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

Yönetim Yeri
Adres / *Address*

Ankara Üniversitesi Veteriner Fakültesi Dekanlığı
Yayın Alt Komitesi
06 110 Ankara, Türkiye
Tel: 90 312 317 03 15, **Fax:** 90 312 316 44 72
Sürdürüm ve iletişim: vfdergi@veterinary.ankara.edu.tr
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Prevalence and rapid identification of *Salmonella* Infantis in broiler production in Turkey

Özlem Şahan YAPICIER[1,a,✉], Barış SAREYYÜPOĞLU[2,b]

[1]Mehmet Akif Ersoy, Faculty of Veterinary Medicine University Department of Microbiology, Burdur, TURKEY; [2]Ankara University, Faculty of Veterinary Medicine, Department of Microbiology, Dışkapı, Ankara, TURKEY
[a]ORCID: 0000-0003-3579-9425; [b]ORCID: 0000-0002-2212 2610

✉Corresponding author: ozlemsahan@mehmetakif.edu.tr
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Abstract: In the present study, the prevalence of *Salmonella enterica* subsp. *enterica* serovar Infantis (*S.* Infantis) and other serovars were investigated in samples collected from commercial broiler chicken flocks in Turkey according to the ISO 6579:2002/Amd 1:2007, Annex D, standard method. Furthermore, previously developed *S.* Infantis-specific polymerase chain reaction (PCR)-based methods with primers targeting *fljB, fliC*, *IMP1-IMP2-IMP3* and *sinI* were conducted in different media (BPW, MRSV, MKTTN, XLD, and XLT_4 agars) and during four incubation stages (6, 12, 18, and 24 h) of the ISO 6579 procedure to develop rapid and reliable diagnosis method. One-hundred thirty-three (15.6%) *Salmonella* strains were isolated from a total of 848 samples (240 cecal swabs, 200 cloacal swabs, 190 intestinal contents, 59 feed, 39 dust, and 120 litter). The serovar distribution of isolated strains was as follows: *S.* Infantis, 88.70%; *S.* Agona, 2.3%; *S.* Kentucky 1.50, *S.* Hadar 1.50, and *S.* Tennessee 1.50; *S.* Mbandaka 0.75 %, *S.* Montevideo 0.75 %, *S.* Enteritidis 0.75 %, *S.* Adelaide 0.75 %, *S.* Liverpool 0.75 %, and *S.* Derby 0.75 %. Primers targeting *fljB, fliC*, and *IMP1-IMP2-IMP3* were not able to detect all *S.* Infantis isolates, therefore, a novel PCR technique was developed and validated in the study. It was concluded that it is a fast, reproducible and low-cost alternative to the gold standard method by detecting the *S.* Infantis isolates on the 3rd day at the earliest by PCR (*sinI* PCR). using primers specific to *S.* Infantis species.
Keywords: Broilers, ISO 6579, PCR, *Salmonella Infantis*.

Türkiye'deki broyler üretiminde *Salmonella* Infantis'in yaygınlığı ve hızlı tanısı

Özet: Bu çalışmada, Türkiye'de bulunan ticari tavuk sürülerinden toplanan toplam 848 örnek (240 sekal ve 200 kloakal svap, 190 bağırsak içeriği, 59 yem, 39 toz ve 120 altlık), ISO 6579: 2002 / Amd 1: 2007, Ek D standart yöntemine göre *S.* Infantis ve diğer serovarların prevalansı yönünden araştırıldı. Buna ek olarak, hızlı ve güvenilir bir teşhis amacıyla, *S.* Infantis'e özgü genleri (*fljB, fliC, IMP1-IMP2-IMP3* ve *sinI*) hedefleyen PCR temelli metodlar (PCR ve multipleks PCR), ISO 6579 prosedürünün farklı inkübasyon aşamaları (6, 12, 18 ve 24 saat) ve ortamlarında (BPW, MSRV, MKTTN, XLD ve XLT_4 agarları) kullanıldı. Toplam 848 örnekten 133(%15,6) *Salmonella* suşu izole edildi. İzole edilen suşların serovar dağılımı, *S.* Infantis % 88,70; *S.* Agona % 2,3; *S.* Kentucky % 1,50, *S.* Hadar % 1,50 ve *S.* Tennessee % 1,50; *S.* Mbandaka % 0,75, *S.* Montevideo % 0,75, *S.* Enteritidis % 0,75, *S.* Adelaide % 0,75, *S.* Liverpool % 0,75 ve *S.* Derby % 0,75'dir. Bu çalışmada, *fljB, fliC* ve *IMP1-IMP2-IMP3* primerleri tüm *S.* Infantis izolatlarını tespit edemedi ve bu nedenle yeni bir PCR tekniği geliştirilip validasyonu yapıldı. *S.* Infantis türüne spesifik primerlerler yapılan (*sinI* PCR) PCR ile *S.* Infantis izolatlarını en erken 3. günde tespit ederek altın standart yönteme alternatif hızlı, tekrarlanabilir ve düşük maliyetli metot olduğu sonucuna varıldı.
Anahtar sözcükler: Broyler, ISO 6579, PCR, *Salmonella* Infantis.

Introduction

Salmonella agents cause the most common foodborne diseases in the world (48). Some serotypes show host-specific characteristics, but the vast majority cause cross-species infections. Hence, warm-blooded animal origin serotypes are considered as potential pathogens for humans (37). *Salmonella* nomenclature is complex in the Enterobacteriaceae family, and there are more than 2500 serotypes according to the Kauffman-White scheme (20).

S. enterica subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) and *S. enterica* subsp. *enterica* serovar Enteritidis (*S.* Enteritidis) are the most common serotypes leading to Salmonellosis in poultry (9, 12, 44). However, in the past two decades, studies in developing countries also in Turkey have reported the dominance of these two

serotypes has gradually decreased, while the frequency of other serotypes, especially *Salmonella enterica* subspecies *enterica* serovar Infantis (*S*. Infantis), have increased (3, 24, 46). Surveys conducted by the European Food Safety Authority (EFSA) have shown that the isolation rates for *S.* Infantis originating from broiler chickens and carcasses in the EU, have recently been emerging (9-11). According to the National Salmonella Control Program, *S*. Infantis is the most frequently isolated serovar in Turkey (30).

