicles

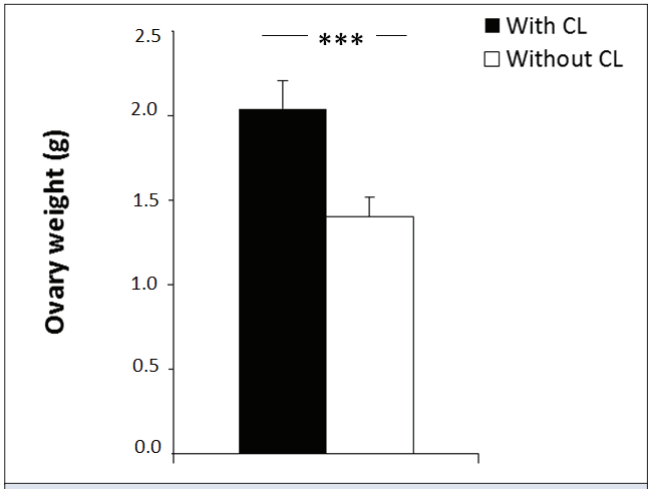

Fig 3. Weight of the ovaries with and without corpus luteum of Bedouin goats reared in Arid environment. Data are presented as mean ± SEM. ***P<0.001

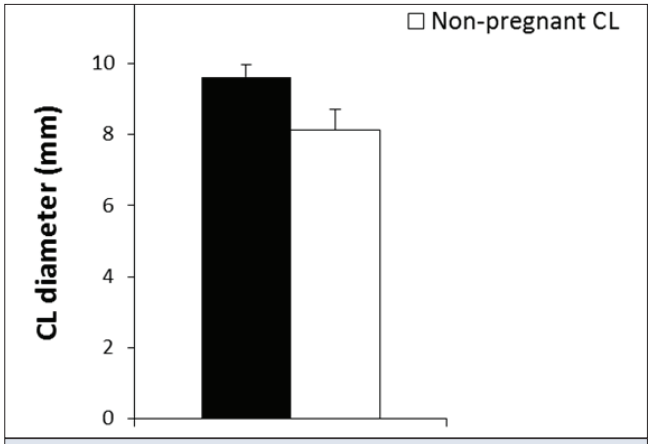

Fig 5. Diameter of corpus luteum from pregnant and non-pregnant Bedouin goats reared in arid environment. Data are presented as mean ± SEM

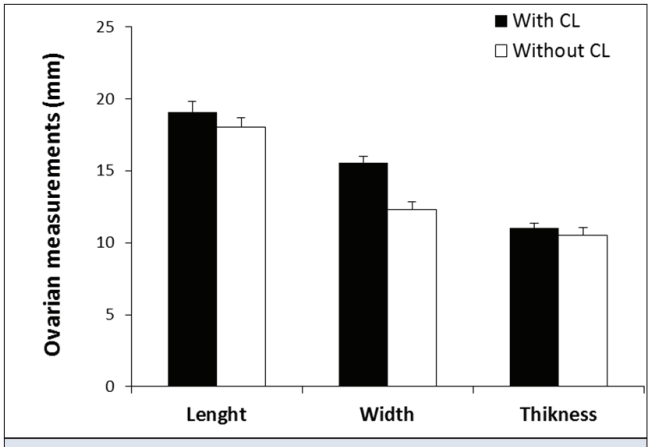

Fig 4. Measurements of the ovaries with and without the corpus luteum of Bedouin goats reared in arid environment. Data are presented as mean ± SEM

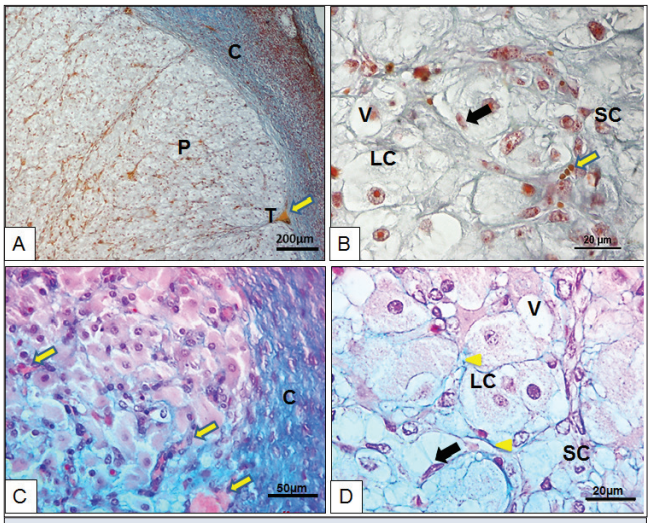

Fig 6. Histology of corpus luteum of Bedouin goats reared in arid environment. A: Photomicrograph showing capsule and trabeculae richly vascularised *(yellow arrow)* surrounding the parenchyma which is crossed by trabeculae, B: Photomicrograph showing the composition of CL parenchyma, LC are ovoid and enclose vacuole and eccentric nuclei with a prominent nucleolus, small luteal cell (SC), blood cells *(yellow arrow)* and fibroblast cells *(black arrow)*, C-D: Photomicrographs showing the carbohydrates in the CL, the capsule were highly reactive to the alcian blue and were richly vascularized *(yellow arrow)*, the alcian blue reactivity was also observed surrounding the luteal cells *(yellow arrow ahead)* in (D). C: capsule; LC: Large luteal cells; SC: small luteal cells; P: parenchyma; T: trabeculae; V: vacuole. Stained with modified azan (A and B) and alcian blue (C and D)

Ovarian Morphometric Study

The morphometric analysis of the ovaries parameters were reported in the *Fig. 3* and *Fig. 4.* The weight of ovaries with and without CL (2.04±0.16 g *vs* 1.4±0.12 g respectively) showed a highly significant difference (P<0.001) (*Fig. 3*).

Ovaries with and without CL showed a non-significant difference (P>0.05) in length (19.09±0.74 *vs* 18.05±0.63 mm), width (15.55±0.45 *vs* 12.33±0.54 mm) and thickness (11.00±0.38 *vs* 10.53±0.52 mm) *(Fig. 4)*.

The diameter of pregnant CL was higher (P>0.05) than the diameter of non-pregnant CL (9.63±0.34 mm *vs* 8.12±0.59 mm respectively) *(Fig. 5)*.

Histomorphometry of Corpus Luteum

Histologically, the CL was delimited by a capsule which was composed of fibroblasts, mainly comprised of collagen fibbers and blood vessels surrounded the CL parenchyma *(Fig. 6-A)*. Histochemically, this capsule exhibited a positive reaction to the alcian blue *(Fig. 6-C)*. The CL parenchyma was crossed by trabeculae containing connective tissue and constituted by endothelial, blood, small and large luteal cells *(Fig. 6-B,C)*. Both of LC and SC were spherical cells centred by spherical nucleus. However, the LC is characterised by its large size and the presence of vacuoles in its cytoplasm *(Fig. 6-B)*.

The morphometric measurements of the diameter of luteal cells and nuclei between pregnant and non-pregnant CL

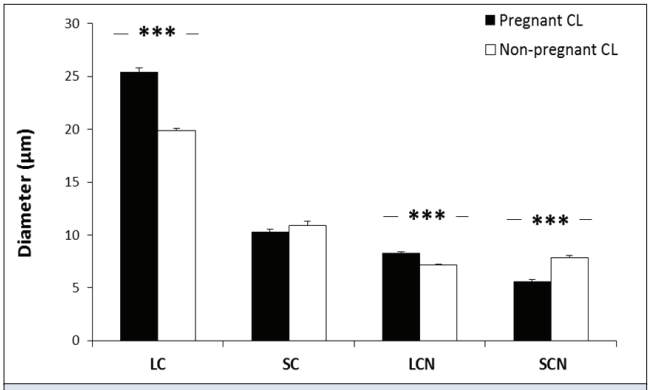

Fig 7. Diameter of luteal cells and nuclei from pregnant and non-pregnant corpus luteum of Bedouin goats reared in arid environment. LC: large luteal cells; LCN: large luteal cells nuclei; SC: small luteal cells; SCN: small cells nuclei. Data are presented as mean ± SEM. ***P<0.001

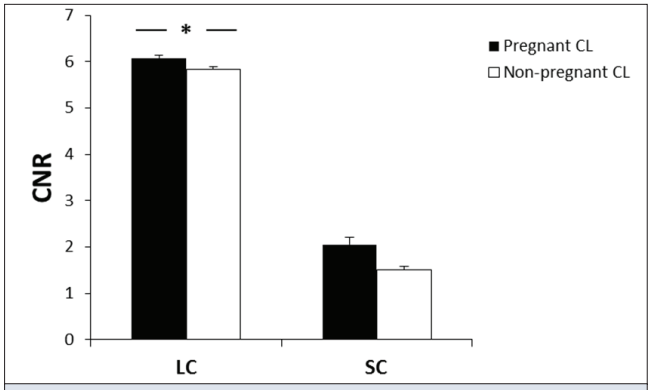

Fig 8. The cytoplasme-to-nucleus ratio of luteal cells from pregnant and non-pregnant corpus luteum of Bedouin goats reared in arid environment. LC: large luteal cells; SC: small luteal cells. Data are presented as mean ± SEM. *P<0.05

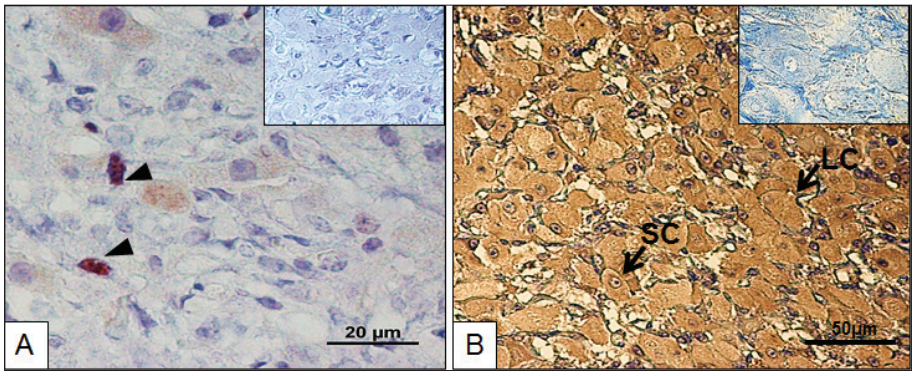

Fig 9. Immunohistochemistry of Ki-67 and active caspase-3 in non-pregnant corpus luteum of Bedouin goats reared in arid environment. A - Immunolocalization of Ki-67 in nuclei of SC *(black arrow)*. B- Immunolocalisation of active caspase-3 in both LC and SC cytoplasm, DAB visualization system. Negative controls insert in A and B

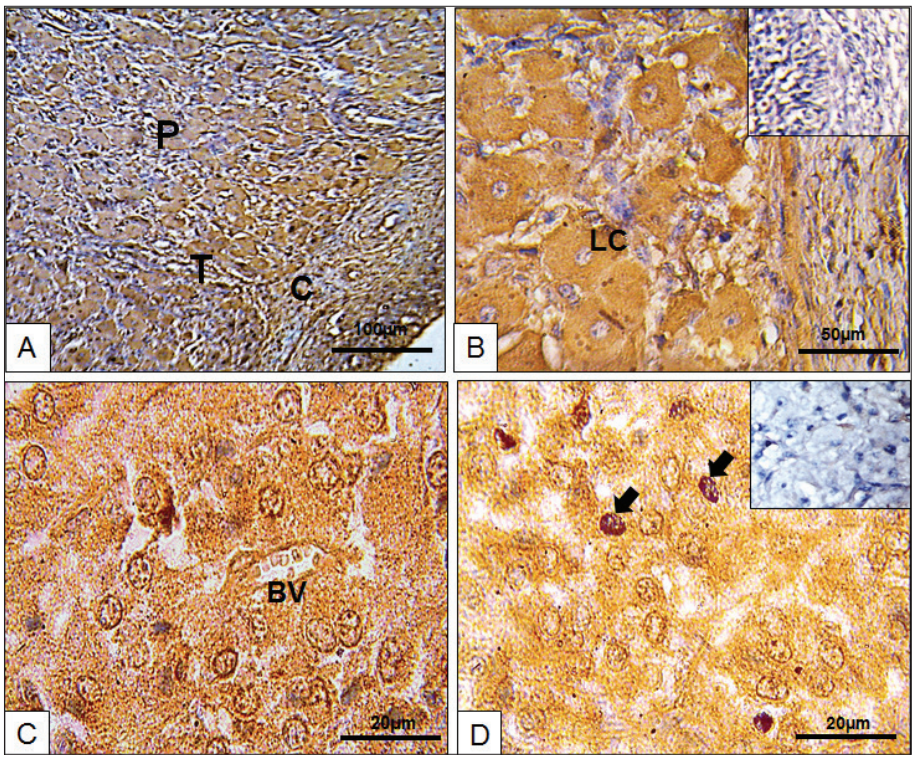

Fig 10. Immunohistochemistry of aromatase and progesterone receptor in non-pregnant corpus luteum of Bedouin goats reared in arid environment. A and B- Immunolocalization of P450-Arom in non-pregnant CL showed cytoplasmic staining in the luteal cells and capsule cells. C and D- immunolocalization of PR in the cytoplasm of CL cells predominantly and in some nuclei *(black arrow)*, DAB visualization system. Negative controls insert in B and D

were reported in *Fig. 7*. The LC diameter from pregnant CL (25.44±0.34 µm) was significantly higher (P<0.001) than LC diameter from the non-pregnant CL (19.85±0.21 µm). The LCN diameter was significantly higher (P<0.001) in the pregnant CL (8.33±0.08 µm) than that of non-pregnant CL (7.14±0.1 µm). Moreover, the SC diameter

from pregnant CL (10.27±0.29 µm) was lower compared to that to the SC from the non-pregnant CL (10.89±0.44 µm) (P>0.05). A significant difference has been observed between the SCN from pregnant CL and that from non-pregnant CL (P<0.001). Additionally, the CNR of LC (6.078±0.06 *vs* 5.85±0.06) (P<0.01) and SC (2.05±0.16 *vs* 1.50±0.09) (P<0.001) was significantly higher in pregnant CL than that of non-pregnant and *(Fig. 8)*.

Immunohistochemistry of Corpus Luteum

The immunohistochemistry of Ki-67 and active caspase-3 in non-pregnant CL was reported in *Fig. 9*. The immuno-localization of Ki-67 was observed only in nuclei of SC *(Fig. 9-A)*; the other CL cells did not exhibit any immunostaining. However, the active caspase-3 was immunolocalized in the cytoplasm of both LC and SC of non-pregnant CL *(Fig. 9-B)*.

The immunohistochemistry of P450-Arom and PR in non-pregnant CL was reported in *Fig. 10.* The CL expressed P450-Arom in all of the CL cells cytoplasm and capsule fibroblasts *(Fig. 10-A)*. The PR was observed in SC and LC cytoplasm with a variable intensity of immunostaining at the level of the nucleus of these cells *(Fig. 10-B)*.

Discussion

In this study, the morphological aspects of the goat ovaries collected from local slaughterhouses have been described; we reported that all of the ovaries were similar in shape and colour. Macroscopically, the description of ovaries was similar to those reported in other goats [16]. Regarding the presence or absence of CL, these ovaries were recorded as ovaries with and without CL. On the other hand, the mean weight of ovaries was significantly higher (P<0.01) and length, width and thickness were comparatively higher in the ovaries with CL than that without CL. It has been revealed that the presence of CL in ovaries increases ovarian biometric measurements. The same observations have been reported in other goat breeds [17-19]. Shathi et al.[20] and Mervat et al.[21] demonstrated the effect of CL presence on the morphometry of goat and cow ovaries respectively which affects the weight and dimensions. Miranda-Moura et al.[22] showed the existence of positive correlation between the dimensions of the ovary and the CL and between the weight of the ovary and the CL. Jablonka-Shariff et al.[23] explained that the higher value of biometric measurements of ovaries were due to the hypertrophy of luteinized granulosa cells, hyperplasty of fibroblasts of the connective tissues and vascularity of the CL.

The diameter of the non-pregnant CL found in this study was lower than that of pregnant CL. The non-pregnant CL of Bedouin goat was similar of that of the Alpine goat in dioestrus phase [24]. This increase in CL diameter, during pregnancy, suggest that the growth of luteal tissue is positively correlated with CL functionality which is represented by synthesis and production of progesterone [24].

Histologically, it was observed that the CL in the Bedouin goat is formed by heterogeneous population cells (small luteal, large luteal, fibroblastic and endothelial cells) in accordance with the composition of the CL observed in the Alpin goat [19], Nelore sheep [25] and cows [10].

Our study demonstrates that the morphometric diameters of the large and small luteal cells was higher compared to the luteal cells from Angora goat which varied as CL aged [26]. The CNR of both large and small luteal cells was significantly higher in the pregnant CL than in the non-pregnant CL; it is admitted that in growing cells, the cytoplasm is continuously expanding from amino-acid and nutrient import, and also protein synthesis [27]. Moreover, the CNR was affected by numerous factors: protein and ribosome synthesis, transport across both cell surface and the nuclear envelop and protein degradation and ribosome disassembly [28]. This may explain why the luteal cells of the pregnant CL were more active than the luteal cells of the cyclic CL , this activity seems to be related to steroidogenic synthesis to maintain the pregnancy which is essentially dependent on the presence of CL in goat [9] .

In this study, we have highlighted some factors such as Ki-67 as a cell proliferation marker, active caspase¬-3 a luteolytic factor, P450-Arom and PR. Indeed, the activity of CL was influenced by the balance between luteotropic and luteolytic factors which affected the structural and morphological appearance of the CL [29]. Investigating the CL activity, we found that the Ki-67 was localized in the nucleus of steridogenic cells, more precisely in the SC in the non-pregnant CL. The same observation was reported by Yoshioka et al.[30] in cattle CL which demonstrated that only SC proliferate during luteal development. Indeed, during the ruminant CL life, granulosa-derived luteal cells were predominantly non-proliferative while theca-derived luteal cells were proliferative during the early luteal phase and become non-proliferative by the late luteal phase [31]. In addition, it was reported that in the early luteal phase the development of CL is supported by robust angiogenesis which is accompanied by dynamic extracellular matrix remodelling that affects deeply the CL development and maturation [32]. As a result, the mature CL is a highly vascular gland and luteal endothelial cells comprise the larger part of its cells [32].

In the other hand, we reported that the luteal cells in some of CL were positive to active caspase-3 suggesting that these luteal cells initiated the cell death pathway via a caspase dependent mechanism. In this study, caspase-3 activity (marker of apoptosis) was detected in the large and small

luteal cells cytoplasm. It was reported in a previous study in the Bedouin goat, that morphological changes reach atretic follicles wall by apoptosis via caspase-3 signalling pathway in breeding and non-breeding seasons [3]. The active caspase-3 being expressed in the granulosa and theca cells [3] suggested that these cells retain the same cell death mechanism after their differentiation into luteal cells in CL. Caspase-3 has been also shown to be involved in luteal regression in cows [33], sheep [34], rabbits [35], women [36] and mice [37]. However, morphologically we did not observe any structural changes indicating the luteolysis, it seems probably a functional luteolysis as reported in the study of Hiti et al.[38] yet, the transition from functional luteolysis to structural luteolysis has not been precisely presumed. In another study, It was reported that the activity of caspase-3 was involved in initial process of cell death and observed before morphological changes [32]. In addition, It seems that the expression of the active caspase-3 interfered with functional luteolysis including cessation [39]. Accumulating evidence indicates that luteolysis was divided into two phases, namely functional luteolysis characterized by a decline in progesterone concentration and structural luteolysis characterized by the degradation of luteal tissues from the ovary [40].

The present study shows that both large and small luteal cells exhibited positive cells immunostaining to P450-Arom, this finding agreed with the study done in Japanese Shiba goat [41], Criollo goat, sheep [42] and pigs [43]. Previous studies demonstrated the expression of P450-Arom in both luteal cell type which increased as the luteal phase progresses [44]. Gregoraszczuk reported a few positive cells to P450-Arom in the early porcine CL cells but no reactivity was detected in mid luteal CL [44].The presence of positive signal for P450-Arom in the non-pregnant CL of the Bedouin goat suggested that this tissue has the capacity to produce oestrogen.

As a result of our study, we observed strong immunostaining for PR in goat luteal cells in non-pregnant CL; the immunolabeling was essentially cytoplasmic with few occasional immunostaining nuclei which suggest specific genomic response. It is well known that the primary function of the CL is the secretion of progesterone (P4), which is required for maintenance of normal pregnancy in mammals [42]. The P4 exerts its main function by binding with progesterone receptors PR to induce cellular responses through genomic or non-genomic signalling cascades[45]. The increase of the diameter of large luteal cell in our study seems due to its steroidogenic activity to produce more progesterone during CL progression to prepare and maintain the gestation. It is reported in the previous data that both SC and LC, are capable of producing this steroid, however, LC are more secretory-active [46]. In the Nelore cow, it is suggested that there is a stimulatory effect of progesterone in a paracrine/autocrine manner on the formation and the initial secretory activity of the CL. In addition, in the pseudo pregnant rabbit, the atretic large lutein cells of the regressed corpus luteum showed negative immunostaining for PR [47]. Indeed, While the majority of the existing research on Progesterone focuses on classic P4/PR paired actions such as nuclear transcriptional factors, there is new evidence suggesting that P4 also induces a wide variety of P4 actions through non-classic membrane PR receptors [45].

In conclusion, the presence of CL caused a significant increase in the ovarian wight and also increased various ovarian biometric parameters. The large luteal cells increased in diameter in the pregnant CL compared to the non-pregnant CL suggesting that the steroidogenic activity may be provided by the large luteal cells more than the small ones which are characterised by proliferative ability. The functioning of the non-pregnant CL is represented by the oestrogen production via the aromatase activity and P4/PR regulation. The luteolysis in CL of the Bedouin goat undergoes the apoptotic mechanism via caspase-3 pathway.

Availability of Data and Materials

The datasets generated and/or analysed during the current study are available from the corresponding author upon reasonable request.

Acknowledgements

We thank Drs BOUDJELLABA and SALHI (Veterinary doctors), SALMI, YAICHE and all of Bechar slaughterhouse's workers for their helpful in tissue sampling.

Financial Support

The authors declared that there is no financial support

Conflict of Interest

The authors declared that there is no conflict of interest

Author Contributions

S.K.M.: Conceptualization, Methodology, Validation, Formal analysis, investigation, Writing - Original Draft, Writing - Review & Editing, Visualization. N.B.F.: Methodology, Formal analysis, Visualization, Review & Editing. L.A.A.A.: Visualization and Review. E.M.: Conceptualization, Methodology, J.M.E.: Visualisation and Revision. F.K.: Resources, Visualization & Supervision.

References

1. Choshniak I, Ben-Kohav N, Taylor CR, Robertshaw D, Barnes RJ, Dobson A, Belkin V, Shkolnik A: Metabolic adaptations for desert survival in the Bedouin goat. *Am J Physiol Regul Integr Comp Phys,* 268 (5): R1101-R1110, 1995. DOI: 10.1152/ajpregu.1995.268.5.R1101

2. Charallah S, Lakhdari Y, Amirat Z, Khammar F, Sempere A: Variations saisonnières hormonales de l'activité sexuelle chez la chèvre bédouine. *Bul Soc Ecophysio,* 18 (1-2): 67-70, 1993.

3. Kassouri-Maouche S, Boukenaoui-Ferrouk N, Charallah S, Moudilou E, Chakhma A, Exbrayat JM, Amirat Z, Khammar F: Atretic ovarian follicles morphology and immunolocalization of active caspase-3 in algerian bedouin goat *(Capra hircus)* ovaries. *Kafkas Univ Vet Fak Derg,* 25, 147-156, 2018. DOI: 10.9775/kvfd.2018.20292

4. Chakhma A, Khaldoun-Benabbas M, Charallah-Cherif S, Kassouri S, Khammar F, Amirat Z: Annual changes in plasma progesterone and estradiol-17β concentrations compared to pituitary-adrenal axis activity in the female goat reared under arid environment. *Biol Rhythm Res,* 52, 1394-1411, 2019. DOI: 10.1080/09291016.2019.1630920

5. Charallah S, Khammar F, Amirat Z, Lakhdari Y: Sexual activity of Bedouin goat *(Capra hircus):* Zootechnical and nutritional characterisation. *7th International Conference on Goats, 15-21 May,* France, 2000.

6. Farsi H, Mhani M, Achaaban MR, Boukhliq R, Tibary A, Allali KE: Environmental cues and seasonal patterns of reproduction in goats. *Rev Mar Sci Agron Vét,* 6 (2): 158-167, 2018.

7. Couse JF, Yates MM, Deroo BJ, Korach KS: Estrogen receptor-β is critical to granulosa cell differentiation and the ovulatory response to gonadotropins. *Endocrinology,* 146 (8): 3247-3262, 2005. DOI: 10.1210/en.2005-0213

8. Mlyczynska E, Kiezun M, Kurowska P, Dawid M, Pich K, Respekta N, Daudon M, Rytelewska E, Dobrzyn K, Kaminska B, Kaminski T, Smolinska N, Dupont J, Rak A: New aspects of corpus luteum regulation in physiological and pathological conditions: involvement of adipokines and neuropeptides. *Cells,* 11 (6):957, 2022. DOI: 10.3390/cells11060957

9. Sheldrick EL, Ricketts AP, Flint APF: Placental production of progesterone in ovariectomized goats treated with a synthetic progesterone to maintain pregnancy. *J Reprod Fertil,* 60 (2): 339-348, 1980. DOI: 10.1530/jrf.0.0600339

10. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW: Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev,* 80 (1): 1-29, 2000. DOI: 10.1152/physrev.2000.80.1.1

11. Stouffer RL, Hennebold JD: Chapter 23 - Structure, function, and regulation of the corpus luteum. **In,** Plant TM, Zeleznik AJ (Eds): Knobil and Neill's Physiology of Reproduction. Fourth ed., 1023-1076, Academic Press, San Diego, 2015.

12. Inbaraj KC, Yu Z, Chai S, Alagamuthu KK, Vallavan R, Meyyazhagan A, Balasubramanian B, Chinappan G, Xu S, Yang G: Regression of corpus luteum in cetaceans: A systematic review. *Zoology (Jena),* 149:125960, 2021. DOI: 10.1016/j.zool.2021.125960

13. Meidan R, Girsh E, Mamluk R, Levy N, Farberov S: Luteolysis in Ruminants: Past Concepts, New Insights, and Persisting Challenges. **In,** Meidan R (Ed): The Life Cycle of the Corpus Luteum; 159-182, 2017.

14. Kassouri S, Boukenaoui N, Charallah S, Amirat Z, Khammar F: Immunolocalization of androgen receptor in the ovary of Bedouine goat in non breeding season. **In,** Farooq SA, Abed R, Bagir S (Eds): Biotechnology and Conservation of Species from Arid Regionsed. 43-47, Nova Science Publishers, New york, USA, 2014.

15. Raquet M, Brun C, Exbrayat JM: Patterns of apoptosis and proliferation throughout the biennial reproductive cycle of viviparous female *Typhlonectes compressicauda* (Amphibia, Gymnophiona). *Int J Mol Sci,* 18 (1):16, 2016. DOI: 10.3390/ijms18010016

16. Tizhe MA, Kubkomawa HI, Butswat IRS, Addass PA, Kwaji DT: Characterization of reproductive organs of female goats in Guinea Savannah Zone of North-Eastern Nigeria. *Sky J Agric Res,* 4 (3): 47-59, 2015.

17. Islam MR, Khandoker MA, Afroz S, Rahman MG, Khan RI: Qualitative and quantitative analysis of goat ovaries, follicles and oocytes in view of in vitro production of embryos. *J Zhejiang Univ Sci B,* 8 (7): 465-469, 2007. DOI: 10.1631/jzus.2007.B0465

18. Asad L, Rahman ANMI, Hossain MM, Akter M: Ovarian category, follicles and oocytes analysis of Goat ovaries in view of *in vitro* production of embryos. *Int J Anim Resour,* 1, 27-34, 2016. DOI: 10.1631/jzus.2007.B0465

19. Groppetti D, Pecile A, Frattini S, Pagnacco G, Arrighi S: Histological feature of ovarian structures throughout the reproductive cycle in alpine goats *(Capra hircus). Maced Vet Rev,* 42, 23-34, 2019. DOI: 10.2478/macvetrev-2018-0027

20. Shathi SJ, Khandoker M, Debnath S, Mahzabin R, Jahan MA, Islam MR, Khatun A: Evaluation of goat ovaries and follicles by histological study. *Bangladesh J Anim Sci,* 51 (4): 145-151, 2022. DOI: 10.3329/bjas.v51i4.63565

21. Mervat SH, EL-Zeftawy M: Evaluation of corpora lutea effect on ovarian morphometry, follicular population and biochemical profile in follicular fluid and blood of slaughtered cows. *Assiut Vet Med J,* 65 (160): 80-89, 2019. DOI: 10.21608/AVMJ.2019.167316

22. Miranda-Moura MTM, Fonseca VU, Silva NB, Freitas ML, Almeida OB, Rocha HAO, Papa PC, Moura CEB: Morphological features and vascularization study of caprine cyclic corpus luteum. *Pesqui Vet Brasil,* 30 (4): 351-357, 2010. DOI: 10.1590/S0100-736X2010000400012

23. Jablonka-Shariff A, Grazul-Bilska AT, Redmer DA, Reynolds LP: Growth and cellular proliferation of ovine corpora lutea throughout the estrous cycle. *Endocrinology,* 133 (4): 1871-1879, 1993. DOI: 10.1210/endo.133.4.8404629

24. Arashiro EKN, Viana JHM, Fonseca JF, Almeida Camargo LS, Carvalho Fernandes CA, Zandonadi Brandão F: Luteal dynamics in goats: Morphological and endocrine features. *R Bras Zootec,* 39 (9): 1937-1942, 2010. DOI: 10.1590/S1516-35982010000900011

25. Supriya BV, Jamuna KV, Ramayya PJ, Prasad R, Sathyanarayana ML, Kumar A: Histological and micrometrical exploration of corpus luteum in cyclic Nellore sheep. *Int J Chem Stud,* 8 (2): 155-157, 2020.

26. Kalender H, Arikan Ş: Size distribution of dispersed luteal cells during oestrous cycle in angora goats. *Reprod Domest Anim,* 42 (5): 457-460, 2007. DOI: 10.1111/j.1439-0531.2006.00806.x

27. Daghash SM, Yasin NAE, Abdelnaby EA, Emam IA, Tolba A, Abouelela YS: Histological and hemodynamic characterization of corpus luteum throughout the luteal phase in pregnant and non-pregnant buffalos in relation to nitric oxide levels based on its anatomical determination. *Front Vet Sci,* 9:896581, 2022. DOI: 10.3389/fvets.2022.896581

28. Wu Y, Janmey P, Sun SX: The correlation between cell and nucleus size is explained by an eukaryotic cell growth model. *PLoS Comput Biol,* 18 (2):e1009400, 2022. DOI: 10.1371/journal.pcbi.1009400

29. Stouffer R, Hennebold J: Structure, function, and regulation of the corpus luteum. **In,** Knobil and Neill's Physiology of Reproduction. 1023-1076, Elsevier Inc., 2015.

30. Yoshioka S, Abe H, Sakumoto R, Okuda K: Proliferation of luteal steroidogenic cells in cattle. *PLoS ONE,* 8 (12):e84186, 2013. DOI: 10.1371/journal.pone.0084186

31. Farin C, Moeller C, Sawyer H, Gamboni F, Niswender G: Morphometric analysis of cell types in the ovine corpus luteum throughout the estrous cycle. *Biol Reprod,* 35, 1299-1308, 1986. DOI: 10.1095/biolreprod35.5.1299

32. Shrestha K, Rodler D, Sinowatz F, Meidan R: Corpus luteum formation. **In,** Leung PCK, Adashi EY (Eds): The Ovary. Third ed., 255-267, Academic Press, 2019.

33. Okuda K, Korzekwa A, Shibaya M, Murakami S, Nishimura R, Tsubouchi M, Woclawek-Potocka I, Skarzynski DJ: Progesterone is a suppressor of apoptosis in bovine luteal cells. *Biol Reprod,* 71 (6): 2065-2071, 2004. DOI: 10.1095/biolreprod.104.028076

34. Rueda BR, Hendry IR, Tilly JL, Hamernik DL: Accumulation of caspase-3 messenger ribonucleic acid and induction of caspase activity in the ovine corpus luteum following prostaglandin F2α treatment *in vivo. Biol Reprod,* 60 (5): 1087-1092, 1999. DOI: 10.1095/biolreprod60.5.1087

35. Abdo MA, Richards A, Atiya N, Singh B, Parkinson S, Hisheh S, Dharmarajan AM: Inhibitors of caspase homologues suppress an apoptotic phenotype in cultured rabbit corpora lutea. *Reprod Fertil Dev,* 13, 395-403, 2001. DOI: 10.1071/rd01035

36. Khan SM, Dauffenbach LM, Yeh J: Mitochondria and caspases in induced apoptosis in human luteinized granulosa cells. *Biochem Biophys Res Commun,* 269 (2): 542-545, 2000. DOI: 10.1006/bbrc.2000.2321

37. Carambula SF, Matikainen T, Lynch MP, Flavell RA, Dias Gonc Alves PB, Tilly JL, Rueda BR: Caspase-3 Is a pivotal mediator of apoptosis during regression of the ovarian corpus luteum. *Endocrinol,* 143 (4): 1495-1501, 2002. DOI: 10.1210/endo.143.4.8726

38. Hitit M, Kose M, Kocak N, Atli MO: Expression patterns of genes in steroidogenic, cholesterol uptake, and liver x receptor-mediated cholesterol efflux pathway regulating cholesterol homeostasis in natural and PGF2α induced luteolysis as well as early pregnancy in ovine corpus luteum. *Anim Reprod Sci,* 240: 106988, 2022. DOI: 10.1016/j.anireprosci.2022.106988

39. Luz MR, Cesário MD, Binelli M, Lopes MD: Canine corpus luteum regression: Apoptosis and caspase-3 activity. *Theriogenology,* 66 (6-7): 1448-1453, 2006. DOI: 10.1016/j.theriogenology.2006.02.025

40. Hojo T, Skarzynski DJ, Okuda K: Apoptosis, autophagic cell death, and necroptosis: different types of programmed cell death in bovine corpus luteum regression. *J Reprod Dev,* 68 (6): 355-360, 2022. DOI: 10.1262/jrd.2022-097

41. Weng Q, Medan MS, Ren L, Watanabe G, Tsubota T, Taya K: Immunolocalization of steroidogenic enzymes in the corpus luteum and placenta of the Japanese Shiba goat. *J Reprod Dev,* 51 (2): 247-252, 2005. DOI: 10.1262/jrd.16081

42. Mondragon JA, Miranda C, Garcia-Mena J, Ocadiz-Delgado R, Gariglio P, Romano MC: P450-aromatase mRNA is expressed in the corpus luteum (CL) of the non-pregnant sheep and goat: The expression of the enzyme is present throughout pregnancy in the goat CL. *Reprod Domest Anim,* 48 (1): 85-89, 2013. DOI: 10.1111/j.1439-0531.2012.02035.x

43. Słomczyñska M, Tabarowskib Z: Localization of androgen receptor and cytochrome P450 aromatase in the follicle and corpus luteum of the porcine ovary. *Anim Reprod Sci,* 65, 127-134, 2001. DOI: 10.1016/S0378-4320(00)00225-6

44. Gregoraszczuk EL: Large and small cells of the porcine corpus luteum: Differential capacity to secrete estradiol and aromatize exogenous androgen during mid- and late luteal phase. *Exp Clin Endocrinol Diabetes,* 104: 278-283, 1996. DOI: 10.1055/s-0029-1211454

45. Aickareth J, Hawwar M, Sanchez N, Gnanasekaran R, Zhang J: Membrane progesterone receptors (mPRs/PAQRs) are going beyond its initial definitions. *Membranes,* 13 (3):260, 2023. DOI: 10.3390/membranes13030260

46. Mlyczyńska E, Kieżun M, Kurowska P, Dawid M, Pich K, Respekta N, Daudon M, Rytelewska E, Dobrzyń K, Kamińska B, Kamiński T, Smolińska N, Dupont J, Rak A: New aspects of corpus luteum regulation in physiological and pathological conditions: involvement of adipokines and neuropeptides. *Cells,* 11 (6):957, 2022. DOI: 10.3390/cells11060957

47. Abd-Elkareem M, Abou-Elhamd AS: Immunohistohemical localization of progesterone receptors alpha (PRA) in ovary of the pseudopregnant rabbit. *Anim Reprod,* 16 (2): 302-310, 2019. DOI: 10.21451/1984-3143-ar2018-0128

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 265-271, 2023
DOI: 10.9775/kvfd.2023.29162

Research Article

Determination of Biofilm Formation, Antibacterial Resistance and Genotypes of *Bacillus cereus* Isolates from Raw Milk

Sadık SAVAŞAN [1] Çağatay NUHAY [2] Volkan Enes ERGÜDEN [3] Serap SAVAŞAN [4 (*)]

[1] Aydın Adnan Menderes University, Graduate School of Health Sciences, Food Hygiene and Technology Department, TR-09100 Aydın - TÜRKİYE

[2] Bornova Veterinary Control Institute, Bacteriology Laboratory, TR-35040 İzmir - TÜRKİYE

[3] Ondokuz Mayıs University, Faculty of Veterinary Medicine, Veterinary Microbiology Department, TR-55200 Samsun - TÜRKİYE

[4] Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Veterinary Microbiology Department, TR-09100 Aydın - TÜRKİYE

ORCIDs: S.S. 0000-0002-5077-3189; Ç.N. 0000-0002-1475-3041; V.E.E. 0000-0003-2215-2868; S.S. 0000-0002-9826-077X

Article ID: KVFD-2023-29162 Received: 15.01.2023 Accepted: 29.04.2023 Published Online: 05.05.2023

Abstract: *Bacillus cereus* is a foodborne pathogen that has a widespread presence in the environment and frequently found in foods especially in dairy products. Raw milk contaminated with *B. cereus* could be the cause of its widespreadness in the environment. In this study, it was aimed to determine the genotypes, biofilm formation, antimicrobial susceptibilities, and antibiotypes of *B. cereus* isolates from raw milk. For this aim, *B. cereus* isolated and identified from 10 of 250 raw milk samples were investigated. Biofilm forming abilities were determined *in vitro* by Congo Red Agar Method. Kirby Bauer Disc Diffusion Method was used for determining the antibiotic susceptibilities of the isolates. According to the antibiotic susceptibility results, quantitative antibiotyping was implemented. Genotyping of the isolates were performed by RAPD-PCR. Biofilm formation was determined in 40% of the isolates. The resistances against amoxicillin-clavulanic acid, gentamicin, erythromycin, vancomycin, chloramphenicol, tetracycline, trimethoprim-sulfamethoxazole were determined in 100%, 0%, 30%, 0%, 0%, 0%, and 50% of the isolates, respectively. In the quantitative antibiotyping, the isolates showed similarity between 0.75 to 1.00. The phylogenetic similarities were calculated between 29% to 82%. In conclusion, raw milks might threat the public health because of having potential of containing the antibiotic resistant *B. cereus*.

Keywords: *Antibacterial resistance, B. cereus, Biofilm, Genotyping, Raw milk*

Çiğ Süt Kökenli *Bacillus cereus* İzolatlarının Biyofilm Oluşturma, Antibakteriyel Direnç ve Genotiplerinin Belirlenmesi

Öz: *Bacillus cereus*, doğal ortamlarda yaygın olarak bulunan, gıdalarda, özellikle süt ürünlerinde sıklıkla bulunan, gıda kaynaklı patojendir. Çiğ süt, çevrede yaygın olarak bulunmasından dolayı *B. cereus* ile kolaylıkla kontamine olmaktadır. Bu çalışmada çiğ sütten izole edilen *B. cereus* izolatlarının genotip, biyofilm, antimikrobiyal duyarlılıkları ve antibiyotiplerinin belirlenmesi amaçlandı. Bu amaçla 250 adet çiğ süt örneğinden izole ve identifiye edilen 10 adet *B. cereus* izolatı incelendi. Biyofilm oluşturma yetenekleri Kongo Red Agar Metodu ile belirlendi. İzolatların antibiyotik duyarlılıklarının belirlenmesinde Kirby Bauer Disk Difüzyon testi kullanıldı. Antibiyotik duyarlılık sonuçlarına göre kantitatif antibiyotiplendirme gerçekleştirildi. İzolatların genotiplendirilmesi RAPD-PCR ile yapıldı. Suşların %40'ında biyofilm oluşumu saptandı. İzolatların amoksisilin-klavulanik asit, gentamisin, eritromisin, vankomisin, kloramfenikol, tetrasiklin, trimetoprim-sülfametoksazol dirençleri sırasıyla %100, %0, %30, %0, %0, %0 ve %50 olarak belirlendi. Kantitatif antibiyotiplendirme sonucunda izolatlar 0,75 ile 1,00 arasında benzerlik gösterdi. Filogenetik benzerlikler %29 ile %82 arasında hesaplandı. Sonuç olarak, çiğ sütler antibiyotiğe dirençli *B. cereus* içerme potansiyeline sahip olduğundan halk sağlığını tehdit edebilir bulunmuştur.

Anahtar sözcükler: *Antibakteriyel direnç, B. cereus, Biofilm, Çiğ süt, Genotiplendirme*

How to cite this article?

Savaşan S, Nuhay Ç, Ergüden VE, Savaşan S: Determination of biofilm formation, antibacterial resistance and genotypes of *Bacillus cereus* isolates from raw milk. *Kafkas Univ Vet Fak Derg,* 29 (3): 265-271, 2023.
DOI: 10.9775/kvfd.2023.29162

(*) Corresponding author: Serap SAVAŞAN

Phone: +90 256 2206000 Cellular phone: +90 532 5694957 Fax: +90 256 2206299
E-mail: ssavasan@adu.edu.tr

Introduction

Composition of raw milk is a key point that influence the quality of milk and milk products. Microbiological ingredients play a major role in spoilage of raw milk. Some of the microorganisms, such as *Bacillus cereus,* affect on the safety and quality of raw milk [1]. *B. cereus* is a foodborne pathogen spread in environment and found in foods especially in dairy products [2]. The contamination of *B. cereus* has a higher rate than the other foodborne pathogens and its growth resulted to various dairy defects [3,4]. *B. cereus* remains a problem for dairy products in terms of shelf life and public health safety. Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) announced that *B. cereus* is the third agent reason of communal foodborne infections in Europe. In 2016, European Union announced that 5.5% of the outbreaks by foodborne pathogens are related to *Bacillus* [5]. The goal of dairy producers is to supply good quality products, but psychrotrophic bacteria in raw milk complicates this goal.

Microorganisms in milk have the ability like adhering and aggregating on stainless steel surfaces, resulted by biofilm formation in storage tanks and process surfaces. Adhesive biofilms formed by *B. cereus* can provide a source of contamination during production and processing. Biofilm formation increases the risk of cross-contamination by negatively affecting shelf life and reliability of dairy products. The structure of the biofilm also increases the resistance to immune system and antimicrobial agents as well as causes mechanical damage [6]. *B. cereus* can adhere to a wide variety of materials used in food processes to form biofilms. Biofilms often cannot be removed during Cleaning in Places (CIP) procedures. This makes *B. cereus* a foodborne pathogen causing deterioration of food quality and health hazards. *Bacillus* spp. can form resistant biofilms. This can result in continued contamination and is a significant risk for food quality and safety [7].

It is important not only to indicate the presence of *B. cereus* in foods, but also to detect disease-causing factors. Except beta-lactam antibiotics, most of the *B. cereus* isolates are susceptible to commonly used antimicrobial agents [8]. Antibiotic-resistant *B. cereus* strains are the cause of horizontal gene transfer [4]. *B. cereus* can also cause problems in the dairy industry due to its resistance to disinfectants. Establishing a *B. cereus* antibiotic resistance profile is important for public health [9,10]. Reports announced that *B. cereus* isolated from different foods are resistant to many antibiotics like ceftriaxone, tetracycline, streptomycin, trimethoprim, ampicillin, and penicillin. It is important to determine the resistance of foodborne *B. cereus* to antibiotics in order to better manage infectious diseases [11]. Mobile genetic elements lead to the spread of antibiotic resistance between *Bacillus* and the other pathogens through horizontal gene transfer [12]. Investigation of antibiotic resistance of *B. cereus* is important for food safety and public health.

Genome analyzes reveal that *B. cereus* was activated in protein metabolism, suggesting the adaptation of *B. cereus* to a symbiotic or parasitic life cycle. Evaluation of the relationships of microbial changes through chemical analyzes or analytical technologies should allow for the identification of the metabolic activity of the active degradation microbiota [13]. Methods for genotyping of *B. cereus* have been reported as multiple-locus variable-number tandem repeat analysis (MLVA), amplified fragment length polymorphism (AFLP), repetitive element palindromic-PCR (rep-PCR), and randomly amplified polymorphic DNA PCR (RAPD-PCR) [9]. Molecular biology methods determine the specific genes of *B. cereus* and it is time-saving and highly specific, but have difficulties like equipment and personnel needs and in achieving constant temperature sensing. Methods based on PCR like RAPD-PCR are more common in use [14]. The VITEK2 BCL card method has made significant progress in the reliable identification of *Bacillus* spp. and related genera [15]. This method is an automatic microbial identification system that provides accurate and reproducible results, and is also a fast and reliable application for pathogen identification. The VITEK2 method is advantageous over PCR [16,17].

The aim of this study was to determine the biofilm formation, antimicrobial resistance, genotypes and antibiotypes of *B. cereus* strains isolated from raw milk.

Material and Methods

Isolation and Identification of *B. cereus*

Raw milk samples (n=250) from İzmir, Türkiye were brought to Bornova Veterinary Control Institute, Bacteriology Laboratory under cold chain conditions.

The raw milk samples were plated onto Columbia Agar (5% sheep blood, Liofilchem) and incubated at 37°C for 24-48 h. After the incubation period, colonies with strong β-hemolytic activity were applied Gram staining [18]. Gram-positive bacilli colonies were purified and then identified with the BCL ready card on the VITEK 2 (bioMérieux) instrument [16].

The identifications of the isolates were confirmed by PCR. The specific *motB* gene targeting PCR for the identification of *B. cereus* was carried out with the protocol as reported [19]. The presence of a 575 bp band after imaging was considered positive for *B. cereus*.

Determination of Biofilm Formation

Biofilm formation of isolates was detected *in vitro* by Congo Red Agar (CRA) method. CRA method was carried

out with the reported method [20]. By taking a single colony from the pure colonies in Trypticase Soy Agar (TSA), it was inoculated to CRA, and incubated at 37°C for 24-48 h under aerobic conditions. After incubation, color changes were observed. Isolates forming black-gray colony on CRA were determined as positive for the biofilm production and the pink-red colonies were determined as negative.

Determination of Antimicrobial Resistance and Antibiotyping

Antimicrobial resistances of *B. cereus* isolates were tested by Kirby-Bauer Disc Diffusion Method. A bacterial suspension was prepared from fresh cultures of the isolates in Physiological Buffer Solution (PBS) with a density of 0.5 McFarland. The prepared suspension (100 µL) was inoculated on Mueller Hinton Agar (MHA) surface. The 7 antibiotics including chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (30 µg), vancomycin (5 µg), trimethoprim-sulfamethoxazole (25 µg), amoxicillin-clavulanic acid (20 µg/10 µg) and tetracycline (30 µg) were selected. Antibiotic discs were placed on the medium and incubated at 37°C for 24 h. Zone diameters formed after incubation period were measured and evaluated according to Clinical and Laboratory Standards Institute (CLSI) 2020 (for chloramphenicol, gentamicin, trimethoprim-sulfamethoxazole, tetracycline), CLSI 2012 (for amoxicillin-clavulanic acid) and to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) 2023 (for erythromycin, vancomycin) guidelines. The interpretive categories and zone diameter breakpoints of the antibiotics (except erythromycin and vancomycin) against *S. aureus* ATCC 25923 were used for testing resistances of the isolates according to CLSI guidelines. For erythromycin and vancomycin breakpoints against *Bacillus spp*., EUCAST guideline was used [10,21-23].

The antibiotyping was performed according to the resistance profiles of the strains by means of the Unweighted Pair Group Method using arithmetic averages (UPGMA) cluster analysis, and the dendrogram was created for evaluation of relatedness between the strains [24,25]. For this method, the antibiotic resistance patterns were recorded to a table as susceptible (S), intermediate resistant (I) or resistant (R) for each antibiotics tested. This table was converted to a figure showing bands like a genotyping pattern. Then, this figure was analyzed in commercial band analyzes software (e.g. Quantitity One, BioRad). The phylophenotypic tree were drawn with using the software depending on the antibiotic resistance profiles.

Genotyping of *Bacillus cereus*

The ERIC-2 (Enterobacterial Repetitive Intergenic Consensus-2) primer (5'-AAG TAA GTG ACT GGG GTG AGC G-3') was used to evaluate RAPD-PCR patterns of *B. cereus* isolates. For PCR, a 25 µl RAPD master mix containing 1X PCR Buffer, 2.5 mM $MgCl_2$, 200 µM each dNTP, 2.5 U *Taq* DNA polymerase, 25 pmol primer and 5 µL template DNA was prepared. This mixture was pre-denatured for 5 min at 94°C followed at 94°C by 1 min denaturation, at 40°C 1 min bonding, at 72°C 3 min elongation at 40 cyclus and for 7 min at 72°C were subjected to amplification at final elongation conditions. Amplification products were visualized by UV transilluminator with 1.5% agarose gel electrophoresis containing ethidium bromide (2 µg/mL) [26]. The UPGMA clustering method was used to generate dendrograms of RAPD patterns by using image analysis program. Genetic relationship between the isolates was also evaluated by considering the 70% similarity coefficient.

Results

Isolation and Identification of *B. cereus*

The Gram-positive bacilli colonies were purified after incubation period, and identified with BCL ready card on VITEK 2 (bioMérieux) instrument. The identifications of the isolates were confirmed by PCR, and all 10 isolates gave specific bands of 575 bp for B. cereus *(Fig. 1)*. *B. cereus* was isolated and identified from 10 of 250 raw milk samples at the rate of 4%.

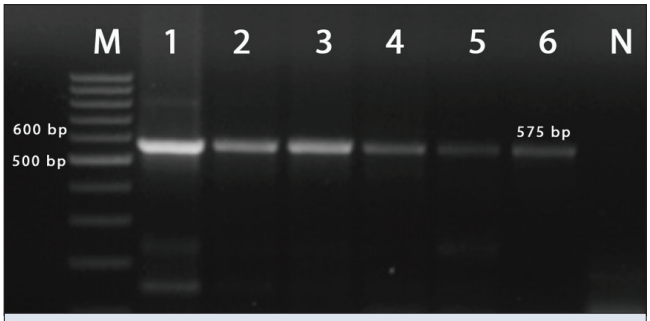

Fig 1. Confirmation of identifications by PCR. M: marker, 1-6: *B. cereus* isolates, N: negative control *(Escherichia coli)*

Determination of Biofilm Formation

Biofilm formations of *B. cereus* isolates (n=10) were evaluated with CRA method. It was determined that 4 of 10 *B. cereus* isolates at the percentage of 40% had biofilm activity.

Determination of Antimicrobial Resistance and Antibiotyping

B. cereus isolates were tested for antimicrobial resistance profiles against 7 selected antibiotics. All the isolates (n=10) were susceptible to chloramphenicol, gentamicin,

Table 1. *Antibiotic resistance/susceptibility of 10 Bacillus cereus isolates in this study*

Antibiotic	Susceptible*		Intermediate**		Resistant***	
	n	%	n	%	n	%
Amoxicillin-Clavulanic acid	0	0	0	0	10	100
Gentamicin	10	100	0	0	0	0
Erythromycin	7	70	0	0	3	30
Vancomycin	10	100	0	0	0	0
Chloramphenicol	10	100	0	0	0	0
Tetracycline	10	100	0	0	0	0
Trimethoprim-sulfamethoxazole	2	20	3	30	5	50

**Susceptible: indicates the diameter of inhibition zone (DIZ) against B. cereus strain was larger than the quality control strain; **Intermediate: indicates the DIZ against B. cereus strain was between SUS and RES; ***Resistant: indicates the DIZ against B. cereus strain was less than the quality control strain*

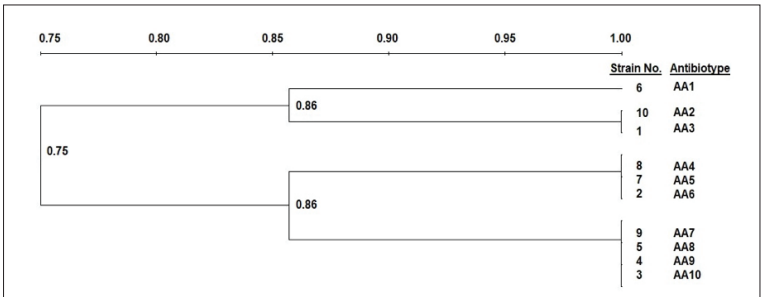

Fig 2. Dendrogram of 10 *Bacillus cereus* isolates according to their antibiotic susceptibility profiles

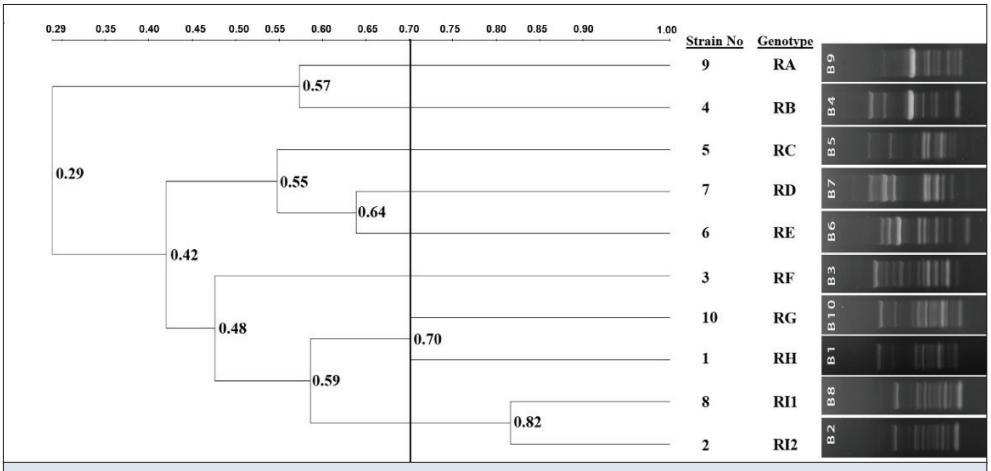

Fig 3. Dendrogram of 10 *Bacillus cereus* isolates after phylogenetic analysis for genotyping

tetracycline and vancomycin as well as resistant to amoxicillin-clavulanic acid *(Table 1)*. After determining antibiotic resistance/susceptibility status of the isolates, their phylophenotypicv similarities were determined by considering the resistance to the relevant antibiotics. The phylophenotypicv similarities of *B. cereus* isolates were calculated and they showed 75-100% similarity *(Fig. 2)*. The phylophenotypic relationship between isolates was evaluated by considering the 70% similarity coefficient. As the result of the evaluation, it was determined that isolates had 1 multiple antibiotype (AA).

Genotyping of *Bacillus cereus*

The phylogenetic analysis of *B. cereus* isolates by RAPD-PCR for genotyping was determined that the isolates showed similarity between 29-82% *(Fig. 3)*. Genetic relationships among isolates were evaluated by considering the 70% similarity coefficient. It was determined that

isolates had 8 single genotypes (RA-RH) and 1 multiple genotype (RI). It was observed that 2 isolates in the RI multiple genotypes showed 82% similarity.

Discussion

B. cereus has long been a health threat to human and animals, also have a significant impact on the food industry and agriculture [2,27]. *B. cereus* can cause hygiene problems and economic losses due to the deterioration of dairy products and sticking on process equipment [3]. *B. cereus* can weaken the bactericidal effect of disinfectants [10]. *B. cereus* has a higher contamination ability than other foodborne pathogens [4]. *B. cereus* isolates from pasteurized milk placed in same cluster, indicates that they came from a similar source, on the other hand raw milk isolates varied at a level showing different sources [28]. The studies can determine the distribution and genetic diversity of *B. cereus* strains found in raw milk and can provide a theoretical basis for controlling potential harms of this pathogen in dairy and dairy products [29]. In this study, *B. cereus* was detected in 10 of 250 raw milk samples at the rate of 4%. Compared to previous studies, it is the lowest contamination level of raw milk samples compared with 6.66% in a cheese plant at Alexandria [30], 9.8% in dairy farms in China [31], 23.3% in 60 raw milk samples [1], 26% in China [4], 29.5% in milk samples from the dairy animals [32], 33.3% in Guangxi, Yunnan, and Guizhou, the provinces of China [10], 37.5% in household milk from dairy environments [33], 40% in Pakistan [34], 40% in Zagazig city [35], 47% in Ghana [36], and 85% in Egypt [37].

In our research, it was determined that 4 of the 10 *B. cereus* isolates at the percentage of 40% had biofilm activity. Compared to previous studies, it is lower compared with the isolates in Victoria, Australia those had 53.7% biofilm forming ability [28]. In a previous study, they measured the ability to form biofilms of 5 groups of *B. cereus* with 41 ST (sequence type) on stainless-steel tubes [10]. In another study, all the isolates of *B. cereus* bacteria were selected to verify biofilm formation ability in microtiter plates and results showed that all isolates (100%) could form biofilm. Data highlights that dairy industry needs to reinforce control in the initial quality of raw material and in CIP cleaning applications [38].

In this study, it was determined that all isolates were susceptible to chloramphenicol, gentamicin, tetracycline and vancomycin as well as resistant to amoxicillin-clavulanic acid. Compared to a previous study, *B. cereus* isolates had resistance to amoxicillin at the rate of 80% whereas our rate was 100%. The isolates were susceptible to erythromycin, vancomycin at the rates of 100%, 93.33% [35] respectively, whereas our rates were respectively 70% and 100%. In another study, *B. cereus* isolates showed resistance to amoxicillin and tetracycline with 68.9% and 51.1% [33] whereas our rates were 100% and 0%, respectively. The study in Ghana presented that, *B. cereus* isolates were resistant to amoxicillin at the rate of 100% that was same in our study. The isolates susceptible to gentamicin and chloramphenicol at the rates 100% and 99% [36] whereas similar to our study 100% and 100%, respectively. The study in Southwestern China introduced that all isolates were susceptible to gentamicin and chloramphenicol [10] same as in our study. The antibiotic susceptibility of 54 *B. cereus* isolates to 17 antibiotics was tested and all isolates were determined susceptible to chloramphenicol and gentamicin [4] same as our study. Isolates were susceptible to tetracycline, trimethoprim-sulfamethoxazole, erythromycin at the rates of 98.15%, 85.18% and 83.33% [4] whereas 100%, 20% and 70% in our study, respectively. Accept trimethoprim-sulfamethoxazole, the other rates are quite similar with our study. In another study, *B. cereus* isolates were susceptible to gentamicin at the rate of 100% as same in our study [39].

The phylogenetic analysis of *B. cereus* isolates determined that the isolates showed similarity between 29-82%. It was determined that the isolates had 8 single genotypes (RA-RH) and 1 multiple genotype (RI). It was observed that 2 isolates in the RI multiple genotype showed 82% similarity. A previous study determined that the 96 *B. cereus* strains containing 41 ST (sequence type) were divided into 5 clusters using 90% similarity for the critical threshold [10]. Another study presented that 56 *B. cereus* strains were detected from 300 environmental samples and 50 raw milk samples divided into 18 sequence types (STs) using multilocus sequence typing method. The results could reveal the distribution and genetic diversity of *B. cereus* strains in raw milk and cattle farm environments, and provide a theoretical basis for controlling the potential harm of these pathogenic bacteria in dairy products [29]. It was presented that 54 strains of *B. cereus* isolates were divided into 24 ST in raw milk samples. An elaborated phylogenetic relationship of the 54 *B. cereus* strains was clustered into 3 groups. The results showed no obvious association between *B. cereus* genotype and collection regions [4]. A previous study introduced that 14 sequence types of *B. cereus* were found in raw bovine milk samples and all isolates in the research and 13 selected reference strains showed phylogenetic relationship [40].

As the biofilm formation ability positive strains (no. 3,6,7,9) were evaluated, it was determined that the phylophenotypic similarities of antibiotic resistance/susceptibility of the strains 3 and 9 showed 100% similarity while their genetic relationship showed 29% similarity. The strains 6 and 7 showed 64% genetic similarity while their phylophenotypic similarity of antibiotic resistance/susceptibility was determined as 75% similarity. The strains 2 and 8 which were biofilm formation ability

negative, showed the highest genetic similarity rate as 82% while their phylophenotypic similarity of antibiotic resistance/susceptibility was 100%.

In conclusion, the widespread presence in the natural environment, high biofilm formation ability, resistance to heat treatment and antibiotics make *B. cereus* an important bacterium to follow in terms of public health. *B. cereus* shows a wide diversity from raw milk to the final product. In order to reduce the risks of this foodborne pathogen in terms of raw milk, dairy products and public health, it is very important to implement sanitation rules at all stages from raw milk to the final product. Determination of critical control points such as transport tanks, storage tanks, production equipment, in places and personnel from the raw milk stage to the final product stage and the implementation of sanitation practices at these points is highly important against *B. cereus* contamination.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (S. Savaşan) on reasonable request.

Funding Support

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

Ethical Statement

The study does not require ethical approval from Animal Experiments Local Ethics Committee Funding Support.

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

SS, ÇN, VEE and SS planned, designed and performed the analysis. The manuscript was written by SS and SS. All authors have interpreted the data, revised the manuscript for contents, and approved the final version.

References

1. Liang L, Wang P, Qu T, Zhao X, Ge Y, Chen Y: Detection and quantification of *Bacillus cereus* and its spores in raw milk by qPCR, and distinguish *Bacillus cereus* from other bacteria of the genus *Bacillus*. *Food Qual Saf*, 6, 1-10, 2022. DOI: 10.1093/fqsafe/fyab035

2. Liu XY, Hu Q, Xu F, Ding SY, Zhu K: Characterization of *Bacillus cereus* in dairy products in China. *Toxins (Basel)*, 12 (7):454, 2020. DOI: 10.3390/toxins12070454

3. Kumari S, Sarkar PK: *Bacillus cereus* hazard and control in industrial dairy processing environment. *Food Control*, 69, 20-29, 2016. DOI: 10.1016/j.foodcont.2016.04.012

4. Zhao S, Chen J, Fei P, Feng H, Wang Y, Ali MA, Li S, Jing H, Yang W: Prevalence, molecular characterization, and antibiotic susceptibility of *Bacillus cereus* isolated from dairy products in China. *J Dairy Sci*, 103 (5): 3994-4001, 2020. DOI: 10.3168/jds.2019-17541

5. Vidic J, Chaix C, Manzano M, Heyndrickx M: Food sensing: Detection of *Bacillus cereus* spores in dairy products. *Biosensors*, 10 (3):15, 2020. DOI: 10.3390/bios10030015

6. Kowalska J, MaĆkiw E, Stasiak M, Kucharek K, Postupolski J: Biofilm-forming ability of pathogenic bacteria isolated from retail food in Poland. *J Food Protect*, 83 (12): 2032-2040, 2020. DOI: 10.4315/JFP-20-135

7. Angela MC, Tiziana C, Pierluigi ADC, Maria AG, Patrizia M, Alessandra D: Characterization of vegetative *Bacillus cereus* and *Bacillus subtilis* strains isolated from processed cheese products in an Italian dairy plant. *Foods*, 10 (11):2876, 2021. DOI: 10.3390/foods10112876

8. Park KM, Kim HJ, Jeong M, Koo M: Enterotoxin genes, antibiotic susceptibility, and biofilm formation of low-temperature-tolerant *Bacillus cereus* isolated from green leaf lettuce in the cold chain. *Foods,* 9 (3):249, 2020. DOI: 10.3390/foods9030249

9. Ohkubo Y, Komori K, Uchida K, Motoshima H, Katano N: Seasonal variation in spore levels of *Bacillus cereus* and its psychrotrophic strains in raw milk in Hokkaido, Japan, and evaluation of strain diversity. *Int Dairy J*, 97, 209-215, 2019. DOI: 10.1016/j.idairyj.2019.06.011

10. Chang Y, Xie Q, Yang J, Ma L, Feng H: The prevalence and characterization of *Bacillus cereus* isolated from raw and pasteurized buffalo milk in southwestern China. *J Dairy Sci*, 104 (4): 3980-3989, 2021. DOI: 10.3168/jds.2020-19432

11. Yıbar A, Çetinkaya F, Soyutemiz E, Yaman G: Prevalence, enterotoxin production and antibiotic resistance of *Bacillus cereus* isolated from milk and cheese. *Kafkas Univ Vet Fak Derg*, 23 (4): 635-642, 2017. DOI: 10.9775/kvfd.2017.17480

12. Zhai Z, Cui C, Li X, Yan J, Sun E, Wang C, Guo H, Hao Y: Prevalence, antimicrobial susceptibility, and antibiotic resistance gene transfer of *Bacillus* strains isolated from pasteurized milk. *J Dairy Sci*, 106 (1): 75-83, 2022. DOI: 10.3168/jds.2022-22199

13. Moschonas G, Lianou A, Nychas GE, Panagou EZ: Spoilage potential of *Bacillus subtilis* in a neutral-pH dairy dessert. *Food Microbiol,* 95:103715, 2021. DOI: 10.1016/j.fm.2020.103715

14. Zhou Z, Lan X, Zhu L, Zhang Y, Chen K, Zhang W, Xu W: Portable dual-aptamer microfluidic chip biosensor for *Bacillus cereus* based on aptamer tailoring and dumbbell-shaped probes. *J Hazard Mater*, 445:130545, 2022. DOI: 10.1016/j.jhazmat.2022.130545

15. Halket G, Dinsdale AE, Logan NA: Evaluation of the VITEK2 BCL card for identification of *Bacillus* species and other aerobic endosporformers. *Lett Appl Microbiol*, 50, 120-126, 2010. DOI: 10.1111/j.1472-765X2009.02765.x

16. Aboyadak IM, Sabry NM, Ali NG, El-Sayed HS: Isolation of *Staphylococcus epidermidis*, *Bacillus cereus* and *Pseudomonas stutzeri* from diseased European sea bass *(Dicentrarchus labrax)* for the first time in Egypt. *Egypt J Aqua Biol Fish Artic,* 11, 20 (4): 103-114, 2016. DOI: 10.21608/ejabf.2016.11182

17. Khyralla H, Marouf S, Hanafy MH, Hussein A, Saad ASA, Yazeed HAE: Characterization of *Lactobacillus* species recovered from raw dromedary milk in relation to its antimicrobial activity. *Int J Vet Sci,* 11 (3): 373-377, 2022. DOI: 10.47278/journal.ijvs/2021.087

18. Fuchs E, Raab C, Brugger K, Ehling-Schulz M, Wagner M, Stessl B: Performance testing of *Bacillus cereus* chromogenic agar media for improved detection in milk and other food samples. *Foods,* 11 (3):288, 2022. DOI: 10.3390/foods11030288

19. Oliwa-Stasiak K, Molnar CI, Arshak K, Bartoszcze M, Adley CC: Development of a PCR assay for identification of the *Bacillus cereus* group species. *J Appl Microbiol,* 108 (1): 266-273, 2010. DOI: 10.1111/j.1365-2672.2009.04419.x

20. Atshan SS, Shamsudin MN, Lung LT, Sekawi Z, Ghaznavi-Rad E, Pei CP: Comparative characterisation of genotypically different clones of MRSA in the production of biofilms. *J Biomed Biotechnol*, 201, 417-427, 2012. DOI: 10.1155/2012/417247

21. CLSI: Performance Standards for Antimicrobial Susceptibility Testing. 30th ed., CLSI Supplement M100.Wayne, PA: Clinical and Laboratory Standards Institute, 2020.

22. CLSI: Performance Standards for Antimicrobial Susceptibility Testing, 12th ed., Wayne, PA: Clinical and Laboratory Standards Institute, 2012.

23. The European Committee on Antimicrobial Susceptibility Testing: *Bacillus spp.* calibration of zone diameter breakpoints to MIC values. Version 2.1, February 2023. EUCAST Bacillus_v_2.1_February_2023.pdf; 2023.

24. Gülhan T, Boynukara B, Çiftci A, Söğüt MÜ, Fındık A: Determination

of biofilm production, genotype and antibiotic resistance profiles of *Enterococcus feacium* isolates originated from dog, cat and human. *Kafkas Univ Vet Fak Derg*, 21 (4): 553-561, 2015. DOI: 10.9775/kvfd.2015.12956

25. Çiftci A, Onuk EE, Çiftci G, Fındık A, Söğüt MÜ, Didinen BI, Aksoy A, Üstünakın K, Gülhan T, Balta F, Altun S: Development and validation of glycoprotein-based native-subunit vaccine for fish against *Aeromonas hydrophila*. *J Fish Dis*, 39 (8): 981-992, 2016. DOI: 10.1111/jfd.12499

26. Versalovic J, KoeuthT, Lupski JR: Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res*, 19 (24): 6823-6831, 1991. DOI: 10.1093/nar/19.24.6823

27. Büyük F, Çelebi Ö, Coşkun MR, Şahin M: The clues of the discovery of new pathogens eventuated by the horizontal gene transfer among the *Bacillus cereus* group members. *Kafkas Univ Vet Fak Derg,* 28 (4): 447-454, 2022. DOI: 10.9775/kvfd.2022.27401

28. Radmehr B, Zaferanloo B, Tran T, Beale TT, Palombo EA: Prevalence and characteristics of *Bacillus cereus* group isolated from raw and pasteurised milk. *Curr Microbiol*, 77, 3065-3075, 2020. DOI: 10.1007/s00284-020-02129-6

29. Fei P, Yuan X, Zhao S, Yang T, Xiang J, Chen X, Zhou L, Ji M: Prevalence and genetic diversity of *Bacillus cereus* isolated from raw milk and cattle farm environments. *Curr Microbiol*, 76, 1355-1360, 2019. DOI: 10.1007/s00284-019-01741-5

30. Michel D, Amin A, Amer A, Abo El-Makarem HS, Ashry S: Enterotoxigenic profiles of *Bacillus Cereus* isolated from manufacturing steps of traditional soft cheese plant. *Alex J Vet Sci*, 66 (1): 45-51, 2020 DOI: 10.5455/ajvs.113229

31. Cui Y, Liu X, Dietrich R, Märtlbauer E, Cao J, Ding S, Zhu K: Characterization of *Bacillus cereus* isolates from local dairy farms in China. *FEMS Microbiol Lett*, 363 (12):fnw096, 2016. DOI: 10.1093/femsle/fnw096

32. Bruk A, Teshome B, Getnet F: Detection and antimicrobial profile of *Bacillus cereus* in milk from lactating farm animals. *East Afr J Vet Anim Sci*, 4 (2): 39-46, 2020.

33. Ahmed AS, Alsayeqh AF, Diab HM: Enterotoxigenic profiles of virulent *Bacillus cereus* isolated from dairy environments: antimicrobials resistant pattern and sporicidal disinfectants efficacy. *Adv Anim Vet Sci,* 8 (5): 543-557, 2020. DOI: 10.17582/journal.aavs/2020/8.5.543.557

34. Rafique A, Luqman M, Nawaz Z, Ashraf A, Nasir S, Asif AR, Mahmood MS, Abbas RZ, Jabeen F, Sultana T, Sultana S, Naz S, Asad F: Multiplex PCR based detection of toxin producing *Bacillus cereus* from different milk samples retailed in Pakistan. *Pak J Agri Sci*, 57 (3): 887-891, 2020. DOI: 10.21162/PAKJAS/20.9246

35. Amer IH, Awad EI, Abd El-Aal SF, Kamal RM, Algendy RM: Toxigenicity and antimicrobial resistance of *Bacillus cereus* isolated from raw and pasteurized milk. *Adv Anim Vet Sci*, 7 (Suppl. 2): 123-128, 2019. DOI: 10.17582/journal.aavs/2019/7.s2.123.128

36. Owusu-Kwarteng J, Wuni A, Akabanda F, Tano-Debrah K, Jespersen L: Prevalence, virulence factor genes and antibiotic resistance of *Bacillus cereus sensu lato* isolated from dairy farms and traditional dairy products. *BMC Microbiol*, 17 (1):65, 2017. DOI: 10.1186/s12866-017-0975-9

37. Hammad AM, Eltahan A, Khalifa E, Abbas NH, Shimamoto T: Toxigenic potential of *Bacillus cereus* strains isolated from retail dairy products in Egypt. *Foodborne Pathog Dis*, 18 (9): 655-660, 2021. DOI: 10.1089/fpd.2020.2920

38. Alonso VPP, de Oliveira Morais J, Kabuki DY: Incidence of *Bacillus cereus*, *Bacillus sporothermodurans* and *Geobacillus stearothermophilus* in ultra-high temperature milk and biofilm formation capacity of isolates. *Int J Food Microbiol,* 354:109318, 2021. DOI: 10.1016/j.ijfoodmicro.2021.109318

39. Abouelhag HA, Khairy EA, Marie HS, Khalaf DD: Prevalence, antibiogram pattern and virulence genes profile of *Bacillus cereus* isolated from buffalo milk. *Int J Vet Sci* 10 (3): 234-239, 2021. DOI: 10.47278/journal.ijvs/2021.045

40. Meng L, Zhang R, Dong L, Hu H, Liu H, Zheng N, Wang J, Cheng J: Characterization and spoilage potential of *Bacillus cereus* isolated from farm environment and raw milk. *Front Microbiol*, 13:940611, 2022. DOI: 10.3389/fmicb.2022.940611

Kafkas Universitesi Veterinar Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 273-279, 2023
DOI: 10.9775/kvfd.2023.29165

Research Article

Desflurane 6% Inhalation Inhibits Erythrocyte Deformability and Alters Oxidative Stress in Rat Lung and Kidney in a Time-Dependent Manner

Ilknur Hatice AKBUDAK [1a,*], Ozgen KILIC-ERKEK [2b], Melek BOR-KUCUKATAY [2c]

[1] Pamukkale University, Faculty of Medicine, Department of Anesthesiology and Reanimation, TR-20160 Kınıklı, Denizli - TÜRKİYE

[2] Pamukkale University, Faculty of Medicine, Department of Physiology, TR-20160 Kınıklı, Denizli - TÜRKİYE

ORCIDs: I.H.A. 0000-0001-9937-9169; Ö.K.E. 0000-0001-8037-099X; M.B.K. 0000-0002-9366-0205

Article ID: KVFD-2023-29165 Received: 10.02.2023 Accepted: 27.05.2023 Published Online: 27.05.2023

Abstract: Time-dependent effects of 6% desflurane applied for 6 h on erythrocyte deformability and tissue oxidative stress levels within 24 h period are unknown. This study aimed to investigate the influences of 6% desflurane, on erythrocyte deformability and serum, heart, lung, kidney oxidative stress in a time-dependent manner (6 h intervals) following exposure. 10-12 week-old Wistar-albino male rats were divided into five groups (n=6 per group). Rats of the 0h group were not exposed desflurane. The other rats were named in terms of time (6 h, 12 h, 18 h, 24 h) passed following anesthesia. Desflurane was administered at concentration of 6% in 6L min−1 flow-rate of 100% oxygen for 6 h in an anesthetic chamber. Erythrocyte deformability was determined using an ectacytometer. Total oxidant status (TOS), total antioxidant status (TAS) were determined using commercial kits, while oxidative stress index (OSI) was calculated. Desflurane inhalation caused a general decrement in erythrocyte deformability, the effect being more prominent at 18th h following anesthesia. Oxidative stress was not altered in serum, heart. TOS was increased at 6th and 18th h following desflurane exposure in lung. TOS and OSI in kidney were decreased at 18th and 24th h compared to 6th. Our results suggest that the oxidative potential and adverse effect of deflurane on tissue oxygenation by inhibiting RBC deformability in the first 24 h should be kept in mind especially in the presence of comorbidities.

Keywords: *Desflurane, Erythrocyte deformability, Oxidative stress, Lung, Kidney*

%6 Desfluran İnhalasyonu Eritrosit Deformabilitesini İnhibe Eder ve Sıçan Akciğeri İle Böbreğindeki Oksidatif Stresi Zamana Bağlı Olarak Değiştirir

Öz: Altı saat boyunca uygulanan %6'lık desfluranın, 24 saatlik periyotta zamana bağlı olarak eritrosit deformabilitesi ve doku oksidatif stres seviyeleri üzerindeki etkileri bilinmemektedir. Bu çalışma, %6'lık desfluranın eritrosit deformabilitesi ve serum, kalp, akciğer, böbrek oksidatif stres indeksleri üzerindeki etkilerini, desfluran inhalasyonunu takiben 24 saatlik sürede zamana bağlı bir şekilde (6 saat aralıklarla) araştırmayı amaçladı. 10-12 haftalık Wistar-albino erkek ratlar beş gruba ayrıldı (her grupta n=6). 0.saat grubundaki sıçanlar desflurana maruz bırakılmadı. Diğer sıçanlar anestezi inhalasyonundan sonra geçen süreye (6. saat, 12. saat, 18. saat ve 24. saat) göre isimlendirildi. Anestezi odasında altı saat boyunca 6 L dk-1 akım hızında %100 oksijen içinde %6 desfluran uygulandı. Eritrosit deformabilitesi, bir ektasitometre kullanılarak belirlendi. Toplam oksidan kapasitesi (TOK) ile toplam antioksidan kapasitesi (TAK) ticari kitler kullanılarak belirlendi ve oksidatif stres indeksi (OSI) hesaplandı. Desfluran inhalasyonu eritrosit deformabilitesinde genel bir azalmaya neden oldu ve bu etki anesteziden sonraki 18. saatte daha belirgin hale geldi. Oksidatif stres serum ve kalpte değişmedi. Akciğerde desfluran maruziyetini takiben 6. ve 18. saatlerde TOS arttı. Böbrekte TOS ve OSİ 18. ve 24. saatlerde 6. saate göre azaldı. Sonuçlarımız, desfluranın oksidatif hasara neden olma potansiyelinin ve ilk 24 saatte eritrosit deformabilitesini inhibe ederek doku oksijenasyonu üzerindeki olumsuz etkisinin özellikle komorbidite varlığında önemini düşündürmektedir.

Anahtar sözcükler: *Desfluran, Eritrosit deformabilitesi, Oksidatif stres, Akciğer, Böbrek*

How to cite this article?

Akbudak IH, Kilic-Erkek O, Bor-Kucukatay M: Desflurane 6% inhalation inhibits erythrocyte deformability and alters oxidative stress in rat lung and kidney in a time-dependent manner. *Kafkas Univ Vet Fak Derg,* 29 (3): 273-279, 2023.
DOI: 10.9775/kvfd.2023.29165

(*) Corresponding author: İlknur Hatice AKBUDAK

Phone: +90 258 296 1698 Fax: +90 258 296 2433
E-mail: ilhakbudak@gmail.com

Introduction

Desflurane, one of the third generation inhaled anesthetic drugs, is frequently preferred in clinical use for providing safe and effective anesthesia. Recovery and extubation are also rapid following desflurane anesthesia [1]. Desflurane is minimally metabolized but it is known to affect various systems such as the central nervous, respiratory, neuromuscular and cardiovascular systems. Desflurane generally depresses these systems in a dose-dependent manner [2]. Like other anesthetics, desflurane can cause a decrease in cardiac and urinary output, and glomerular filtration. Although there was a significant decrease in perfusion pressure following desflurane anesthesia; based on the mixed venous oxyhemoglobin saturation, oxygen consumption, oxygen transport and oxygen transport/consumption ratio, it has been suggested that tissue perfusion may be sufficient [3].

Microcirculation is essential for adequate tissue oxygenation and therefore organ function. Red blood cell (RBC) deformability is an important determinant of resistance to flow since erythrocytes need to change their shape (deformability) in order to pass through narrow capillaries [4]. Thus, erythrocyte deformability is not only a very important feature for the cell to perform its gas transport function, but also for the cell's circulating half-life [5]. Although a few studies in the literature report alterations in RBC deformability after desflurane inhalation [6,7], it is not clear yet how long these changes persist following anesthesia.

Oxidative stress is one of the determinants of erythrocyte deformability. Membrane proteins forming cross-bridges with each other and/or with hemoglobin due to oxidative stress adversely affect erythrocyte deformability [8]. It is known that the effects of desflurane on oxidative stress occur depending on the concentration and duration of anesthesia [9-13]. 6% desflurane is commonly used in clinics, and the amount of anesthetic substance exposed in long-term surgeries also increases proportionally with time. Time-dependent oxidative status of different organs following anesthesia may be an important factor for post-op recovery.

In this study, we aimed to investigate the time-dependant effects of desflurane at 6% concentration in 100% oxygen 6 L min-1 for 6 h on erythrocyte deformability and oxidative stress in serum, heart, lung and kidney tissues. Tissues were selected considering the organs and systems that may be affected by desflurane as mentioned above. Samples were obtained at 6-h intervals (0, 6, 12, 18 and 24 h) within 24 h following anaesthesia. Blood flow affected by anesthetics at various parts of the body may lead to organ dysfunction in the postoperative period. It is anticipated that our data may provide contribution to this clinical issue.

Material and Methods

Ethical Approval

All experimental procedures were carried out according to Pamukkale University animal care guidelines and Animal Experiments Ethics Committee of the same University (PAUHADYEK-2022/20, 08.08.2022-06) approved the study.

Animals

200-250 g, 10-12 week-old Wistar albino male rats (n=30, Pamukkale University Animal Laboratory, Denizli, Türkiye) were used in the experiments. Rats were housed in a temperature and humidity controlled (22-23°C, 50±5%) room under a 12 h light-dark cycle. Standard diet food and water were available ad libitum. The animals were divided into five groups (n=6 per group).