Diagnosis of *Salmonella* infections is based on ISO 6579:2002/Amd 1:2007, Annex D, standard methods and this procedure takes approximately 11 days to complete (17-19). Gold standard *Salmonella* detecting method may result in an increased false-negative rate, which under an on-farm hazard analysis critical control point program would lead to no action when a corrective action is required (25). Therefore, many laboratories around the world have supported molecular methods to shorten this method, which requires intensive labour and experienced staff. During the past decade, there have been many advances in the molecular detection of *Salmonella*, especially polymerase chain reaction (PCR)-based methods [e.g., conventional and real-time] (19, 28), and several previous studies have used several genes, such as *invA*, *invE*, *himA*, *phoP* (23, 37) as targets for PCR investigation of *Salmonella* DNA in natural environmental and faecal samples.

Routine PCR-based testing and identification of *Salmonella* in diagnostic and microbiology laboratories must be rapid, reliable, and cost-effective (6, 22). *Salmonella* PCR assays have been combined with pre-enrichment, nonselective, and/or selective enrichment stages required to improve sensitivity, elimination, and identification of PCR-inhibitory substances. Therefore, many have been successful in detecting *Salmonella* DNA following a minimum 6 to 8 h or 24 to 30 h precultivation step (15, 33, 42-44). The present study investigated the prevalence of *S.* Infantis and other serovars in samples (intestinal content, feed, dust, litter, cecal and cloacal swabs) from commercial broiler chicken flocks and different field materials in Turkey. In addition, *S.* Infantis-specific PCR-based techniques with primers targeting different genes using different media during four incubation stages of the ISO 6579 procedure were used to develop an alternative molecular diagnostic method that is rapid and reliable.

Materials and Methods

Salmonella strains: To investigate the specificity of PCR assays, Infantis, Liverpool, Enteritidis, Mbandaka, Typhimurium, Gallinarum, Heidelberg, Agona, Newport, Stanleyville, Hadar, Colombo, Muenchen, Kentucky, Virchow, and Anatum serovars were used as target control strains and obtained from Ankara University Veterinary Faculty, Department of Microbiology culture collection. Strains were kept at 4 °C in stock agar, inoculated in tryptic soy broth (TSB), and incubated for 18–24 h at 37 °C prior to PCR.

Sampling procedure: A total of 848 samples (240 cecal swabs, 200 cloacal swabs, 190 intestinal contents, 59 feed, 39 dust, and 120 litter) were collected from 27–38-day-old broilers in 238 broiler houses (11,000-90,000 poultry capacity) and 48 slaughterhouses in three different regions (Southeastern Anatolia, Marmara and Black Sea) of Turkey. Each broiler house was traversed in a zigzag pattern to ensure random collection of litter samples around feed lines, water lines, and side areas using sterile drag swabs. Sterile sponge swabs were used for random sampling of dust across feeders, drinkers, and walls of each poultry house. At least 25 g of feed samples were collected in sterile sampling bottles. Cloacal swabs were collected from recently deceased animals in farms using Amies transport medium. Intestinal contents were collected in faecal cups, and cecal swabs were collected in Whirl-Pak bags after evisceration in the slaughterhouses. At least five cloacal and cecal swabs were pooled in sterile bags. All samples were cooled to 4-8 °C in an icebox and immediately transported to the *Salmonella* Research Laboratory in the Department of Microbiology, Faculty of Veterinary Medicine, Ankara University for processing.

Isolation and Identification: All samples were analysed for *Salmonella* using ISO 6579:2002/Amd 1:2007. Specifically, samples were inoculated in buffered peptone water (BPW) as pre-enrichment medium and incubated at 37 °C for 18–24 h. After incubation, samples were transferred to Muller-Kauffmann tetrathionate-novobiocin broth (MKTTN) and modified semi-solid Rappaport-Vassiliadis (MSRV) medium and enriched for 18–24 h at 37 °C and 24 h at 41.5 °C, respectively (16, 17, 19). The cultures obtained were plated onto xylose lysine deoxycholate (XLD) and xylose lysine Tergitol-4 (XLT_4), incubated at 37°C, and examined after 24 h (16, 17, 19). All presumptive *Salmonella* colonies were characterized biochemically (triple sugar iron (TSI), H_2S, gas formation, Voges Proskauer (VP), urea, lysine decarboxylase, and β-galactosidase tests) (16, 17, 19-21).

Serotyping: The serogroup and serotyping of the strains that are biochemically compatible with *Salmonella* spp. were made by slide agglutination using polyvalent and monovalent *Salmonella* "O" and "H" antisera (Statens Serum Institut, Denmark and Denka Seiken, Japan) and serotyped according to the Kauffman-White scheme (16).

DNA extraction: Samples were taken at 6, 12, 18, and 24 h during incubation at different stages of the ISO 6579 method (before inoculation, pre-enrichment, and selective enrichment) (Figure 1). DNA extraction was performed according to the instructions of the GeneJET Genomic DNA Purification Kit and QIAamp DNA Stool Kit. DNA for use as template DNA was stored at -20 °C until amplification.

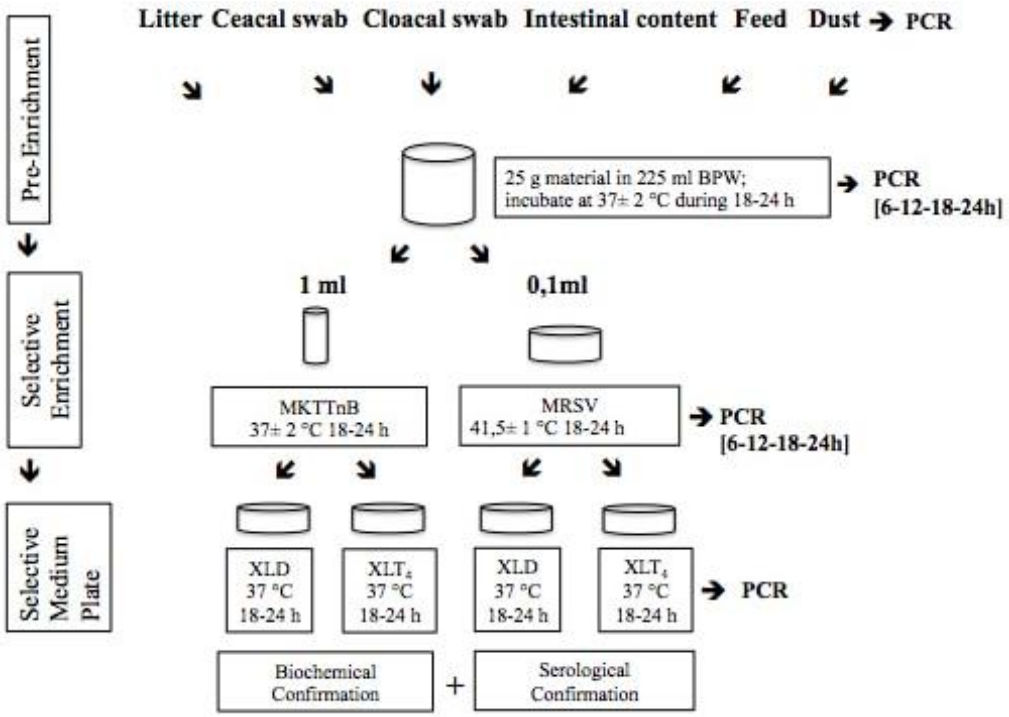

Figure 1. Flowchart of the diagnostic method of *S.* Infantis.