Anesthesia Protocol

Rats of the 0 h group were not exposed to desflurane but were placed in the anesthetic chamber to minimaze the stress and then were sacrified at 07.00 am. The other rats were administered desflurane at 6% concentration in 6 L min-1 flow rate of 100% oxygen and divided in terms of time passed following anesthesia inhalation as; 6 h group (Rats were exposed to desflurane at 07:00-13:00 am and sacrified 6 h after anesthesia), 12 h group (Rats were exposed to desflurane at 07:00-13:00 am and sacrified 12 h after anesthesia), 18 h group (Rats were exposed to desflurane at 07:00-13:00 am and sacrified 18 h after anesthesia) and 24 h group (Rats were exposed to desflurane at 07:00-13:00 am and sacrified 24 h after anesthesia).

Rats were placed in the anesthetic chamber approximately for 1 h, 4 days before beginning the experimental procedure for adaptation. Desflurane at a concentration of 6% in 6 L min-1% 100% oxygen for 6 h was applied to anesthesia groups in a transparent anesthesia box between 07:00-13:00 am. After anesthesia, the fresh gas flow was decreased to 1 L/min. These procedures were performed under dark conditions, and under a dim red light that did not affect the circadian rhythm. The rats were sacrified every 6 h following anesthesia within a 24-h period and blood, heart, lung, kidney tissues were immediately obtained. Blood samples from the tail vein of the animals were collected into standard tubes containing EDTA (3.5 mg/mL) for the determination RBC deformability. Serum obtained by centrifugation (6450 g, 5 min) was used for measuring oxidative stress indices. RBC deformability was determined within 4 h; while serum, heart, lung and kidney were stored at –80°C until use.

Determination of RBC Deformability

The deformability of erythrocytes was determined at nine

shear stresses between 0.3 and 30 Pa using an ectacytometer (LORCA; RR Mechatronics, Hoorn, The Netherlands) at 37°C, and similar patterns of RBC deformability alterations were obtained between groups at all stress levels [14]. A suspension of low hematocrit (Hct) RBC in an isotonic viscous medium (4% polyvinylpyrrolidone 360 solution; MW 360 kD; Sigma P 5288; St. Louis, MI) was sheared in a Couette system consisting of a glass beaker and a close-fitting flask with a gap of 0.3 mm between the cylinders. Through the sheared sample, a laser beam was directed and the diffraction pattern produced by the deformed RBC was examined by a computer. Based on the geometry of the elliptical diffraction pattern, an elongation index (EI) was calculated as EI = (L−W)/(L+W), where L and W are the length and width of the diffraction pattern, respectively.

Measurement of TOS, TAS and Calculation of OSI

Serum, heart, lung and kidney TOS and TAS were determined using novel automated colorimetric measurement method (Thermo Scientific, Multiskan Go) using commercial kits (Rel Assay Diagnostics, Turkey). Results were expressed in micromolar hydrogen peroxide equivalents per liter (mol H_2O_2 Eq/L) for TOS and mmol Trolox/L for TAS. OSI was calculated according to the following formula; OSI (arbitrary unit)=TOS (mol H_2O_2 Eq/L)/TAS (mmol Trolox Equiv./L) X 100 [15,16].

Statistical Analysis

As a result of the power analysis we performed, it was calculated that a power of 80% at a confidence level of 95% could be obtained if at least 6 rats (at least totally 30 rats for all groups) were included in the study and the effect size was d=0.69. All calculations and power analysis were performed by the G-power program (version 3.1.9.2. Heinrich-Heine-Universitat. Duesseldorf. Germany). All statistical analyses were performed using SPSS 25.0 (IBM SPSS Statistics 25 software (Armonk, NY: IBM Corp.). Continuous variables were defined by the mean ± standard deviation. Shapiro Wilk tests were used for determination of normal distribution. For independent groups comparisons, we used One Way Analysis of Variance (post hoc: Tukey method) when parametric test assumptions were provided, Kruskal Wallis Variance Analysis (post hoc: Mann Whitney U test with Bonferroni Correction) were used when parametric test assumptions were not provided. The level of statistical significance was set at P≤0.05.

Results

Table 1 demonstrates time-dependant alterations in RBC deformability at 9 different shear stresses between 0.30 and 30 Pa following desflurane anesthesia. When all shear stresses are evaluated together, the effect of desflurane anesthesia on reducing erythrocyte deformability was most evident at the 18th h. Namely; RBC deformability measured at 0.3-3 Pa at the 18th h was lower than both 0 and 6 h groups (P<0.05). On the other hand, erythrocyte deformability determined under at 5.33-16.87 Pa shear stresses at the 18th h following anesthesia was decreased compared to the group which did not receive desflurane (P<0.05). The desflurane anesthesia applied, resulted in a decrement in RBC deformability under 0.95 and 1.69 Pa

Table 1. *Time-dependant effects of desflurane anesthesia on RBC deformability under different physiological shear stresses*

EI Shear Stress (Pa)	0 h	6 h	12 h	18 h	24 h
0.30	0.186±0.022	0.157±0.032	0.126±0.035	0.096±0.007*,#	0.113±0.008
0.53	0.252±0.021	0.227±0.031	0.188±0.039	0.158±0.008*,#	0.178±0.006
0.95	0.337±0.007	0.314±0.025	0.2765±0.037*	0.248±0.007*,#	0.268±0.004
1.69	0.420±0.006	0.397±0.017	0.365±0.031*	0.342±0.004*,#	0.361±0.003
3.00	0.483±0.009	0.469±0.010	0.4445±0.026	0.426±0.002*,#	0.441±0.005
5.33	0.532±0.008	0.520±0.016	0.5055±0.02	0.492±0.006*	0.502±0.007
9.49	0.566±0.004	0.554±0.023	0.549±0.015	0.54±0.006*	0.548±0.008
16.87	0.597±0.002	0.578±0.029	0.582±0.011	0.577±0.007*	0.582±0.007
30.00	0.622±0.001	0.597±0.034	0.608±0.011	0.610±0.007	0.608±0.007*

*Values are expressed as mean ± SD. * P<0.05, difference from 0 h group; # P<0.05, difference from 6 h group*

shear stress at 12 h post-anesthesia and under 30 Pa at 24 h following desflurane compared to the 0 h group (P<0.05) *(Table 1)*.

Fig. 1 demonstrate that serum oxidative stress was not altered within a 24 h period after desflurane anesthesia. When the oxidative stress states of different organs were exemined, it was observed that, desflurane anesthesia applied herein, did not affect oxidant-antioxidant levels in the heart, as well *(Fig. 2)*.

Lung TOS, TAS and OSI levels following desflurane inhalation are shown in *Fig. 3*. Although desfluare inhalation resulted in increment of lung TOS the alteration being statistically significant at 6 and 18 h groups compared to 0 h group (P<0.05), no statistically significant alteration was observed in lung TAS and OSI. Desflurane at 6% concentration resulted in a decrement in kidney TOS and OSI at 18 and 24 h following anesthesia compared to 6 h group. Additionally, OSI of 24 h group was less than 12 h group (P<0.05). TAS of kidney was not affected by desflurane *(Fig. 4)*.

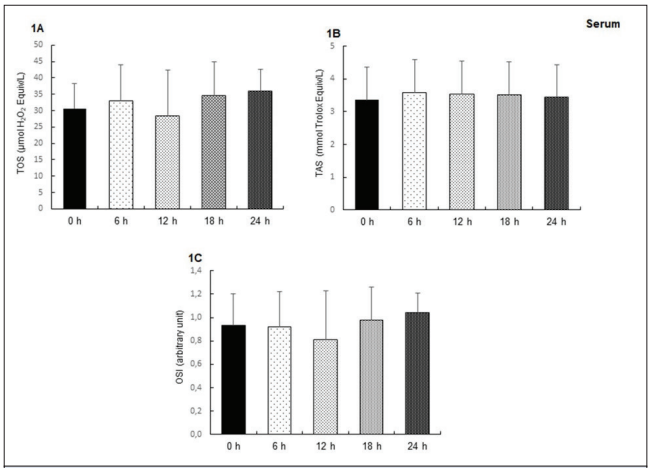

Fig 1. Serum TOS, TAS and OSI of the groups. **A.** Total oxidant status (TOS) levels of serum in the control and experimental groups. **B.** Total antioxidant status (TAS) levels of serum in the control and experimental groups. **C.** Oxidative stress index (OSI) levels of serum in the control and experimental groups. Values are expressed as mean ± SD

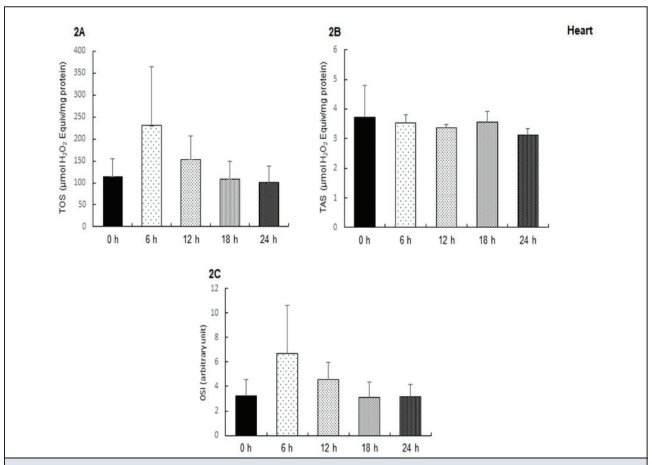

Fig 2. Heart TOS, TAS and OSI of the groups. **A.** Heart total oxidant status (TOS) levels following desflurane anesthesia. **B.** Heart total antioxidant status (TAS) levels following desflurane anesthesia. **C.** Heart oxidative stress index (OSI) levels following desflurane anesthesia. Values are expressed as mean ± SD

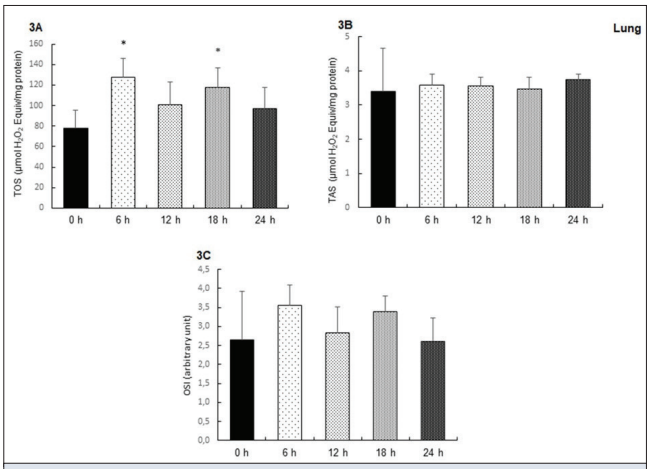

Fig 3. Lung TOS, TAS and OSI of the groups. **A.** Lung total oxidant status (TOS) levels of the groups. **B.** Lung total antioxidant status (TAS) levels of the groups. **C.** Lung total oxidative stress index (OSI) levels of the groups. Values are expressed as mean ± SD. :P<0.05, difference from 0 h group

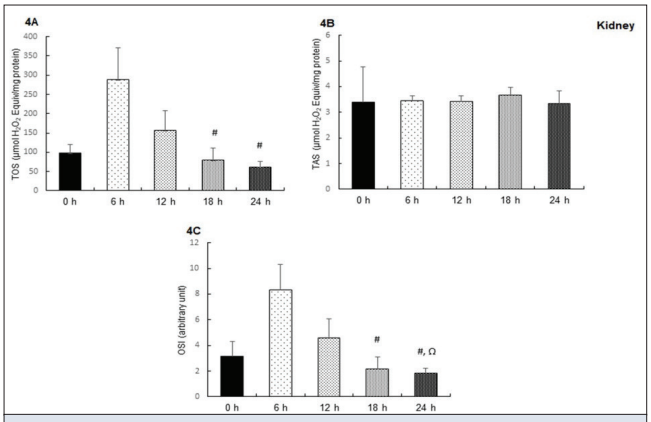

Fig 4. Kidney TOS, TAS and OSI of the groups. **A.** Kidney total oxidant status (TOS) levels of the groups. **B.** Kidney total antioxidant status (TAS) levels of the groups. **C.** Kidney oxidative stress index (OSI) levels of the groups. Values are expressed as mean ± SD. Values are expressed as mean ± SD. # :P<0.05, difference from 6 h group: Ω: P<0.05 difference from 12 h group

Discussion

Sevoflurane, isoflurane and desflurane are the most commonly used inhalation anesthetics in clinical practice [17,18]. Lungs, kidney and vessel rich group of organs are especially important to find out the time constants for wash-out of an anesthetic drug [19]. Renal and hepatic toxicity of halogenated ethers result from biotransformation to toxic

metabolites [20]. Although desflurane has lower solubility in blood and tissues compared to other halogenated agents [1], it was also suggested that, the estimated tissue distribution of desflurane may not differ significantly from that of isoflurane [20]. On the other hand, the low fat solubility of desflurane provides benefits in long surgeries. Studies have shown that midazolam, sufentanil, propofol, desflurane and sevoflurane may result in alterations in sublingual microcirculation [21,22]. In the present study, the effects of 6% desflurane applied for 6 h were investigated on RBC deformability and oxidative stress in serum, heart, lung and kidney tissues of rats within a 24-h period. The dose of desflurane was selected in accordance with the dose commonly used in surgeries in our hospital, and the duration of anesthesia was chosen as 6 h in order to observe the alterations in cases requiring long-term anesthesia such as some cancer and bypass surgeries. It was observed that, desflurane inhalation resulted in a general decrement in RBC deformability, the effect being more prominent at the 18th h (12-24 h) following anesthesia. Although oxidative stress was not altered in serum and heart, TOS was increased at 6th and 18th h following exposure to desflurane in lung. Kidney TOS was also increased at the 6th h but this alteration was not statistically significant. On the other hand, TOS in kidney tissue was decreased at the 18th and 24th h measurements compared to the 6th h value. OSI in kidney was also decreased at 18th and 24th.

Circulatory disorders caused by anesthetic agents may cause organ failure by disrupting tissue oxygenation due to their systemic cardiovascular and direct hemorheological influences [23]. On the other hand, although desflurane has dose-dependent depressive effects on cardiovascular functions and myocardial contractility, it was demonstrated that hemodynamic stability may be maintained after desflurane inhalation [20]. RBC deformability affects both the microcirculation and the macrocirculation. At the microcirculatory scale, RBC deformability is essential for perfusion of small vessels, in which capillary diameters are smaller than that of the eryrtrocyte [24]. In larger vessels, the ability to change its shape aids in the orientation of the erythrocyte to the streamlines as well as their migration towards the central regions of the vessel. These factors contribute to improvement of blood fluidity [25]. Yerer et al.[7] observed an increment in erythrocyte deformability using a laser diffractometer (Myrenne Rheodyne SSD) in male and female rats in response to 6% desflurane anesthesia administered for 1 h. In another study, it was observed that erythrocyte deformability increased in young rats, while it decreased in aged rats following the administration of 6% desflurane for 1 h [6]. Authors have commented that this anesthetic agent may reduce erythrocyte deformability due to changes in membrane structure with age and inhalation of desflurane may cause more serious problems during surgery by affecting hemorheological parameters in the elderly [6]. In the current study, we used 10-12 week-old adult male rats and showed for the first time that, 6% desflurane anesthesia applied for 6 h causes decrement in RBC deformability, the alteration being more prominent at all shear stresses 18 h after the inhalation. RBC deformability was measured at nine shear stresses between 0.3 and 30 Pa using an ectacytometer (LORCA) in our study. 9 different shear stresses are selected to mimic the different flow conditions encountered by RBC in different parts of the body. We observed that, at smaller shear stresses (0.95 and 1.69 Pa) desflurane anesthesia resulted in a decrement in RBC deformability earlier (12 and 18 h), while at higher shear stresses (30 Pa) at 24 h. The instrument (LORCA) we used to measure erythrocyte deformability is accepted as a trustworthy device with a good repetition of consecutive measurements. Our results recommend that the patient should be followed closely in terms of circulatory disorders for at least 24 h after exposure to desflurane and emphasize once again the importance of dose and time dependent effects of anesthesia.

Both erythrocyte deformability and osmotic fragility are physical features applied to diagnostics of RBC. Deformability determines the extensibility of a RBC upon mechanical stress, while osmotic fragility shows membrane extensibility upon hypotonic stress [26]. Therefore, erythrocyte osmotic fragility would be a good parameter to support the results of deformability. We did not investigate osmotic fragility in this study, and to the best of our knowledge, there is no study measuring this parameter following desflurane anesthesia. On the other hand, halothane or its metabolites, at concentrations occurring during anaesthesia, were demonstrated not to alter erythrocyte fragility in malignant hyperthermia-susceptible and resistant pigs [27].

Determinants of RBC deformability may be listed as "passive" properties in response to external forces, RBC morphology, internal viscosity and membrane flexibility [5]. Oxidative stress impairs RBC deformability through disruption of the biconcave disc structure by affecting the erythrocyte membrane lipids and especially proteins such as hemoglobin [8]. Studies examining the alterations in oxidative stress parameters following desflurane anesthesia in the literature have obtained contradictory results depending on the species and tissue studied, the measured oxidant/antioxidant levels, the duration and dose of desflurane, the sampling period following anesthesia, and the method used [9,11-13]. The measurement of TOS and TAS used in our study practically represents the cumulative action of oxidant and antioxidants and their synergistic interaction, which reflects the total oxidant-antioxidant status as well as OSI of the organism. We observed that,

serum TOS, TAS and OSI were not altered significantly in response to 6% desflurane anesthesia for 6 h. It may be concluded that, the decrement in RBC deformability is far from beeing explained by blood oxidative stress levels. Nogueira et al.[12] investigated blood oxidative stress markers in humans undergoing septoplasty surgery based on protein carbonyls, lipid peroxidation and antioxidant defense following 6% desflurane anesthesia, and similar to our results found no alteration within 18 h after the induction of anesthesia. On the other hand, measurement of oxidative indices directly on erythrocyte membrane would be a more suitable way to discuss effects of desflurane-induced oxidative stress on RBC deformability compared to measuring serum oxidative status. Unfortunately, we did not determine oxidative stress on erythrocyte membrane directly; but Turkan et al. examined erythrocyte malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) up to 3 days following desflurane anesthesia and observed no change, supporting our comment above that the change in RBC deformability is not related to oxidative stress [10].

There is a growing interest in the field of anesthesiology on the cardioprotective effects of myocardial preconditioning provoked by halogenated anesthetics especially in cases of ischemic myocardial damage. Preconditioning with desflurane was demonstrated to reduce oxidative stress-induced cardiomyocyte death [9]. We have observed that, desflurane administered at a dose of 6%, for 6 h does not alter TOS, TAS and OSI at heart. The tissue directly exposed to anesthetic gases is the lung. Lung MDA levels were shown to be increased in response to desflurane (4%) inhalation [10]. Additionally, desflurane inhalation for 2 h was demonstrated not only to decrease GSH, but also cause neutrophil and macrophage infiltration, hemorrhage, alveolar damage, and edema in the rat lung [13]. Another study suggested that isoflurane and sevoflurane prevent ventilator-induced lung injury, while desflurane does not [11]. Here, we have demonstrated that 6% desflurane applied for 6 h causes oxidative stress by increasing TOS in lung until at least the 18th h post exposure. Blood and urine fluoride metabolites are considered as markers of fluorinated ether anaesthetic metabolism. Exposure to desflurane was demonstrated to cause slight rises in urine and serum trifluoroacetic acid levels [28]. When we examined the effects of desflurane on the kidneys, we observed that the main effect was again on total oxidants and OSI, causing a non-significant increase at the 6th h and a time-dependent decrease afterwards, the decrements being most pronounced at the 18th and 24th h. Post-exposure first 24 h seems remarkable in terms of desflurane's effects on oxidative status as well as erythrocyte deformability. Türkan et al.[29] evaluated liver, brain, kidney, and lung oxidative stress in rats exposed to 4%. desflurane. They demonstrated that lung MDA levels increased (in line with our results), while liver decreased after four h of exposure. Liver SOD level decreased and brain increased in response to desflurane exposure. Brain is one of the organs that will be mostly affected by the alteration of erythrocyte deformability. Unfortunately, liver and brain oxidative status following desflurane anesthesia were not assessed in the current study due to technical reasons.

Findings of the current study are important in terms of giving information to anesthetists about a few issues they should pay attention to when choosing the anesthetic agent they will use. First one is that, while preferring desflurane they should be aware that, RBC deformability may be inhibited and thus tissue oxygenation may be adversely affected for at least 24 h. Other points to be considered are that desflurane may cause oxidative damage, especially in the lungs, in the first 18 h following exposure, and may increase oxidants in the kidneys in the early period while decreasing them over time within 24 h. These data may be particularly important in the presence of additional cardiovascular, respiratory and/or renal disorders. The physiological mechanisms of these effects are not fully clarified yet. This study was conducted in adult, male, healthy rats. The time-dependent influences of different desflurane concentrations on hemorheological parameters and oxidative stress indices in humans of different ages especially in the presence of some comorbidities may also be examined. Another limitation of the study is that; erythrocyte osmotic fragility was not examined herein. Investigation of oxidative stress indices in brain and other tissues expected to be affected by desflurane anesthesia as well as erythrocyte osmotic fragility in future studies may contribute to the clarification of the subject. Determination of oxidative stress directly on RBC membrane may also be a more suitable approach to interpret the alteration in erythrocyte rheological properties in future studies.

Availability of Data and Materials

The authors declare that data supporting the study findings are also available from the corresponding author (İ. H. Akbudak) on reasonable request.

Funding

No source of funding is provided.

Conflict of Interest

The authors declare that there is no conflict of interest in publishing this article.

Author Contribution

I.H.A, O.K.E.: Conceptualization, methodology, data curation, investigation, resources, project administration; M.B.K.: Methodology, writing, review, and editing. All authors read and approved the final manuscript.

References

1. Gaya da Costa M, Kalmar AF, Struys MMRF: Inhaled anesthetics: Environmental role, occupational risk, and clinical use. *J Clin Med,* 10 (6):1306, 2021. DOI: 10.3390/jcm10061306

2. Devlin-Hegedus JA, McGain F, Harris RD, Sherman JD: Action guidance for addressing pollution from inhalational anaesthetics. *Anaesthesia,* 77 (9): 1023-1029, 2022. DOI: 10.1111/anae.15785

3. Daniel M, Mcgrady EM: Epidural diamorphine. *Anaesthesia*, 50 (1): 14-16, 1995. DOI: 10.1111/j.1365-2044.1995.tb04505.x

4. McMahon TJ: Red blood cell deformability, vasoactive mediators, and adhesion. *Front Physiol,* 10:1417, 2019. DOI: 10.3389/fphys.2019.01417

5. Richardson KJ, Kuck L, Simmonds J: Beyond oxygen transport: Active role of erythrocytes in the regulation of blood flow. *Am J Physiol-Hear Circ Physiol*, 319 (4): 866-872, 2020. DOI: 10.1152/AJPHEART.00441.2020

6. Beaussier M, Mouren S, Souktani R: Role of nitric oxide and cyclooxygenase pathways in the coronary vascular effects of halothane, isoflurane and desflurane in red blood cell-perfused isolated rabbit hearts. *Br J Anaesth*, 88 (3): 399-407, 2002. DOI: 10.1093/bja/88.3.399

7. Yerer MB, Aydogan S, Comu FM: Gender-related alerations in erythrocyte mechanical activities under desflurane or sevoflurane anesthesia. *Clin Hemorheol Microcirc*, 39 (1-4): 423-427, 2008. DOI: 10.3233/CH-2008-1113

8. Gyawali P, Richards RS, Bwititi PT, Nwose EU: Association of abnormal erythrocyte morphology with oxidative stress and inflammation in metabolic syndrome. *Blood Cells Mol Dis*, 54 (4): 360-363, 2015. DOI: 10.1016/j.bcmd.2015.01.005

9. Guerrero-Orriach JL, Carmona-Luque MD, Gonzalez-Alvarez L: Heart failure after cardiac surgery: The role of halogenated agents, myocardial conditioning and oxidative stress. *Int J Mol Sci,* 23 (3):1360, 2022. DOI: 10.3390/ijms23031360

10. Turkan H, Aydin A, Sayal A, Karahalil B: The effect of sevoflurane and desflurane on markers of oxidative status in erythrocyte. *Toxicol Ind Health*, 27 (2): 181-186, 2011. DOI: 10.1177/0748233710384911

11. Strosing KM, Faller S, Gyllenram V, Engelstaedter H, Buerkle H, Spassov S, Hoetzel A: Inhaled anesthetics exert different protective properties in a mouse model of ventilator-induced lung injury. *Anesth Analg*, 123 (1): 143-151, 2016. DOI: 10.1213/ANE.0000000000001296

12. Nogueira FR, Braz LG, Souza KM, vd. Aun AG, Arruda NM, Carvalho LR, Chen CO, Braz JRC, Braz MG: Comparison of DNA damage and oxidative stress in patients anesthetized with desflurane associated or not with nitrous oxide: A prospective randomized clinical trial. *Anesth Analg*, 126 (4): 1198-1205, 2018. DOI: 10.1213/ANE.0000000000002729

13. Tosun M, Olmez H, Unver E, Arslan YK, Cimen FK, Ozcicek A, Aktas M, Suleyman H: Oxidative and pro-inflammatory lung injury induced by desflurane inhalation in rats and the protective effect of rutin. *Adv Clin Exp Med*, 30 (9): 941-948, 2021. DOI: 10.17219/ACEM/136194

14. Baskurt OK, Hardeman MR, Uyuklu M, Ulker P, Cengiz M, Nemeth N, Shin S, Alexy T, Meiselman HJ: Comparison of three commercially available ektacytometers with different shearing geometries. *Biorheology*, 46 (3): 251-264, 2009. DOI: 10.3233/BIR-2009-0536

15. Erel O: A new automated colorimetric method for measuring total oxidant status. *Clin Biochem*, 38 (12): 1103-1111, 2005. DOI: 10.1016/j.clinbiochem.2005.08.008

16. Erel O: A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem,* 37 (4): 277-285, 2004. DOI: 10.1016/j.clinbiochem.2003.11.015

17. Straat M, Van Bruggen R, De Korte D, Juffermans NP: Red blood cell clearance in inflammation. *Transfus Med Hemotherapy*, 39 (5): 353-360, 2012. DOI: 10.1159/000342229

18. Ronzani M, Woyke S, Mair N, Gatterer H, Oberacher H, Plunser D, Haller T, Ströhle M, Rugg C: The effect of desflurane, isoflurane and sevoflurane on the hemoglobin oxygen dissociation curve in human blood samples. *Sci Rep,* 12 (1):13633, 2022. DOI: 10.1038/s41598-022-17789-6

19. Caldwell JE: Desflurane clinical pharmacokinetics and pharmacodynamics. *Clin Pharmacokinet*, 27, 6-18, 1994. DOI: 10.2165/ 00003088-199427010-00002

20. Patel SS, Goa KL: Sevoflurane: A review of its pharmacodynamic and pharmacokinetic properties and its clinical use in general anaesthesia. *Drugs*, 51 (4): 658-700, 1996. DOI: 10.2165/00003495-199651040-00009

21. Lamblin V, Favory R, Boulo M, Mathieu D: Microcirculatory alterations induced by sedation in intensive care patients: Effects of midazolam alone and in association with sufentanil. *Crit Care*, 10 (6): 1-9, 2006. DOI: 10.1186/cc5128

22. De Blasi RA, Palmisani S, Boezi M, Arcioni R, Collini S, Troisi F, Pinto G: Effects of remifentanil-based general anaesthesia with propofol or sevoflurane on muscle microcirculation as assessed by near-infrared spectroscopy. *Br J Anaesth*, 101 (2): 171-177, 2008. DOI: 10.1093/bja/aen136

23. Alet AI, Basso SS, Castellini H V, Delannoy M, Alet N, D'Arrigo M, Riquelme BD: Hemorheological in vitro action of propofol on erythrocytes from healthy donors and diabetic patients. *Clin Hemorheol Microcirc*, 64 (2): 157-165, 2016. DOI: 10.3233/CH-162054

24. Ebrahimi S, Bagchi P: A computational study of red blood cell deformability effect on hemodynamic alteration in capillary vessel networks. *Sci Rep*, 12 (1): 1-19, 2022. DOI: 10.1038/s41598-022-08357-z

25. Namgung B, Ng YC, Leo HL, Rifkind JM, Kim S: Near-wall migration dynamics of erythrocytes in vivo: Effects of cell deformability and arteriolar bifurcation. *Front Physiol*, 8:963, 2017. DOI: 10.3389/fphys.2017.00963

26. Kuo MS, Chuang CH, Cheng HC, Lin, HR., Wang JS, Hsu K: Different involvement of band 3 in red cell deformability and osmotic fragility-A comparative GP. Mur Erythrocyte Study. *Cells*, 10 (12):3369, 2021. DOI: 10.3390/cells10123369

27. Heffron JJ, Mitchell G: Influence of pH, temperature, halothane and its metabolites on osmotic fragility of erythrocytes of malignant hyperthermia-susceptible and resistant pigs. *Br J Anaesth,* 53 (5): 499-504, 1981. DOI: 10.1093/bja/53.5.499

28. Sutton TS, Koblin DD, Gruenke LD, Weiskopf RB, Rampil IJ, Waskell L: Fluoride metabolites after prolonged exposure of volunteers and patients to desflurane. *Anesth Analg*, 73 (2): 180-185, 1991. DOI: 10.1213/00000539-199108000-00011

29. Türkan H, Aydin A, Sayal A, Eken A, Akay C, Karahalil B: Oxidative and antioxidative effects of desflurane and sevoflurane on rat tissue *in vivo. Arh Hig Rada Toksikol*, 62 (2): 113-119, 2011. DOI: 10.2478/10004-1254-62-2011-2096

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 281-288, 2023
DOI: 10.9775/kvfd.2023.29187

Research Article

Evaluation of the Immunopathological Response to BCG Vaccine in a Xenogeneic Immunocompetent Animal

Salma A. SHOULAH [1] Mohamed M. GABALLA [2] Said M. ELSHAFAE [2] Maha A. MOUSSA [3] Abdelfattah SELIM [1 (*)] Majed H. WAKID [4,5]

[1] Department of Animal Medicine (Infectious Diseases), Faculty of Veterinary Medicine, Benha University, Moshtohor, Tukh, Qalyobiya, 13736 EGYPT

[2] Department of Pathology, Faculty of Veterinary Medicine, Benha University, Moshtohor, Tukh, Qalyobiya, 13736 EGYPT

[3] Department of Statistics, Faculty of Commerce, Benha University, Benha, Qalyobiya, EGYPT

[4] Department of Medical Laboratory Sciences, Faculty of Applied Medical Sciences, King Abdulaziz University, Jeddah, SAUDI ARABIA

[5] Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, SAUDI ARABIA

ORCIDs: S.A.S. 0000-0002-6453-5587; M.M.G. 0000-0003-2949-430X; S.M.E. 0000-0001-6285-5483; M.A.M. 0000-0002-6885-1456; A.S. 0000-0002-0211-2853; M.H.W. 0000-0003-4941-5373

Article ID: KVFD-2023-29187 Received: 15.01.2023 Accepted: 26.04.2023 Published Online: 03.05.2023

Abstract: *Mycobacterium bovis,* the causative agent of bovine tuberculosis (BTB), is one of the most significant endemic diseases confronting government, veterinary professionals, and farming industry worldwide nowadays. *M. bovis* has not only a negative impact on bovine health and economy, but also poses a threat to public health as a zoonotic disease that could be transmitted from animal to human. Although, bacillus Calmette-Guérin (BCG) vaccine of *M. bovis* has been extensively used in many animals, only few studies had reported its side effects in these animals. In this study, the systemic pathological lesions and immunoglobulin levels associated with intranasal (IN) and subcutaneous (SC) injection of BCG vaccine in Swiss male mice have been evaluated. The results revealed an elevation in IgM and IgA levels in both routes (nebulization, subcutaneous injection) while there was a dramatic increase in IgG levels in subcutaneously injected mice. Aerosolization of BCG vaccine using a nebulizer resulted in severe pulmonary lesions with numerous megakaryocytes in the spleens of mice. On the other hand, SC injection had mild effect on pulmonary tissues and induced moderate extramedullary hematopoiesis in the hepatic tissues of mice. In conclusion, inadvertent vaccination of BCG in Swiss mice, triggered adverse tissue reaction and remarkable increase in Ig level. The severity of tissue lesions corresponded to the injection route in mice.

Keywords: *Aerosol, BCG, Extramedullary hematopoiesis, Mice, Vaccine*

Ksenojenik İmmünokompetan Bir Hayvanda BCG Aşısına İmmünopatolojik Yanıtın Değerlendirilmesi

Öz: Sığır tüberkülozunun (BTB) etkeni olan *Mycobacterium bovis*, günümüzde dünya genelinde hükümetlerin, veteriner hekimlerin ve tarım endüstrisinin karşı karşıya kaldığı en önemli endemik hastalıklardan birisidir. *M. bovis* sadece sığır sağlığı ve ekonomisi üzerinde olumsuz bir etkiye sahip olmakla kalmayıp, aynı zamanda hayvandan insana bulaşabilen zoonotik bir hastalık olarak halk sağlığı için de tehdit oluşturmaktadır. *M. bovis* basilinin Calmette-Guérin (BCG) aşısı birçok hayvanda yaygın olarak kullanılmasına rağmen, sadece birkaç çalışma bu hayvanlarda yan etkilerini bildirmiştir. Bu çalışmada, İsviçre erkek farelerinde BCG aşısının inhalasyon ve subkutan (SC) enjeksiyonu ile ilişkili sistemik patolojik lezyonlar ve immünoglobulin seviyeleri değerlendirilmiştir. Sonuçlar, her iki yolla da (nebülizasyon, subkutan enjeksiyon) IgM ve IgA seviyelerinde artış olduğunu ortaya koyarken, subkutan olarak enjekte edilen farelerde IgG seviyelerinde dramatik bir artış olduğunu göstermiştir. BCG aşısının bir nebülizör kullanılarak aerosolize edilmesi, farelerin dalaklarında çok sayıda megakaryosit içeren ciddi pulmoner lezyonlarla sonuçlanmıştır. Öte yandan, SC enjeksiyonunun pulmoner dokular üzerinde hafif bir etkisi olmuş ve farelerin hepatik dokularında orta derecede ekstramedüller hematopoezi indüklemiştir. Sonuç olarak, İsviçre farelerinin BCG ile yanlışlıkla aşılanması, olumsuz doku reaksiyonunu ve Ig seviyesinde kayda değer bir artışı tetiklemiştir. Doku lezyonlarının şiddeti, farelerdeki enjeksiyon yolu ile uyumluluk göstermiştir.

Anahtar sözcükler: *Aerosol, BCG, Ekstramedüller hematopoez, Fare, Aşı*

How to cite this article?

Shoulah SA, Gaballa MM, Elshafae SM, Moussa MA, Selim A, Wakid MH: Evaluation of the immunopathological response to BCG vaccine in a xenogeneic immunocompetent animal. *Kafkas Univ Vet Fak Derg,* 29 (3): 281-288, 2023.
DOI: 10.9775/kvfd.2023.29187

(*) Corresponding author: Abdelfattah SELIM

Phone: +20 101 7447734
E-mail: abdelfattah.selim@fvtm.bu.edu.eg

Introduction

Mycobacterium bovis is a member of *Mycobacterium tuberculosis* complex (MTBC), the main causes of tuberculosis (TB) in livestock and wildlife across the globe and most noticeably in Africa and Asia [1-6]. Bovine tuberculosis (BTB) is a zoonotic disease that not only has a substantial impact on the world economy and animal health, but also poses a hazard to both animals and humans [7]. It has been estimated that cases of BTB among cattle worldwide will be greater than 50 million annually with $3 billion loss in economy [8]. Therefore, there is an urgent need for BTB control strategies, especially in low- and middle-income countries and other regions where test-and-slaughter methods are not feasible and standardized [9]. MTBC are known to induce granulomatous-caseous-necrotizing lesions mainly in the lungs and regional lymph nodes, but they could also induce lesions in the liver, spleen, kidneys, mammary glands, pericardium, uterus, and brain [10].

In Egypt, BTB is considered one of the most significant animal health hazards since cattle represent the main source of meat and milk in Egyptian community and their health status have a great impact on economy and social life [11]. In the recent years, there was an increase in the incidence of BTB due to the export of live animals from endemic regions with a high prevalence of *M. bovis* [12, 13].

The most effective method of controlling BTB is known to be vaccination combined with reliable diagnostic testing. Currently, the only alternative for protecting humans and livestock against tuberculosis is the live attenuated Bacillus Calmette-Guérin (BCG) vaccine [14]. BCG was originally intended as a cattle vaccine, and its efficacy against BTB is still debated [4]. However, modest clinical symptoms have been observed on rare occasions during BCG vaccine testing in a few animal species [15]. The individual's age, immunological status, vaccination dose, strain, and route of administration of BCG vaccine are the main variables that could predict the severity of tissue reaction to BCG vaccine [16]. Most tissue reactions against BCG were reported to be local/regional and self-limiting, whereas suppurative lymphadenitis and abscessations were the most severe tissue reactions that occurred in some cases [15]. A large dose of BCG administered subcutaneously to cattle resulted in topical lesions with no further problems, and the TB bacilli were cleared from the body, but following oral administration of BCG to mice, mild side effects were observed including infrequent cervical lymphadenitis [17, 18]. Although large doses of intranasal BCG provided better lung protection, it also caused BCG postvaccinal granulomatous pneumonia [19]. Moreover, few studies focused on the parenchymatous lesions and altered Ig levels caused by live attenuated BCG vaccine.

Consequently, the aim here is to study the histopathological alterations in parenchymatous organs after intranasal aerosolization (IN, via nebulization) and subcutaneous injection (SC) of BCG vaccine in xenogeneic Swiss mice and evaluate the changing in Ig level of vaccine mice.

Materials and Methods

Ethical Statement

The Benha University ethical committee for animal experiments approved all procedures that involved the handling and collection of blood samples (approval Nr. BUFVTM 02-09-22).

Vaccine

Servac Freeze-dried BCG vaccine used in trials of the present study, was prepared from a living attenuated BCG and *M. bovis* strain. It was produced by the Veterinary Serum and Research Institute (VSRI, Abbasia, Egypt).

Experimental Animals

For this study, 15 Swiss male mice with one and half month age, weighing 20 g were purchased from the Center for Laboratory Animals at Benha University's Faculty of Veterinary Medicine in Egypt. Before the experiment, all mice were acclimated for two weeks (in a light/dark cycle of 25±2°C and 12:12 h) and given a standardized pellet meal and free access to water.

Vaccination Protocol

As stated in *Table 1*, the 15 mice were divided into three groups, each with five mice. Group 1 (control) mice were not immunized. Mice in groups 2 received 0.1 mL Servac BCG vaccine by SC injection, while mice in group 3 received 0.1 mL from the vaccine via IN using a nebulizer (Uhde GmbH, Germany) for 5 min. After 21 days, all of the mice in the three groups were scarified.

Blood Sampling and Serological Analysis

Before the mice were euthanized, they received an intraperitoneal (IP) dosage of 120 mg/kg of ketamine (100 mg/mL) to anaesthetize them, then blood samples were obtained from the retro-orbital plexus in collecting tubes containing dipotassium ethylenediamine tetra acetic acid. Serum was separated from the collected blood samples after centrifugation of the blood tubes for 15 min at

Table 1. *Animal groups and vaccines used in the study*

Group	Animals	Route and Dose
1 (control)	Five mice	Non-vaccinated
2 (SC- vaccinated mice)	Five mice	0.1 mL SC and booster dose 14 days later
3 (IN- vaccinated mice)	Five mice	0.1 mL IN and booster dose 14 days later

1200xg. Immunoglobulins (Ig) IgM, IgA, and IgG levels were assessed. Each Ig was tested using enzyme-linked immunosorbent assays (ELISAs): rat IgG, IgA, and IgM ELISA kits (Creative Biolabs, USA).

Histopathological Analysis

After 21 days of vaccination, all mice were euthanized and tiny samples of lung, spleen, kidney, liver, and lymph node tissue were taken from each mice. The specimens were fixed for 72 h in 10% neutral-buffered formalin, dehydrated, cleaned, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin (H&E) dye. Using a Nikon eclipse E800 microscope with an OMAX eye-piece camera, photomicrographs of histopathological changes were taken.

Statistical Analysis

The data was analyzed using the Graph Pad Prism 6.0 software (San Diego, USA). Parametric one-way analysis of variance (ANOVA) and Dunnett's multiple comparisons tests were used to compare outcome variables between groups.

Results

Serum Immunoglobulin Levels

The BCG vaccine induced remarkable changes in the level of immunoglobulins in xenogeneic mice via both routes. In comparison to control unvaccinated mice, there was a significant rise in the level of IgG in the sera of both IN (P=0.0015) and SC (P<0.0001) vaccinated mice *(Fig. 1-A; Table 2)*. Notably, mice with the SC immunization of BCG had much higher IgG levels than mice with the IN administration. The level of IgM and IgA in the blood of both IN and SC vaccinated mice was also significantly higher than that in the unvaccinated mice (P<0.0001) *(Fig. 1-B,C; Table 2)*.

Histopathology

In most of the examined parenchymatous organs, the control group exhibited no degenerative alterations or inflammation. In contrast, mice vaccinated via both routes had varying degrees of cell damage and inflammation. There were no significant microscopic lesions in the lungs of control mice *(Fig. 2-A)*.

The IN-vaccinated mice had more severe pulmonary lesions than the SC-vaccinated mice. Bronchioles had significant hyperplasia/hypertrophy of the epithelial lining, as well as intraluminal homogeneous eosinophilic material infiltrated with cellular debris *(Fig. 2-B)*. The majority of the mice in this group had significant peribronchial mononuclear cellular infiltration. Congestion of interalveolar blood vessels was the frequent finding in all the mice. There was also multifocal interalveolar inflammatory cellular aggregations specially lymphocytes in many examined lungs *(Fig. 2-C)*. Intra alveolar oedema and hemorrhages were reported in 75% of the mice *(Fig. 2-D)*. Hyperplasia of pneumocytes type II was also prominent in all the lungs where the hemorrhage was extensive *(Fig. 2-D)*.

In SC vaccinated mice, BCG had less adverse effects compared to IN vaccinated mice. Mild bronchial mucosal hyperplasia was evident in many mice in SC vaccinated

Table 2. *The means and standard deviations of immunoglobulin concentrations in control, vaccinated mice by BCG vaccine*

Immunoglobulin	Control		IN-vaccinated Mice		SC-vaccinated Mice	
	Mean	SD	Mean	SD	Mean	SD
IgG	390.8	64.3	755.2	50.9	1710.8	209.3
IgM	215.0	26.7	584.2	26.7	779.8	86.5
IgA	357.8	48.4	606.4	19.6	700.4	32.3

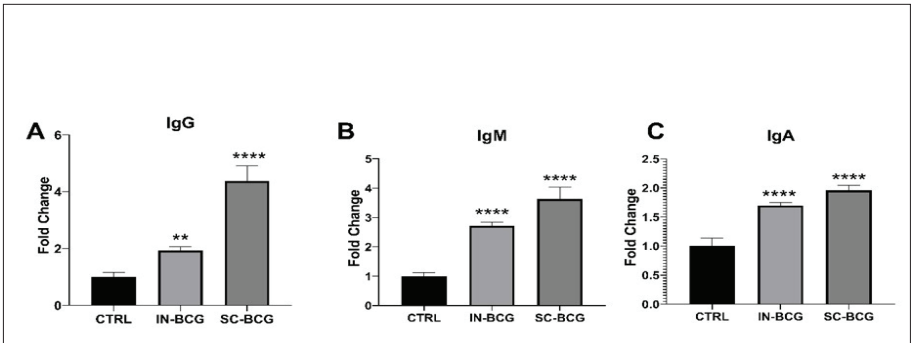

Fig 1. Serum levels of immunoglobulins (IgG, IgA and IgM) in vaccinated and unvaccinated mice. The graph represent the fold increase in Ig level following vaccination compared to unvaccinated mice. Significance difference is indicated as ****P≤0.0001, **P≤0.01

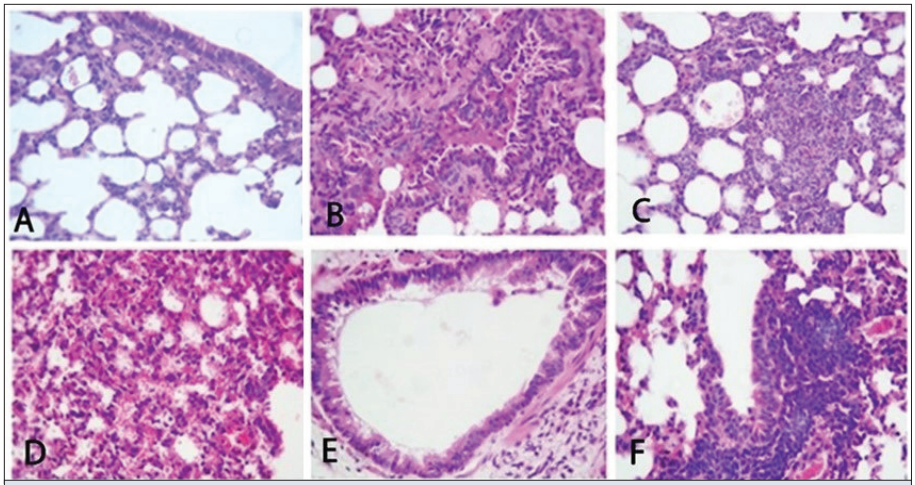

Fig 2. Pulmonary photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** lung of control group showing nearly normal alveoli and interstitial tissue. **B-D)** Lungs of IN vaccinated mice showing hyperplasia/hypertrophy of the epithelial lining with intraluminal homogeneous eosinophilic material (B), interalveolar inflammatory cellular aggregations (C), and Intra alveolar oedema and hemorrhages (D). **E-F)** Lungs of SC vaccinated mice showing mild bronchial mucosal hyperplasia (E), and foci of peribronchial mononuclear inflammatory cellular infiltration (F)

mice *(Fig. 2-E)*. Foci of peribronchial mononuclear inflammatory cellular infiltration were also seen in few mice in this group *(Fig. 2-F)*.

The control group's livers showed no significant microscopic alterations *(Fig. 3-A)*. The adverse effect of BCG vaccination was more pronounced in the mice vaccinated via IN compared to SC route. The hepatic parenchyma of IN-vaccinated mice showed foci of extramedullary haematopoiesis (EH) and Kupffer cell infiltrations. Many of the examined livers in this group had hepatocellular deterioration in the form of hydropic degeneration *(Fig. 3-B)*. In many mice, there were many hepatocytes with basophilic cytoplasm in the hepatic parenchyma *(Fig. 3-C)*. Some mice in this group had multifocal areas of hepatic necrosis mixed in with inflammatory cells (mostly mononuclear and a few giant cells) *(Fig. 3-D)*. Congestion of hepatic sinusoids with multifocal areas of hemorrhage were also observed in IN-vaccinated mice *(Fig. 3-E)*. Thrombosis of some central veins was among the incidental findings in few mice. Furthermore, periductal moderate infiltration of mononuclear cells was detected in a few IN-vaccinated mice *(Fig. 3-F)*. In SC vaccinated mice, mild hepatocellular degeneration with few foci of hepatic necrosis were evident in some examined livers. Many mice had central vein congestion with perivascular focal mononuclear cellular aggregations *(Fig. 3-G)*. Mild hyperplasia of the bile ducts with periductal fibrosis and inflammatory cellular aggregation were seen *(Fig. 3-H)*.

The control mice had no noticeable kidney lesions,

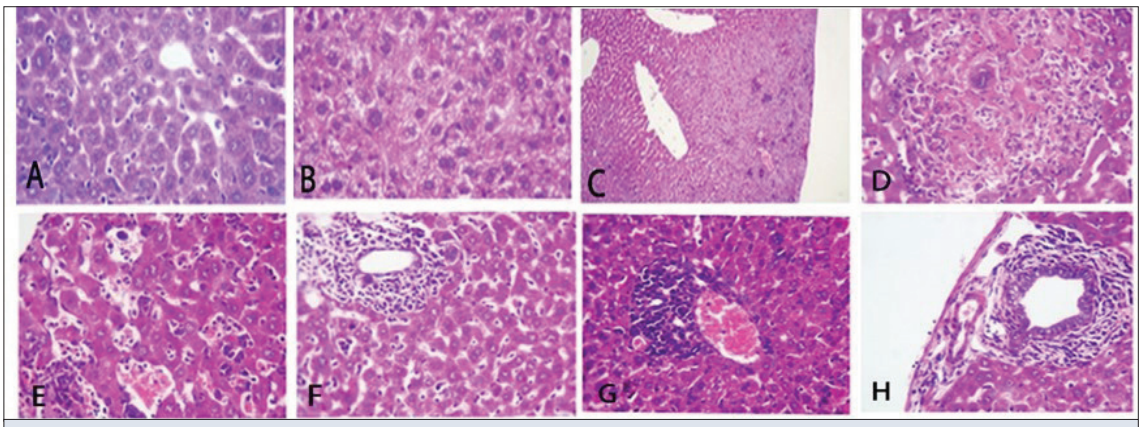

Fig 3. Liver photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** Liver of control group showing normal hepatocyte and patent sinusoids. **B-F)** Liver of I/N vaccinated mice showing hydropic degeneration (B), hepatocytes with basophilic cytoplasm in the hepatic parenchyma (C), multifocal areas of hepatic necrosis mixed in with inflammatory cells (D), congestion of hepatic sinusoids with multifocal areas of hemorrhage (E), and moderate periductal mononuclear cells infiltration (F). **G-H)** Liver of SC vaccinated mice showing central vein congestion with perivascular focal mononuclear cellular aggregations (G), and mild hyperplasia of the bile ducts with periductal inflammatory cellular aggregation (H)

whereas both vaccinated groups had similar renal lesions *(Fig. 4-A)*. Proliferative glomerulonephropathy was the most common microscopic lesion in the kidneys of IN-vaccinated mice *(Fig. 4-B)*. In IN-vaccinated mice, necrotic cellular debris was seen in the bowman's space of some glomeruli *(Fig. 4-C)*. Periglomerular and intertubular hemorrhage were noted in some mice in this group *(Fig. 4-C)*. The IN-vaccinated mice showed a lot of basophilic cytoplasm in their renal tubular epithelia *(Fig. 4-D,E)*. In SC-vaccinated mice, there were a few foci of infiltrating intertubular and periglomerular mononuclear inflammatory cells in the kidney *(Fig. 4-F)*.

Control mice showed no significant microscopic changes in their heart *(Fig. 5-A)*. Degeneration (vacuolation) of cardiomyocytes was prominent in IN-vaccinated mice compared to SC-vaccinated mice *(Fig. 5-B)*. Congestion and oedema of intermuscular blood vessels were evident in IN-vaccinated mice *(Fig. 5-C)*. Cardiomyocytes with basophilic cytoplasm and marked loss of striations were evident in many IN-vaccinated mice *(Fig. 5-D)*. On the other hand, no degeneration was detected in the cardiomyocytes in SC-vaccinated mice. Similar to IN-vaccinated mice, cardiac muscles with basophilic cytoplasm were also seen in some mice in this group.

No significant microscopic alterations were seen in the spleens of the control mice *(Fig. 5-E)*. Megakaryocyte

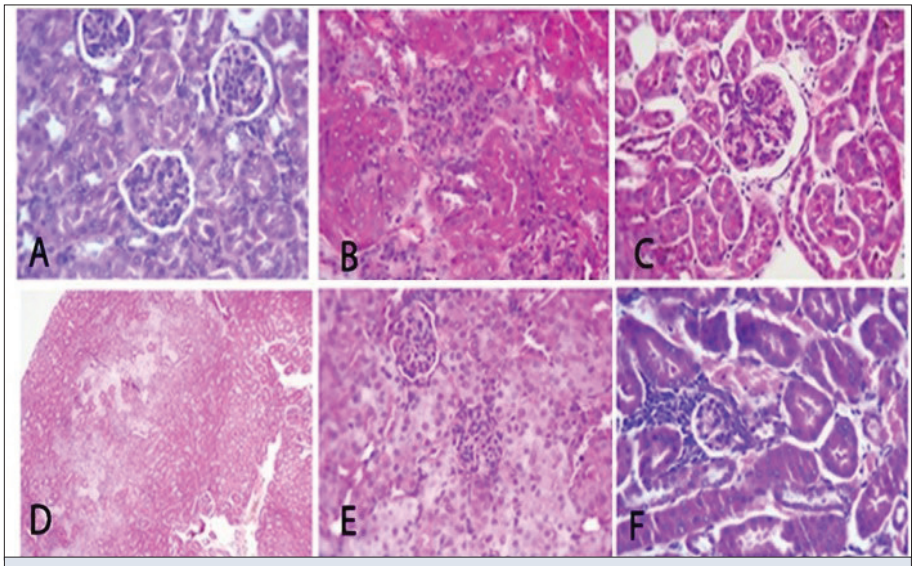

Fig 4. Kidney photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** Kidney of control group showing normal renal glomeruli as well as normal renal tubules. **B-E)** Kidney of IN vaccinated mice showing proliferative glomerulonephropathy (B), necrotic cellular debris in the bowman's space (C), and basophilic cytoplasm in the renal tubular epithelia (D-E). **F)** kidney of SC vaccinated mice showing intertubular and periglomerular mononuclear inflammatory cells

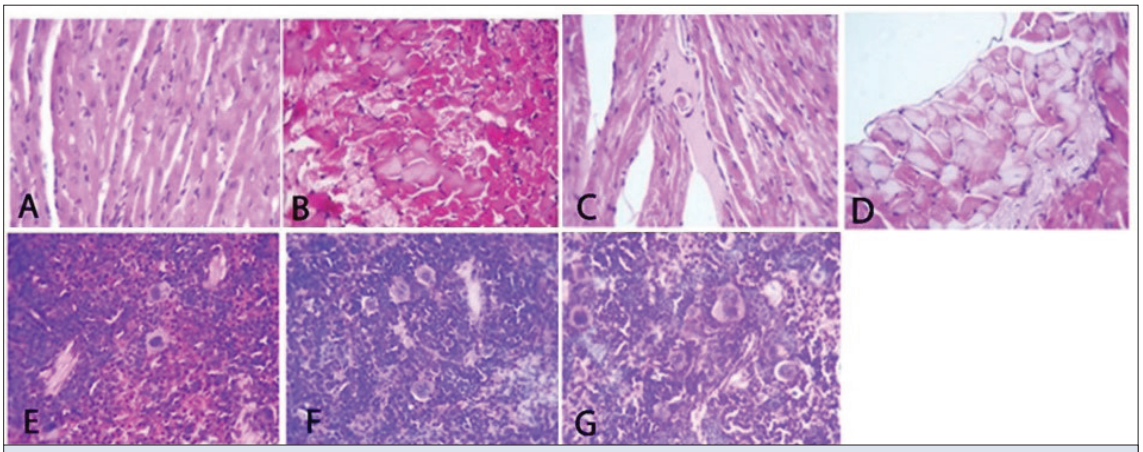

Fig 5. Heart and spleen photomicrographs of control, IN, and SC vaccinated mice with BCG. **A)** Heart of control group showing cardiomyocyte. **B-D)** Heart of IN vaccinated mice showing vacuolation of cardiomyocytes (B), congestion and oedema of intermuscular blood vessels (C), and basophilic cytoplasm and marked loss of cardiomyocytes striations (D). **E)** Spleen of control group showing normal microscopic structure. **F)** Spleen of IN vaccinated mice showing low number of megakaryocyte proliferation. **G)** Spleen of SC vaccinated mice showing high number of megakaryocyte proliferation

proliferation was evident in both IN and SC-vaccinated mice, but it was more significant in SC-vaccinated mice than in IN-vaccinated mice *(Fig. 5-F,G)*.

Discussion

BCG is one of the most popular and safest human vaccine against tuberculosis worldwide [14]. Although BCG has 70-80% effectiveness against the most serious complications of TB, i.e TB meningitis, it is less potent in preventing pulmonary tuberculosis [20]. Reports of adverse reactions to BCG are relatively rare in healthy immunocompetent individuals while serious adverse reactions were recorded in some immuno-compromised individuals [21]. Many factors affect the development of various adverse reactions to BCG vaccine including the potency and dose of the vaccine strain, administration route, age, and host immunity [21]. Post vaccinal adverse reactions and complications in humans are mainly in the form of mild and transient fever, injection site abscesses, lymphadenitis, skin rash, and systemic disseminated BCG infection [22]. Although BCG has also been widely used in vaccine research in laboratory animal hosts, and it is currently being developed for usage in a range of livestock and wild animals, the detrimental effects of BCG on lab animals i.e mice, however, are not well understood [23]. In this study, we studied the adverse effect of BCG vaccine in xenogeneic mice using two different routes (IN and SC) and assessed the alteration in the immunoglobulin levels post vaccination.

The results confirmed that BCG vaccine significantly increased the level of IgG in the sera of both IN and SC vaccinated mice and caused notable alterations in the level of immunoglobulins in xenogeneic mice via both routes. Additionally, SC immunization caused a greater rise in all immunoglobulins than nebulized BCG vaccination and compared to control mice.

The obtained results come in accordance with previous findings of Husain, Kashyap [24] and Husain, Warke [25] where they observed increased IgG level in comparison with IgA and IgM in mice after BCG vaccination. In addition, Medeiros, Armôa [26] found significant increase in IgG1 following vaccination of BALB/c mice with BCG vaccine. Contrary to these results, other studies has found that intranasal BCG vaccination significantly increases IgA upregulation compared to subcutaneous BCG vaccination and unvaccinated mice [27]. Mice are thought to be more resistant to *M. tuberculosis* infection than humans, and they could tolerate large numbers of *Mycobacterium* in their lungs for months without apparent progression of disease [28].

Our results suggest that BCG vaccination have adverse effects on parenchymatous organs which are route-dependent. The severity of the effects was more pronounced in mice vaccinated via the IN route, implying that aerosolization of BCG induces an inflammatory response with moderate irritation of the respiratory tract. The hepatic lesions demonstrate that BCG vaccination using IN route alters hepatic architecture and promotes more extramedullary hematopoiesis and Kupffer cell infiltrations compared to the SC route in rats.

The present findings showed that BCG vaccine induced moderate granulomatous response in the lungs of mice after 21 days of vaccination without showing intragranulomatous necrosis. This finding has been observed in some of previous studies [29]. As an argument for this, mice immunized with mucosal vaccination had a higher lymphocyte proliferation than mice immunized with SC route [30].

Consistent with our findings, intraperitoneal BCG vaccination induced significant inflammatory cellular infiltration and hepatocyte damage in 3 weeks post vaccination [31]. Moreover, it has been reported that BCG induced granulomas in some organs, mainly lungs and liver, though these granulomas may represent a natural immune response [32].

Megakaryocyte proliferation and extramedullary hematopoiesis in spleen and livers were prominent in all BCG vaccinated mice in this study. Similarly, other studies have reported these findings in the mice post vaccination [33,34]. In addition, platelets and the megakaryocyte of which they were developed, perform a range of immune functions including activating and adhering leukocytes and endothelium, creating extracellular traps for neutrophils, sensing pathogens, and clearing them [35,36]. As part of an immune response to pathogens, extramedullary hematopoiesis occurs as well. This response is mainly directed towards the spleen and liver, where antigen-presenting cells and phagocytes take place [35].

Both vaccinated groups endured comparable renal lesions. However, cardiac degeneration was one of the most severe lesions observed in IN vaccinated mice compared to SC vaccinated mice. BCG can be phagocytosed and degraded by macrophages, giving rise to various immunogenic components [37] that can strongly stimulate an inflammatory response through the activation of different pattern recognition receptors (PRRs) [38].

The limitation of the current study was the vaccine should be evaluated with more routes and study the immunological and pathological effects of these routes.

The present findings clearly showed that a change in the route of administration of BCG to mimic the natural route of infection might be a successful strategy to prevent pulmonary tuberculosis. However, further researches are needed to avert aggravating undesirable concerns.

Availability of Data and Materials

The datasets analyzed during the current study are available from

the corresponding author (A. Selim) on reasonable request.

Financial Support

This reserach did not receive external fund

Conflict of Interest Competing Interests

The authors declare that they have no conflicts of interest.

Author Contributions

Conceptualization, methodology, formal analysis, investigation, resources, data curation, writing-original draft preparation, S.A.S., A.S., M.M.G., S.M.E., M.H.W and M.A.M.; writing-review and editing, S.A.S., A.S., M.M.G., M.H.W., S.M.E. and M.A.M.; project administration, S.A.S., A.S., M.M.G., S.M.E. and M.A.M. All authors have read and agreed to the published version of the manuscript.

References

1. Selim A, Ali AF, Ramadan E: Prevalence and molecular epidemiology of Johne's disease in Egyptian cattle. *Acta Trop,* 195, 1-5, 2019. DOI: 10.1016/j.actatropica.2019.04.019

2. Selim A, Attia KA, Alsubki RA, Kimiko I, Sayed-Ahmed MZ: Cross-sectional survey on *Mycobacterium avium* Subsp. *paratuberculosis* in Dromedary Camels: Seroprevalence and risk factors. *Acta Trop,* 226:106261, 2022. DOI: 10.1016/j.actatropica.2021.106261

3. Selim A, Elhaig M, Taha S, Nasr E: Antibacterial activity of silver nanoparticles against field and reference strains of *Mycobacterium tuberculosis, Mycobacterium bovis* and multiple-drug-resistant tuberculosis strains. *Rev Sci Tech,* 37 (3): 823-830, 2018. DOI: 10.20506/rst.37.3.2888

4. Srinivasan S, Conlan AJK, Easterling LA, Herrera C, Dandapat P, Veerasami M, Ameni G, Jindal N, Raj GD, Wood J, Juleff N, Bakker D, Vordermeier M, Kapur V: A meta-analysis of the effect of Bacillus Calmette-Guérin vaccination against bovine tuberculosis: Is perfect the enemy of good? *Front Vet Sci,* 8:637580, 2021. DOI: 10.3389/fvets.2021.637580

5. Elsohaby I, Arango-Sabogal JC, Selim A, Attia KA, Alsubki RA, Mohamed AM, Megahed A: Bayesian estimation of sensitivity and specificity of fecal culture, fecal PCR and serum ELISA for diagnosis of *Mycobacterium avium* subsp. *paratuberculosis* infections in sheep. *Prev Vet Med,* 206:105712, 2022. DOI: 10.1016/j.prevetmed.2022.105712

6. Selim A, Halim R, Galila E, Hamouda F: Seroprevalence and associated risk factors for bovine paratuberculosis in dairy cattle. *J Hell Vet Medical Soc,* 72 (1): 2647-2652, 2021. DOI: 10.12681/jhvms.26746

7. Selim A, El-Haig M, Galila ES, Geade W: Direct detection of *Mycobacterium avium* subsp. *paratuberculosis* in bovine milk by multiplex real-time PCR. *Anim Sci Pap Rep,* 31 (4): 291-302, 2013.

8. Bernitz N, Kerr TJ, Goosen WJ, Chileshe J, Higgitt RL, Roos EO, Meiring C, Gumbo R, de Waal C, Clarke C, Smith K, Goldswain S, Sylvester TT, Kleynhans L, Dippenaar A, Buss PE, Cooper DV, Lyashchenko KP, Warren RM, van Helden PD, Parsons SDC, Miller MA: Review of diagnostic tests for detection of *Mycobacterium bovis* infection in South African wildlife. *Front Vet Sci,* 8:588697, 2021. DOI: 10.3389/fvets.2021.588697

9. Cadmus S, Fujiwara P, Shere J, Kaplan B, Thoen C: The control of *Mycobacterium bovis* infections in Africa: A one health approach. *Tuberculosis in Animals: An African Perspective*: 41-55, 2019. DOI: 10.1007/978-3-030-18690-6_4

10. Natarajan A, Beena P, Devnikar AV, Mali S: A systemic review on tuberculosis. *Indian J Tuberc,* 67 (3): 295-311, 2020. DOI: 10.1016/j.ijtb.2020.02.005

11. Wahdan A, Riad EM, Enany S: Genetic differentiation of *Mycobacterium bovis* and *Mycobacterium tuberculosis* isolated from cattle and human sources in, Egypt (Suez Canal area). *Comp Immunol Microbiol Infect Dis,* 73:101553, 2020. DOI: 10.1016/j.cimid.2020.101553

12. Abdellrazeq G, Elnaggar M, Osman H, Davis W, Singh M: Prevalence of bovine tuberculosis in Egyptian cattle and the standardization of the interferon-gamma assay as an ancillary test. *Transbound Emerg Dis,* 63 (5): 497-507, 2016. DOI: 10.1111/tbed.12291

13. Selim A, Abdelhady A, Abdelrahman A: Ovine paratuberculosis: Seroprevalence and comparison of fecal culture and direct fecal PCR assay. *Comp Immunol Microbiol Infect Dis,* 74:101526, 2021. DOI: 10.1016/j.cimid.2020.101526

14. Chandran A, Williams K, Mendum T, Stewart G, Clark S, Zadi S, Lanni F, McLeod N, Williams A, Villarreal-Ramos B, Vordermeier M, Maroudam V, Prasad A, Bharti N, Banerjee R, Manjari Kasibhatla S, McFadden J: Development of a diagnostic compatible BCG vaccine against bovine tuberculosis. *Sci Rep,* 9 (1): 1-11, 2019. DOI: 10.1038/s41598-019-54108-y

15. Buddle BM, Vordermeier HM, Chambers MA, de Klerk-Lorist LM: Efficacy and safety of BCG vaccine for control of tuberculosis in domestic livestock and wildlife. *Front Vet Sci,* 5:259, 2018. DOI: 10.3389/fvets.2018.00259

16. Li J, Zhan L, Qin C: The double-sided effects of *Mycobacterium bovis* bacillus Calmette–Guérin vaccine. *NPJ Vaccines,* 6 (1): 14, 2021. DOI: 10.1038/s41541-020-00278-0

17. Eickhoff CS, Blazevic A, Killoran EA, Morris MS, Hoft DF: Induction of mycobacterial protective immunity by sublingual BCG vaccination. *Vaccine,* 37 (36): 5364-5370, 2019. DOI: 10.1016/j.vaccine.2019.07.034

18. Lesellier S, Boschiroli ML, Barrat J, Wanke C, Salguero FJ, Garcia-Jimenez WL, Nunez A, Godinho A, Spiropoulos J, Palmer S, Dave D, Anderson P, Boucher JM, de Cruz K, Henault S, Michelet L, Gowtage S, Williams GA, Nadian AK, Monchâtre-Leroy E, Boué F, Chambers MA, Richomme C: Detection of live *M. bovis* BCG in tissues and IFN-γ responses in European badgers *(Meles meles)* vaccinated by oropharyngeal instillation or directly in the ileum. *BMC Vet Res,* 15:445, 2019. DOI: 10.1186/s12917-019-2166-4

19. Tree J, Williams A, Clark S, Hall G, Marsh P, Ivanyi J: Intranasal bacille Calmette-Guérin (BCG) vaccine dosage needs balancing between protection and lung pathology. *Clin Exp Immunol,* 138 (3): 405-409, 2004. DOI: 10.1111/j.1365-2249.2004.02648.x

20. Ábalos P, Valdivieso N, Pérez de Val B, Vordermeier M, Benavides MB, Alegría-Morán R, Saadi K, Wistuba M, Ortega C, Sánchez N, Retamal P: Vaccination of calves with the *Mycobacterium bovis* BCG strain induces protection against bovine tuberculosis in dairy herds under a natural transmission setting. *Animals,* 12 (9):1083, 2022. DOI: 10.3390/ani12091083

21. Murphy D, Corner L, Gormley E: Adverse reactions to *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccination against tuberculosis in humans, veterinary animals and wildlife species. *Tuberculosis,* 88 (4): 344-357, 2008. DOI: 10.1016/j.tube.2007.11.010

22. Venkataraman A, Yusuff M, Liebeschuetz S, Riddell A, Prendergast AJ: Management and outcome of Bacille Calmette-Guérin vaccine adverse reactions. *Vaccine,* 33 (41): 5470-5474, 2015. DOI: 10.1016/j.vaccine.2015.07.103

23. Zhang L, Ru HW, Chen FZ, Jin CY, Sun RF, Fan XY, Guo M, Mai JT, Xu WX, Lin QX, Liu J: Variable virulence and efficacy of BCG vaccine strains in mice and correlation with genome polymorphisms. *Mol Ther,* 24 (2): 398-405, 2016. DOI: 10.1038/mt.2015.216

24. Husain AA, Kashyap RS, Kalorey DR, Warke SR, Purohit HJ, Taori GM, Daginawal HF: Effect of repeat dose of BCG vaccination on humoral response in mice model. *Indian J Exp Biol,* 49 (1): 7-10, 2011.

25. Husain AA, Warke SR, Kalorey DR, Daginawala HF, Taori GM, Kashyap RS: Comparative evaluation of booster efficacies of BCG, Ag85B, and Ag85B peptides based vaccines to boost BCG induced immunity in BALB/c mice: A pilot study. *Clin Exp Vaccine Res,* 4 (1): 83-87, 2015. DOI: 10.7774/cevr.2015.4.1.83

26. Medeiros MA, Armôa GR, Dellagostin OA, McIntosh D: Induction of humoral immunity in response to immunization with recombinant *Mycobacterium bovis* BCG expressing the S1 subunit of *Bordetella pertussis* toxin. *Can J Microbiol,* 51 (12): 1015-1020, 2005. DOI: 10.1139/w05-095

27. Tanner R, Villarreal-Ramos B, Vordermeier HM, McShane H: The humoral immune response to BCG vaccination. *Front Immunol,* 10:1317, 2019. DOI: 10.3389/fimmu.2019.01317

28. McMurray DN: Disease model: Pulmonary tuberculosis. *Trend Mol Med,* 7 (3): 135-137, 2001. DOI: 10.1016/s1471-4914(00)01901-8

29. Turner J, Rhoades ER, Keen M, Belisle JT, Frank AA, Orme IM: Effective preexposure tuberculosis vaccines fail to protect when they are

given in an immunotherapeutic mode. *Infect Immun,* 68 (3): 1706-1709, 2000. DOI: 10.1128/IAI.68.3.1706-1709.2000

30. Kramnik I, Beamer G: Mouse models of human TB pathology: Roles in the analysis of necrosis and the development of host-directed therapies. *Semin Immunopathol*, 38 (2): 221-237, 2016. DOI: 10.1007/s00281-015-0538-9

31. Chapman R, Shephard E, Stutz H, Douglass N, Sambandamurthy V, Garcia I, Ryffel B, Jacobs W, Williamson AL: Priming with a recombinant pantothenate auxotroph of *Mycobacterium bovis* BCG and boosting with MVA elicits HIV-1 Gag specific $CD8^+$ T cells. *PLoS One,* 7 (3):e32769, 2012. DOI: 10.1371/journal.pone.0032769

32. Tajima Y, Takagi R, Nakajima T, Kominato Y: An infant with asymptomatic hepatic granuloma probably caused by bacillus Calmette-Guérin (BCG) vaccination found incidentally at autopsy: A case report. *Cases J,* 1 (1): 1-5, 2008. DOI: 10.1186/1757-1626-1-337

33. Mansour S, Elshahedy M, Rabie T, Fetaih H, Obaid J: Post-vaccination studies on mice vaccinated against uropathogenic *Escherichia coli. Catrina: Int J Environ Sci,* 11 (1): 73-79, 2015.

34. Shoulah SA, Elshafae SM, Gaballa MM, Moussa MA, Selim A, Attia K, AlKahtani MD, Albohairy FM: Adverse effect of vaccination in xenogeneic animals. *Microb Pathog,* 166:105541, 2022. DOI: 10.1016/j.micpath.2022.105541

35. Kim S-J, Davis RP, Jenne CN: Platelets as modulators of inflammation. *Semin Thromb Hemost,* 44 (2): 91-101, 2018. DOI: 10.1055/s-0037-1607432

36. Koupenova M, Clancy L, Corkrey HA, Freedman JE: Circulating platelets as mediators of immunity, inflammation, and thrombosis. *Circ Res,* 122 (2): 337-351, 2018. DOI: 10.1161/CIRCRESAHA.117.310795

37. Dockrell HM, Smith SG: What have we learnt about BCG vaccination in the last 20 years? *Front Immunol,* 8:1134, 2017. DOI: 10.3389/fimmu.2017.01134

38. Tsuji S, Matsumoto M, Takeuchi O, Akira S, Azuma I, Hayashi A, Toyoshima K, Seya T: Maturation of human dendritic cells by cell wall skeleton of *Mycobacterium bovis* bacillus Calmette-Guerin: Involvement of toll-like receptors. *Infect Immun,* 68 (12): 6883-6890, 2000. DOI: 10.1128/IAI.68.12.6883-6890.2000

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 289-292, 2023
DOI: 10.9775/kvfd.2022.28672

SHORT COMMUNICATION

The Use of Artificial Neural Networks for Prediction of Milk Productivity of Cows in Ukraine

Mykhailo MATVIEIEV [1] Yuriy ROMASEVYCH [2] Andriy GETYA [3 (*)]

[1] National University of Life and Environmental Sciences of Ukraine, Faculty of Livestock Raising and Water Bioresources, Department of Animal Feeding and Feed Technology named after Pshenychnyi, 03041 Kyiv, UKRAINE

[2] National University of Life and Environmental Sciences of Ukraine, Faculty of Design and Engineering, Department of Machines and Equipment Design, 03041 Kyiv, UKRAINE

[3] National University of Life and Environmental Sciences of Ukraine, Faculty of Livestock Raising and Water Bioresources, Department of Animal Genetics, Breeding and Animal Biotechnology, 03041 Kyiv, UKRAINE

ORCIDs: M.M. 0000-0003-1281-9032; Y.R. 0000-0001-5069-5929; A.G. 0000-0002-4747-9261

Article ID: KVFD-2022-28672 Received: 24.10.2022 Accepted: 06.04.2023 Published Online: 12.04.2023

Abstract: Aim of this work was to study effectiveness of prediction of cows' milk productivity (yield, fat and protein content) using artificial neural network (ANN) technology for data sets with missing values. Four variants of data sets, which consist of different numbers of monthly test-day milk records (MTDMRs) were chosen. Calculated milk productivity using Test Interval Method (TIM) was chosen as control value. Obtained results showed that milk productivity can be predicted using ANN even if missing data occurs. Rank correlation coefficients between control and predicted results were 0.918, 0.949, and 0.852 for milk yield, protein, and fat content, respectively.

Keywords: *Artificial Neural Networks, Dairy Productivity Prediction*

Ukrayna'da İneklerin Süt Verimliliğinin Tahmininde Yapay Sinir Ağlarının Kullanımı

Öz: Bu çalışmanın amacı, ineklerin süt verimliliğinin (verim, yağ ve protein içeriği) tahmin edilmesinde eksik değerlere ait veri setleri için yapay sinir ağı (YSA) teknolojisini kullanılarak etkinliğini incelemektir. Farklı sayıda aylık test-günlük süt kayıtlarından (MTDMR) oluşan dört farklı veri seti seçilmiştir. Test Aralık Yöntemi (TIM) kullanılarak hesaplanan süt verimliliği kontrol değeri olarak kullanılmıştır. Elde edilen sonuçlar, eksik veriler olsa bile süt verimliliğinin YSA kullanılarak tahmin edilebileceğini göstermiştir. Kontrol ile tahmin edilen sonuçları arasındaki sıralama korelasyon katsayıları, süt verimi, protein ve yağ içeriği için sırasıyla 0.918, 0.949 ve 0.852 olarak saptanmıştır.

Anahtar sözcükler: *Yapay Sinir Ağları, Süt Verimliliği Tahmini*

Introduction

The improvement of modern breeds of farm animals occurs with the widespread use of mathematical methods integrated into software. One of the basic elements of successful application of programs is the availability of data on the productivity of animals, collected as a result of organized reliable breeding [1]. The list of traits is usually regulated by national and international associations and unions. Maintenance of such recording scheme is important for both, traditional breeding and genomic [2].

In the world, there is a tendency for a constant increase in the number of traits, particularly in dairy farming [3], which necessitated the use of modern methods of data processing, including ANN, machine learning algorithms, and other methods [4].

In Ukraine ANN did not find their application in breeding practice. However, the main problem in Ukraine is not a large amount of data, but the presence of incomplete data (missing values) in productivity records, as in many farms it is not recorded regularly.

How to cite this article?

Matvieiev M, Romasevych Y, Getya A: The use of artificial neural networks for prediction of milk productivity of cows in Ukraine. *Kafkas Univ Vet Fak Derg,* 29 (3): 289-292, 2023.
DOI: 10.9775/kvfd.2022.28672

(*) Corresponding author: Andriy GETYA

Phone: +38 050 1950125 Fax: +38 050 1950125
E-mail: getya@ukr.net

Therefore, it is often not possible to assess milk productivity by classical methods. In this case, there are several ways to predict milk productivity of cows [5].

Based on the above mentioned, it was decided to study the effectiveness of prediction of milk productivity of cows using the capabilities of ANN technology for the cases with missing values about milk productivity.

Material and Methods

Ethical Statement

This study was approved by the Commission on bioethics of Research Institute of Animal Health of the National University of Life and Environmental Sciences of Ukraine (Approval no: 103-07 from 01.07.2019).

General Conditions

Cows of different origins, years of birth, and lactation were involved in the study. Their milk yield exceeded 6000 kg per standard lactation (lactation duration 305 days). Analysis of milk, collected on the farm, was performed in the laboratory. Contents of fat (%), protein (%), lactose (%), and somatic cell counts (SCC, thousand/cm^3) were determined [6].

To transform the somatic cell counts (SCC) into the somatic cell scores (SCS) the formula by Wiggans and Shook [7] was applied.

For prediction of cows' productivity 4 variants of the data sets were tested, which included different MTDMRs counted from the beginning of lactation:

1) first, second, fifth, eighth, and tenth MTDMRs; 2) first three, ninth and the tenth MTDMRs; 3) first five MTDMRs; 4) second, fifth, and tenth MTDMRs.

Obtained results were compared with results, calculated according to TIM [8], using the data of 10 MTDMRs of 144 cows.

The animals whose data were included in the prediction of productivity were the same for all four variants.

Development of ANNs and Their Training

Procedures for training ANNs and prediction were carried out for each data set separately. For training of each ANN, milk productivity data, calculated using TIM on the basis on 10 MTDMRs, were used.

A feedforward ANN was used for training. The number of inputs of each ANN corresponded to the dimension of the input vector for a certain task (36 - for ANN-1, ANN-2 and ANN-3, 24 - for ANN-4). For all tasks, the output of the ANN was represented by a four-component vector of the following traits (data): milk yield, fat and protein content per standard lactation.

The number of hidden layers of the ANN was equal to one, and the number of neurons in the hidden layer was 10. The activation function for all neurons is a hyperbolic tangent. For all of the neurons biases were used as well.

The estimation of unknown ANNs' parameters corresponds to the total number of biases and weights. The number of biases in the hidden layer equals 10 and in the output layer - 4. The ANNs' weights form two matrices: the first one for the hidden layer, and the second one - for the output layer. For the ANN-1, ANN-2, and ANN-3 cases, the hidden layer weights matrix is of 36x10 dimension. For the ANN-4 the matrix is of 24x10 dimension. The output layer weights matrix for all of the ANNs is of 10x4 dimension. This data allows the determination of a total number of needed parameters: 414 weights and biases for ANN-1, ANN-2, and ANN-3, 294 weights and biases for ANN-4.

The function that reflected the quality of training (loss function) was the root mean square deviation of the prediction and training data. Moving on the surface of the loss function a training algorithm must determine such values of weights and biases, that the corresponding value of the loss function reaches a minimum (the best variant - the global one). This problem is quite difficult, because of the huge dimensionality of the loss function, and its topology complexity (non-linearity, multimodality, non-separability, etc.). Thus, the selection of a training algorithm is a very important stage of the study, it influences ANN prediction quality. Here we applied the gradient-base ADAM algorithm [9], which is a common and effective method of ANNs training. In the ADAM algorithm, the size of the data sets, on which one gradient was calculated, was chosen to be 50. The number of training rounds was 5000 (this number was defined to be sufficient to minimize the loss function).

The training pair in the prediction tasks described above was represented by data in the following format: input vector (traits of the cow) - scalar (productivity of the cow per standard lactation). The input vector included a different number of components for different task statements.

In all samples, the individual cow number, year of birth of the cow, sire number (coded with numbers from 1 to 51), calving age (in days from birth of cow), day of MTDMR (in days from calving) and performance data for each MTDMR (milk yield, fat-, protein-, lactose content, and SCS) were considered.

All data were normalized. So, numerical values in both groups (for training and for prediction) for all traits vary from 0 to 1. This makes it possible to train ANNs since input vector components vary in the limited domains, and no activation function saturation will occur. The feature of the current work, which should be stressed, is connected with quite a big number of traits in data. This provides additional factors to involve in prediction. Their influence

we might assess indirectly by analyzing the prediction performance of a trained ANN.

Verification of the Accuracy of the Prediction

To compare the received (predicted by ANN) data with the control data (TIM), coefficient of variation (C_v) [10] and Spearman's rank correlation coefficients (r_s) [11] were calculated.

Results

All average predicted values of milk yield for all ANN variants were higher than the control values. Results of the first and fourth ANNs most deviated from the control values, and the average values of the second and third ANNs were the closest to the control ones *(Table 1)*.

A comparison of the results obtained using ANNs showed that individual predictions of cows' milk yield, calculated by ANN-1 were the most precise (deviation from the control value - C_v=5.16%). Least precise were the predictions, calculated by ANN-4 (C_v=7.91). Analysis of rank correlation coefficients, confirm the previous conclusion.

Since the breeding value of cows is usually calculated involving different traits, the predictions for other traits like fat and protein content also were made, because these traits directly affect the price of milk.

Coefficient of variation of differences between control and predicted values of protein content was from 2.17 to 3.07%, for ANN-1, and ANN-4, respectively. Statistically significant Spearman's rank correlation coefficients (P<0.001) were established between predicted and control values for all ANNs (r_s ranged from 0.88 to 0.95). ANN-4 was characterized by the smallest r_s (0.888), while the highest value of r_s between predicted and control values had ANN-1 (0.949).

Calculation of prediction of fat content in the milk, showed the advantage in the accuracy of ANN-2 over other variants. The coefficient of variation of ANN-2 was smaller by 1.09, 1.39, and 3.64 percentage points compared to ANN-1, ANN-3, and ANN-4, respectively.