Table 1. Primer used for conventional PCR determination of *S.* Infantis.

NCBI accession no.	Primer name	Primer sequence (5'- 3')	Amplicon size (bp)
J03391.1	*sinI*	CGTTGCGTGAAACCATAACT CTTACGACGAGTTGCATGGG	201

Sequences: For specific detection of *S.* Infantis DNA in field samples, *fljB*, *fliC* (17, 31), and *IMP1-IMP2-IMP3* (1) primers were used in conventional and multiplex PCR, respectively, using methods described previously (1). An additional *invA* PCR was used as a confirmatory test for detection of *Salmonella* (43).

Primer design and PCR assay optimization: A new primer pair specific to a 201-bp sequence of *sinI* (*S.* Infantis modification methylase gene; Accession no. J03391.1) of *S.* Infantis (Table 1) was designed using Primer 3 software (41). PCR amplification was optimized and the PCR assay was carried out in a 25 µL reaction solution containing 3 µL of MgCl (25 mM), 0.5 µL dNTP (10 mM), 10 pmol of primers, and 0.2 µL Taq polymerase (5 U/µL). The following cycling conditions were used: 3 min at 94 °C, followed by 30 cycles of 1 min at 94 °C (denaturation) and 1 min at 54 °C (primer annealing), 1 min at 72 °C (extension), and 7 min at 72 °C (final extension).

Specificity and detection limit: The detection limit was determined using a 10-fold serial dilution of a broth culture (in BPW incubated for 24 h at 37 °C) of one of the *S.* Infantis strains sequenced for primer design. The CFU number was determined by quantitative culture of these dilutions (26).

Detection of S. Infantis in naturally contaminated samples: The validity and reliability of the *sinI* PCR assay for *S.* Infantis was tested to confirm that the method was a rapid alternative to the reference culture method. Naturally contaminated samples (n = 16) consisted of 5 litter, 4 cecum swabs, 1 cloaca swab, 1 intestinal content, 3 feed, and 2 dust samples collected from broiler houses (two broiler houses from two different geographic locations) with a previous history of being *S.* Infantis-positive and -negative and examined according to ISO 6579 (16, 17, 19) and PCR in parallel. Samples were taken at 6, 12, 18, and 24 h during incubation at different stages (initial, pre-, and selective enrichment) of the culture method for PCR analysis as described above. The results of ISO 6579 and PCR methods were compared.

Results

Isolation and identification: A total of 133 (15.6%) *Salmonella* strains were isolated from litter 56/120 (46.7%), feed 23/59 (39%), cloacal swabs 21/40 (52.5%), cecal swabs 15/240 (6.25%), intestinal content 14/190

(7.47%), and dust 4/39 (10.25%). We identified 11 different *Salmonella* serotypes from the 133 *Salmonella* isolates. The most common serotype was *S.* Infantis 118 (88.70%) followed by *S.* Agona 3 (2.3%); *S.* Kentucky 2 (1.50%), *S.* Hadar 2 (1.50%), and *S.* Tennessee 2 (1.50%); and *S.* Mbandaka 1 (0.75%), *S.* Montevideo 1 (0.75%), *S.* Enteritidis 1 (0.75%), *S.* Adelaide 1 (0.75%), *S.* Liverpool 1 (0.75%), and *S.* Derby 1 (0.75%). *S.* Infantis was isolated from all sample types; *S.* Kentucky, S. Montevideo, *S.* Mbandaka, and *S.* Enteritidis were isolated from litter; *S.* Agona was isolated from litters and intestinal contents; *S.* Hadar and *S.* Adelaide were isolated from cecal swabs; and *S.* Liverpool and *S.* Derby were isolated from feed. The obtained isolation rate of *S.* Infantis was 39.16% (47/120) in litters, 10.25% (4/39) in dust, 10.5% (21/200) in cecal swabs, 6.84% (13/190) in intestinal content, and 35.59% (21/59) in feed.

Determination of PCR assay specificities: In studies determining the specificity of conventional and multiplex PCR techniques, *fljB* and *fliC* primers were not able to detect all *S.* Infantis isolates, nonspecific band profiles were observed, and *IMP1-IMP2-IMP3* primers gave common bands with *S.* Infantis, *S.* Liverpool, *S.* Enteritidis, *S.* Mbandaka, *S.* Typhimurium, and *S.* Gallinarum. Because these results conflicted, new primers for *S. Infantis* DNA detection were designed. Conventional PCR was performed on a total of 21 *Salmonella* serotypes [*S.* Infantis (n = 6); *S.* Typhimurium (n = 2); *S.* Heidelberg, *S.* Agona, *S.* Newport, *S.* Stanleyville, *S.* Hadar, *S.* Mbandaka, *S.* Colombo, *S.* Muenchen, *S.* Kentucky, *S.* Enteritidis, *S.* Virchow, *S.* Anatum, and *S.* Mbandaka (all n = 1)] with novel *sinI*F and *sinI*R primers. Designed primers amplified all *S.* Infantis serotypes, while no PCR product was obtained with other serotypes (Figure 2). The diagnostic specificity was accepted to be 100%, as no false-negative or -positive results were obtained from the PCR.

S. Infantis PCR detection limit: The detection limit of *S.* Infantis following optimization of the PCR assay was 1×10^3 CFU/mL.

Salmonella detection using invA: *InvA* primers (605-bp DNA fragments) could be amplified from all *Salmonella* isolates tested by conventional PCR. Therefore, all *Salmonella* isolates were detected at a genus level.

Detection of S. Infantis in naturally contaminated samples: A total of 7 (2 litter, 1 cecal swabs, 1 cloacal swab, 1 feed, 1 intestinal content, and 1 dust sample) out of 16 naturally contaminated samples gave positive results for *S.* Infantis by both culture and PCR methods. PCR performed during the pre-enrichment and selective enrichment stages, it was determined that the *S.* Infantis rates detected by the design primer at the 6, 12 and 18th hours varied between 0-42.85% and 28.57-57.14% in the sampling performed at the 24th hour. On the 3rd day of isolation *Salmonella*-suspected colonies occurred in XLD and XLT_4. The *sinI* PCR assay detected 100% of culture-positive *S.* Infantis (Figure 3) correctly on day 3, without false-positive or -negative test results (Table 2).

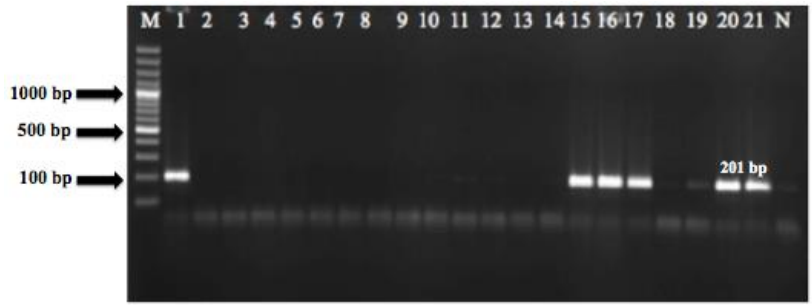

Figure 2. PCR assay was performed using *sinIF and sinIR* primers (M: 100bp marker, 1, 15-17, 20, 21; *S.* Infantis, 2; *S.* Heidelberg, 3; *S.* Agona, 4; *S.* Newport, 5; *S.* Stanleyville, 6; *S.* Hadar, 7; *S.* Mbandaka, 8, 18; *S.* Typhimurium, 9; *S.* Colombo, 10; *S.* Muenchen, 11; *S.* Kentucky, 12; *S.* Enteritidis, 13; *S.* Virchow, 14; *S.* Anatum, 19; *S.* Mbandaka; N: negative control).

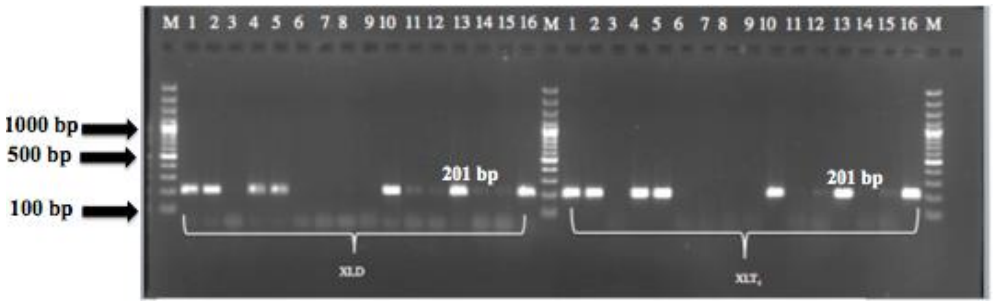

Figure 3. *S.* Infantis isolates were detected using *sinIF and sinIR* primers on the XLD and XLT_4 agar (M: 100 bp marker; 1, 2, 4,5,10,13,16; *S.* Infantis; 3,6-8,11,12,14,15; negative).

Table 2. Detection of *S.* Infantis in naturally contaminated samples.

Test day	Test Time (hour)	ISO 6579 Medium / PCR Results; Positives(%)	
Day 0	0	Direct Analysis (Negative)	
Day 1	6th	BPW (Negative)	
	12th	BPW (1/7)	
	18th	BPW (2/7)	
	24th	BPW (2/7)	
Day 2	6th	MSRV (1/7; 14.8%)	MKTTN (Negative)
	12th	MSRV (1/7)	MKTTN (Negative)
	18th	MSRV (3/7)	MKTTN (2/7; 28.57%)
	24th	MSRV (4/7; 57.15%)	MKTTN(2/7; 28.57%)
Day 3	-	XLD (7/7; 100%)	XLT_4 (7/7; 100%)
Day 4	-	Nutrient Agar (7/7; 100%)	
Day 11	-	Serotype Identification (7/7; 100%)	

BPW: Buffered Peptone Water; MSRV: MKKTN; XLD; XLT_4.

Discussion and Conclusion

Poultry production is increasing every year in the world and becoming more common than any other animal protein source due to heightened consumption. Thus, intense efforts are being made to increase the quality standards of poultry products. Turkey is the 8th largest broiler meat (2.25 million tons) and egg producer (>20 billion chicken eggs annually) and the 6th largest poultry meat exporter (425,000 tons) [FAO report, April 2018] in the world (51). Regarding its $6-billion-dollars annual endorsement, the poultry sector has a significant share in overall animal production in Turkey. According to data obtained from a study investigating the epidemiology of *Salmonella* serotypes carried out from 2014 to 2017 as part of the "Project for the Development of Monitoring and Control Programs for *Salmonella* from Poultry and Food" in cooperation with the Faculty of Veterinary Medicine, Ankara University (Turkey) and the Ministry of Food Agriculture and Livestock and supported by the Scientific and Technological Research Council of Turkey (TUBITAK, 113R036/113R037), a "National Control Program for *Salmonella*" was established and a *Salmonella* Research Laboratory in the Department of Microbiology, Faculty of Veterinary Medicine, Ankara University, was authorized as the National *Salmonella* Reference Laboratory (30).

S. Infantis has become increasingly important in recent years due to increased isolation rates from environmental samples from poultry farms in the European Union and other countries. Furthermore, recent data also showed that *S.* Infantis accounts for 36.5% and 55.7% of all serotyped *Salmonella* isolates from broiler flocks (10).

Salmonella can persist and even multiply in remaining organic matter and show the ability to adapt the special conditions of poultry houses (13, 38). Therefore, significant resources are spent on cleaning and disinfection of poultry houses and it may not be easy to determine which proper control measures need to be taken. Recent studies highlight that broiler production is a critical point of *Salmonella* contamination worldwide, and Turkey is no exception. Concerning the distribution of *Salmonella* serotypes in present broiler samples, the predominant serotype was *S.* Infantis (88.72%) followed by *S.* Agona (2.25%); *S.* Kentucky, *S.* Hadar, and *S.* Tennessee (all 1.50%); and *S.* Mbandaka, *S.* Montevideo, *S.* Enteritidis, *S.* Adelaide, *S.* Liverpool, and *S.* Derby (all 0.75%). Notably, these findings suggest that *S.* Infantis as a dominant serovar may have inhibited colonization of other serovars, thereby enabling it to continuously maintain a higher prevalence rate in those flocks. In contrast to other dominant serovars like *S.* Enteritidis and *S.* Typhimurium which can usually be overcome within a few flock production cycles, unfortunately *S.* Infantis persistence are constantly reported in farms and it is well known that still remain difficult to trace (14, 35). Another concern from Berchieri and Barrow (1990) reported that strains of *S.* Infantis colonized the chicken alimentary and produced inhibition of a wider range of serotypes (4). Several other studies have investigated the presence of *S.* Infantis in broiler production and revealed a high prevalence in Poland [8%] (40), Hungary [2.5%] (32) and in the last two years period number of positive flocks has increased by more than 100% in Slovenia (35). *S.* Infantis as the main serovar in different countries in Asia (34, 39), although in some European countries was *S.* Typhimurium and *S.* Enteritidis (8, 45, 50). In Japan, trend in the number of serotypes are S. Infantis 57.6%, *S.* Manhattan 40.3%, and *S.* Schwarzengrund 2.1% (7). The difference in serovar prevalence by region or country could be due to