Based on the results of analysis, it was found that trained ANNs are able to predict milk productivity with different accuracy. Some ANNs better predicted milk yield, while others - fat or protein content *(Table 2)*.

Discussion

Since the last 5 years the number of articles dealing with the application of artificial intelligence in animal husbandry has increased significantly [12].

The ANN-1 gave the best prediction for milk yield and protein content. ANN-2 was more suitable for prediction of fat content. ANN-4 showed the worst prediction for all traits, however, this can be explained by the smallest number of MTDMRs that were included in the database for its training (3 MTDMRs from each cow compared to 5 in other ANNs).

An important criterion for farmers is the cost of this assessment. Taking into account the cost of one laboratory analysis of milk quality (0.95 USD), the total cost of analyzing of milk productivity of a single cow during lactation (10 MTDMRs) is 9.5 USD. Using ANNs in

Table 1. *Milk yield, protein, and fat content per standard lactation of cows, calculated by ANN's with different variants of data sets (n=49)*

Parameter	Indicator	TIM	ANN-1	ANN-2	ANN-3	ANN-4
Calculation for Milk Yield	M±Se, kg	8467.1±167.61	8569.1±142.65	8474.8±168.28	8505.7±157.95	8590.4±135.19
	C_v, %	-	5.16	5.98	6.54	7.91
	ΔM, kg	-	102.0	7.7	38.6	123.3
	r_s	-	0.918*	0.886*	0.866*	0.815*
Calculation for Protein Content	M±Se, %	3.24±0.033	3.234±0.028	3.246±0.027	3.261±0.030	3.248±0.029
	C_v, %	-	2.17	2.94	2.83	3.07
	ΔM, %	-	0.0060	0.0056	0.0213	0.0085
	r_s	-	0.949*	0.909*	0.901*	0.888*
Calculation for Fat Content	M±Se, %	4.15±0.061	4.116±0.050	4.139±0.055	4.204±0.051	4.075±0.046
	C_v, %	-	5.40	4.31	5.70	7.95
	ΔM, %	-	0.0345	0.0102	0.0544	0.0751
	r_s	-	0.852*	0.910*	0.853*	0.612*

** Correlation is significant at the 0.001 level; r_s - Spearman's rank correlation coefficients (control: prediction); ΔM - differences between the average values for control and predicted values*

TIM: Test Interval Method; ANN-1: Artificial Neural Network-1; ANN-2: Artificial Neural Network-2; ANN-3: Artificial Neural Network-3; ANN-4: Artificial Neural Network-4

Table 2. *Comparative characteristics of accuracy (based on the coefficient of variation) of prediction using different ANN*

Variant of Prediction	Traits		
	Milk Yield	Fat Content	Protein Content
ANN-1	1	1	2
ANN-2	2	3	1
ANN-3	3	2	3
ANN-4	4	4	4

1 - the best, 2 - better, 3 - worse, 4 - the worst
ANN-1: Artificial Neural Network-1; ANN-2: Artificial Neural Network-2; ANN-3: Artificial Neural Network-3; ANN-4: Artificial Neural Network-4

various variations, it is possible to reduce the cost of evaluation of one cow by more than 50% (ANN-1, ANN-2, and ANN-3).

The obtained results allow us to conclude that by using ANN it is possible to reduce the number of MTDMRs during the assessment of milk productivity of cows per standard lactation without losing the accuracy of the assessment.

Obviously, the number of MTDMRs, included in the calculations, is one of the important factors affecting the accuracy of the prediction of each ANNs. A similar conclusion was reached by other researchers, because the precision of the models increases with increase in the number of test-day milk records generally [13].

Other important factor is the month of MTDMRs from the beginning of lactation. So, the most correct results were obtained by ANN-1, which used the data of 5 MTDMRs, measured on different periods during lactation: on the first, second, fifth, eighth, and tenth months.

The application of ANN can help to compute (predict) milk productivity of cows for standard lactation if there are missing values in the database [14]. This method is advisable to use in breeding both at the farm level and in breeding centers.

Availability of Data and Materials

Data sets analyzed during the current study are available from the corresponding author (A. Getya) on reasonable request.

Acknowledgments

The authors would like to thank Mr. Oleh Yemets (Dairy management system), Mr. Vitalii Tkach, and Vira Sikorska for their assistance in the data collecting.

Funding Support

This study was conducted as part of the project, supported by the Ministry of education of Ukraine (0119U102345) "Usage of integrated database for breeding merit evaluation of dairy cows" (Researchers A. Getya, M. Matvieiev).

Competing Interests

The authors report no conflicts of interest.

Authors' Contributions

A.G. and M.M. conceived the design of study. M.M and Y.R. collected and analyzed data. M.M., A.G. and Y.R. have approved and read the final version of the manuscript.

References

1. Cole JB, VanRaden PM: Symposium review: Possibilities in an age of genomics: The future of selection indices. *J Dairy Sci*, 101 (4): 3686-3701, 2018. DOI: 10.3168/jds.2017-13335

2. Fessenden B, Weigel DJ, Osterstock J, Galligan DT, Di Croce F: Validation of genomic predictions for a lifetime merit selection index for the US dairy industry. *J Dairy Sci*, 103 (11): 10414-10428, 2020. DOI: 10.3168/jds.2020-18502

3. Miglior F, Fleming A, Malchiodi F, Brito LF, Martin P, Baes CF: A 100-Year Review: Identification and genetic selection of economically important traits in dairy cattle. *J Dairy Sci*, 100 (12): 10251-10271, 2017. DOI: 10.3168/jds.2017-12968

4. Benos L, Tagarakis AC, Dolias G, Berruto R, Kateris D, Bochtis D: Machine learning in agriculture: A comprehensive updated review. *Sensors (Basel)*, 21 (11):3758, 2021. DOI: 10.3390/s21113758

5. Pollott G, Gootwine E: Appropriate mathematical models for describing the complete lactation of dairy sheep. *Anim Sci*, 71, 197-207, 2000. DOI: 10.1017/S1357729800055028

6. Bentley Instruments I: DairySpec FT User Manual. Bentley Instruments, Inc., Minnesota, USA, 2015.

7. Wiggans G, Shook G: A Lactation measure of somatic-cell count. *J Dairy Sci*, 70 (12): 2666-2672, 1987. DOI: 10.3168/jds.S0022-0302(87)80337-5

8. ICAR: Computing Lactation Yield. Procedure 2 of Section 2 of ICAR Guidelines Computing of Accumulated Lactation Yield. ICAR, 2020.

9. Kingma DP, Ba J: Adam: A method for stochastic optimization. *Proceedings of the 3rd International Conference on Learning Representations (ICLR 2015)* (arXiv:14126980), 2015.

10. Rawlings JO, Pantula SG, Dickey DA: Applied regression analysis: a research tool. Springer, 1998.

11. de Winter J, Gosling S, Potter J: Comparing the pearson and spearman correlation coefficients across distributions and sample sizes: A tutorial using simulations and empirical data. *Psychol Methods*, 21 (3): 273-290, 2016. DOI: 10.1037/met0000079

12. Bao J, Xie Q: Artificial intelligence in animal farming: A systematic literature review. *J Clean Prod*, 331, 2022. DOI: 10.1016/j.jclepro.2021.129956

13. Gorgulu O: Prediction of 305-day milk yield in Brown Swiss cattle using artificial neural networks. *South Afr J Anim Sci*, 42 (3): 280-287, 2012. DOI: 10.4314/sajas.v42i3.10

14. Bhosale MD, Singh TP: Comparative study of feed-forward neuro-computing with multiple linear regression model for milk yield prediction in dairy cattle. *Current Science*, 108 (12): 2257-2261, 2015.

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 293-297, 2023
DOI: 10.9775/kvfd.2022.28784

Case Report

A Case of Endometrial Carcinoma and Pregnancy in a Cat

Atakan ÇORTU [1 (*)] Aybars AKAR [2] Volkan İPEK [3] Leyla Elif Özgü AYÖZGER [3]

[1] Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Obstetrics and Gynecology Department, TR-15100 Burdur - TÜRKİYE

[2] Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Internal Medicine Department, TR-15100 Burdur - TÜRKİYE

[3] Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Pathology Department, TR-15100 Burdur - TÜRKİYE

ORCIDs: A.Ç. 0000-0003-1662-3352; A.A. 0000-0001-7530-8761; V.İ. 0000-0001-5874-7797, L.E.Ö.A. 0000-0003-3320-1688

Article ID: KVFD-2022-28784 Received: 15.11.2022 Accepted: 06.04.2023 Published Online: 17.04.2023

Abstract: This case report describes a rare condition of a pregnant cat that has endometrial carcinoma. A 5-year-old cat was presented because of vaginal discharge on the 40th day after the mating. USG examination revealed small embryonic structures, and thickened endometrium. It was decided to perform an ovariohysterectomy operation. The ovaries and uterus were sent to the laboratory for histopathological examination. The uterine mucosa showed papillary growth and atypical cells with mild to moderate anisokaryosis and occasional mitotic figures. The estrogen beta receptor was mildly positive in some neoplastic epithelium and stromal cells. The progesterone receptor was significantly positive in the lamellar layers of the placenta and myometrium but only slight positivity was observed in neoplastic cells. In summary, the gestational sacs were still at the implantation stage on the 40th day after mating may have been due to the carcinoma in the uterine endometrium and implantation could not occur for this reason. Our case might suggest that estrogen-dependent uterine tissue negatively affects embryonal development in cats and thus pregnancy couldn't be completed properly.

Keywords: *Endometrial carcinoma, Neoplasm, Queen, Pregnancy*

Bir Kedide Endometriyal Karsinoma ve Gebelik Olgusu

Öz: Bu olgu raporu, nadir görülen endometriyal karsinomlu gebe bir kedinin durumunu anlatmaktadır. Beş yaşında bir kedi çiftleşme sonrası 40. günde vajinal akıntı nedeniyle getirildi. USG incelemesinde küçük embriyonik yapılar ve kalınlaşmış endometriyum belirlendi. Ovariohisterektomi operasyonu yapılmasına karar verildi. Ovaryumlar ve uterus histopatolojik inceleme için laboratuvara gönderildi. Uterus mukozasında papiller büyüme ve hafif ila orta derecede anizokaryoz, bazı bölgelerde de mitotik yapılar içeren atipik hücreler görüldü. Östrojen beta reseptörü, bazı neoplastik epitel ve stromal hücrelerde hafif pozitifti. Progesteron reseptörü, plasenta ve miyometriumun lameller tabakalarında önemli ölçüde pozitifti, ancak neoplastik hücrelerde sadece hafif pozitiflik gözlendi. Özetle, gebelik keselerinin çiftleşmeden sonraki 40. günde hala implantasyon aşamasında olması uterus endometriumdaki karsinomdan kaynaklanmış olabilir ve bu nedenle implantasyon gerçekleşememiştir. Olgumuz östrojene bağımlı uterusun kedilerde embriyonal gelişimi olumsuz etkilediğini ve bu nedenle gebeliğin tam olarak tamamlanamadığını düşündürebilir.

Anahtar sözcükler: *Dişi kedi, Endometriyal karsinoma, Gebelik, Neoplazma*

Introduction

Endometrial carcinoma is a tumor formed by the irregular division and proliferation of cells in the endometrium of the uterus. One of the most important factors in the formation of this disease is that the level of estrogen in the circulation is higher than normal [1]. Although cases of endometrial carcinoma are commonly observed in humans, rabbits, and cows, they are rarely observed in cats [2,3].

How to cite this article?

Çortu A, Akar A, İpek V, Ayözger LEÖ: *A case of endometrial carcinoma and pregnancy in a cat. Kafkas Univ Vet Fak Derg,* 29 (3): 293-297, 2023.
DOI: 10.9775/kvfd.2022.28672

(*) Corresponding author: Atakan ÇORTU

Phone: +90 248 213 2231 Cellular phone: +90 539 600 0777
E-mail: atakancortu@mehmetakif.edu.tr

Table 1. *Vital parameters, complete blood count, and biochemical findings*

Parameter	Value	Reference Value	Parameter	Value	Reference Value
WBC (x10^9/L)	16.35	5.5-19.5	GGT (U/L)	2	0-5
RBC (x10^{12}/L)	7.32	5-10	Glucose (mg/dL)	94.48	56-153
HGB (g/dL)	9	8-15	Total Bilirubin (mg/dL)	0.30	56-13
HCT	50.1	24-45	ALT (U/L)	64.3	26-128
MCV (fL)	41	39-55	AST (U/L)	46.1	14-54
PLT (x10^9/L)	430	300-800	Albumin (g/dL)	2.57	2.3-3.9
Ostradiol (pg/mL)	5.2	<20	BUN (mg/dL)	35.51	18-36
Progesterone (ng/mL)	28.3	>2	Body temp (ºC)	38.5	37.5-39
Creatine (mg/dL)	0.64	0.6-2	Heart rate (bpm)	124	120-140
Urea (mg/dL)	76		Respiratory rate (rpm)	18	15-30
Total Protein (g/dL)	6.32	5.9-8.4			

According to the data of one study, endometrial carcinomas accounted for 0.29% of carcinoma cases in cats [4]. The most common symptoms observed in endometrial carcinoma cats include abdominal mass, weight loss, loss of appetite, pain, and vaginal bleeding [3,4].

In the literature review, it was determined that cases of endometrial carcinoma during pregnancy have been reported in humans before, and it was evaluated that it would be significant to prepare a case report of endometrial carcinoma observed during pregnancy in a female cat. [5-8]. In this case, clinical, laboratory, ultrasonographic and histopathological findings of a pregnant cat with endometrial carcinoma and recovery status after an ovariohysterectomy operation are presented.

Case History

Before the patient was examined, the owner approved the informed consent form that she accepted all the procedures to be performed.

The material of this case was a 5-year-old cat brought to Burdur Mehmet Akif Ersoy University Veterinary Faculty Obstetrics and Gynecology clinics on 7 September 2023 with the complaint of vaginal discharge on the 40th day after the mating. It was learned that 40 days ago, the cat stayed with a male cat in another house for three days and left that house after mating, did not run away from the owner's house, and did not mate with another male cat for 40 days. In the physical examination, it was seen that the patient's pulse, respiratory rate, and rectal body temperature were within the reference ranges. It was determined that the cat felt pain on abdominal palpation, the general appearance of the cat was normal, there was a transparent discharge from the vagina, and it was learned that her appetite was good. Complete blood count (H60 Vet, Edan, China) and serum biochemical (Respons®910, DiaSys, Germany) examination were performed by taking blood samples from the cephalic vena. Serum E2 and

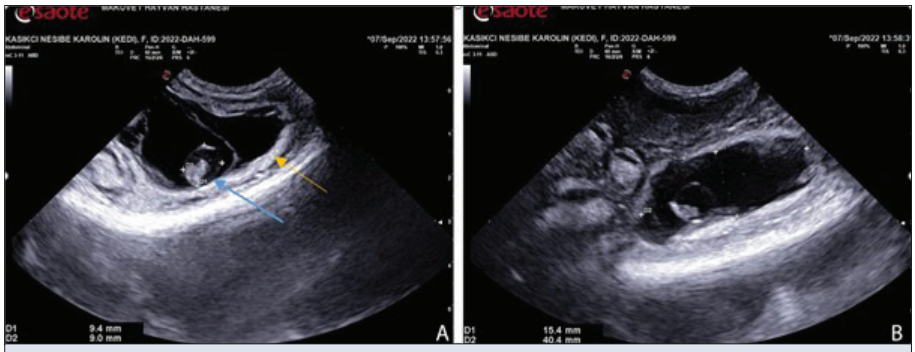

Fig 1. A- Image of uterine endometrium (yellow arrow) and embryonic sac *(blue arrow),* **B-** Size of the uterus

P4 concentrations were measured (Cobas E800, Roche, Switzerland) in accordance with the relevant instructions using electrochemiluminescence immunoassay kits (Elecsys Estradiol III and Elecsys Progesterone III, Roche, USA). All values were determined to be within the reference range *(Table 1)*.

It was decided to perform a B-mode ultrasonography (7.5-MHz micro-convex; MyLab™X8 Platform, Esaote, Italy) examination for pregnancy examination. During the examination, the uterus and gestational sacs were detected. The embryonic structures that could be identified were quite small compared to the 40th day and there was no sign of life [9], the lumen in cervix uteri, corpus uteri and cornu uteri was thicker than those of normal *(Fig. 1-A)*, and the dimensions of the uterus were determined in the area where pregnancy occurred *(Fig. 1-B)*.

Since the patient's general condition was good and the owner did not want his cat to have kittens in the future, it was decided to perform an ovariohysterectomy operation. Chemotherapy could not be performed because the patient owner did not accept chemotherapy treatment.

General anesthesia induction was performed with 5.5 mg/kg propofol (Propofol®, Polifarma, Türkiye). Then the patient was intubated and standard anesthesia monitoring was applied. Anesthesia was maintained with sevoflurane (Sevorane®, Abbvie, USA). The operation was performed with the routine ovariohysterectomy method. The gestational sacs were of different sizes and irregular shapes, and they were taken out. Then, the ovaries and uterus were ligated, removed, and sent to the pathology laboratory in 10% formaldehyde for histopathological examination. For prophylaxis, 10 mg/kg amoxicillin-clavulanic acid (Synulox®, Zoetis, Germany) for 5 days, and 0.2 mg/kg meloxicam (Meloxicam®, Bavet, Türkiye) for 1 day were administered s.c. after the operation. In the follow-up examination performed one week later, the patient had recovered.

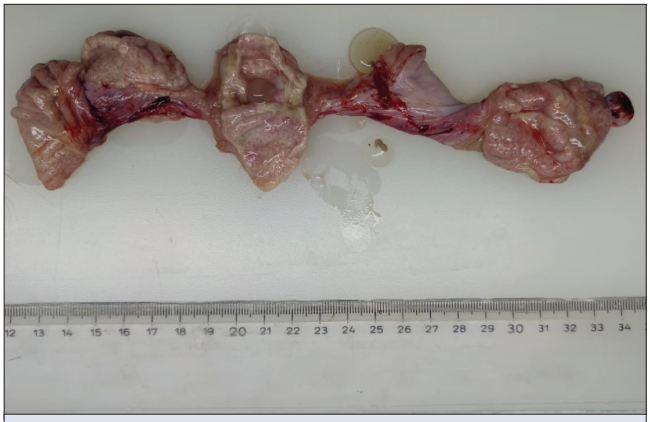

Fig 2. Macroscopical view of the gravid uterus with embryonal sacs

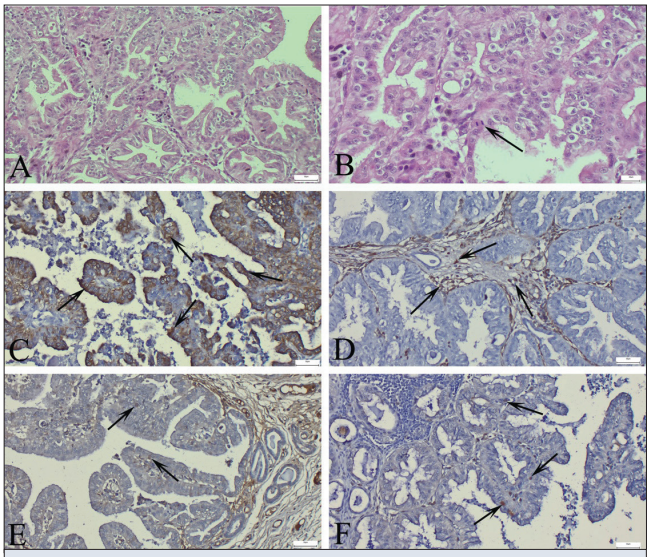

Fig 3. A- Papillary proliferation of endometrial epithelium with merged endometrial glands and stromal invasion, **B**- Papillary growths and occasional mitotic figures (arrow) in atypical neoplastic cells, **C**- Cytokeratin positivity in neoplastic cells (arrows), **D**- Negative vimentin reaction in neoplastic cells but a positive reaction in the stroma (arrows), **E**- Mild ER-beta positivity in some neoplastic epithelial cells (arrows) and prominent positivity in stromal cells, **F**- Mild progesterone receptor positivity in neoplastic epithelial cells (arrows)

Histopathology Findings

Pregnancy was detected on the macroscopic examination of the uterine tissue. The uterus' lumen was seen to be enlarged, contain some purulent material, and occasionally have thickened mucosa *(Fig. 2)*. Four kittens in the early stages of gestation were also present. Uterine samples were fixed in 10% formaldehyde and were embedded in paraffin after routine follow-up procedures. Sections of 5-micrometer thickness were taken from paraffin blocks, stained with hematoxylin-eosin, and evaluated under a light microscope. In the histopathological examinations, the uterine mucosa showed papillary growth and atypical cells with mild to moderate anisokaryosis and occasional mitotic figures. Furthermore, stromal invasion, irregular and merged cribriform pattern of glands, stromal disappearance, and surface syncytial changes were noticed *(Fig. 3-A,B)*. There was no cystic formation which is characteristics of cystic endometrial hyperplasia. Immunohistochemical staining was performed with cytokeratin, vimentin, estrogen receptor alpha (ER-alpha), beta (ER-beta), and progesterone receptor. Neoplastic cells exhibited significant cytokeratin positivity *(Fig. 3-C)*. Vimentin positivity was observed in stromal cells and placental cells but not in the neoplastic epithelium *(Fig. 3-D)*. ER-alpha receptor was negative in all sections. ER-beta was mildly positive in some neoplastic epithelium and stromal cells *(Fig. 3-E)*. The progesterone receptor was significantly positive in the lamellar layers of the placenta and myometrium but only slight positivity was observed in neoplastic cells *(Fig. 3-F)*.

Discussion

It is known that the sudden increase in the level of estrogen in the circulation stimulates the formation of endometrial carcinoma in female mammals and accelerates its development [8].

While progesterone suppresses estrogen-induced endometrial growth during the luteal phase, it also prepares the endometrium for blastocyst implantation. In this balance between growth-stimulating estrogen and growth-suppressing progesterone, the hormone that triggers cancer formation is usually estrogen. In domestic animals, estrogen levels that are not suppressed by progesterone can lead to endometrial hyperplasia or cancer [10]. In this case, the fact that the progesterone level was high and the estrogen level was relatively low during pregnancy suggests that the carcinoma may have formed in the first days of pregnancy or before pregnancy.

Histological differentiation of well-differentiated endometrial carcinoma with atypical hyperplasia is challenging. According Kurman and Norris[11], stromal invasion, irregular infiltration of glands, desmoplastic response, confluent glandular pattern, and extensive papillary pattern are indicators of malignancy. Yet, Silverbeerg[12] suggested that papillary growth is not a reliable criterion but stromal invasion with stromal disappearance, desmoplasia and necrosis or combination of them are the best criteria of low-grade carcinoma. In our case, mild to moderate nuclear atypia, extensive papillary growth with surface syncytial changes, irregular and merged cribriform pattern of glands, stromal invasion, and stromal disappearance were encountered. Thus, well-differentiated carcinoma was diagnosed based on histopathological findings.

The expression of estrogen and progesterone receptors in tumor masses in humans has been linked to the prognosis of endometrial carcinomas [13,14]. In feline endometrial carcinomas, ERs-alpha are positive in 50-83.3% of cases, whereas progesterone receptors are generally positive [3,15]. In another study, loss of ER-alpha expression was detected in the endometrium and myometrium, while progesterone receptor expression was observed in the stroma and myometrium [16], and suggested that alternative pathways involving local growth factors can influence epithelial proliferation. Similarly, in the presented case, the lack of the progesterone receptor in the tumoral tissue and its presence in the myometrium were observed. ER-alpha was also found to be negative throughout the section, however, the ER-beta was revealed to be positive in some tumoral epithelial and many stromal cells. Negative ER-alpha expression has been linked to the loss and methylation of transcriptional activators in tumor tissue, as well as the trans domination of ER-beta [16,17]. It has also been observed that ER-alpha deficiency is associated with a poor tumor prognosis in feline endometrial carcinomas [16]. Furthermore, the ER-beta receptor has been discovered as a uterine proliferation controller [19]. In our case, the negativity of ER-alpha and the very low positivity of ER-beta might be interpreted as a negative prognostic factor.

In this case, it was observed that the embryonic sacs were approximately 9 mm in diameter on the 40th day after the mating. In cats, implantation occurs between 12-14 days of pregnancy. It has been determined in previous studies that the outer embryonic sac diameter is approximately 8 mm in diameter at this stage [9,20]. In the early stages of pregnancy, endometrial carcinoma adversely affects embryo development and implantation [6]. In summary, we think that the reason why the gestational sacs were still at the implantation stage on the 40th day after mating may have been due to the carcinoma in the uterine endometrium and implantation could not occur for this reason. Our case and clinical review of the literature might suggest that estrogen-dependent uterine tissue negatively affects embryonal development in cats and thus embryos may not have developed properly.

Availability of Data and Materials

The datasets analyzed during the study available from the corresponding author (A. Çortu) on request.

Competing Interests

The authors declared that there is no conflict of interest.

Acknowledgements

The authors would like to thank the owner of the cat.

Author Contributions

Ultrasonographic examination was done by AA and AC, histopathological examination was done by VI and LEOA, and the article was written by AC, AA, VI and LEOA.

References

1. Yang B, Chen R, Liang X, Shi J, Wu X, Zhang Z, Chen X: Estrogen enhances endometrial cancer cells proliferation by upregulation of prohibitin. *J Cancer,* 10 (7): 1616-1621, 2019. DOI: 10.7150/jca.28218

2. Hubbard SA, Gargett CE: A cancer stem cell origin for human endometrial carcinoma? *Reprod Fertil,* 140 (1): 23-32, 2010. DOI: 10.1530/REP-09-0411

3. Miller MA, Ramos-Vara JA, Dickerson MF, Johnson GC, Pace LW, Kreeger JM, Turnquist SE, Turk JR: Uterine neoplasia in 13 cats. *J Vet Diagn Invest,* 15 (6): 515-522, 2003. DOI: 10.1177/104063870301500602

4. Cooper TK, Ronnett BM, Ruben DS, Zink MC: Uterine myxoid leiomyosarcoma with widespread metastases in a cat. *Vet Pathol,* 43 (4): 552-556, 2006. DOI: 10.1354/vp.43-4-552

5. Eriksson AG, Fallaas Dahl G, Nesbakken AJ, Lund KV, Amant F: Endometrial cancer during pregnancy: Management strategies. *Int J Gynecol Cancer,* 29 (7): 1221-1224, 2019. DOI: 10.1136/ijgc-2019-000756

6. Hannuna KY, Putignani L, Silvestri E, Pisa R, Angioli R, Signore F: Incidental endometrial adenocarcinoma in early pregnancy: A case report and review of the literature. *Int J Gynecol Cancer,* 19 (9): 1580-1584, 2009. DOI: 10.1111/IGC.0b013e3181a841a7

7. Shiomi M, Matsuzaki S, Kobayashi E, Hara T, Nakagawa S, Takiuchi T, Mimura K, Ueda Y, Tomimatsu T, Kimura T: Endometrial carcinoma in a gravid uterus: A case report and literature review. *BMC Pregnancy Childbirth,* 19 (1): 425, 2019. DOI: 10.1186/s12884-019-2489-y

8. Vaccarello L, Apte SM, Copeland LJ, Boutselis JG, Rubin SC: Endometrial carcinoma associated with pregnancy: A report of three cases and review of the literature. *Gynecol Oncol,* 74 (1): 118-122, 1999. DOI: 10.1006/gyno.1999.5369

9. Zambelli D, Prati F: Ultrasonography for pregnancy diagnosis and evaluation in queens. *Theriogenology,* 66 (1): 135-144, 2006. DOI: 10.1016/j.theriogenology.2006.04.004

10. Rodrigez AC, Blanchard Z, Maurer KA, Gertz J: Estrogen signaling in endometrial cancer: A key oncogenic pathway with several open questions. *Horm Cancer,* 10 (2-3): 51-63, 2019. DOI: 10.1007/s12672-019-0358-9

11. Kurman RJ, Norris HJ: Evaluation of criteria for distinguishing a typical endometrial hyperplasia from well-differentiated carcinoma. *American Cancer Society,* 49 (12): 2547-2559. DOI: 10.1002/1097-0142(19820615)49:12<2547::aid-cncr2820491224>3.0.co;2-0

12. Silverberg SG: Problems in the differential diagnosis of endometrial hyperplasia and carcinoma. *Mod Pathol,* 13 (3): 309-327. DOI: 10.1038/modpathol.3880053

13. Trovik J, Wik E, Werner HM, Krakstad C, Helland H, Vandenput I, Njolstad TS, Stefansson IM, Marcickiewicz J, Tingulstad S, Staff AC; MoMaTEC study group, Amant F, Akslen LA, Salvesen HB: Hormone receptor loss in endometrial carcinoma curettage predicts lymph node metastasis and poor outcome in prospective multicentre trial. *Eur J Cancer,* 49 (16): 3431-3441, 2013. DOI: 10.1016/j.ejca.2013.06.016

14. Tangen IL, Werner HM, Berg A, Halle MK, Kusonmano K, Trovik J, Hoivik EA, Mills GB, Krakstad C, Salvesen HB: Loss of progesterone receptor links to high proliferation and increases from primary to metastatic endometrial cancer lesions. *Eur J Cancer,* 50 (17): 3003-3010, 2014. DOI: 10.1016/j.ejca.2014.09.003

15. Gil da Costa RM, Santos M, Amorim I, Lopes C, Pereira PD, Faustino AM: An immunohistochemical study of feline endometrial adenocarcinoma. *J Comp Pathol,* 140 (4): 254-259, 2009. DOI: 10.1016/j.jcpa.2008.12.006

16. Saraiva AL, Payan-Carreira R, Gärtner F, Fortuna da Cunha MR, Rêma A, Faria F, Lourenço LM, Pires Mdos A: An immunohistochemical study on the expression of sex steroid receptors, Ki-67 and cytokeratins 7 and 20 in feline endometrial adenocarcinomas. *BMC Vet Res,* 11, 204, 2015. DOI: 10.1186/s12917-015-0530-6

17. Leslie KK, Thiel KW, Reyes HD, Yang S, Zhang Y, Carlson MJ, Kumar NS, Dai DD: The estrogen receptor joins other cancer biomarkers as a predictor of outcome. *Obstet Gynecol Int,* 2013:479541, 2013. DOI: 10.1155/2013/479541

18. Hayashi SI, Eguchi H, Tanimoto K, Yoshida T, Omoto Y, Inoue A, Yoshida N, Yamaguchi Y: The expression and function of estrogen receptor alpha and beta in human breast cancer and its clinical application. *Endocr Relat Cancer,* 10 (2): 193-202, 2003. DOI: 10.1677/erc.0.0100193

19. Morani A, Warner M, Gustafsson JA: Biological functions and clinical implications of oestrogen receptors alfa and beta in epithelial tissues. *J Intern Med,* 264 (2): 128-142, 2008. DOI: 10.1111/j.1365-2796.2008.01976.x

20. Denker HW, Eng LA, Hamner CE: Studies on the early development and implantation in the cat. II. Implantation: proteinases. *Anat Embryol (Berl),* 154 (1): 39-54, 1978. DOI: 10.1007/BF00317953

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 299-303, 2023
DOI: 10.9775/kvfd.2023.29100

Case Report

Recurrence of Solitary Spinal Meningothelial Meningioma in a Dog

İlayda PAZARBAŞILAR [1,2] Ömer BEŞALTI [1 (*)]

[1] Ankara University, Faculty of Veterinary Medicine, Surgery Department, TR-06110 Ankara - TÜRKİYE
[2] Ankara University, Graduate School of Health Sciences, TR-06110 Ankara - TÜRKİYE

ORCIDs: İ.P. 0000-0002-4131-8440; Ö.B. 0000-0002-7819-9094

Article ID: KVFD-2023-29100 Received: 27.01.2023 Accepted: 22.04.2023 Published Online: 24.04.2023

Abstract: This report identifies clinical, Magnetic Resonance Imaging (MRI), surgical and histological findings of recurrent solitary spinal meningothelial meningioma in a dog. Five-year-old female Pomeranian dog with a history of severe cervicalgia was subjected. A solitary mass between C3-C4 vertebras were diagnosed by MRI, and surgery was carried out with successful results; however, clinical signs reappeared one year after surgery. Control MRI revealed the recurrence of the mass at the same location. Patient reoperated and it was histopathologically reconfirmed as meningothelial meningioma. In conclusion, reoperation of the recurrence of spinal meningioma can be suggested for the practitioners.

Keywords: *Dog, Meningioma, Recurrence, Spinal tumor*

Bir Köpekte Nüks Eden Soliter Spinal Meningothelial Meningioma

Öz: Bu rapor bir köpekte nüks eden soliter spinal meningothelial meningiomanın klinik, manyetik rezosans görüntüleme (MRG) ve histopatolojik sonuçlarını bildirmektedir. Şiddetli servikalji ile kliniğe getirilen 5 yaşlı dişi Pomerian ırkı köpekte, C3-C4 vertebraları arasında solid yapılı bir kitle MRG ile tespit edildi ve operatif mudahale ile başarılı sonuç alındı. Ancak 1 yıl sonra klinik bulguların tekrar ortaya çıkmasıyla birlikte çekilen kontrol MRG'sinde kitlenin nüks ettiği görüldü. Hasta tekrar operasyona alındı ve alınan kitlenin histopatolojik incelemesinde ilk sonuçla aynı olan meningothelial meningioma tanısı kondu. Sonuç olarak, nüks eden spinal meningioma hastalarına operasyon yapılması pretisyen hekimler için önerilir bulundu.

Anahtar sözcükler: *Köpek, Meningiom, Nüks, Spinal tümör*

Introduction

Spinal meningiomas can be classified as encapsulated like tumors according to their well-defined shape. They constitute between 55-65% of spinal tumors [1]. MRI is the method of choice in diagnosing of meningiomas because the scenery of the tumor is typical which has a well-defined shape and is located intradural-extramedullary or extradurally. They often have a broad-based attachment to the duramater [2,3]. Tumor appears as iso-intense or hypointense in T1W images and mildly hyperintense in T2W images. In addition to that, it has a homogeneous contrast enhancement [4]. However, histopathologic examination is required for definitive diagnosis.

Optimal treatment for spinal meningioma is surgery. Other treatment options for spinal meningioma are chemotherapy, radiotherapy or different combinations of them with surgery. The combined treatments of surgery and radiotherapy can significantly extend the life expectancy and decrease the rate of recurrence [5].

The aim of representing this report was to evaluate the clinical symptoms, advanced diagnostic work up, surgical management and histopathologic characteristics of cervical meningioma in a dog that faced a recurrence one year after surgery, and to determine the successful reoperation for the second time with satisfactory improvement.

Case History

Five-year-old Pomeranian dog was admitted to Ankara University Faculty of Veterinary Medicine Department

How to cite this article?
Pazarbaşılar İ, Beşaltı Ö: Recurrence of solitary spinal meningothelial meningioma in a dog. *Kafkas Univ Vet Fak Derg,* 29 (3): 299-303, 2023.
DOI: 10.9775/kvfd.2023.29100

(*) Corresponding author: Ömer BEŞALTI
Phone: +90 312 317 0315 Cellular phone: +90 535 284 0546
E-mail: besalti@hotmail.com

of Surgery with a history of severe pain in cervical spine and she had a certain loss of appetite. An "informed consent form" involving the whole process was obtained in the presented case. In neurologic examination, the dog was mentally alert and had normal cranial and spinal reflexes. All limbs had intact deep pain perception. Serum biochemistry and cell blood count were checked and they were all in reference. Spinal radiographs were normal.

MRI was carried out with 3 Tesla (Siemens). Anesthesia was induced by butorphanol (0.1 mg/kg) and diazepam (0.5 mg/kg) and it was maintained by Total Intravenous Anesthesia (TIVA) consisted of propofol with a dosage of 0.3 mg/kg/min. Images acquired as sagittal, dorsal and transverse views in T1W, T2W and post contrast T1W (Gadolinium Dimeglumine was used at dose of 0.1 mmol/kg body weight (BW) intravenously). The lesion was observed as a focal mass. It looked hypointense in T1W and isointense to hyperintense in T2W images. Lesion was located at the right side adjacent to the dural margin. Post contrast T1W image revealed a broad-based attachment to the duramater located both extra and intradurally in C3-C4 region *(Fig. 1)*. The images mimicked nerve sheath tumor.

In preoperative period, cefazolin (Equizolin 500 mg/2 mL) was used at dose of 20 mg/kg intravenously for prophylaxy. Butorphenol (0.1 mg/kg) was applied subcutaneously in order to apply preemptive analgesia. Intravenously diazepam (0.5 mg/kg) was used (Diazem 10 mg/2 mL) for premedication. Propofol, with a dosage of 3 mg/kg, was used to make induction of anesthesia and the dog intubated orolaryngeally. The anesthesia was maintained by isoflurane. Constant Rate Infusion (CRI) of ketamine at a dosage of 0.3 mg/kg/h with the speed rate of 5 mL/kg/h was applied for perioperative analgesia.

The dog was positioned in sternal recumbency with the head gently flexed in a neutral position, and operation table was slightly tilted to the left side. Midline incision was made over the C2-C5 spinous processes. Paraspinal muscles were separated by subperiosteal dissection. Hemilaminectomy was performed at C3-C4 intervertebral foramen by surgical burr and hemilaminectomy defect was extended both cranially and caudally based on the size of the mass. A pale yellowish tumor along the nerve roots and invading intradurally was revealed by durotomy. The mass including root was removed by microdissection and aspiration under the operation microscope blood vessels of the meninges were obscured by electrocoagulation. Durotomy defect was not sutured. Fat graft, which was harvested subcutaneously, was placed in hemilaminectomy defect.

In the post-operative period gabapentin (Neruda 250 mg/5 mL suspension) with a dose of 10 mg/kg was administered in every 12 h PO for two weeks after the surgery. Prednisolone at a dose of 0.5 mg/kg, PO, BID for two weeks and amoxicillin and clavulonic acid was used (Augmentin 625 mg) at dosage of 25 mg/kg PO, BID for one week after the surgery. Ten days after operation patient was clinically normal and was followed by telephone communications in certain periods.

The retrieved mass was examined histopathologically *(Fig. 2)*. Uniform meningothelial cells with large, round/oval, normochromatic basophilic nuclei were seen. In some regions, fibrous bundles were identified, as well. These cells tend to come together as lines or small clusters in which the cytoplasmic borders could not be distinguished. Microcalsification was observed in some areas. In addition to that, psammoma bodies were observed. Furthermore, there exists an ascending rate of mitotic activity of the cells without necrosis. The results were at the same line with meningothelial meningioma which was classified as grade 1 tumor of meningioma.

Twelve months after surgery, the similar clinical signs reappeared. In addition to cervicalgia, incoordination and ataxia was observed in the gait analysis. MRI was taken for the second time with the same anesthesia protocol, accordingly *(Fig. 3)*. The tumor became larger than the

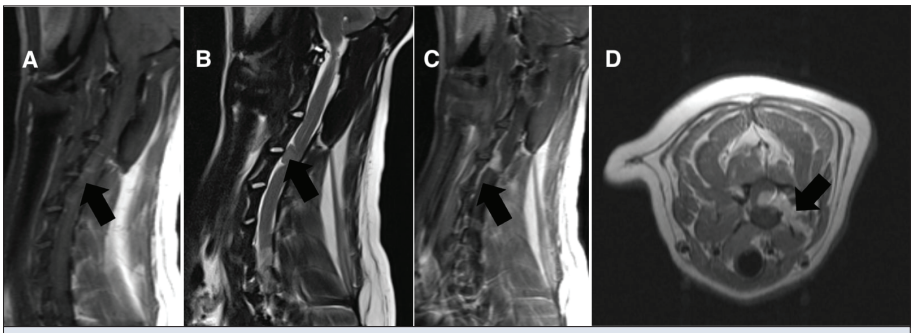

Fig 1. T1W, T2W and post contrast T1W sagittal and transversal images of the patient. **A:** The T1W sagittal image indicates the hypointense lesion at C3-C4 *(black arrow)*, **B:** T2 W sagittal image shows the iso-to-hyperintense lesion *(black arrow)*, **C-D:** shows sagittal and transversal postcontrast T1W images, respectively. The dural tail sign can be seen (black arrow) in the sagittal image

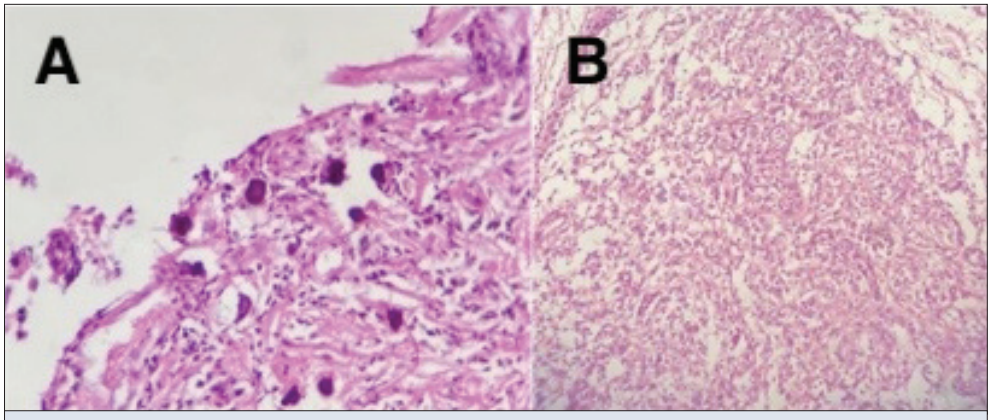

Fig 2. The histopathologic examination of the mass that was retrieved at C3-C4. **A:** Psammoma bodies and fibrous bundles were observed, **B:** Increased mitotic activity of the cells is shown and there does not exist any necrotic area

beginning which was filling nearly about 2/3 diameter of spinal canal. The lesion was hypointense in both T1W and T2W images; however, in T2W image the circumference of the lesion was hyperintense, and well contrast enhanced in postcontrast T1W images.