geographical differences and husbandry practices in occurrence and dominance.

S. Infantis can be isolated at different rates from different samples, both environmental and directly from birds. In this study level of detection is consistent with other reports, where *S.* Infantis in broiler chicken was 21.97% and 43% in fecal samples (25, 26) and 12.12% in feed (31). Taken together, these results indicate that environmental samples, such as from litter and dust, may be more useful than animal cloacal and cecal swabs for the routine screening and identification of *Salmonella*-positive flocks at the house level (36), while colonization and/or contamination can be measured through fecal sampling (15).

In the present study, PCR was first performed with primers designed by researchers who claimed their methods specifically detected *S.* Infantis (1, 21). Nevertheless, nonspecific bands were obtained with *fliC* and *fljB* primers, while the multiplex PCR technique, based on the simultaneous amplification of three different genomic regions specific to *S.* Infantis, produced common bands in PCRs with *S.* Enteritidis, *S.* Typhimurium, *S.* Mbandaka, and *S.* Liverpool (1). Additionally, it was seen that both methods performed with environmental samples failed to detect the *S.* Infantis accurately and sensitively. In this study, we achieved the detection limit of *S.* Infantis as 1×10^3 CFU/mL, however, the method developed by Kardos et al. (21) determined *S.* Infantis at the level of 10^5 cfu/ml^{-1} and sensitivity study was not conducted by Akiba et al. (1) These results not only demonstrate the need for new primer pairs with greater specificity for *S.* Infantis, but also the strength of the novel *sinI* PCR assay, which detected all culture-positive *S.* Infantis correctly and early (on day 3), without false-positive or -negative test results. The latter is most significant because previous *S.* Infantis DNA detection methods involve live cells in colonies formed on XLD and XLT_4 agars, rather than DNA from inactivated and/or injured *S.* Infantis bacteria in the growth media from the early steps of the ISO procedure.

The *invA* gene was detected in all *Salmonella* strains isolated in the study. Invasive *invA* is necessary for *Salmonella* virulence and has been studied by many researchers (28, 29). At the same time, the amplification of the *invA* gene of potential pathogenic *Salmonella* strains is accepted as the international standard procedure for the detection of *Salmonella* species (2).

The ISO 6579 standard *Salmonella* isolation and identification procedure lasts 11 d. Herein, samples were taken at 6, 12, 18, and 24 h during incubation at different stages of the ISO 6579 method and then conventional PCR was performed with designed primers and DNA loss (false-negative PCR results) was observed during the pre-enrichment and selective enrichment stages. This loss is thought to have been caused by PCR inhibition due to the sample composition and/or media content. In the present study to specifically isolate DNA from the environment, it was found important to apply internal control in the initial stages of diagnosis, using 10-fold dilution of materials and / or DNA, and / or modification of DNA extraction or use of magnetic or immunomagnetic separation techniques.

Overall, the present study reports development of a novel PCR technique that was validated for the detection of *S.* Infantis isolates during different stages of the ISO 6579 procedure. Moreover, this new analysis method provides a rapid, repeatable, and economical alternative to the gold standard. It is possible that this same strategy could be applied to other serotypes and has the potential to produce much more effective molecular assays in future, such as the real-time multiplex detection of most prevalent serotypes together and in a shorter time frame.

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Ethical Statement

This study was approved by Ankara University and the institutional ethics committee for the local use of animals in experiments (Protocol No. 2012-16-101). Permission to collect samples was obtained from the poultry houses and slaughterhouses.

Conflict of Interest

The authors declared that there is no conflict of interest.

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The monetary impact of zoonotic diseases on society: The Turkish Case

Hakan Oğuz ARI[1,a], Elif İŞLEK[1,b], Merve Kardelen BİLİR USLU[1,c], Yonca ÖZATKAN[1,d], Fatih KARAKAŞ[1,e], Hasan Hüseyin YILDIRIM[1,f], Emine ALP[2,g,✉]

[1]Ministry of Health, TUSEB Turkish Institute for Health Policies, Ankara, Turkey; [2]Ankara Yıldırım Beyazıt University, Ankara, Turkey

[a]ORCID: 0000-0001-5817-0916; [b]ORCID: 0000-0001-7963-4628; [c]ORCID:0000-0001-8563-2303; [d]ORCID: 0000-0002-1388-1148; [e]ORCID: 0000-0002-9815-4248; [f]ORCID: 0000-0001-7963-4628; [g]ORCID: 0000-0003-0189-6008

✉Corresponding author: eminealpmese@gmail.com
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Abstract: In this study, the burden of disease, costs, and animal losses caused by the seven most common zoonoses in humans and the two most common zoonoses in animals are calculated between 2016-2018 in Turkey. It aims to contribute to the literature by providing a holistic framework on the costs and burden of diseases of zoonoses in Turkey. The methodology of the study was based on the formula of "Disease Burden of Zoonotic Diseases" developed by the FAO. It was calculated under "Burden of Early Mortality in Humans", "Burden of Morbidity in Humans", "Financial Value of Lost Animals" and "Reduction in Production Capacity of Infected Animals". All cases which were registered in 2016, 2017 and, 2018 from the Ministry of Health (MoH) and the Ministry of Agriculture and Forestry (MoAF) concerning the relevant diseases were included in the study. It is found that the DALYs of all related zoonotic diseases increased and the costs for diagnosis, treatment, and prevention also rose between 2016-2018. The share of total social cost in the GDP of Turkey was estimated to be 0.0090% in 2016, 0.0097% in 2017, and 0.0113% in 2018. It is argued that the seven zoonoses in the scope of this study have an increasing burden graph on Turkish society between 2016-2018. Moving from the fact that most infectious diseases that threaten human and community health are of zoonotic origin and difficulties in predicting when, where or how a zoonotic disease will occur, all sectors should continue to carefully monitor events related to zoonoses and carry out joint studies.

Keywords: Burden of diseases, cost of zoonotic diseases, Turkey, zoonotic diseases.

Zoonotik hastalıkların toplum üzerindeki parasal etkisi: Türkiye örneği

Özet: Bu çalışmada Türkiye'de insanlarda en sık görülen yedi ve hayvanlarda en sık görülen iki zoonotik hastalığın 2016-2018 yılları arasında neden olduğu hastalık yükü, hastalık maliyeti ve hayvansal kayıplar nedeniyle oluşan yük hesaplanmıştır. Türkiye'deki zoonotik hastalıklarının toplam maliyeti ve hastalık yükü üzerine bütünsel bir çerçeve sunarak literatüre katkıda bulunmayı amaçlanmaktadır. Çalışmanın metodolojisinde Birleşmiş Milletler Gıda ve Tarım Örgütü (Food and Agriculture Organization of the United Nations -FAO) tarafından geliştirilmiş olan ‘‘Zoonotik Hastalıkların Hastalık Yükü’’ formülü temel alınarak ‘‘İnsanlarda Erken Ölümlerin Topluma Oluşturduğu Yük’’, ‘‘İnsanlarda Morbiditenin Oluşturduğu Yük’’, ‘‘Kaybedilen Hayvanların Mali Değeri’’ ve ‘‘Enfekte Hayvanlarda Üretim Kapasitelerinde Meydana Gelen Azalma’’ olarak dört ayrı başlıkta hesaplanmıştır. Sağlık Bakanlığı ve Tarım ve Orman Bakanlığından ilgili hastalıklar özelinde 2016, 2017 ve 2018 yılında kayıtlı olan tüm olgular çalışmaya dahil edilmiştir. İlgili tüm zoonotik hastalıkların DALY'lerinin 2016-2018 yılları arasında arttığı ve tanı, tedavi ve önleme maliyetlerinin de yükseldiği tespit edilmiştir. Toplam sosyal maliyetinin GSYİH içindeki payı ise 2016 yılında %0,0090 2017 yılında %0,0097 ve 2018 yılında %0,0113 olarak tahmin edilmiştir. Bu çalışma kapsamında incelenen yedi zoonozun 2016-2018 yılları arasında Türk toplumu üzerinde artan bir yüke sahip olduğu görülmektedir. İnsan ve toplum sağlığını tehdit eden bulaşıcı hastalıkların çoğunun zoonotik kökenli olduğu ve bir zoonotik hastalığın ne zaman, nerede ve nasıl ortaya çıkacağını tahmin etmedeki zorluklardan hareketle, tüm sektörler zoonozlarla ilgili olayları dikkatle izlemeye ve ortak çalışmalar yapmaya devam etmelidir.

Anahtar sözcükler: Hastalık yükü, Türkiye, zoonotik hastalıklar, zoonotik hastalıkların maliyeti.

Introduction

Zoonotic diseases (also known as zoonoses) are infectious diseases that can be transmitted from animals to humans. Approximately 60% of all human diseases and 75% of the infectious diseases that occur are derived from zoonotic origin (9). Since zoonoses can cause disease both in humans and animals, their effects and management policies of these diseases concern both areas (8).

Many endemic zoonoses have a double effect on the human health and livestock industry. Human populations that are dependent on animal husbandry are not only at risk of health caused by zoonotic diseases, but they are also vulnerable to indirect effects such as dangers on food safety and low levels of animal production and other risks related to the vicious circle of poverty (18). Zoonotic parasitic infections have become principally an increasing concern with the rise of the concept of "One Health" which has emerged as a public health discipline over the past decade, given the changing interactions between people and animals, and global trade and agriculture (23). Thus, the calculation of the economic and social burden of zoonoses on society has started to appear as a significant issue on the agenda of the countries (18). The approach to zoonoses necessitates the prevention and alleviation of diseases from occurring in humans as well as the control and elimination of diseases in animal reserves where appropriate (28). According to Can's study (2), the annual total financial loss of infected cattle, ovine animals, and humans was calculated as 62,006,200 TL (~ 41.3 million $) in the expected scenario. While It was found as 30,100,314 TL (~ 20 million $) in the optimistic scenario; and it was calculated as 92.567.357 TL (~ 61.7 million $) in the pessimistic scenario in the same study (2).

In this study, the burden of diseases; costs and animal losses of the seven most common zoonoses in humans (brucellosis, anthrax, tularaemia, Crimean-Congo hemorrhagic fever [CCHF], rabies, cystic echinococcosis, toxoplasmosis) and the two most common zoonoses in animals (brucellosis and anthrax) were aimed to estimate in between the years 2016 to 2018 in Turkey. The main purpose of this study is to provide a general framework for policymakers by calculating the burden of zoonoses in Turkey for both humans and animals.

Materials and Methods

Turkey has the presence of certain zoonotic diseases like anthrax, brucellosis, tularemia, and rabies. Until today, there have been reported 107 zoonotic diseases that have different factors from each other. In this respect, Turkey Zoonotic Diseases Action Plan (2019 - 2023) was prepared by the MoH in order to determine strategies and objectives for the elimination of the most common seven zoonotic diseases in Turkey. In this article, the priorities determined in the action plan have been taken into consideration and these seven zoonotic diseases (brucellosis, anthrax, tularaemia, Crimean-Congo hemorrhagic fever [CCHF], rabies, cystic echinococcosis, toxoplasmosis) were addressed (17).

The data required for this study were obtained from both the databases of the MoH and the MoFA. The data of 2016, 2017 and 2018 were included in the study because the detailed data needed to calculate the disease burden and cost are available in the databases of the relevant Ministries in a regular and comparable manner since 2016.

Since zoonoses infect both animals and humans, it creates a great burden both on human and animal health (26). The burden of brucellosis and HPAI (Avian Influenza) in Egypt and the burden of brucellosis, bovine tuberculosis and salmonellosis in Kenya were analyzed by FAO to estimate the economic losses of zoonoses. The following formula has been used to calculate the burden of zoonoses in related studies; because a more comprehensive approach is needed that considers the effects it will have on both humans and animals (5, 6):

Disease Burden of Zoonotic Diseases = Burden of Early Deaths in Society + Burden of Morbidity in Humans + Financial Value of Lost Animals + Decrease in Production Capacity in Infected Animals.