Surgical intervention was carried out with the same protocol. The difference from the first surgical findings was swelling and a purplish color change in the duramater. Based on the differences, hemilaminectomy defect was enlarged, the mass was revealed in greyish color, well demarcated and it pushed the spinal cord to left side. The mass was removed by microdissection and aspiration. The dural attachment was extensively coagulated. The dural defect was not repaired for the possibility of tumor recurrence. Operation wound was closed with the same procedure as the first operation. In the post-operative period, the same pharmacy was used in order to make pain management and prophylaxis for infection. The patient's neurological status was indistinguishable from before the operation at the 2nd day; however, it was deteriorated from second month to the fourth by the time. The owner declined further diagnostic workup and preffered euthanasia, accordingly.

Discussion

Surgery is the optimal treatment option for solid spinal meningiomas, although there is limited data for reoperation of meningioma after recurrence[6,7]. In human studies, the residual volume of meningioma after the operation considered as an important cause of recurrence. Thus, gross total resection is the primary goal of surgeons, however retrieving tumor is carry a high risk of neural damage. In the presented case, the authors did not have information about the residual volume because of absence postoperative control MRI, but it was estimated that there was no residual tumor in both interventions.

MRI provides evaluation of the tumors with their characteristic features to reach presumptive diagnosis. The reported case introduces a meningioma which mimics nerve root tumor. The shape and location of the mass led us to consider nerve sheath tumor rather than meningioma in MRI. In generally, meningiomas do not spread through the intervertebral foramen and they are located as intradural-extramedulary as a solid mass and causing root compression in some cases [6]. However, in this case report meningioma protrudes through intervertebral foramen which is an important finding and

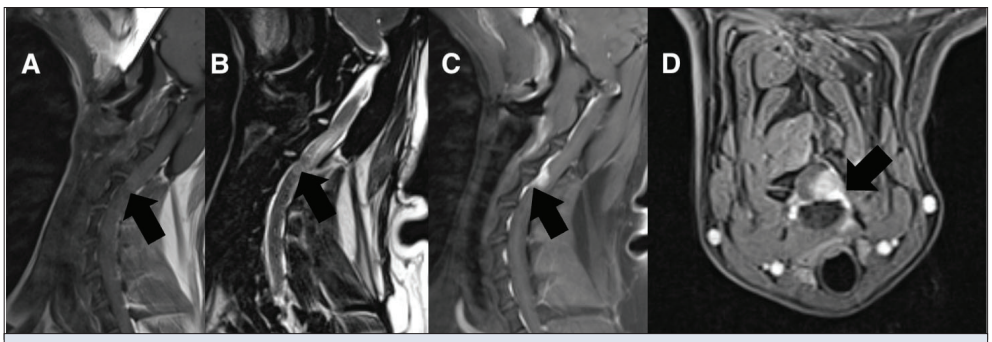

Fig 3. Sagittal T1 and T2 W images as well as sagittal and transversal postcontrast T1W images of the same patient one year after surgery with the recurrence signs. **A:** T1W sagittal image indicates the iso - hypointense lesion, **B:** Indicates the iso-hyperintense lesion in the T2W sagittal image, **C-D:** Well contrast enhanced mass in both sagittal and transversal images, **D:** It relocated intradural- extramedullary in transversal image

it highlights the uniqueness of the study. In accordance with these findings, histopathologic examination should be taken into account so as to come up with the definitive diagnosis. These unusual data should be considered by the practitioners.

Reoperation for spinal disorders carry heavy risk of surgical morbidity. As for the presented case, fibrous tissue was filled and adherent to the free fat graft. It was gently removed. The tumor was appeared as darker color and almost totally located intradurally. It was retrieved under the surgical microscope. In this case, after the second surgical intervention, recovery of the neurologic status of the patient was fast enough like the first surgery. However, the reason of faster deterioration of the neurological status of the case after second surgery compared with the first, could not be clarified because of not having further diagnostic work-up.

In general, histologically benign tumors prove to be non-recurring and non-metastasizing lesions; furthermore, histologically malignant tumors usually behave aggressively. In human grade I meningioma, surgery is the method of choice for the treatment and results are satisfactory and postoperative recurrence is low which is found approximately 3-20% [7]. Thus, for veterinary medicine, it is known that the observation rate of recurrence in grade 2 and grade 3 subtypes are higher than grade 1 subtype as human being. However, the natural history of recurrences of benign meningiomas in veterinary medicine have been described that there exists no specific rate of recurrence for grade 1 meningiomas [2,7-10]. In the presented case with grade 1 meningioma (WHO classification), recurrence was seen one year later, and required a reoperation. This illustrates that tumor regrowth is possible after gross total resection and careful follow-up is important for the recurrence.

Several important histologic variants of meningioma, and even a histopathologically typical meningioma can have unusual radiological features so less frequent and uncharacteristic imaging features should be known so as to suggest the correct pre-operative diagnosis in clinical cases. [11,12]. In this case, tumor has characteristic image which mimicked the nerve sheat tumor. After hemilaminectomy, the mass was seen alongside the nerve roots and adherent to the duramater. The appearance and location of the tumor was at the same line with MRI findings and consistent with nerve sheath tumor. In addition, after durotomy the part of intradural mass was also mixed with rootlets. In contrast, histological examination revealed type 1 meningothelial meningioma rather than nerve sheath tumor. This illustrates the uncharacteristic MRI images for the solitary meningothelial meningioma, and this should be taken into account in the pre-operative period [11].

In conclusion, further studies are needed for determining the rate of recurrence according to the tumor subtypes of meningiomas and for outcome of reoperation. The control MRI and detailed histological evaluation of the tumor are essential for the follow up of the cases. With respect to the experience that was gained from the presented case, reoperation of meningioma can be considered for the practitioners, should it be possible for them to evaluate and follow-up the case more in detail.

Availability of Data and Materials

The data, that support the findings of this study, are available from the corresponding author (O. Besalti) upon reasonable request.

Acknowledgements

For the contribution in obtaining and interpreting the pathology findings of this study, we thank to Gören Pathology Lab.

Competing Interests

The authors declared there is no conflict of interest

Author Contributions

Case examination, evaluation of clinical findings and interpretation of MRI images were done by authors and the article was written, accordingly. Authors submitted the article together.

References

1. Besalti O, Caliskan M, Can P, Vural SA, Algin O, Ahlat O: Imaging and surgical outcomes of spinal tumors in 18 dogs and one cat. *J Vet Sci,* 17 (2): 225-234, 2016. DOI: 10.4142/jvs.2016.17.2.225

2. Montoliu P, Anor S, Vidal E, Pulmarola M: Histological and immunohistochemical study of 30 cases of canine meningioma. *J Comp Pathol*, 135 (4): 200-207, 2006. DOI: 10.1016/j.jcpa.2006.06.006

3. Senturk S, Guzel A, Guzel E, Bayrak AH, Sav A: cervical spinal meningioma mimicking intramedullary spinal tumor. *Spine*, 34 (1): E45-E49, 2009. DOI: 10.1097/BRS.0b013e318189fd20

4. Petersem SA, Sturges BK, Dickinson PJ, Pollard RE, Kass PH, Kent M, Vernau KM, Lecouteur RA, Higgins RJ: Canine intraspinal meningiomas: imaging features, histopathologic classification, and long-term outcome in 34 dogs. *J Vet Intern Med*, 22 (4): 946-953, 2008. DOI: 10.1111/j.1939-1676.2008.0106.x

5. Kippenes H, Gavin PR, Bagley RS, Silver GM, Tucker RL, Sande RD: Magnetic resonance imaging features of tumors of the spine and spinal cord in dogs. *Vet Radiol Ultrasound*, 40 (6): 627-633, 1999. DOI: 10.1111/j.1740-8261.1999.tb00890.x

6. Lacassagne K, Hearon K, Berg J, Seguin B, Hoyt L, Byer B, Selmic LE: Canine spinal meningiomas and nerve sheath tumours in 34 dogs (2008-2016): Distribution and long-term outcome based upon histopathology and treatment modality. *Vet Comp Oncol*, 16 (3): 344-351, 2018. DOI: 10.1111/vco.12385

7. Benes V, Margoldova M, Bradac O, Skalicky P, Vlach D: Meningiomas in dogs. *Surg Neurol Int*, 8 (12): 551-562, 2021. DOI: 10.25259/SNI_675_2021

8. Hortobagyi T, Bencze J, Varkoly G, Kouhsari MC, Klekner A: Meningioma recurrence. *Open Med (Wars)*, 11 (1): 168-173, 2016. DOI: 10.1515/med-2016-0032

9. Viloris K, Katsarides V, Sakellariou P: The recurrence rate in meningiomas: analysis of tumor location, histological grading and extent of resection. *OJMN*, 2 (1): 6-10, 2012. DOI: 10.4236/ojmn.2012.21002

10. Forward AK, Volk HA, Cherubini GB, Harcourt-Brown T, Plessas IN, Garosi L, Decker SD: Clinical presentation, diagnostic findings and outcome

of dogs undergoing surgical resection for intracranial meningioma: 101 dogs. *BMC Vet Res*, 18 (1): 88-98, 2022. DOI: 10.1186/s12917-022-03182-y

11. Kobayashi K, Imagama S, Ito Z, Ando K, Ukai J, Muramoto A, Shinjo R, Matsumoto T, Nakashima H, Matsuyama Y, Ishiguro N: Recurrence of solitary fibrous tumor of the cervical spinal cord. *Nagoya J Med Sci,* 75 (1-2): 217-223, 2014.

12. Ober CA, Chai O, Milgram J, Peştean CP, Danciu C, Soare T, Oana LI, Taulescu M: Meningioma in cervical spinal cord segment 6 of a dog - A case report. *Acta Vet Brno*, 87 (3): 225-229, 2018. DOI: 10.2754/avb201887030225

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 305-309, 2023
DOI: 10.9775/kvfd.2023.29127

Case Report

Progressive Ocular Histiocytosis in a Cat

Aynur DEMİR [1 (*)] Zeynep Nilüfer AKÇASIZ [1] Özge ERDOĞAN BAMAÇ [2]

[1] Istanbul University-Cerrahpaşa, Faculty of Veterinary, Surgery Department, TR-34315 İstanbul - TÜRKİYE
[2] Istanbul University-Cerrahpaşa, Faculty of Veterinary, Pathology Department, TR-34555 İstanbul - TÜRKİYE

ORCIDs: A.D. 0000-0002-5471-1655; Z.N.A. 0000-0003-4857-8891; Ö.E.B. 0000-0002-0352-4841

Article ID: KVFD-2023-29127 Received: 31.01.2023 Accepted: 31.03.2023 Published Online: 09.04.2023

Abstract: Feline progressive histiocytosis is a rare proliferative disorder manifested by solitary or multiple cutaneous nodules and papules with potential late distant metastasis. This case study presents clinical, diagnostic imaging, and histopathologic findings and therapeutic approach to progressive ocular histiocytosis detected in a 1.5-year-old male Domestic Shorthair cat with a complaint of a painless firm mass on the left lower eyelid. Magnetic resonance imaging (MRI) of the orbit revealed a tumoral lesion expanding from the left lower eyelid into the intraorbital space, translocating the bulbus from ventrolateral to dorsomedial. The computed tomography (CT) visualized a destructive lesion on the infraorbital edge, which was histopathologically confirmed to be progressive nodular histiocytosis.

Keywords: *Cat, Eye, Computed tomography, Magnetic resonance imaging, Progressive histiocytosis*

Bir Kedide Progresif Oküler Histiyositozis

Öz: Feline progresif histiyositozis, olası geç dönem uzak metastazlı soliter veya çoklu kutanöz nodüller ve papüllerle kendini gösteren nadir bir proliferatif hastalıktır. Bu olgu sunumunda sol alt göz kapağında ağrısız sert kitle şikayeti ile başvuran 1.5 yaşında erkek evcil kısa tüylü bir kedide saptanan ilerleyici oküler histiyositozun klinik, tanısal görüntüleme, histopatolojik bulgular ve tedavi yaklaşımı sunulmaktadır. Orbitanın manyetik rezonans görüntülemesinde (MRG), sol alt göz kapağından intraorbital boşluğa doğru genişleyen ve bulbusu ventrolateralden dorsomedial'e kaydıran tümöral bir lezyon görüldü. Bilgisayarlı tomografi (BT), infraorbital kenarda histopatolojik olarak progresif nodüler histiyositoz olduğu doğrulanan yıkıcı bir lezyonu görüntüledi.

Anahtar sözcükler: *Bilgisayarlı tomografi, Göz, Kedi, Manyetik rezonans görüntüleme, Progresif histiyositozis*

Introduction

Feline progressive histiocytosis (FPH) is a rare disorder, with limited reports in horses, cattle, dogs, and humans [1,2]. The incidence of the disorder in cats is unknown due to the differential diagnostic challenge. Histiocytic neoplasms are classified as focal and self-restricting (cutaneous histiocytoma), locally aggressive (localized histiocytic sarcoma), and multisystemic lesions (disseminated histiocytic sarcoma) [1,2]. No breed, age, and gender predisposition has been noted [3].

The lesions emerge as solitary or multifocal painless, alopecic, or ulcerated cutaneous nodules on the head, neck, and distal extremities and can be easily confused with other allergic, infectious, and neoplastic types of skin disorders [2-4] . Histiocytosis may show a spontaneous regression in dog [5], on the contrary, it can progress into a malignant histiocytic sarcoma-like neoplasm, with lymph node, liver, spleen, kidney, lung, and even bone marrow metastasis in cat [2,3,6]. It is usually a fatal disorder with the lack of efficient therapeutic options.

This report presents clinical, imaging, and histopathological findings and surgical treatment, and its postoperative outcome of an infrequently encountered diffuse progressive histiocytosis case in a male Domestic Shorthair cat expanding from the lower eyelid skin of the left eye into the soft tissues of the intraorbital cavity.

Case History

A veterinary practitioner submitted a 1.5-year-old neutered male Domestic Shorthair to the Istanbul University-

How to cite this article?
Demir A, Akçasız ZN, Erdoğan Bamaç Ö: *Progressive ocular histiocytosis in a cat. Kafkas Univ Vet Fak Derg,* 29 (3): 305-309, 2023.
DOI: 10.9775/kvfd.2023.29127

(*) Corresponding author: Aynur DEMIR
Phone: +90 506 755 0873
E-mail: aynur.demir@istanbul.edu.tr

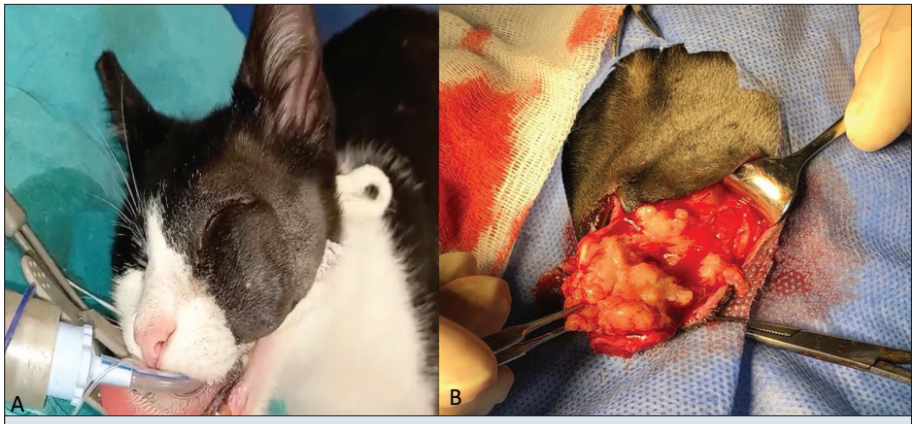

Fig 1. A 1.5 years old Domestic Shorthair cat. **A**- with photophobia and retrobulbar swelling. **B**- This swelling appeared to be a large solid mass attached tightly to the bulbar surface of the left lower eyelid

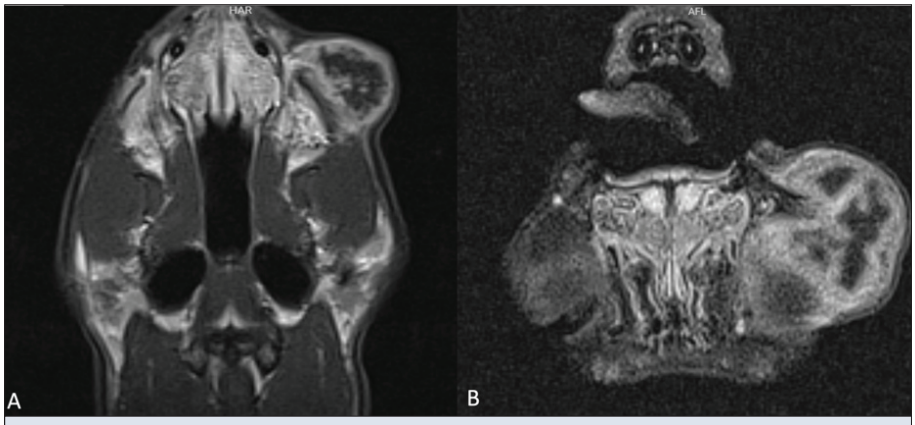

Fig 2. Preoperative magnetic resonance image of progressive histiocytosis. **A**- Coronal T2-weighted image. Image of the mass with heterogeneous hyperintense signals, **B**- Axial T1-weighted contrast image. A nodular lesion with central necrotic hypointense and partial peripheral contrast enhancement.

Cerrahpaşa, Veterinary Teaching and Research Hospital for further evaluation of a fast-growing mass on the left lower eyelid. In the relevant case, an "informed consent form" containing the entire process was obtained. The patient initially received for a week systemic antibiotics and anti-inflammatory drugs with no recovery.

Physical examination revealed no abnormailty in the patient's general health status. However, a round-shaped solid tumoral mass with an approximate diameter of 3 cm was detected on the lower eyelid of the left eye, attached firmly to the underlying zygomatic arch. Conjunctival congestion, hyperemia, and partial dorsomedial eyeball deviation were noted. The affected eye resisted retropulsion, and the protruding third eyelid could not be stretched onto the ocular surface *(Fig.1-A)*. No intraocular lesion was detected. Menace response, pupillary light, and dazzle reflexes were normal. Right (15 mm/min) and left (7 mm/min) eyes Schirmer's tear test and right (22 mmHg) and left (24 mmHg) intraocular pressure values were within reference range, of 11-23 mm/min and 15-25 mmHg, respectively. The left submandibular lymph node was mildly enlarged. Detailed blood testing exhibited no abnormal values, and thoracal and abdominal radiography revealed no evidence of metastasis.

Orbital magnetic resonance imaging (MRI) revealed an exophytic, infiltrative mass with heterogeneous contrast expanding from the subcutaneous space of the left lower eyelid into the intraorbital cavity with conjunctival and muscle involvement. Orbital computed tomography (CT) confirmed destructive lesions on the base of the orbit.

MRI of the brain and orbit was performed by a 1.5 T MRI unit. T1W FLAIR images were obtained after intravenous injection of gadopentetate dimeglumine (0.1 mmol/kg). Sequences of transverse, sagittal, and coronal planes revealed a maximum of 2.6x2.6x2.2cm-sized ventrolaterally located mass with a subcutaneous fatty content on the caudal aspect of the left eye. T2-weighted images showed a mass lesion with heterogeneous hyperintense signals, and T1-weighted contrast images revealed a centrally hypointense nodular lesion with partial peripheral contrast enhancement, particularly indicating a potential malignant mass of aggressive behavior with central necrosis. Fatty planes between the defined lesion and the orbit were undistinguished *(Fig. 2-A,B)*.

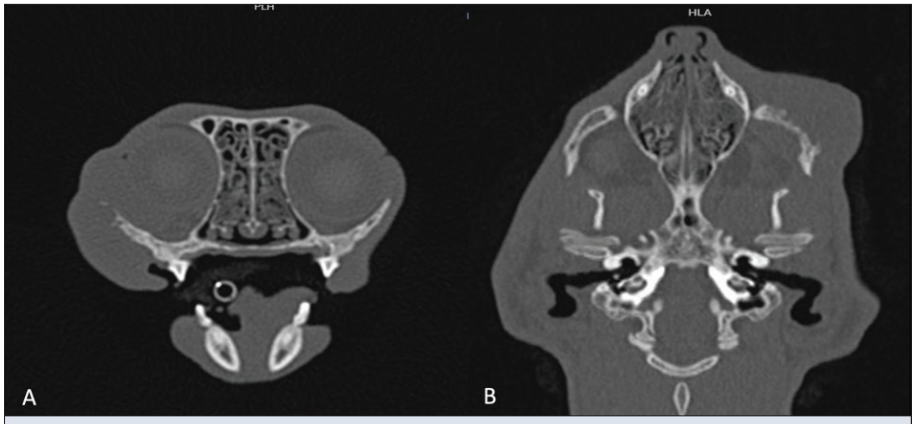

Fig 3. Preoperative computed tomography images. **A**- Ventrolateral appearance of the mass in the axial plane, **B**- Partial destruction of the left orbital bone in the coronal plane

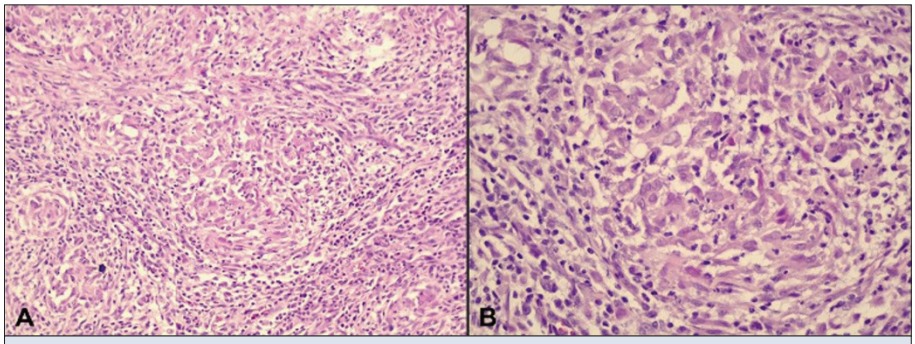

Fig 4. Histopathological findings. **A**- Subcutaneous densely cellular histiocytic nodule with inflammatory cells (H&E, original magnification x200), **B**- Atypical cells with round to polyglonal cell borders, lightly eosinophilic cytoplasm and irregular vesicular nuclei with marginated chromatin. Moderate anisocytosis and anisokaryosis are remarkable (H&E, original magnification x400)

CT imaging was performed by the Siemens SOMATOM Scope VB30 (0.6 mm, 110 Kv, 28 mA with an exposure time of 14 sec). The scans demonstrated a mass of approximately 2.6x2.6x2.2 cm ventro-laterally located within the subcutaneous fat plane on the caudal aspect of the left eye, and partial destruction of the orbital bone was noted *(Fig. 3-A,B)*.

Immediately after MR and CT imagines and while the patient was still under general anesthesia, the mass with extraocular soft tissue and bone involvements along with bulbus oculi were surgically removed. The tumoral lesion on the left lower eyelid was excised through a linear incision on the skin, and then orbital exenteration was performed *(Fig.1-B)*. Honeycomb-like lesions on the inferior wall of the bony orbit were removed by a 16 cm rongeur until macroscopically intact tissue was achieved, and the resected tissues were then submitted in 10% neutral buffered formalin for histopathology. After routine histopathological preparation and sectioning procedures, 4 µm thick sliced samples were stained with hematoxylin and eosin (H&E) and examined by a light microscope. The histologic examination of the tumor revealed multinodular histiocytic infiltrates composed of round to polygonal cells with a light eosinophilic cytoplasm and irregular vesicular nuclei. Moderate anisocytosis and anisokaryosis were observed, and a reactive inflammatory response was prominent *(Fig. 4-A,B)*. After ocular enucleation, a drain was placed in the medial canthus. Following full anesthetic recovery, the patient was discharged prescribing ceftriaxone (50 mg/kg SID IM), and meloxicam (0.01 mg/kg SID SC) for one week. An Elizabethan collar was recommended for two weeks. The drain and the sutures were removed on the fourth and twelfth postoperative day, respectively, and periodic follow-ups were scheduled to evaluate potential recurrence and infection. The owner declined postoperative radiotherapy and chemotherapy. During five months follow-up period no health complaint was noted.

Discussion

This is the first comprehensive case study to present clinical findings, including diagnostic imaging scans (MRI and CT), histopathological features, and a therapeutic approach to feline progressive ocular histiocytosis among few previously reported cases in cats [4,6]. This is also the first and only case in a cat where ocular tissues were affected, and advanced imaging techniques were utilized for clinical diagnosis. Therefore, this report is

considered to provide a better understanding of the lesion and contribute to the literature. A comparative evaluation of imaging scans of its human counterpart suggests the potential efficacy of these techniques in feline practice.

Progressive histiocytosis is usually encountered in young dogs [7], whereas in cats it typically affects middle-aged to older cats (Age range=7-17) [2]. Despite the lack of known breed and gender predisposition, females are slightly more prone to develop the disorder [8]. In a study by Costa et al.[8], 21 of 26 cases were represented by approximately 9.3-year-old Domestic Shorthair cats. FPH is initially manifested by well-circumscribed solitary skin nodules that may extend to 4 cm in diameter [9]. Nevertheless, it is usually smaller than 2 cm, showing progressive growth as multiple papules, nodules, and plaques [2]. Skin lesions are most likely to have developed in different species diagnosed with progressive histiocytosis; however, there are only few reported cases of FPH, particularly with ocular involvement [1]. Unlike the previously documented cases, progressive histiocytosis occurred in a young (1.5 years of age) male cat, developing as a primary ocular mass showing rapid growth in two months in the presented report. Moreover, the lower eyelid skin was affected, including the intraorbital soft tissues with inferior orbital wall involvement. The solid lesion was more extensive than the previously reported cases and showed exophytic growth, compressing the bulbus oculi on the orbit's caudal portion.

Clinical findings in FPH differ due to the affected organ systems. Yaygıngül et al.[10] reported that unilateral exophthalmos, nictitating membrane protrusion, secondary conjunctival hyperemia, keratitis, strabismus, dysphagia, blindness, glaucoma, retinoschisis, circulatory alterations or edema, changes in pupillary light or corneal reflexes were detected in a calf due to progressive paraorbital histiocytosis. In our case restricted ocular and lower eyelid movements of the left eye due to the lesion's localization and tumoral infiltration of the extraocular muscles were determined. Decreased palpebral reflex resulting in lagophthalmos, exophthalmos, protruded nictitating membrane, conjunctival hyperemia, the eyeball's partial dorsomedial deviation, and partial ventral resistance to retropulsion were also noted.

Histological examinations of tumor masses often reveal proliferation of pleomorphic histiocytes with marked hemophagocytosis [2]. It is well known that FPH and feline histiocytic sarcoma (FHS) have common histologic features [2,7,8]. Especially, the morphological features of the advance form of FPH resemble of those of FHS [2]. In our case, the round to polygonal cells that constituted the multinodular histiocytic infiltrates with moderate anisocytosis and anisokaryosis and the reactive inflammatory response was compatible with the histologic findings of the previous reports of FPH [2,7]. Also, absence of high mitotic index and numerous multinucleated giant cells with bizarre mitotic figures differentiated the histologic findings of our case from those of FHS [8].

Advanced imaging techniques (MRI and CT scan) are utilized to identify the depth and extent of the ocular and orbital lesions aiding in differential clinical diagnosis, which facilities the most efficient therapeutic protocol, and also to monitor the prognosis of the defined disorder [9]. Despite the valuable diagnostic and surgical guidance of CT scan and MRI [11], Park et al.[12] suggested that both imaging techniques might fail to make a specific radiological diagnosis in malignant fibrous histiocytoma (MFH) cases due to the poor or inconsistent signaling. In CT scans, MFH usually appeared to be a large multilobulated soft-tissue mass isoattenuating to muscle due to necrosis, hemorrhage, or myxoid material at the center, and calcification or ossification could be imaged with relatively poor preciseness like 5-20%. In MRI, MFH is typically visualized as isoattenuating mass to muscle tissue in T1-weighted scans and heterogenous hyperintense mass in T2-weighted sequences. Khan and Sepadhari [9] reported that a CT scan of the brain demonstrated osteolytic lesions in the right frontal and zygomatic bones in a patient with ocular histiocytosis, and MRI revealed a heterogenous exophytic infiltrative mass extending from the intraorbital cavity to the subcutaneous space. In the presented case, we obtained similar data concerning the radiographic imaging of the lesion. The closeness of the mass to the orbit necessitated MRI for clinical diagnosis. MRI of the patient's orbit and periocular areas revealed a left lower eyelid-originating mass near the left medial-inferior orbital wall, extending into the left orbital cavity and intraorbital soft tissues, pushing out the eye bulb from ventrolateral to dorsomedial. The indistinct moth-eaten appearance of the medial-inferior orbital wall suggestive of lytic areas was confirmed as the destruction of the orbital bone on CT scans.

While localized eyelid lesions are successfully treated by excisional biopsy, surgical management of FPH varies. Kim et al.[5] suggested that the primary therapeutic approach to canine cutaneous histiocytomas is surgical excision, and local recurrence or occurrence of a new lesion on the other body parts is highly unlikely. Coste et al.[8] showed that a wide local excision with clear surgical margins generated favorable outcomes in seven cats with FPH located on different body regions other than the orbit. On the contrary, in a study by Affolter and Moore [2], local recurrence was noted in four out of 8 cats with a solitary mass or limited cutaneous lesions with a few nodules that underwent surgical excision and new tumoral lesions developed in other body parts in all surgically treated cats. The results of the presented case are

compatible with the outcomes of some of the previously mentioned therapeutic approaches. Our treatment protocol comprised exenteration of the affected eye and partial ostectomy of the orbital floor. Although the mean survival time for progressive ocular histiocytosis in cats documented only in few case studies is unknown, survival for FPH encountered in different body sites other than the orbit was shown to be 96 days (range=41-238 days). In another study, the mean survival time was reported to be 13.5 months [13]. Neither complications nor metastases were detected in the presented case study in which we evaluated an ocular form of FPH in a 5-month-follow up period. Therefore, a radical surgical removal in FPH with ocular involvement might be a good treatment choice like its human counterpart.

In conclusion, progressive histiocytosis, is very rare in cats, usually occurs as a skin lesion and sometimes self-limited and regressed, while it can become very aggressive and metastasize to internal organs. For this reason, early and differential diagnosis of the disease is very important for the prognosis of the patient. This case report showed that FPH can also occur in the paraorbital region and orbit. In addition, it is aimed to emphasize here the diagnostic value of cross-sectional imaging and the efficacy of surgical excision and curettage in the treatment of ocular lesion.

Availability of Data and Materials

The authors declare that the data supporting the study findings are also presented to the corresponding author (A. Demir).

Acknowledgements

The authors thank the owner of the animal and the surgical team who assisted in the conduct of this study.

Competing Interest

The authors declared no conflict of interest regarding this report.

Author's Contributions

The authors have equally contributed to the preparation of this manuscript. AD and NA designed and supervised this study. AD, NA, and OEB collected and analyzed data. OEB wrote the pathological examination section, AD and NA wrote the first draft of the rest of the text. All authors contributed to the critical revision of the article and read and approved the final version.

References

1. Hülskötter K, Pfankuche VM, Rocksin A, Junginger J, Lei WKJ, Vries E, Kruppa J, Baumgartner W, Wohlsein P: Feline progressive histiocytosis - A case report with a review of the literature. *WTM,* 105 (1/2): 41-47, 2018.

2. Affolter VK, Moore PF: Feline progressive histiocytosis. *Vet Pathol,* 43 (5): 646-655, 2006.

3. Treggiari E, Ressel L, Polton GA, Benoit J, Desmas I, Blackwood L: Clinical outcome, PDGFRβ and KIT expression in feline histiocytic disorders: A multicentre study. *Vet Comp Oncol,* 15 (1): 65-77, 2017. DOI: 10.1111/vco.12142

4. Trost ME, Ramos AT, Masuda EK, dos Anjos BL, Cunha MGMCM, Graça DL: Malignant histiocytosis in a cat. Case report. *Braz J Vet Pathol,* 1 (1): 32-35, 2008.

5. Kim B, Lim J, Shim JH, Seo K, Kang S: Spontaneous regression of eyelid histiocytoma in a Maltese dog. *J Vet Clin,* 39 (3): 121-125, 2022.DOI: 10.17555/jvc.2022.39.3.121

6. Scurrell E, Trott A, Rozmanec M, Belford CJ: Ocular histiocytic sarcoma in a cat. *Vet Ophthalmol,* 16, 173-176, 2013. DOI: 10.1111/vop.12052

7. Moore PF: A review of histiocytic diseases of dogs and cats. *Vet Pathol,* 51 (1): 167-184, 2014. DOI: 10.1177/0300985813510413

8. Coste M, Prata D, Castiglioni V, Minoli L, Etienne-Raffestin CL, Boulouha L, Lagadic M: Feline progressive histiocytosis: A retrospective investigation of 26 cases and preliminary study of Ki67 as a prognostic marker. *J Vet Diagn Invest,* 31 (6): 801-808, 2019. DOI: 10.1177/1040638719884950

9. Khan SN, Sepahdari AR: Orbital masses: CT and MRI of common vascular lesions, benign tumors, and malignancies. *Saudi J Ophthalmol,* 26 (4): 373-383, 2012. DOI: 10.1016/j.sjopt.2012.08.001

10. Yaygingül R, Kiliç N, Epikmen ET, Akin İ, Avci H: Paraorbital malignant histiocytosis in a Holstein calf. *Atatürk Üniv Vet Bil Derg,* 12 (1): 71-75, 2017. DOI: 10.17094/ataunivbd.309777

11. Kanazawa T, Karatsu K, Kuramae T, Ishihara M: Langerhans cell histiocytosis in the frontal and zygomatic bones of an adolescent girl. *BMJ Case Rep,* 14 (9):e246367, 2021. DOI: 10.1136/bcr-2021-246367

12. Park SW, Kim HJ, Lee JH, Ko YH: Malignant fibrous histiocytoma of the head and neck: CT and MR imaging findings. *AJNR,* 30 (1): 71-76, 2009. DOI: 10.3174/ajnr.A1317

13. Borucki RB, Neskey DM, Lentsch EJ: Malignant fibrous histiocytoma: database review suggests a favorable prognosis in the head and neck. *The Laryngoscope*, 128 (4): 885-888, 2018. DOI: 10.1002/lary.26909

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 311-315, 2023
DOI: 10.9775/kvfd.2023.29274

Case Report

Contagious Pustular Dermatitis in a Wild Sheep *(Ovis orientalis)* in Iran

Ehsan SAEIDI [1 (*)] Foozhan KHERADMAND [2]

[1] Collaborator veterinarian of Fars Provincial Office of Department of Environment, Shiraz, IRAN

[2] Laboratory of Shahin Small Animal Veterinary Clinic, Shiraz, IRAN

ORCIDs: E.S. 0000-0003-1582-6553: F.K. 0000-0002-4349-4560

Article ID: KVFD-2023-29274 Received: 25.02.2023 Accepted: 16.04.2023 Published Online: 22.04.2023

Abstract: Contagious Ecthyma is an infectious disease of sheep and goats that causes dermatitis primarily on the lips, mouth and muzzle. In this article, we describe a case report of Contagious Ecthyma in a wild sheep *(Ovis orientalis)* in Fars province of Iran. One wild lamb was found in the border of Bamou National Park near human communities. The lamb died during the transfer to the rehabilitation center. Gross lesions were characterized by multifocal scabs, proliferative and crusty wart-like multiple lesions on the muzzle, nose, between the eyes, ears, neck and coronary band. Skin samples were taken from lesions and sent to the collaborator laboratory of Veterinary Organization for DNA extraction and analysis by PCR tests. Laboratory results confirmed *Contagious Ecthyma (Orf) virus* in the wild sheep. This is the first documented report of Orf in wild sheep from Bamou National Park.

Keywords: *Bamou National Park, Contagious Ecthyma, Wild sheep, PCR*

İran'da Bir Yaban Koyununda (*Ovis orientalis)* Bulaşıcı Püstüler Dermatit

Öz: Bulaşıcı Ektima koyun ve keçilerde görülen ve esasen dudaklarda, ağızda ve burunda dermatite neden olan bulaşıcı bir hastalıktır. Bu çalışmada, İran'ın Fars eyaletinde bir yaban koyununda *(Ovis orientalis)* görülen bir Bulaşıcı Ektima vakası bildirilmiştir. Bamou Ulusal Parkı sınırında insan topluluklarının yakınında bir yaban kuzusu bulunmuştur. Kuzu rehabilitasyon merkezine nakli sırasında ölmüştür. Makroskobik lezyonlar, ağız, burun, gözler arası, kulaklar, boyun ve koroner bantta multifokal kabuklanmalar, proliferatif ve kabuklu siğil benzeri çoklu lezyonlar şeklindeydi. Lezyonlardan deri örnekleri alınmış ve DNA ekstraksiyonu ve PCR analizi için Veteriner Teşkilatının ilgili laboratuvarına gönderilmiştir. Laboratuvar sonuçları yabani koyunda *Bulaşıcı Ektima (Orf) virüsünü* doğrulamıştır. Bu, Bamou Ulusal Parkı'ndaki yaban koyunlarında belgelenmiş ilk Orf raporudur.

Anahtar sözcükler: *Bamou Ulusal Parkı, Bulaşıcı Ektima, Yaban koyunu, PCR*

Introduction

Orf (contagious pustular dermatitis or contagious ecthyma) is one of the most widespread, contagious, communicable, zoonotic, economically important viral diseases caused by *Parapoxvirus* that is a genus of virus in the family Poxviridae, in the subfamily Chordopoxvirinae [1]. The disease has been recognized as an entity since the last century and was shown to be caused by a specific virus in 1923 [2]. Orf virus infections in humans typically occur when broken skin comes into contact with the virus from infected animals or contaminated equipment [3].

In general, in domestic animals, the disease is more severe in goat than sheep [4]. Transmission is by contact with affected animals or with contaminated objects or surfaces (fomites) [5]. Transmission probably follows a similar pattern in wild ungulates [3]. This virus primarily causes acute pustular lesion, where the severe oral and facial effects can be seen in goats rather than sheep [6]. Interestingly, the virus can also spread to other parts of the body such as the vulva, udder, under the tail and scrotal sac. In more severe cases, the skin of the eyes and feet also may be affected [7]. During outbreak, morbidity can approach 100% while mortality is usually less than 1%. However, mortality can increase by 20%~50% as a result of secondary bacterial infection, stress, immunosuppression or concomitant disease and may exceed 90% in case of malignant Orf [8].

How to cite this article?

Saeidi E, Kheradmand F: Contagious pustular dermatitis in a wild sheep *(Ovis orientalis)* in Iran. *Kafkas Univ Vet Fak Derg,* 29 (3): 311-315, 2023. DOI: 10.9775/kvfd.2023.29274

(*) Corresponding author: Ehsan SAEIDI

Phone: +98 713 6238421 Cellular phone: +98 939 845 3734
E-mail: info_saeidi@yahoo.com

Fig 1. The location where the Infected lamb was found (Google Map. 2021)

The 486 km² Bamou (also transliterated as Bamoo or Bamu) National Park is in Fars Province, north-east of Shiraz. Established in 1967 and upgraded to National Park in 1970, it encompasses three parallel mountain ridges extending in an east-west direction and the hilly plains between. The flora comprises 350 vascular plant species, including 51 endemics and the fauna includes 143 species of vertebrates [9]. The western part of Bamu is separated by the Isfahan-Shiraz highway and its large mammalian fauna has been depleted by poaching. Only the eastern part (356 km²) is effectively protected [10].

In the present paper, described a clinical aspect of Contagious ecthyma infection in a wild sheep *(Ovis orientalis)* that had found by rangers in Bamou National Park.

Case History

The permission of publishing this article has been given by Wildlife Department of Fars Environmental Organization of Iran.

On July, 2021 Bamou National Park rangers, during the daily control of the protected area in one of the border areas of the park in the surrounding of The Bardej village, observed a three-month-old wild sheep lamb that was decubitus and agonized *(Fig.1)*. The rangers transferred the lamb to wildlife rehabilitation center at the entrance of the National Park. Unfortunately, the lamb died before reaching the rehabilitation center. The carcass of the lamb was examined and severe skin lesions including proliferative lesions, severely crusted wart-like multiple

Fig 2. Orf virus infection is evident lik scaby and proliferative lesions on the muzzle of the lamb

lesions and multifocal necrotic scabs on the muzzle, nose, between the eyes, ears, neck and coronary band and also inflammation of three joints in the forelimb and hindlimb were observed *(Fig. 2, Fig. 3, Fig. 4, Fig. 5, Fig. 6)*.

Despite of the daily protection and control of the whole area of Bamou National Park, in order to find more infected individuals and/or carcasses a more extensive surveillance was advised to the rangers of the National Park, especially in the border area of Park and the Bardej village. But during the 15 days of searching in the border areas, no other cases of the infected were found.

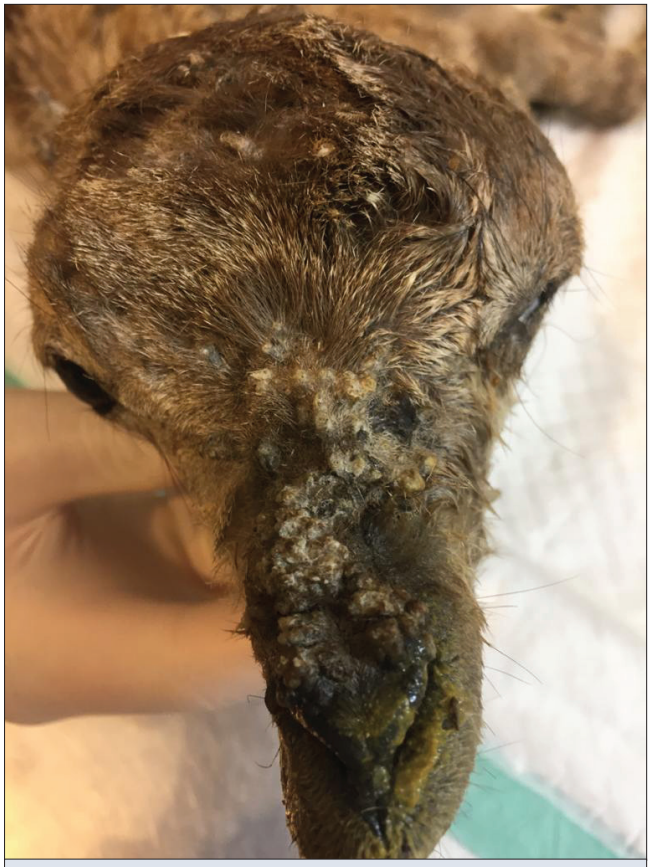

Fig 3. Severely crusted wart-like multiple lesions on the muzzle, nostril area and between eyes in the lamb

Fig 4. Proliferative lesions on the coronary bands of the lamb

Initially, several diseases like contagious ecthyma and foot and mouth disease were suspected as the primary possible diagnoses. Therefore, for differential diagnosis and sampling for diagnosis of the causative agent of the disease, the carcass of the lamb was sent to the Iran Veterinary Organization laboratory of Fars province where necropsy was performed and tissue sections were collected: joint fluid was collected aseptically and scabs were taken from lesions of lips and the muzzle of the lamb. For histopathological examination, tissue specimens were taken from the junction between normal skin and the lesions on the lips and muzzle and fixed in 10% buffered formalin. The samples were processed aseptically and then sent to collaborator laboratory of Fars Provincial Office of Veterinary Organization for DNA extraction by PCR tests.

During the necropsy, no specific internal lesions were detected. Standard bacteriological examination on blood agar plates was carried out on samples from joint fluid. The plates were incubated aerobically at 37.8°C and examined after 24-48 h. *Staphylococcus aureus* and *Streptococcus* spp. were isolated from samples of joint fluid.

Electron microscopy analysis from lesions revealed epithelial hyperplasia *(Fig.7),* necrotic crusts and marked epidermal proliferation with elongated rete ridges covered by a thick crust. The affected epidermis showed multifocal ulcerations, intraepidermal covered by serocellular crust. Furthermore, hyperemia, diffuse infiltration of lymphocytes and variable amounts of neutrophils were found.

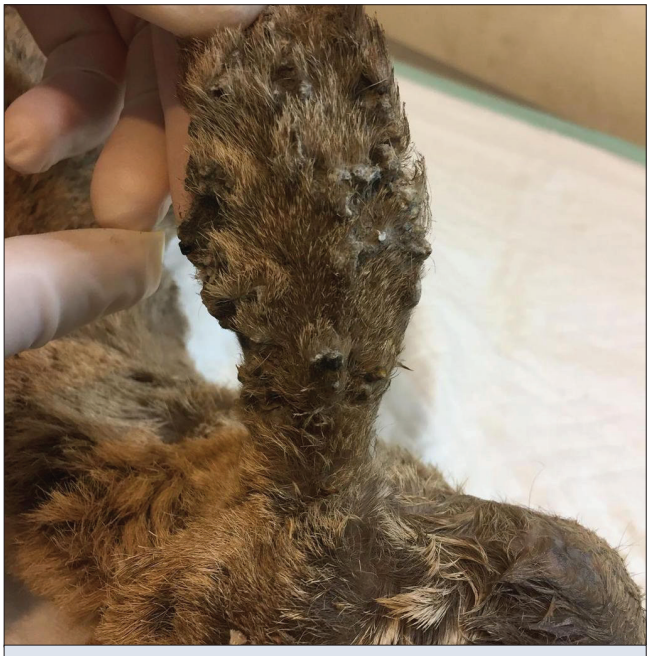

Fig 5. Growth of tumor-like lesions on the ear of the lamb

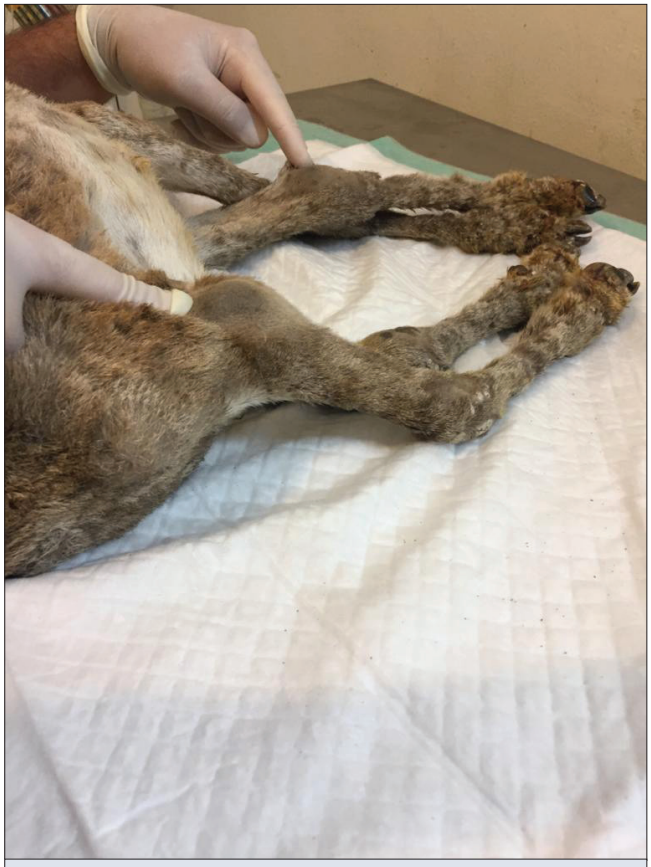

Fig 6. Multiple Arthritis seen in the wild lamb

Envelope membrane glycoprotein (B2L) gene of the orf virus was targeted using PCR assay as per the standard protocol described by Inoshima et al.[11]. The high pure PCR template preparation kit (Roche Company, Germany) was used for extracting DNA from 200 µL samples based on the manufacturer's guidelines. a set of three primer pairs in a semi nested PCR format was used. In the first PCR, a set of pan-parapox primer (PPP-1) and pan-parapox primer (PPP-4) primers was used to generate the product. In the semi nested PCR, a set of PPP-4 and PPP-3 was used. In the result, the analysis of the semi nested PCR revealed the confirmation of the Orf virus only in the scab samples collected from wild sheep.

Discussion

Since secondary bacterial contamination in Orf virus infection is common [12], some secondary bacterial infections like staphylococci, streptococci, fusobacterium, cornyebacterium and less often dermatophilius have been also recorded along with Contagious ecthyma on sheep and goats [13]. Therefore, isolation of *Staphylococcus aureus* and *Streptococcus* spp. from joint fluid of this case can be considered as a secondary infection and probably had happened due to the weakness of immune system of the lamb and also the long period of the virus infection.

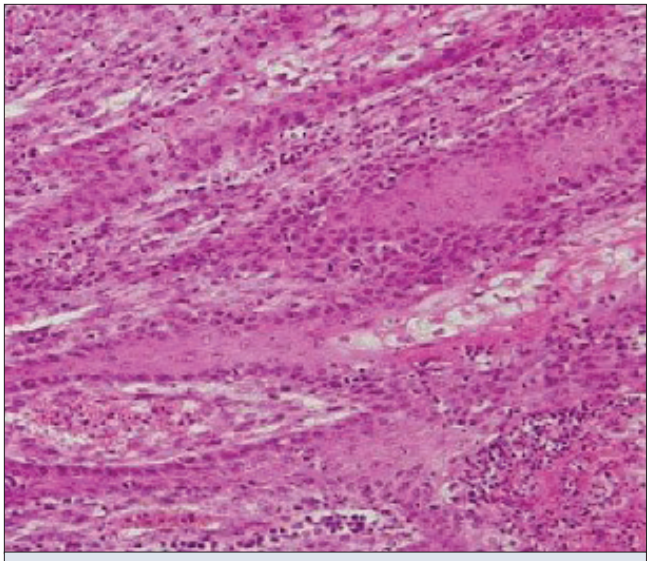

Fig 7. Histological appearance of Orf virus disease with epithelial hyperplasia

The disease is widespread in variety species of artiodactyls like alpacas, reindeer *(Rangifer tarandus),* Japanese serows *(Capricornis crispus),* musk oxen *(Ovibos moschatus),* bighorn sheep *(Ovis canadensis),* Sichuan takin *(Budorcas taxicolor tibetana),* deer, pronghorn *(Antilocapra americana)* and wapiti/elk *(Cervus canadensis),* and it is suspected to occur in some wild chamois *(Rupicapra rupicapra)* [3].

Contagious ecthyma infects a broad range of wild artiodactyls. The total confirmed species from order of artiodactyla in Fars Province of Iran comprises 6 species in 5 genera of 3 families living in different habitats that 4 out of these 6 species are present in Bamou National Park [14].

The virus is very resistant in the environment, particularly in dry atmosphere areas where the virus has been shown to be infective for up to 17 years [15], and no clinical disease in persistently Orf virus infected animals have also been described, and it is possible that such animals contribute to inter-epidemic survival of the virus. The lesions on such persistently infected animals may not be readily detected. Thus, apparently normal animals may have trivial lesions that are capable of being a source of infection for other fully susceptible animals [1]. Therefore, there are concerns about the possibility of disease transmission to susceptible species of the park.

Based on a research, fifty suspected clinical samples were analyzed in Fars province for the Orf DNA presence using PCR technique, and 50% positivity was shown [16]. Furthermore, one of the old problems in the way of protecting Bamou National Park that has never solved is the illegal grazing of livestock inside the protected area especially in areas where there are the borders of Park with human communities. To investigate the possibility of disease transmission from livestock to wild species, through a letter, we asked the Iran Veterinary Organization

of Fars province if there are any reports of this disease in the livestock of Bardej village or not and realized during last six months there were two reports of infected livestock by Contagious ecthyma in that village. So as a result, it can be said the presumption of disease transmission from livestock animals to the wildlife of Bamou National Park is high.

In conclusion, the domestic animals/wildlife interface is an important global problem and solving this requires to expansion studies on different aspect of interactions between wild and domestic animals. The transmission of infectious or some zoonotic diseases like contagious ecthyma due to frequent contact between humans, domestic and wild animals is becoming an issue of major interest. Reducing and eliminating the direct and indirect contact of domestic animals with wildlife species is probably the most effective and important way to prevent the transmission of infectious diseases.

Acknowledgements

The author thanks the Fars Provincial Office of the Department of Environment, especially Leila Joolaee (Head of the Wildlife Bureau) for her continued support of our investigates.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Approval

The permission of publishing this article has been given by Wildlife Department of Fars Environmental Organization of IRAN.

References

1. Sunder J, Sujatha T, De AK, Bhattacharya D, Bhowmick S, Perumal P, Kundu A: First report of contagious ecthyma (orf) outbreak in goats of Andaman and Nicobar Islands. *Indian J Anim Res*, 54 (4): 503-507, 2019. DOI: 10.18805/ijar.B-3787

2. Reid HW, Rodger SM: Orf. **In**, Aitken ID (Ed): Diseases of Sheep. 4th ed., 297-302, Blackwell Publishing, 2007.

3. Spickler RA: Contagious Ecthyma, 2015. http://www.cfsph.iastate.edu/DiseaseInfo/ factsheets.php; *Accessed: 23 September 2015.*

4. Manzoor S, Bahadur SUK, Talib U, Arshad MJ, Abubakar M, Zahur AB: Differential diagnosis of orf from peste des petits ruminants: An example from field. *Res J Vet Pract*, 6, 10-13, 2018. DOI: 10.17582/journal.rjvp/2018/6.2.10.13

5. Gündoğdu E, Arpacik A, Sari A, Başkaya S: Orf virus infection in wild goats *(Capra aegagrus,* Erxleben 1777) of Saricicek Mountain. *Appl Ecol Environ Sci*, 16, 4515-4521, 2018. DOI: 10.15666/aeer/1604_45154521

6. Kumar R, Ramakant, Kumar P, Diwakar RP, Husain S, Alam K: Therapeutic management of contagious ecthyma (Orf) in goat: A case report. *Vet Clin Sci*, 8, 8-10, 2020.

7. Sadiq MA, Abba Y, Jesse FFA, Chung ELT, Bitrus AA, Abdullah AA, Balakrishnan KN, Bala JA, Mohd Lila A: Severe persistent case of contagious ecthyma (Orf) in goats. *JAHP*, 5, 24-28, 2017. DOI: 10.14737/journal.jahp/2017/5.1.24.28

8. Abbas G, Mughal MN: Case report on Orf in sheep in Faisalabad Pakistan. *Int J Mol Vet Res*, 4, 1-2, 2014.

9. Ghoddousi A, Khaleghi-Hamidi A, Ghadirian T, Ashayeri D, Khorozyan I: The status of the endangered Persian leopard *Panthera pardus saxicolor* in Bamu National Park, Iran. *Oryx*, 44, 551-557, 2010. DOI: 10.1017/S0030605310000827

10. Nowzari H, Rad BB, Hemami M: Habitat use by Persian gazelle *(Gazella subgutturosa subgutturosa)* in Bamoo National Park during autumn and winter. *Acta Zool Mex*, 23, 109-121, 2007. DOI: 10.21829/azm.2007.231560

11. Inoshima Y, Morooka A, Sentsui H: Detection and diagnosis of parapoxvirus by the polymerase chain reaction. *J Virol Methods*, 84, 201-208, 2000. DOI: 10.1016/s0166-0934(99)00144-5

12. Teshale A, Alemayehu A: Contagious ecthyma and its public health significance. *Dairy Vet Sci J*, 7 (3): 55571, 2018. DOI: 10.19080/JDVS.2018.07.555711

13. Spyrou V, Valiakos G: Orf virus infection in sheep or goats. *Vet Microbiol*, 181, 178-182, 2015. DOI: 10.1016/j.vetmic.2015.08.010

14. Zarei F, Kafaei S, Esmaeili HR: Annotated checklist and conservation status of mammals of Fars Province, southern Iran. *J Threat Taxa*, 11, 13087-13113, 2019. DOI: 10.11609/jott.4231.11.1.13087-13113

15. Lacasta D, Reina R, De Arcaute MR, Ferrerl M, Benito AA, Tejedor MT, Echeverria I, Ruiz H, Cardenas SM, Windsor PA: Effect of a topical formulation on infective viral load in lambs naturally infected with orf virus. *Vet Med (Auckl)*, 12, 149-158, 2021. DOI: 10.2147/VMRR.S306355

16. Esmaeili H, Ghorani M, Arani EB, Shakeri AP: Detection of contagious ovine ecthyma (Orf) and risk factors for infection in small ruminants in Iran. *Comp Immunol Microbiol Infect Dis*, 79:101714, 2021. DOI: 10.1016/j.cimid.2021.101714

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
29 (3): 317-325, 2023
DOI: 10.9775/kvfd.2023.29241

Review

Strongyloides Infections Among Human and Non-Human Host in Indonesia: A Systematic Review

Nanis NURHIDAYAH [1 (*)] Anuraga JAYANEGARA [2] Fadjar SATRIJA [3]

[1] Research Center for Veterinary Science, National Research and Innovation Agency, Bogor 16911, INDONESIA
[2] Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Bogor 16680, INDONESIA
[3] School of Veterinary Medicine and Biomedical Science, IPB University, Bogor 16680, INDONESIA

ORCIDs: N.N. 0000-0001-7416-281X; A.J. 0000-0001-7529-9770; F.S. 0000-0001-8512-5862

Article ID: KVFD-2023-29241 Received: 22.02.2023 Accepted: 20.04.2023 Published Online: 03.05.2023

Abstract: *Strongyloides* have been known to infect many hosts, including humans and animals around the world. The two, *S. stercoralis* and *S. fuellborni,* were zoonotic species that cause human strongyloidiasis. The disease induces a hyper infection syndrome in an immunocompetence person, while the clinical signs of infections in animals vary from asymptomatic to sudden death in severe and fatal cases. However, integrated data on *Strongyloides* infection among human and animal hosts in Indonesia is sparse. The present study aims to provide information on the prevalence of *Strongyloides* concerning host type and geographical region in Indonesia. Literature searches were conducted to identify epidemiological data on the occurrence of *Strongyloides* in humans and animals from 1985-2022 in seven regions of Indonesia, including 1) Sumatra; 2) Java; 3) Kalimantan; 4) Bali and Nusa Tenggara; 5) Sulawesi; 6) Maluku; and 7) Papua. The data were described and mentioned as percent prevalence and confidence intervals (CIs) of 95%. A total of 46 articles confirmed the *Strongyloides* infection in Indonesia during 1985-2022. The articles consisted of nine on humans (12 data) and 37 on animals (59 data). Human strongyloidiasis could be confirmed in five regions (Bali and Nusa Tenggara, Kalimantan, Maluku, Papua, and Sulawesi). *Strongyloides* infected some animal group hosts, such as pigs, ruminants (beef cattle, swamp buffalo, sheep, and goat), rodents, non-human primates (orangutan, lutung *(Trachypithecus auratus),* and long-tailed macaque), and others (rabbit, snake, and chicken) in different regions. This study provides the prevalence data on *Strongyloides* infection in human and animal hosts. The limited data on *Strongyloides* in Indonesia indicated that the disease is still being neglected and under-reported. More epidemiological studies with the improved diagnostic method are needed to determine the societal burden of the parasites.

Keywords: *Strongyloides, Indonesia, Human strongyloidiasis, Animal strongyloidiasis*

Endonezya'da İnsan ve İnsan Dışı Konaklar Arasında Strongyloides Enfeksiyonları: Sistematik Bir Değerlendirme

Öz: *Strongyloides*'in dünya çapında insanlar ve hayvanlar da dahil birçok konağı enfekte ettiği bilinmektedir. *S. stercoralis* ve *S. fuellborni*, insan strongyloidiasis'ine neden olan zoonotik türlerdir. Hastalık, immünokompetanslı bir insanda hiper enfeksiyon sendromuna neden olurken, hayvanlardaki enfeksiyonların klinik belirtileri asemptomatikten şiddetli ve ölümcül vakalarda ani ölüme kadar değişir. Bununla birlikte, Endonezya'da insan ve hayvan konakçıları arasında *Strongyloides* enfeksiyonuna ilişkin entegre veriler nadirdir. Bu çalışma, Endonezya'da konak tipi ve coğrafi bölgeye göre *Strongyloides* prevalansı hakkında bilgi sağlamayı amaçlamaktadır. 1985-2022 yılları arasında insan ve hayvanlarda *Strongyloides* görülme sıklığına ilişkin epidemiyolojik verileri belirlemek için Endonezya'nın 1) Sumatra; 2) Java; 3) Kalimantan; 4) Bali ve Nusa Tenggara; 5) Sulawesi; 6) Maluku ve 7) Papua olmak üzere yedi bölgesinde literatür taraması yapılmıştır. Veriler, yüzde prevalans ve %95'lik güven aralıkları (CI) olarak tanımlanmış ve belirtilmiştir. 1985-2022 yılları arasında Endonezya'da toplam 46 makalede *Strongyloides* enfeksiyonu bildirilmiştir. Makalelerin 9'u insanlar (12 veri) ve 37'si hayvanlar (59 veri) üzerinedir. İnsan Strongyloidiasis'i 5 bölgede (Bali ve Nusa Tenggara, Kalimantan, Maluku, Papua ve Sulawesi) doğrulanabilmiştir. *Strongyloides*, farklı bölgelerde domuz, geviş getiren hayvanlar (sığır, bataklık mandası, koyun ve keçi), kemirgenler, insan olmayan primatlar (orangutan, lutung (*Trachypithecus auratus*) ve uzun kuyruklu makak) ve diğerleri (tavşan, yılan ve tavuk) gibi bazı hayvan grubu konakçılarını enfekte etmiştir. Bu çalışma, insan ve hayvan konakçılarında *Strongyloides* enfeksiyonuna ilişkin prevalans verilerini sunmaktadır. Endonezya'da *Strongyloides* ile ilgili verilerin sınırlı olması, hastalığın hala ihmal edildiğini ve yeterince rapor edilmediğini göstermektedir. Parazitlerin toplumsal yükünü belirlemek için gelişmiş tanı yöntemleriyle daha fazla epidemiyolojik çalışmaya ihtiyaç vardır.

Anahtar sözcükler: *Strongyloides, Endonezya, İnsan strongyloidiasisi, Hayvan strongyloidiasisi*

How to cite this article?

Nurhidayah N, Jayanegara A, Satrija F: *Strongyloides* infections among human and non-human host in Indonesia: A systematic review. *Kafkas Univ Vet Fak Derg,* 29 (3): 317-325, 2023.
DOI: 10.9775/kvfd.2023.29241

(*) Corresponding author: Nanis NURHIDAYAH

Phone: +62 8111 9333631 Cellular phone: +62 8151 5652622
E-mail: nanis.nurhidayah@brin.go.id

Introduction

Strongyloides is a genus of gastrointestinal nematodes infecting many hosts, such as mammals, reptiles, amphibians, and birds worldwide [1]. Around fifty species have been identified, with two major species responsible for human strongyloidiasis, a zoonosis disease transmitted from companion animals such as dogs and cats, namely *S. stercoralis* and *S. fuellborni* [2]. *Strongyloides stercoralis* was first reported from the stool samples of French soldiers who have severe diarrhea during their duty in Vietnam in 1876. The disease was also known as Cochin-China diarrhea [3]. The disease was typically chronic and non-pathognomonic, such as indigestion, diarrhea, constipation, irritable bowel syndrome, urticaria, and larva currents. The infection could induce hyper infection syndrome in the immunocompetence group induced by the large numbers of larvae in organs. The clinical manifestations involving diarrhea, intestinal bleeding, alveolar hemorrhages, heart failure, jaundice, bacteremia with fatality rate near to 90% [4].

Strongyloides species that infect animals are more varied but specific to each animal group. For example, *S. stercoralis* is species that infect dogs, while cats are infected with *S. felis*, *S. tumefaciens*, *S planiceps*, and perhaps *S. stercoralis*. In farm animals, pigs enabled to be infectedwith *S. ransomi*. On the other hand, *S. papillosus* has been an infecting agent for cattle and other ruminants in many countries [5].

As a large and developing country, Indonesia has become a house of more than 270 million people with a high diversity of flora and fauna. Transmission of soil-transmitted helminth (STHs), including *Strongyloides*, is potentially found among humans and animals occurs due to minimal hygiene practice and low awareness of the transmission route of the disease. In addition, the close habitat of animal and human are increasing the potency of zoonotic disease transmission. *Strongyloides* infections in humans and animals have been previously reported, but the national distribution following region and host are sparse. This study provides information on *Strongyloides* prevalence concerning host type and geographical region in Indonesia.

Methods

Study Area

Indonesia is the largest country in Southeast Asia, located on 6°08' N-11°15'S and 95°45 W'-141°05'E. It consists of 17.504 islands in the area of 1.913.578,68 km^2 [6]. The country borders are Malaysia, Singapore, the Philippines, and the Pacific Ocean to the north, Papua New Guinea and the Pacific Ocean to the east, the Indian Ocean to the west, and the Democratic Republic of Timor-Leste and the Indian Ocean to the south. Kalimantan, Sumatera, Java, and Sulawesi are among the largest islands in the country. Furthermore, Indonesia has a large area of rainforest and the house of the highest biological diversity in the world. The geographical condition varies from west to the east, including lowland to the mountainous region. As a tropical country, Indonesia has an extended period of dry and wet seasons within a year, providing a suitable environment for parasites including the gastrointestinal parasites.

Literature Selection and Data Extraction

A literature search was conducted to identify an epidemiological field survey of the infection of *Strongyloides* among human and non-human hosts in Indonesia. We conducted a literature search on the website of Google scholar and PubMed with the date restriction of the study set from January 1, 1985, to August 1, 2022, and the search terms: *Strongyloides* Indonesia, OR Strongyloidiasis Indonesia, OR gastrointestinal nematode Indonesia, both in Bahasa Indonesia and English. The whole search output is provided in *Attachment 1* and *Attachment 2*. During the literature selection, the title and abstract were screened based on the following criteria:

1. Dealing with infection of *Strongyloides* spp. in humans and animals in Indonesia;
2. Providing the primary data based on an epidemiological study or questionnaire survey;
3. Data reported from January 1, 1985, to August 1, 2022;
4. The full text was published in Bahasa Indonesia and English in a peer-reviewed journal or indexed proceeding.

According to the geographical condition, the region is divided into: (i) Sumatera, (ii) Java, (iii) Bali and Nusa

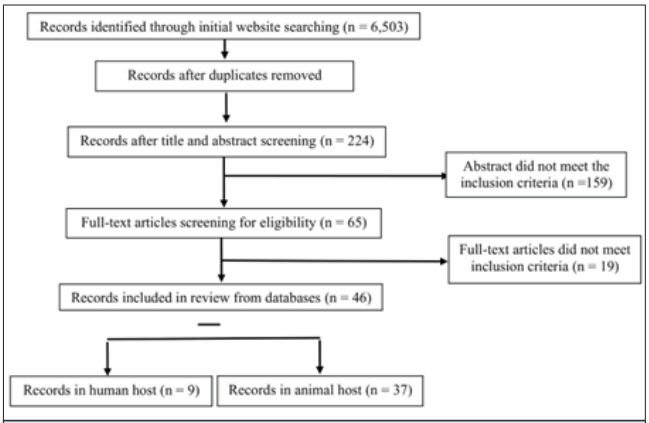

Fig 1. Flow diagram of the search strategy for strongyloidiasis in human and animal host

Table 1. *Eligible data revealed during 1985-2022*

Publication Years	Human	Animals				
		Pig	Ruminants	Rodents	Non-human Primates	Others
1985-2000	6	na	na	2	6	na
2001-2015	na	5	3	1	10	1
2016-2022	6	7	17	na	4	3
Total	**12**	**12**	**20**	**3**	**20**	**4**

na= not available

Tenggara, (iv) Kalimantan, (v) Sulawesi, (vi) Maluku, and (vii) Papua. Data added to the analysis were treated individually, even when published within one article. In addition, the data analysis was separated into humans and animals. *Figure 1* describes the flow diagram of the literature search and selection strategy.

Data Analysis

Data synthesis and calculation on prevalence of *Strongyloides* spp. in human and animals was done in the Microsoft Excel. The prevalence provided in percent and confidence intervals (95% CIs).

Results

The search initially revealed 6.503 articles, of which only 46 articles contained sufficient epidemiological data on infection of *Strongyloides* in Indonesia from 1985 to 2022 (attachments 1 and 2). The articles could be divided into nine articles (including 12 data) on humans and 37 articles (59 data) on animals *(Table 1)*. Unfortunately, the data on *Strongyloides* at the province or national level cannot be extracted for humans and animals, but the regional prevalence of *Strongyloides* is available in *Table 2*.

Nine articles (containing 12 data) confirmed *Strongyloides* in humans in five regions, including Bali and Nusa Tenggara, Maluku, Kalimantan, Sulawesi, and Papua. The highest prevalence of human strongyloidiasis was found in Papua, followed by Kalimantan and Maluku *(Table 2)*. Eleven out of 12 data on humans were obtained from a conventional coprology study, such as the Kato-Katz smear, Harada Mori coproculture, and Agar Plate Culture (APC). Only one data on humans in Mimika District, Papua, confirmed the infection agent into species level, *S. stercoralis*, by a real-time PCR technique [11].

Strongyloides spp. also infected numerous groups of animals on the farm, village, or district level *(Table 2)*. Most data on ruminants and pigs followed the standard coprology techniques for gastrointestinal nematodes, such as native, sedimentation, simple flotation and McMaster, and continued with Baermann coproculture method. The host range included Bali cattle, Madura cattle, Bligon goat, Kacang goat, swamp buffalo, crossbred sheep, and pigs. Most data on ruminants were obtained in the center of ruminant production in Java, Bali, and Nusa Tenggara region, while infection in pigs was most prevalent in Papua, Bali and Nusa Tenggara and a small part of Java where Islam is not predominantly practiced.

We identified 20 data on captive, semi-captive, and free-ranging endemic non-human primates, such as lutung *(Trachypithecus auratus)*, orangutan *(Pongo pigmaeus)*, and long-tailed macaque *(Macaca fascicularis)*. The data was collected from zoo, captivity, rehabilitation center, or national park in Java, Sumatera, Kalimantan, Bali and Nusa Tenggara. Moreover, the helminth was also found in animals that have close contact with humans, such as rodents, dogs, layer chickens, and captive phyton-snake. Since the golden standard method was unavailable for helminth identification on non-human primates, reptiles, and exotic animals, non-invasive methods such as scotch-tape perianal smear, Kato-Katz, McMaster, and sugar flotation could be chosen. On the other hand, a necropsy was feasible for rodents and chickens (domesticated fowl) following trapping in their habitat. None of the molecular epidemiology was recorded in animals in all regions, so the species of *Strongyloides* infecting each group of animals could not be determined.

Discussion

Human Strongyloidiasis

To our knowledge, the present study was the first systematic review of the epidemiology and distribution of *Strongyloides* in human and animal hosts in Indonesia. Our finding shows that the report on infection of *Strongyloides* in a human was very limited in the last three decades. Although study on human has covered at least five out of seven regions, none of the eligible studies were published for Java and Sumatera region, which has the highest human population in Indonesia. Moreover, the absence of data at the national level indicated that human strongyloidiasis is still being neglected and underreported; even disease control was prioritized in the national strategy for neglected tropical diseases control by the Ministry of Health of Indonesia in 2020-2024 [7].

Table 2. *Regional prevalence and techniques for diagnosing Strongyloides spp. infection among human and animals in Indonesia*

Region	Human (n=12)	Animals				
		Pig (n=12)	Ruminants (n=20)	Rodents (n=3)	Non-human Primates (n=20)	Others (n=4)
	% Prevalence (CI 95%)					
Bali and Nusa Tenggara	1.02 (0-2.21)	9.71 (2.98-16.44)	11.52 (1.11-21.92)	na	25.33 (3.12-47.55)	33.35 (0-85.64)
Java	na	6.1 (-)	13.47 (4.85-22.08)	53.00 (-)	71.92 (32.78-100)	15.67 (0-39.84)
Kalimantan	8.90 (0-18.30)	na	na	na	35.52 (22.36-48.69)	na
Maluku	5.59 (3.31-7.86)	na	na	48.98 (32.99-64.97)	na	na
Papua	9.42 (0-24.27)	18.25 (4.87-31.63)	65.22 (0-100)	na	na	na
Sulawesi	1.19 (-)	na	20.05 (-)	na	na	na
Sumatera	na	na	na	na	59.79 (33.68-85.89)	na
	Parasitological Techniques					
Bali and Nusa Tenggara	Kato-katz, Baermann coproculture, Koga Agar Plate, Harada Mori	Sodium Acetic Formaldehyde	Sugar Flotation	na	Formalin ethyl acetate concentration technique, Sugar Flotation	Sedimentation, Sugar Flotation
Java	na	Sugar Flotation	Sedimentation, Sugar flotation, McMaster Flotation, native	Kato-katz	Sugar Flotation	Sugar Flotation, Whitlock
Kalimantan	Kato-katz, agar plate culture	na	Na, Whitlock	na	McMaster Flotation	na
Maluku	Kato-katz	na	na	Necropsy	na	na
Papua	Direct smear, PCR	Necropsy, Sugar Flotation, McMaster Flotation	Sugar Flotation, McMaster Flotation	na	na	na
Sulawesi	Kato-katz	na	Sugar Flotation	na	na	na
Sumatera	na	na	na	na	Scotch-tape perianal smear	na

n: *number of data;* ***na:*** *not available*

In general, the prevalence of human strongyloidiasis conducted with the conventional parasitological technique was low [8]. Diagnosis of *Strongyloides* infection is challenging due to the low parasite load, irregular larval output, so the detection rate may be low, and multiple samples must be examined to achieve adequate sensitivity [2]. Results of a single stool examination using conventional techniques fail to detect larvae in up to 70% of cases [9]. The development of molecular diagnostics gave a chance to reach an accurate and timely diagnosis. In 2011, a real time-PCR was developed to diagnose the infection of *Strongyloides* spp. The 95 % detection limit, as determined by probit analysis, was one larva per PCR, equivalent to 100 larvae per 200 mg stool with 100% specificity [10]. The same technique was used in one epidemiological study on humans in Mimika Papua, which resulted in a high prevalence (32.00 %) of humans [11]. More epidemiological surveys with enhanced coprological methods from across the region and province are required to determine Indonesia's prevalence and disease burden. Estimation of prevalence at the country level has been developed by using a spatiotemporal statistic modeling approach, but it needs the large number of datasets including Gross Domestic Product (GDP), percentage of rural population, territory roughness, sanitation, annual mean temperature, and annual precipitation [8].

Another factor presumed to be linked to the prevalence of *Strongyloides* spp. is geo-climatic factors, such as temperature, humidity, rainfall, soil textures and farming ecosystem [12,13]. The combination of some climatic factors (such as temperature [10-21°C], humidity [40-75%], and annual rainfall [1 001-1 500 mm]), countries or regions with low-personal income, and availability contact to infected animals [14]. As a developing country, Indonesia shares a similar situation. Most studies on human strongyloidiasis were recorded from rural and

urban areas of Indonesia, targeting students, farmers, and housewives who have a high potential to contact the soil in their daily activities. Most face hygiene and sanitary problems due to poverty, limited access to proper public health care, and minimum intervention of health education to increase social awareness of the diseases. In addition, the low-personal income also directly affects the low-quality management of companion animals, such as the provision of caging and feeding. Through this study, we could see the zoonotic potency of *Strongyloides* spp. from possible carrier animals, such as the dog. Although only one eligible study on the dog was reported in the Java region in 2018, the disease is threatening the dog's owner due to the presence of zoonotic species on humans belonging to *S. stercoralis* [1]. As a possible transmission route is through skin penetration by the infective larvae, education on personal hygiene after contact with companion animals was crucial. More epidemiological investigation and transmission risk of *Strongyloides* spp. in dogs in another region was needed with enhanced diagnostic methodology.

Non-human Primates

The highest number of *Strongyloides* spp. infection data in animals were reported from the non-human primates' group. High prevalence of *Strongyloides* has been confirmed in foci habitat of endemic non-human primates, such as in Java, Kalimantan, Sumatera and Bali and, Nusa Tenggara. This situation must be considered a severe threat to human and animal health since the close habitat of animals to human as well as increasing number of ecotourism which involving non-human primates as the object [15]. The first molecular identification confirmed *S. fuellborni* transmission from an adult female with frequent contact with long-tailed macaque in Thailand and Lao-PDR [16]. According to the national conservation program perspective, research on *Strongyloides* in non-human primates needs to focus on early detection and developing a control strategy to avoid severe and fatal cases. A previous study reported the severe clinical symptoms of *Strongyloides* infection in 5-month-old Sumatran Orangutan, such as acute lethargy, dry and non-productive cough, pneumonia, and icterus [17]. *Strongyloides* caused histopathological changes in infected Bornean orangutans, characterized by multiple nodular elevations of the mucosa of the colon, hemorrhages in lungs, and ulcerations in the mucosa of cecum, appendix, and proximal colon [18].

- Ruminants

A total of 19 data of *Strongyloides* in ruminant was revealed through the present study. *Strongyloides* spp. was prevalent in Java, Bali and Nusa Tenggara, Sulawesi, and Papua region, which play the center of national ruminant production. The diseases may cause economic loss due to health disturbance and even mortality in livestock, including ruminants. For example, the prevalence of *Strongyloides* spp. in dairy calves (4-month-old) in Costa Rica was 4-20 % [19], and sudden death has also been reported by heavy experimental infection by 10.000 L3 on days 11-17 post-infection [20]. However, the prediction of sudden death also could be made through coproscopy if the egg count per gram of fecal samples (EPG) is more than 10.000 [5].

A young-infected goat might perform anorexia, cachexia, anemia, foaming at the mouth, and nervous system disturbance (ataxia, stupor, nystagmus), dehydration and diarrhea. In addition, some histological changes might be seen, such as liver rupture, nephrosis, pulmonary edema, interstitial pneumonia, and pneumonia. Six percents of goats died due to acute and fatal hepatic rupture. Undeveloped immunity in young calves and goats makes them susceptible to infection [21].

Infection was also detected in swamp buffalo in Java and Bali, and Nusa Tenggara region. Most of them are used for saving, working animals to plow paddy fields, and meat producers for the community [22]. However, close habitat to other farm animals, such as cattle, gave a chance for disease transmission. Therefore, further investigation needs to be conducted to provide information on cross-transmission among the livestock group in the same area.

- Pigs

The highest report on the pigs was obtained in Papua, Bali and Nusa Tenggara, and a small part of Java (Tangerang, Banten Province), where Islam is not predominantly practiced. A high prevalence of *Strongyloides* spp. in pigs was reported from high latitude in Jayawijaya, Papua, the center of the pig market, which sells both healthy and sick or dead (referred to as dead pig hereafter). The pigs are usually kept under the traditional farming system and slaughtered for consumption and cultural purposes, so the collection of helminth sample through necropsy or fecal sample collection were feasible even in the mountainous area in Papua [23].

In urban areas such as Denpasar, Bali (Bali and Nusa Tenggara region), and Tangerang, Banten (Java region), pig farming plays an essential role in economic activity. The pig population in Bali has become the second largest at the national level since Balinese Hindu people preferred to consume pig meat over cattle. Fecal collection usually obtained from slaughterhouse taken before the slaughtering process [24-26].

- Rodents

The report of *Strongyloides* spp. in rodents from Indonesia are few, but it is also likely that prevalence is high in urban

and rural areas. According to the collected data, there were two data collected from palm plantations and forests in Maluku, and one data from house rats in Surabaya (Java region) with the prevalence of both regions was 48.98% and 53.00%, respectively. The most recent study conducted in Malang City, East Java showed the lowest prevalence, but low-level of knowledge of people participating in the Knowledge, Attitude and Practice (KAP) study on strongyloidiasis are enhancing the potency of zoonotic transmission of parasite to human [27]. Eventhough all studies conducted in Indonesia used the coprological techniques with microscopic examination as the gold standart for egg determination, but those methods were insufficient to provide the data on the *Strongyloides*' species level. According to previous study, two most prevalence species found in rats were identified as *S. ratti* and *S. venezuelensis* which also become the rodent models of *Strongyloides* infection [28].

- Other Animals

Strongyloides spp. also infected exotic animals such as rabbits and phyton, as well as fowl in the region of Bali and Nusa Tenggara, and Java. More epidemiological surveys should be performed in another region since many exotic animals are kept in captivity and used as a pet animal.

Conclusion

The data on infection of *Strongyloides* in humans and animals at the national level was absent. The limited study on humans and each animal group suggest that parasite is under-reported in Indonesia. More epidemiological studies with improved diagnostic methodology are needed.

Moreover, the government at the local and national level must conduct active surveillance since mapping occurrences in humans and animals within a region will provide an integrated description of the disease as a basis for disease control at the national level. Support from private sector as well as community are needed as a key to combat *Strongyloides* infection among humans and animals in Indonesia.

Conflict of Interest

The authors declared that there is no conflict of interest

References

1. Eslahi AV, Hashemipour S, Olfatifar M, Houshmand E, Hajialilo E, Mahmoudi R, Badri M, Ketzis JK: Global prevalence and epidemiology of *Strongyloides stercoralis* in dogs: A systematic review and meta-analysis. *Parasite Vector*, 15 (1): 1-13, 2022. DOI: 10.1186/s13071-021-05135-0

2. Viney ME, Lok JB: The biology of *Strongyloides* spp. *WormBook*, 16, 1-17, 2015. DOI: 10.1895/wormbook.1.141.2

3. Grove DI: Human strongyloidiasis. *Adv Parasitol*, 38, 251-309, 1996. DOI: 10.1016/s0065-308x(08)60036-6

4. Vasquez-Rios G, Pineda-Reyes R, Pineda-Reyes J, Marin R, Ruiz EF, Terashima A: *Strongyloides stercoralis* hyperinfection syndrome: A deeper understanding of a neglected disease. *J Parasit Dis,* 43 (2): 167-175, 2019. DOI: 10.1007/s12639-019-01090-x

5. Thamsborg S, Ketzis J, Horii Y, Matthews J: *Strongyloides* spp. infections of veterinary importance. *Parasitology,* 144 (3): 274-284, 2017. DOI: 10.1017/S0031182016001116

6. Statistics Indonesia: Total Area and Number of Islands by Province, 2021. https://www.bps.go.id/indikator/indikator/view_data_pub/0000/api_pub/UFpWMmJZOVZlZTJnc1pXaHhDV1hPQT09/da_01/1Jakarta: Indonesia, 2022. *Accessed:* 20 June 2022.