The Burden of Disease Calculation: In the study, the burden of disease methodology developed by WHO was used to calculate the burden of zoonoses on humans. According to the WHO methodology; DALY is a health gap measure, that extends the concept of potential years of life lost due to early death to include equivalent years of healthy life lost under individuals being in states of poor health or disability (27). In this framework, the method of attributing a monetary value to a DALY was used in countries where there is no data on the willingness to pay (WTP) a DALY to get its value in monetary terms. Therefore, the Value of a Statistical Life (VSL) estimated in the US was used with a benefit transfer methodology to estimate the willingness to pay for a DALY averted in Turkey. The VSL expresses the numerical value of individuals giving up their income to reduce the risk of death. If the willingness to pay for DALY is to be calculated, it can be represented by the value per Statistical Life Year (VSLY) obtained by dividing the VSL by the discounted expected number of remaining life years (10).

The three following steps were applied in order within the framework of this study. Firstly, the monetary equivalent of the request to pay to avoid DALY was calculated using the VSL value predicted by the US Department of Health and Social Services. Secondly, the benefits transfer method was applied to consider differences in income levels among the US and other countries. This methodology assumes that there is income elasticity of VSL of 1, 1.5, and 2. At this point, the formula that considers the income gap between the US and Turkey

was used to calculate the value of VSLY. The value stated as income refers to the purchasing power parity (PPP) value of the per capita income of the countries in the relevant years (10).

*VSLY T = VSLY the US * (Income T / Income the US)* [1,5]

Finally, the monetary value of these diseases on human health was calculated by multiplying the DALY values of zoonoses in 2016, 2017, and 2018 and the VSL values in 2016, 2017, and 2018. All results are calculated in international dollars using primarily the purchasing power parity exchange rates. The value obtained was calculated separately in TRY and USD using the foreign exchange and purchasing power parity values of the relevant years.

Costs of Disease Analysis: In costs of disease analysis, expenses are generally classified as direct, indirect, and external costs, depending on how the expenses are attributed to the disease. While direct costs are the costs associated with the treatment or prevention of disease; indirect costs are social costs caused by disease, disability, or early deaths. External costs are defined as the effect of economic activity on another economic activity or individuals (12, 16, 21). In this study, direct costs were considered in calculating the human costs of the disease. For this, the following data and their costs are used, which directly form the cost items.

- Prevention costs (vaccination for the emergence of zoonoses and prevention of transmission).
- Diagnosis costs (medical examination, consultation, laboratory and radiological procedures).
- Treatment costs (outpatient and inpatient treatment, medicines and medical devices, supplies, emergency services, surgical procedures, rehabilitation, home care, etc. with other expenses related to the treatment processes).
- Monitoring costs (outpatients examination and other expenses related to laboratory procedures).

Animal Losses: To calculate animal losses; ‘‘Losses Caused by Animal Deaths’’, ‘‘Losses Caused by the Disease (Carcass)’’, ‘‘Compensations Paid Due to Animal Death’’, ‘‘Losses Caused by Unborn Calves/Lambs Lost Due to Animal Deaths’’ and ‘‘Losses Caused by Conditional Slaughter’’ are estimated. Animals that are infected but undead suffer from reduced productivity, especially weight loss, decreased milk production, and loss of fertility. To estimate the value of the total decrease in production to evaluate the economic impact of a disease, ‘‘Meat Losses Caused by the Disease’’, ‘‘Milk Losses Caused by Decreased Lactation Time’’, and ‘‘Milk Losses Caused by Decreased Milk Yield’’ are calculated.

Results

Results Related to Burden of Diseases: As in the Global Burden of Disease 2016 study, while YLDs were analyzed, age weighting and time discounts were not included in the calculations and prevalence data were used in the calculations instead of incidence. Estimates of YLL and YLD values and total DALY values caused by zoonoses are given in Table 1. As of 2016, the disease that created the highest DALY was brucella (860); CCHF was 446; rabies risk contact was 171, anthrax was 30, cystic echinococcosis was 10, and tularemia and toxoplasmosis were found to have 1 DALY. The highest DALY in 2017 belonged to the brucella with 1,083. CCHF was 429, rabies risk contact was 72, toxoplasmosis was 61, cystic echinococcosis was 40, tularemia was 1 caused DALY. In 2018, brucella continued to cause the highest DALY with 1,262. CCHF was 639, anthrax was 119, rabies risk contact was 95, cystic echinococcosis was 22, toxoplasmosis was 2, and tularemia was 1 DALY. Monetary value estimates of the burden of disease of these zoonoses for 2016, 2017 and, 2018 are presented in Table 2. In 2016, DALY which consisted of the seven zoonoses corresponded to a monetary equivalent of $ 188.7 million PPP and 0.0090% of GDP; in 2017, a monetary provision of $ 219.3 million PPP and 0.0097% of GDP. In 2018, it was estimated to be a monetary equivalent of $ 261.6 million PPP and 0.0113% of GDP.

Table 1. YLL, YLD and DALY Values of Zoonoses by Years.

Year	2016			2017			2018		
Zoonoses	**YLL**	**YLD**	**DALY**	**YLL**	**YLD**	**DALY**	**YLL**	**YLD**	**DALY**
Brucella	0	860	860	0	1,083	1,083	50	1,213	1,262
Anthrax	30	0	30	0	0	0	119	0	119
Tularemia	0	1	1	0	1	1	0	1	1
CCHF	428	18	446	415	14	429	619	20	639
Rabies	148	23	171	45	27	72	64	31	95
Cystic Echinococcosis	0	10	10	17	23	40	0	22	22
Toxoplasmosis	0	1	1	59	2	61	0	2	2

Data on the disability weight was taken from the Global Burden of Disease Study 2017 (7), the European Center for Disease Prevention and Control's toolkit, and the studies of Piroozi et al. (15) and Moradi et. al. (13).

Table 2. Monetary Value of Burden of Disease of Zoonoses in 2016-2018.

	2016		2017		2018	
Zoonozes	**Total Value (PPP $)**	**GDP Rate (%)**	**Total Value (PPP $)**	**GDP Rate (%)**	**Total Value (PPP $)**	**GDP Rate (%)**
Brucella	106,738,164	0.0051	140,824,768	0.0062	154,291,324	0.0067
Anthrax	3,766,284	0.0002	1,038	0.0000	14,547,450	0.0006
Tularemia	166,429	0.0000	169,755	0.0000	75,664	0.0000
CCHF	55,367,456	0.0027	55,799,342	0.0025	78,108,402	0.0034
Rabies	21,228,194	0.0010	9,317,312	0.0004	11,557,547	0.0005
Cystic Echinococcosis	1,289,170	0.0001	5,150,407	0.0002	2,726,907	0.0001
Toxoplasmosis	123,774	0.0000	7,969,401	0.0004	269,170	0.0000
TOTAL	188,679,471	0.0090	219,232,023	0.0097	261,576,465	0.0113

Table 3. Diagnosis, Treatment and Control Costs for Selected Zoonoses in 2016-2018 (PPP $).