7. Ministry of Health of the Republic Indonesia: Peraturan Menteri Kesehatan Republik Indonesia Nomor 21 tahun 2020 Tentang Rencana Strategis Kementerian Kesehatan tahun 2020-2024 (Regulation of the Minister of Health of the Republic of Indonesia Number 21 of Year 2020 concerning the Strategic Plan of the Ministry of Health for 2020-2024). Jakarta: Ministry of Health of the Republic of Indonesia, 2020.

8. Buonfrate D, Bisanzio D, Giorli G, Odermatt P, Fürst T, Greenaway C, French M, Reithinger R, Gobbi F, Montessor A, Bisoffi Z: The global prevalence of *Strongyloides stercoralis* infection. *Pathogens*, 9 (6):468, 2020. DOI: 10.3390/pathogens9060468

9. Ericsson CD, Steffen R, Siddiqui AA, Berk SL: Diagnosis of *Strongyloides stercoralis* infection. *Clin Infect Dis*, 33 (7): 1040-1047, 2001. DOI: 10.1086/322707

10. Kramme S, Nissen N, Soblik H, Erttmann K, Tannich E, Fleischer B, Panning M, Brattig N: Novel real-time PCR for the universal detection of *Strongyloides* species. *J Med Microbiol*, 60 (4): 454-458, 2011. DOI: 10.1099/jmm.0.025338-0

11. Kridaningsih TN, Sukmana DJ, Mufidah H, Diptyanusa A, Kusumasari RA, Burdam FH, Kenangalem E, Poespoprodjo JR, Fuad A, Mahendradhata Y, Supargiyono S, Utzinger J, Becker SL, Murhandarwati EEH: Epidemiology and risk factors of *Strongyloides stercoralis* infection in Papua, Indonesia: A molecular diagnostic study. *Acta Trop*, 209:105575, 2020. DOI: 10.1016/j.actatropica.2020.105575

12. Dey AR, Begum N, Alim MA, Malakar S, Islam MT, Alam MZ: Gastro-intestinal nematodes in goats in Bangladesh: A large-scale epidemiological study on the prevalence and risk factors. *Parasite Epidemiol Control*, 9:e00146, 2020. DOI: 10.1016/j.parepi.2020.e00146

13. Widjana DP, Sutisna P: Prevalence of soil-transmitted helminth infections in the rural population of Bali, Indonesia. *Southeast Asian J Trop Med Public Health*, 31 (3): 454-459, 2000.

14. Eslahi AV, Badri M, Nahavandi KH, Houshmand E, Dalvand S, Riahi SM, Johkool MG, Asadi N, Ahangari SAH, Taghipour A, Zibaei M, Khademvatan S: Prevalence of strongyloidiasis in the general population of the world: A systematic review and meta-analysis. *Pathog Glob Health*, 115 (1): 7-20, 2021. DOI: 10.1080/20477724.2020.1851922

15. Kurniawati DA, Suwanti LT, Lastuti NDR, Koesdarto S, Suprihati E, Pratiwi A: Zoonotic potential of gastrointestinal parasite in long-tailed macaque *Macaca fascicularis* at Baluran National Park, Situbondo, East Java, Indonesia. *Aceh J Anim Sci*, 5 (1): 47-56, 2020. DOI: 10.13170/ajas.5.1.15397

16. Thanchomnang T, Intapan P, Sanpool O, Rodpai R, Sadaow L, Phosuk I, Somboobpatarakun C, Laymanivong S, Tourtip S, Maleewong W: First molecular identification of *Strongyloides fuelleborni* in long-tailed macaques in Thailand and Lao People's Democratic Republic reveals considerable genetic diversity. *J Helminthol,* 93 (5): 608-615, 2019. DOI: 10.1017/S0022149X18000512

17. Kleinschmidt LM, Kinney ME, Hanley CS: Treatment of disseminated *Strongyloides* spp. infection in an infant Sumatran orangutan *(Pongo abelii). J Med Primatol*, 47 (3): 201-204, 2018. DOI: 10.1111/jmp.12338

18. Uemura E, Houser WD, Cupp CJ: Strongyloidiasis in an infant orangutan *(Pongo pygmaeus)* 1. *J Med Primatol*, 8 (5): 282-288, 1979. DOI: 10.1159/000460212

19. Jiménez AE, Fernandez A, Alfaro R, Dolz G, Vargas B, Epe C, Schnieder T: A cross-sectional survey of gastrointestinal parasites with dispersal stages in feces from Costa Rican dairy calves. *Vet Parasitol,* 173, 236-246, 2010. DOI: 10.1016/j.vetpar.2010.07.013

20. Tsuji N, Itabisashi T, Nakamura Y, Taira N, Kubo M, Ura S, Genno A: Sudden cardiac death in calves with experimental heavy infection of *Strongyloides papillosus*. *J Vet Med Sci*, 54 (6): 1137-1143, 1992. DOI: 10.1292/jvms.54.1137

21. Pienaar JG, Sasson PA, Du Plessis JL, Collins HM, Naude TW, Boyazoglu PA, Boomker J, Reyers F, Pienaar WL: Experimental studies with *Strongyloides papillosus* in goats. *Onderstepoort J Vet Res,* 66,191-235, 1999.

22. Nurhidayah N, Satrija F, Retnani EB: Gastrointestinal parasitic infection of swamp buffalo in Banten Province, Indonesia: Prevalence, risk factor, and its impact on production performance. *Trop Anim Sci J*, 42 (1): 6-12, 2019. DOI: 10.5398/tasj.2019.42.1.6

23. Nugroho W, Cargill CF, Putra I, Kirkwood RN, Trott DJ, Salasia SIO, Slipranata M, Reichel MP: Investigations of selected pathogens among village pigs in Central Papua, Indonesia. *Trop Anim Health Prod*, 48 (1): 29-36, 2016. DOI: 10.1007/s11250-015-0913-5

24. Akbar MW, Suratma NA, Oka IBM: Prevalensi dan intensitas infeksi cacing *Strongyloides ransomi* pada Babi yang Dipotong di Rumah Potong Hewan Pesanggaran Kota Denpasar. *Bul Vet Udayana,* 14 (3): 238-245, 2022. DOI: 10.24843/bulvet.2022.v14.i03.p07

25. Widisuputri NKA, Suwanti LT, Plumeriastuti H: A Survey for zoonotic and other gastrointestinal parasites in pig in Bali Province, Indonesia. *IJTID*, 8 (1): 54-65, 2020. DOI: 10.20473/ijtid.v8i1.10393

26. Wardhana AH, Sawitri DH, Ekawasti F, Martindah E, Apritadewi D, Shibahara T, Kusumoto M, Tokoro M, Sasai K, Matsubayashi M: Occurrence and genetic identifications of porcine *Entamoeba*, *E. suis* and *E. polecki*, at Tangerang in West Java, Indonesia. *Parasitol Res*, 119 (9): 2983-2990, 2020. DOI: 10.1007/s00436-020-06806-0

27. Kusumarini S, Danuarta MF, Karami F, Yesica R, Wisesa IBGR, Yudhana A, Nurhidayah N: Screening *Strongyloides* spp. infection from wild rodents implications for public awareness and attitudes on zoonotic diseases in Malang City, Indonesia. *JMV*, 5 (2): 196-206, 2022. DOI: 10.20473/jmv.vol5.iss2.2022.196-206

28. Viney M, Kikuchi T: *Strongyloides ratti* and *S. venezuelensis* - Rodent models of *Strongyloides* infection. *Parasitology,* 144 (3): 285-294, 2017. DOI: 10.1017/S0031182016000020

Attachment 1. *Infection of Strongyloides spp. in human host in Indonesia*

Author	N	Positive	Prevalensi	Host Group	Technique	Region
Widjana dan Sutisna [2000]	2394	39	1.63%	Human	Kato Katz	Bali and Nusa Tenggara
Onesiforus et al.[2020]	238	1	0.42%	Human	Baermann, KAP, Haradamori	Bali and Nusa Tenggara
Sedionoto et al.[2019]	213	0	0.00%	Human	Kato Katz	Kalimantan
Sedionoto et al.[2019]	213	35	16.43%	Human	Agar Plate Culture	Kalimantan
Sedionoto et al.[2021]	107	11	10.28%	Human	Kato Katz	Kalimantan
Mangali et al.[1994]	226	10	4.42%	Human	Kato Katz	Maluku
Mangali et al.[1994]	163	11	6.75%	Human	Kato Katz	Maluku
Bangs et al.[1996]	278	3	1.08%	Human	Direct smear	Papua
Bangs et al.[1996]	200	1	0.50%	Human	Direct smear	Papua
Yuwono et al.[2019]	147	6	4.08%	Human	Direct smear	Papua
Kridaningsih et al.[2020]	331	106	32.02%	Human	RT PCR	Papua
Toma et al.[1999]	589	7	1.19%	Human	Kato Katz	Sulawesi

Attachment 2. *Infection of Strongyloides spp. in animal host in Indonesia*

Author	N	Positive	Prevalensi	Host Group	Technique	Region
Akbar et al.[2022]	12	2	15.67	Pig	Flotation	Bali and Nusa Tenggara
Akbar et al.[2022]	90	8	8.89	Pig	Flotation	Bali and Nusa Tenggara
Akbar et al.[2022]	63	0	0	Pig	Flotation	Bali and Nusa Tenggara
Akbar et al.[2022]	35	0	0	Pig	Flotation	Bali and Nusa Tenggara
Antara et al.[2017]	50	1	2	Ruminants (Bali cattle)	Flotation	Bali and Nusa Tenggara
Antara et al.[2017]	50	1	2	Ruminants (Bali cattle)	Flotation	Bali and Nusa Tenggara
Apsari et al.[2022]	55	13	23.64	Ruminants (beef cattle)	Flotation	Bali and Nusa Tenggara
Awaludin et al.[2020]	175	14	8	Ruminants (sheep)	Sedimentation	Java
Baihaqi et al.[2015]	50	25	50	Ruminants (swamp buffalo)	Flotation	Bali and Nusa Tenggara
Collet et al.[1986]	22	20	90.91	Non-human primates	Scotch-tape perianal smear	Java
Collet et al.[1986]	14	13	92.86	Non-human primates	Scotch-tape perianal smear	Java
Collet et al.[1986]	21	14	66.67	Non-human primates	Scotch-tape perianal smear	Kalimantan
Collet et al.[1986]	11	4	36.36	Non-human primates	Scotch-tape perianal smear	Kalimantan
Collet et al.[1986]	13	13	100	Non-human primates	Scotch-tape perianal smear	Sumatera
Collet et al.[1986]	8	6	75	Non-human primates	Scotch-tape perianal smear	Sumatera
Dwinata et al.[2018]	100	4	4	Ruminants (Bali cattle)	Flotation	Bali and Nusa Tenggara
Ekawasti et al.[2019]	289	37	12.8	Ruminant (beef cattle)	Sugar Flotation	Java
Fadli et al.[2014]	100	4	4	Ruminants (Bali cattle)	Flotation	Bali and Nusa Tenggara
Guna et al.[2014]	20	6	30	Pig	Necropsy	Papua
Guna et al.[2014]	10	0	0	Pig	Necropsy	Papua
Hasegawa et al.[1995]	49	20	40.82	Rodents	Necropsy	Maluku
Hasegawa et al.[1995]	49	28	57.14	Rodents	Necropsy	Maluku
Hasyim et al.[2019]	150	30	20.05	Ruminants (Bali cattle)	Flotation	Sulawesi
Joesoef et al.[2018]	50	7	14	Non-human primates	FECT	Bali and Nusa Tenggara
Joesoef et al.[2018]	50	7	14	Non-human primates	FECT	Bali and Nusa Tenggara
Kurniawati et al.[2020]	100	32	32	Non-human primates	Flotation	Java
Kusuma et al.[2021]	150	42	28	Non-human primates	Whitlock	Java

Labes et al.[2010]	163	62	38.04	Non-human primates	McMaster	Kalimantan
Labes et al.[2010]	61	20	32.79	Non-human primates	McMaster	Kalimantan
Labes et al.[2010]	38	22	57.89	Non-human primates	McMaster	Kalimantan
Mul et al.[2007]	54	44	81.48	Non-human primates	Ridley Method	Sumatera
Mul et al.[2007]	32	15	46.88	Non-human primates	Ridley Method	Sumatera
Mul et al.[2007]	19	9	47.37	Non-human primates	Ridley Method	Sumatera
Murdayasa et al.[2019]	50	24	48	Non-human primates	Flotation	Bali and Nusa Tenggara
Nasution et al.[2018]	30	1	3.33	Dog	Flotation	Java
Nugroho et al.[2016]	103	16	15.7	Pig	McMaster	Papua
Nugroho et al.[2016]	92	25	27.3	Pig	Flotation	Papua
Nurcahyo and Prastowo [2013]	171	41	24	Non-human primates	McMaster	Kalimantan
Nurcahyo and Prastowo [2013]	80	11	13.75	Non-human primates	McMaster	Kalimantan
Nurcahyo and Prastowo [2013]	75	11	14.67	Non-human primates	McMaster	Kalimantan
Nurhidayah et al.[2019]	340	10	2.94	Ruminants (swamp buffalo)	McMaster	Java
Oka and Dwinata [2011]	501	37	7.4	Pig	Sodium Acetic Formaldehid	Bali and Nusa Tenggara
Prasetyo [2016]	98	52	53.00	Rodents	Kato-Katz	Java
Purwaningsih and Sumiarto [2012]	1432	90	6.28	Ruminants (beef cattle)	Native	Java
Purwaningsih et al.[2017]	32	32	100	Ruminants (Kacang goat)	Flotation	West Papua
Purwaningsih et al.[2020]	120	37	30.43	Ruminants (beef cattle)	McMaster	West Papua
Purwati et al.[2021]	35	12	34	Ruminants (Bligon goat)	Native	Java
Rahmi et al.[2010]	25	2	8	Non-human primates	Flotation	Sumatera
Ridwan et al.[2018]	263	12	4.56	Ruminants (beef cattle)	McMaster	Java
Ridwan et al.[2018]	270	19	7.04	Ruminants (beef cattle)	McMaster	Java
Sajuri et al.[2017]	100	8	8	Ruminants (Bali cattle)	Flotation	Bali and Nusa Tenggara
Sawitri et al.[2019]	109	35	32.1	Ruminants (beef cattle)	Flotation	Java
Sismami et al.[2014]	15	9	60.03	Reptile	Sedimentation	Bali and Nusa Tenggara
Suastini et al.[2021]	150	4	2.7	Ruminants (Bali cattle)	Flotation	Bali and Nusa Tenggara
Suastini et al.[2021]	150	11	7.3	Ruminants (Bali cattle)	Flotation	Bali and Nusa Tenggara
Telnoni et al.[2016]	30	2	6.67	Reptile	Flotation	Bali and Nusa Tenggara
Wardhana et al.[2020]	196	12	6.1	Pig	Flotation	Java
Widisuputri et al.[2020]	50	13	26	Pig	Flotation	Bali and Nusa Tenggara
Widisuputri et al.[2020]	50	5	10	Pig	Flotation	Bali and Nusa Tenggara

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 2, Year: 2023

ETHICAL PRINCIPLES AND PUBLICATION POLICY

Kafkas Universitesi Veteriner Fakultesi Dergisi follows and implements internationally accepted ethical standards to provide the necessary support to original scientific ideas and to publish high quality, reliable scientific articles in this direction. The journal's publication policy and ethical principles include the ethical standards of conduct that should be followed by author(s), journal editor(s), associate editors, subject editors, reviewers, and publishers who are the participants of this action.

The ethical statement of Kafkas Universitesi Veteriner Fakultesi Dergisi is based on the principles indicated in the "COPE Code of Conduct and Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/Code_of_conduct_for_journal_editors_Mar11.pdf) and "COPE Best Practice Guidelines for Journal Editors" (http://publicationethics.org/files/u2/Best_Practice.pdf).

GENERAL ETHICAL PRINCIPLES

• Objectivity and Independence

Editor-in-chief, editors, associate editors, and referees conduct the evaluation process of the manuscript sent to the journal objectively and in coordination within the framework of ethical principles. Editorial decisions are independent, and internal or external factors cannot influence these decisions. In accordance with the principle of impartiality, academics working in our institution are not deemed eligible to work as a section editor in Kafkas Universitesi Veteriner Fakultesi Dergisi, in order not to be effective in the evaluation of articles due to conflict of interest.

• Privacy

The content of the articles and the personal information of the authors such as name, e-mail address, and telephone numbers that are sent to Kafkas Universitesi Veteriner Fakultesi Dergisi are used only for the scientific purposes of the journal and not for other purposes, and cannot be shared with third parties. Article evaluation processes are also carried out confidentially.

• Authorship and Authors Rights

The authors of the manuscripts sent to Kafkas Universitesi Veteriner Fakultesi Dergisi must have contributed significantly to the design, execution or interpretation of the study. For example, in view of the research and publication ethics as well as authors rights, it is not acceptable to include those as authors who do not actively contribute to the research but just only help in writing or data collection processes, which may not require any scientific knowledge. All the authors in a publication should be in agreement of the names and the orders of the authors in the manuscript.

The competence of the authors to the subject of the study is evaluated by the editor within the framework of deontological rules and the professional fields of each author.

The corresponding author of the article should declare the contributions of the authors to the work under the title of "Author contributions". The corresponding author is primarily responsible for the problems that may arise in this regard.

In multidisciplinary studies, 2 authors who are from different disciplines can be "equivalent first name authors" and up to most 3 authors who are also from different disciplines can be "equivalent second name authors".

• Originality of Research Findings

The authors should declare that the article they presented contained the original research results, that the study data were analyzed correctly, and that they were prepared for publication using adequate and appropriate references, in the "cover letter" section of the on-line system at the submission stage. Using expressions such as "it is the first study done", "there has been no previous study on this subject" and "there is a limited number of studies" to add originality and importance to the article is not acceptable and may cause prevention of the scientific evaluation of the article by the editor.

• Similarity

Articles submitted to the journal are subjected to similarity analysis using appropriate software (iThenticate by CrossCheck) at the beginning and at every required stage. If unethical similarities are detected regardless of the rate of similarity, this situation is reported to the authors and corrections are requested or articles containing excessive similarities are rejected at the first evaluation stage without being evaluated.

• Plagiarism/Self-Plagiarism, Duplicate Publication

Kafkas Universitesi Veteriner Fakültesi Dergisi applies publication ethics and verifies the originality of content submitted before publication and checks all submitted manuscripts for plagiarism/self-plagiarism, similarity and duplication. All submitted manuscripts are meticilously screened by a similarity detection software (iThenticate by CrossCheck). Papers previously presented at scientific meetings and published only as an "abstract" should be indicated in the Title Page file as stated in the "Guidance for Authors". Authors do not have the right to use entire paragraphs from their previous publications into a new submission. These actions are also considered as a plagiarism. In any case, the manuscript should be original in terms of scientific contents and writing. In the event of alleged or suspected research misconduct, the Editorial Board will follow and act in accordance with "COPE Guidelines".

• Multi-part Publication (Piecemeal Publication)

Some authors may tend to divide study data into two or more articles and publish the results in different journals also having different authors names and orders. In principal, Kafkas Universitesi Veteriner Fakultesi Dergisi is against multi-part publication. When necessary, the ethical committee approval information of the study, project information, congress presentations, etc. are checked and such situations that will create an ethical problem are identified and reported to the authors.

Authors may think that their work should be published in multi parts that complement each other. For this, each part of the article should be titled "Part-I", "Part-II" and submitted to the journal "simultaneously". This issue can be evaluated by the editor-in-chief/subject editors/ referees who may suggest that the article can be published in parts or as a whole. In addition, rejection of a submission presented in parts means that all parts will be rejected.

• Animal Rights and Ethics

The authors are responsible for conducting experimental and clinical studies on animal experiments within the framework of existing international legislation on animal rights. Authors must also obtain permission from the Animal Experiment Ethics Committees and provide relevant information in the Material and Method section to experiment with animals. In clinical studies, as well as the approval of the ethics committee, an "informed consent form" should be obtained from the animal owners and the information related to it should be declared in the Material and Method section. Declaration of "informed consent form" is sufficient for the articles in the "Case report" and "Letter to the Editor" category.

Ethics committee permission taken for a study can only be used in one article. It is unacceptable to use the same ethics committee approval number in articles with different names and contents. The editor/subject editors can request from the corresponding author, if necessary, to send a copy of the ethics committee approval form to the journal (electronically or by post).

In cases of violation ethical rules, the article is not taken into consideration or if it is in the evaluation stage, the procedure is terminated and the article is rejected.

• Conflicts of Interest/Competing Interests

The editor-in-chief pays attention to whether there is a conflict of interest or union of interest between editors, reviewers and author (s) for an objective and unbiased evaluation of the article. In addition, the authors should disclose any financial interests or links or any conditions that may raise the bias issue in research and article under the above heading.

• Copyright

Authors retain the copyright to their published work licensed under the Creative Commons Attribution-NonCommercial 4.0 International license (CC BY-NC 4.0) (https://creativecommons.org/licenses/by-nc/4.0/) and grant the Publisher non-exclusive right to publish the work. CC BY-NC 4.0 license permits unrestricted, non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

The authors must fill in the "Copyright Agreement Form" and sign it with a wet signature. Authors who submit articles from abroad should scan the signed form and send it to the editor via the system or by e-mail. Original forms that are wet signed for articles sent domestically should be submitted to the journal via mail or cargo. The works of the authors who do not submit the Copyright Agreement Form on time are not published.

• Withdrawal of a Submission

In case of if the authors detect a significant error or deficiency in their article under review or if this error is reported to them by the editor/ subject editor/referees they can contact immediately to the editor-in-chief and ask the request to withdraw the article by stating the reason. The decision on this issue is up to the editorial board.

• Erratum

After an article has been published, the corresponding author may request the editor to publish "erratum" for any errors or inaccuracies noticed by the authors, editors or readers. In collaboration with the authors, the editor prepares and publishes the Erratum article in the first upcoming issue. These articles, like other publications, should contain the publication tag and DOI number.

• Retraction

If any ethical problem is detected about the article that cannot be compensated and cannot be eliminated with erratum after the article is published, the editor-in-chief and associate editors prepare a justification about the article and apply the retraction procedure to the article. The text file on the web page of a retracted article is blocked and the reason for retraction is added to the system as a file, ensuring that it is constantly in the archive.

• Advertising

Kafkas Universitesi Veterinar Fakultesi Dergisi do not accept advertising and sponsorships that are believed to create a potential conflict of interest. If the article sent to Kafkas Universitesi Veterinar Fakultesi Dergisi is for the promotion of a commercial product and/or the work carried out is directly supported by a company, it is rejected without consideration.

OPEN ACCESS STATEMENT

Kafkas Universitesi Veterinar Fakultesi Dergisi is an open access publication. The journal's publication model is based on Budapest Open Access Initiative (BOAI) declaration. Articles published in Kafkas Universitesi Veterinar Fakultesi Dergisi are available online, free of charge at https://vetdergikafkas.org/archive.php.

ARTICLE EVALUATION AND PUBLICATION PROCESS

• Initial Evaluation Process

Articles submitted to Kafkas Universitesi Veterinar Fakultesi Dergisi are primarily evaluated by the editors and associate editors. At this stage, articles not having suitable scope and aims, with low original research value, containing scientific and ethically important errors, having low potential to contribute to science and the journal, and having poor language and narration are rejected by the editor without peer-review process. Initial evaluation process takes up to most 2 weeks.

• Preliminary Evaluation Process

Articles that are deemed appropriate for editorial evaluation are sent to the subject editor related to the category of articles to be examined in terms of scientific competence and to the statistics editor for evaluation in terms of statistical methods. The suject editors examine the article in all aspects and report their decisions (rejection, revision or peer-review) to the chief editor. This stage takes about 1 month.

• Peer-review Process

Double-blind peer-review is applied to the articles that have completed preliminary evaluation process. Suggestions of subject editors are primarily considered in referee assignment. In addition, reviews can be requested from the referees registered in the journal's referee pool. At least 2 referees are assigned for peer-review. Opinion of more referees can be required depending on the evaluation process. At this stage, referees send their decision (reject, revision or accept) about the article to the editor-in-chief. If the rejection decision given by a referee reflects sufficient examination and evidence-based negativities or ethical problems about the scientific content and accuracy of the article, this decision is checked by the editor-in-chief and associate editors and submitted to the authors regardless of the other referees' decisions. The time given to referees to evaluate an article is ~4 weeks.

• Publication Process of an Article

Total evaluation period of an article, which is completed in the peer-review phase after completing the initial and preliminary evaluation process, takes 4-6 months. The articles that have completed the subject editorial and peer-review evaluation stages and accepted by the editorial are sent to the corresponding author for final checks and necessary final additions. After the acceptance, the article designed in the publication format of the journal is given an DOI number and published immediately on the Article in Press page. When it is time to publish the periodic edition of the journal, a selection is made from the articles kept on the Article in Press page, taking into account the submission date. The time it takes for the article to be published by taking the page number is 6-12 months.

NO PUBLICATION FEE

Processing and publication are free of charge with the journal. There is no article processing char-ges, submission fees or any other fees for any submitted or accepted articles.

RESPONSIBILITIES OF THE PUBLISHER, EDITORS AND ASSOCIATE EDITORS

The publisher (Dean of the Faculty of Veterinary Medicine of Kafkas University) contributes to the execution of the journal's routine processes such as printing, archiving, and mailing, in line with requests from the editor.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and assistants in the article evaluation process and decisions.

The publisher undertakes to carry out an independent and fair decision-making mechanism for its editors and associate editors in the article evaluation process and decisions.

Editor-in-chief/editors/associate editors of Kafkas Universitesi Veteriner Fakultesi Dergisi evaluate the articles submitted to the journal regardless of their race, gender, religious belief, ethnicity, citizenship or political views. In addition, it undertakes not to give any information about the article except for the authors, subject editors and referees.

Kafkas Universitesi Veteriner Fakultesi Dergisi follows internationally accepted principles and criteria and takes the necessary decisions to apply in the journal.

Editor-in-chief/editors/associate editors conduct the evaluation and decision process in the journal in coordination within the principles of confidentiality and have independent decision-making authority and responsibility without being affected by any internal or external factors.

Editor-in-chief/editors/associate editors make and implement all kinds of planning for the development of the journal and its international recognition. They also follow national and international meetings or events on the development of journals and article evaluation, and ensures that the journal is represented on these platforms.

The editor-in-chief/editors/associate editors make every effort to ensure that the journal's subject editors and referee pool have international qualifications. Likewise, it makes the necessary attempts to strengthen the author's profile.

Editor-in-chief/editors/associate editors make plans to improve the quality of the articles published in the journal and carry out the necessary process.

Editor-in-chief/editors/associate editors regularly conduct and control the initial evaluation, preliminary evaluation, peer review and acceptance-rejection decisions of articles submitted to the journal. While carrying out these procedures, features such as the suitability of the study for the aims and scope of the journal, its originality, the up-to-date and reliability of the scientific methods used, and the potential it will contribute to the development of the journal as well as its benefit to science/practice are taken into consideration.

Editor-in-chief/editors/associate editors systematically review, inspect and make decisions about the articles submitted to the journal in terms of features such as author rights, conflict of interest, observance and protection of animal rights, and compliance with research and publication ethics.

The editor-in-chief conducts the evaluation/revision process between the authors and subject editors and referees, and ensures that it is completed within the prescribed time.

ARCHIVE POLICY

The editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi and the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) keep all the articles (electronic and printed) published in the journal in their archives. All articles and their attachment files sent to the journal are kept securely in the archive. In light of the technological developments, the editorial office of the Kafkas Üniversitesi Veteriner Fakültesi Dergisi regularly performs electronic processes for the development and updating of materials in digital environment and presents them to its readers on condition of keeping in safe the original documents and information regarding the articles.

Even if the journal ceases to be published for any reason, the publisher (Dean's Office of the Faculty of Veterinary Medicine, Kafkas University) will continue to protect the journal content in the long term and provide convenient access to users. Electronic services of Kafkas University Information Technologies Department will be used for the journal to maintain this responsibility.

RESPONSIBILITIES OF SUBJECT EDITORS

Subject editors do reviews and evaluations in accordance with the main publication goals and policies of the journal and in line with the criteria that will contribute to the development of the journal.

Author information is kept confidential in articles sent to the subject editor for preliminary evaluation by the editor.

Subject editors thoroughly examine the sections of the introduction, materials and methods, results, discussion and conclusion, in terms of journal publication policies, scope, originality and research ethics. Subject editor submits its decision (rejection, revision or peer-review) after evaluation to the chief editor in a reasoned report.

Subject editor may request additional information and documents related to the study from the authors, when necessary.

In multidisciplinary studies, the article can be submitted for the evaluation of multiple subject editors.

RESPONSIBILITIES OF REFEREES

Double-blinded peer-review procedure is applied in Kafkas Universitesi Veteriner Fakultesi Dergisi in order to evaluate the articles submitted to the journal in accordance with the principle of impartiality and in objective criteria; that is, referees and writers do not know about each other.

The referees submit their opinions and reports to the editor-in-chief to ensure the control and suitability of a submitted article, its scientific content, scientific consistency and compliance with the principles of the journal. When a referee makes a decision "reject" about an article, he/she prepares the reasons for the decision in accordance with the scientific norms and presents it to the editor.

The referee(s) also gives the authors the opportunity to improve the content of the article. Accordingly, the revisions requested from the authors should be of a quality that explains/questions specific issues rather than general statements.

Referees appointed for the evaluation of the articles agree that the articles are confidential documents and will not share any information about these documents with third parties, except for the editors participating in the evaluation.

Referees should place their criticism on scientific infrastructure and write their explanations based on scientific evidence. All comments made by the referees to improve the articles should be clear and direct, and should be written away from disturbing the feelings of the author. Insulting and derogatory statements should be avoided.

If a referee has an interest relationship with the author(s) on one or more issues, he/she must report the situation to the editor and ask his/her to withdraw from the referee position. The same is also applicable when the authors illegally obtain information about the referees of the article and try to influence them.

The editor-in-chief can share the comments and reports from the referees with the editors/associate editors and the relevant subject editor, as necessary, to ensure that the decision on the article is optimal. If necessary, the editor may share the critical decision and its grounds that a referee has sent about the article with the other referee(s) and present them to their attention.

Referee(s) may request revision many times for the article they evaluated.

The content of the referee reports is checked and evaluated by editor-in-chief/editors/associate editors. The final decision belongs to the editorial.

RESPONSIBILITIES OF AUTHOR(S)

It is not tolerable for the author (s) to send an article, which has been already sent to another journal, to Kafkas Universitesi Veteriner Fakultesi Dergisi within the scope of "which accepts" or "which publishes first" approach. If this is detected, the article is rejected at any stage of the evaluation. As a possible result of these actions, in the process following the previous acceptance of the article sent to another journal, the withdrawal request with this excuse that the authors submit for this article, the evaluation process of which is going on in our journal, is evaluated by the editors and associate editors of the journal and disciplinary action on the grounds of ethical violations about those responsible is started. This unethical action is also informed to the journal editor (if known) who accepted the article.

It is essential that the articles to be sent to Kafkas Universitesi Veteriner Fakultesi Dergisi include studies that have up-to-date, original and important clinical/practical results and prepared in accordance with the journal's writing rules.

Authors should choose the references they use during the writing of the article in accordance with the ethical principles and cite them according to the rules.

The authors are obliged to revise the article in line with the issues conveyed to them during the initial evaluation, preliminary evaluation and peer-review phases of the article and to explain the changes they made/did not make sequentially in the "response to editor" and "response to reviewer comments" sections.

If information, documents or data regarding to the study are requested during the evaluation process, the corresponding author is obliged to submit them to the editorial.

Authors should know and take into account the issues listed in the "General Ethical Principles" section regarding scientific research and authors.

The authors do not have the right to simultaneously submit multiple articles to Kafkas Universitesi Veteriner Fakultesi Dergisi. It is more appropriate to submit them with acceptable time intervals for the journal's policy.

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
Volume: 29, Issue: 2, Year: 2023

INSTRUCTION FOR AUTHORS

1- Kafkas Universitesi Veteriner Fakultesi Dergisi (abbreviated title: Kafkas Univ Vet Fak Derg), published bi-monthly (ISSN: 1300-6045 and e-ISSN: 1309-2251). We follow a double-blind peer-review process, and therefore the authors should remove their name and any acknowledgment from the manuscript before submission. Author names, affiliations, present/permanent address etc. should be given on the title page only.

The journal publishes full-length research papers, short communications, preliminary scientific reports, case reports, observations, letters to the editor, and reviews. The scope of the journal includes all aspects of veterinary medicine and animal science.

Kafkas Universitesi Veteriner Fakultesi Dergisi is an Open Access journal, which means that all content is freely available without charge to the user or his/her institution. Users are allowed to read, download, copy, distribute, print, search, or link to the full texts of the articles, or use them for any other lawful purpose, without asking prior permission from the publisher or the author. This is in accordance with the BOAI definition of Open Access.

The official language of our journal is English. Additionally, all the manuscripts must also have Turkish title, keywords, and abstract (translation will be provided by our journal office for foreign authors).

2- The manuscripts submitted for publication should be prepared in the format of Times New Roman style, font size 12, A4 paper size, 1.5 line spacing, and 2.5 cm margins of all edges. The legend or caption of all illustrations such as figure and table and their appropriate position should be indicated in the text. Refer to tables and figures in the main text by their numbers. Also figure legends explanations should be given at the end of the text.

The figures should be at least 300 dpi resolution.

The manuscript and supplementary files (figure etc.) should be submitted by using online manuscript submission system at the address of http://vetdergi.kafkas.edu.tr/

During the submission process, the authors should upload the figures of the manuscript to the online manuscript submission system. If the manuscript is accepted for publication, the Copyright Agreement Form signed by all the authors should be sent to the editorial office.

3- The authors should indicate the name of the institute approves the necessary ethical commission report and the serial number of the approval in the material and methods section. If necessary, the editorial board may also request the official document of the ethical commission report. In case reports, a sentence stating that "informed consent" was received from the owner should be added to the main document. If an ethical problem is detected (not reporting project information, lack of ethical committee information, conflict of interest, etc.), the editorial board may reject the manuscript at any stage of the evaluation process.

4- Authors should know and take into account the issues listed in the "Ethical Principles and Publication Policy" section regarding scientific research and authors.

5- Types of Manuscripts

Original (full-length) manuscripts are original and proper scientific papers based on sufficient scientific investigations, observations and experiments.

Manuscripts consist of the title, abstract and keywords, introduction, material and methods, results, discussion, and references and it should not exceed 12 pages including text. The number of references should not exceed 50. The page limit does not include tables and illustrations. Abstract should contain 200±20 words.

Short communication manuscripts contain recent information and findings in the related topics; however, they are written with insufficient length to be a full-length original article. They should be prepared in the format of full-length original article but the abstract should not exceed 100 words, the reference numbers should not exceed 15 and the length of the text should be no longer than 6 pages in total. The page limit does not include tables and illustrations. Additionally, they should not contain more than 4 figures or tables.

Preliminary scientific reports are a short description of partially completed original research findings at an interpretable level. These should be prepared in the format of full-length original articles. The length of the text should be no longer than 4 pages in total.

Case reports describe rare significant findings encountered in the application, clinic, and laboratory of related fields. The title and abstract of these articles should be written in the format of full-length original articles (but the abstract should not exceed 100 words) and the remaining sections should be followed by the Introduction, Case History, Discussion and References. The reference numbers should not exceed 15 and the length of the text should be no longer than 4 pages in total. The page limit does not include tables and illustrations.

Letters to the editor are short and picture-documented presentations of subjects with scientific or practical benefits or interesting cases. The length of the text should be no longer than 3 pages in total. The page limit includes tables and illustrations.

Reviews are original manuscripts that gather the literature on the current and significant subject along with the commentary and findings of the author on a particular subject (It is essential that the author/s have international scientific publications on this subject). The title and summary of this manuscript should be prepared as described for the full-length original articles and the remaining sections should be followed by introduction, text (with appropriate titles), conclusion, and references.

"Invited review" articles requested from authors who have experience and recognition in international publishing in a particular field are primarily published in our journal.

Review articles submitted to our journal must be prepared in accordance with any of the three categories listed below.

Narrative reviews describe current published information on a scientific topic. However, it does not include a specific methodological process.

Systematic reviews include the search for original studies published in that field on a specific topic, the evaluation of validity, synthesis and interpretation within a systematic methodology.

Meta-analysis is a method of evaluating the results of many studies on a subject with the methods defined in this category and statistical analysis of the obtained findings.

6- The necessary descriptive information (thesis, projects, financial supports, etc.) scripted as an italic font style should be explained below the manuscript title after placing a superscript mark at the end of the title.

7- At least 30% of the references of any submitted manuscript (for all article categories) should include references published in the last five years.

References should be listed with numerical order as they appear in the text and the reference number should be indicated inside the parentheses at the cited text place. References should have the order of surnames and initial letters of the authors, title of the article, title of the journal (original abbreviated title), volume and issue numbers, page numbers and the year of publication and the text formatting should be performed as shown in the example below.

Example: Yang L, Liu B, Yan X, Zhang L, Gao F, Liu Z: Expression of ISG15 in bone marrow during early pregnancy in ewes. *Kafkas Univ Vet Fak Derg*, 23 (5): 767-772, 2017. DOI: 10.9775/kvfd.2017.17726

If the reference is a book, it should follow surnames and initial letters of the authors, title of the book, edition number, page numbers, name and location of publisher and year of publication. If a chapter in a book with an editor and several authors is used, names of chapter authors, name of chapter, editors, name of the book, edition number, page numbers, name and location of publisher and year of publication and the formatting should be performed as shown in the example below.

Example: Mcllwraith CW: Disease of joints, tendons, ligaments, and related structures. **In,** Stashak TS (Ed): Adam's Lameness in Horses. 4th ed., 339-447, Lea and Febiger, Philadelphia, 1988.

DOI number should be added to the end of the reference.

In the references can be reached online only, the web address and connection date should be added at the end of the reference information. The generally accepted scientific writing instructions must comply with the other references. Abbreviations, such as "et al" and "and friends" should not be used in the list of the references.

Follow the link below for EndNote Style of Kafkas Universitesi Veteriner Fakultesi Dergisi;
https://researchsoftware.com/downloads/journal-faculty-veterinary-medicine-kafkas-university

8- Latin expression such as species names of bacteria, virus, parasite, and fungus and anatomical terms should be written in italic character, keeping their original forms.

9- The editorial board has the right to perform necessary modifications and a reduction in the manuscript submitted for publication and to express recommendations to the authors. The manuscripts sent to authors for correction should be returned to the editorial office within a month. After pre-evaluation and agreement of the submitted manuscripts by the editorial board, the article can only be published after the approval of the field editor and referee/s specialized in the particular field.

10- All responsibilities from published articles merely belong to the authors. According to the ethical policy of our journal, plagiarism/self-plagiarism will not be tolerated. All manuscripts received are checking by plagiarism checker software, which compares the content of the manuscript with a broad database of academic publications.

11- There is no copyright fee for the authors.

12- The authors are charged a fee on acceptance of the manuscript to cover printing costs and other expenses. This payment information can be found at http://vetdergi.kafkas.edu.tr/

SUBMISSION CHECKLIST

Please use below list to carry out a final check of your submission before you send it to the journal for review. Ensure that the following items are present in your submission:

- Cover letter

• Importance and acceptability of the submitted work for the journal have been discussed (Please avoid repeating information that is already present in the abstract and introduction).

• Other information has been added that should be known by the editorial board (e.g.; the manuscript or any part of it has not been published previously or is not under consideration for publication elsewhere.

- Title page

• Title, running title (should be a brief version of the title of your paper, no exceed 50 characters)

• The author's name, institutional affiliation, Open Researcher and Contributor ID (ORCID)

• Congress-symposium, project, thesis etc. information of the manuscript (if any)

• Corresponding author's address, phone, fax, and e-mail information

- Manuscript

• Title, abstract, keywords and main text

• All figures (include relevant captions)

• All tables (including titles, description, footnotes)

• Ensure all figure and table citations in the text match the files provided

• Indicate clearly if color should be used for any figures in print

- Availability of Data and Materials

- Acknowledgements

- Funding Support

- Competing Interests

- Authors' Contributions

Further considerations

• Journal policies detailed in this guide have been reviewed

• The manuscript has been "spell checked" and "grammar checked"

• Relevant declarations of interest have been made

• Statement of Author Contributions added to the text

• Acknowledgment and conflicts of interest statement provided