Zoonozes	**2016**	**2017**	**2018**	**TOTAL**
Brucella	3,695,409	8,508,086	5,900,376	18,103,872
Anthrax	54,731	56,032	84,050	194,813
Tularemia	67,313	175,267	109,099	351,679
CCHF	204,748	186,337	350,971	742,056
Rabies	6,455,025	8,522,646	17,235,674	32,213,344
Cystic Echinococcosis	2,082,846	5,016,932	2,043,361	9,143,139
Toxoplasmosis	196,159	665,668	306,561	1,168,388
TOTAL	12,585,183	23,397,012	25,894,631	61,876,826

Table 4. Total Economic Loss Caused by Brucella and Anthrax in Animals by years (PPP $).

Zoonoses		**2016**	**2017**	**2018**	**TOTAL**
Brucella	Large Ruminant	9,170,180	15,388,268	36,264,523	60,822,971
	Small Ruminant	1,302,000	2,889,920	9,064,147	13,256,067
	Total	10,472,179	18,278,188	45,328,670	74,079,037
Anthrax	Large Ruminant	1,063,393	1,271,022	2,621,348	4,955,763
	Small Ruminant	501,068	609,817	1,211,237	2,322,121
	Total	1,564,461	1,880,839	3,832,585	7,277,885
Total		12,036,640	20,159,027	49,161,255	81,356,922

Table 5. Total Economic Loss Caused by Zoonoses by Years (PPP $).

	2016			**2017**			**2018**			**TOTAL**
Zoonozes	**Value of DALY**	**Treatment and Medicine Costs**	**Animal Losses**	**Value of DALY**	**Treatment and Medicine Costs**	**Animal Losses**	**Value of DALY**	**Treatment and Medicine Costs**	**Animal Losses**	
Brucella	106,839,479	3,698,917	10,482,119	141,137.666	8,526.991	18,318,800	154,083,366	5,892.424	45,267,575	494,247,337
Anthrax	3,769,859	54,783	1,565,946	1,041	56,157	1,885,018	14,527,843	83,937	3,827,419	25,772,003
Tularemia	166,587	67,377		170,132	175,656		75,562	108,952		764.266
CCHF	55,420,010	204,942		55,923,323	186,751		78,003,125	350,498		190,088,650
Rabies	21,248,344	6,461,152		9,338,014	8,541,582		11,541.970	17,212,443		74,343,505
Cystic Echinococcosis	1,290,394	2,084,823		5,161,851	5,028,080		2,723.232	2,040,607		18,328,985
Toxoplasmosis	123,891	196,345		7,987,108	667,147		268,807	306,148		9,549,447
TOTAL	188,858,565	12,768,337	12,048,065	219,719,134	23,182,364	20,203,818	261,223,906	25.995.008	49,094,994	813,094,191
	Total of 2016		213,674,967	Total of 2017		263,105,316	Total of 2018		336,313,908	

Cost of Disease Analysis: The cost of disease analysis of these seven zoonoses selected within the scope of the study was conducted in 2016, 2017, and 2018. Outpatient and inpatient treatment, medical procedures, drugs, and vaccines were taken into consideration. In these seven zoonoses, rabies risk contact ($ 32,213,344 PPP) ranks first, brucella ($ 18,103,872 PPP), and cystic echinococcosis ($ 9,143,139 PPP) in the third place in terms of cost analysis. However, increases in costs varied in line with 2016-2018 years (Table 3).

Animal Losses: Estimates of the economic burden of brucella and anthrax in bovine and ovine are given in Table 4. In 2016, there was a total economic loss of $ 12 million PPP, including approximately $ 10.5 million PPP derived from brucella and $ 1.6 million PPP caused by anthrax. In 2017, an economic loss of $ 18.3 million PPP from brucella and $ 1.8 million PPP from anthrax was found. In 2018, it was estimated that an economic loss of a total of $ 49.2 million PPP including approximately $ 45.3 million PPP from brucellosis and $ 3.8 million PPP from anthrax. In a total of 3 years examined within the scope of the study, it was determined that a total of $ 81.4 million PPP economic losses occurred due to brucellosis and anthrax. The total economic loss due to zoonoses is presented in Table 5 which also summarizes the findings of the study. Accordingly, the zoonoses caused an economic loss of $ 213.7 million PPP in 2016, $ 263.1 million PPP in 2017 and it increased to $ 336.3 million PPP in 2018. The sum of the three years covered by the study; the economic loss of zoonoses reached up to $ 813 million PPP. When this economic loss is evaluated in terms of human and animal origin, the total economic loss ($ 813 million PPP) consisted of $ 731.7 million PPP from human-related losses (90%) and $ 81.3 million PPP animal-related losses (10%).

Discussion and Conclusion

Zoonotic infections have global importance and they have caused great economic losses not only in low and middle-income countries but also high-income countries. The impact of these diseases on public health and the economy can affect even more negatively in developing countries and Turkey which are already under pressure in economic terms. For example, it is known that brucella, which is considered as one of the most common zoonoses in the world by WHO, OIE and FAO, creates an important public health problem by affecting people who are in direct contact with these animals or consume contaminated milk and dairy products in addition to the economic losses in animals (2).

It was found from the data of 2016 that brucella in Egypt constituted 29 DALY and avian flu constituted 214 DALY, and the total social cost of this was 164.458 and 1.209.976 USD (according to Purchasing Power Parity), respectively (5). Similarly, according to a study conducted in Kenya with the data of 2016, brucella caused 502.801; bovine tuberculosis caused 41.590 and salmonella caused 131.160 DALY burden. The social costs of these diseases to Kenya were 4.06 million; 336.5 million; and 1.06 billion USD (based on Purchasing Power Parity), respectively (6). Singh et al. (24) stated that the current economic burden of human brucellosis in India is 627.5 million Indian Rupees per year and it creates a loss of 0.15 DALY per thousand people per year. Piroozi et al.'s study on examining the disease burden of brucella in Iranian society was found that the DALY burden, which was 34.6 per hundred thousand in 2009, increased to 71.4 in 2015 (22).

Although it varies from country to country and the structure of health systems, the estimated cost for each brucellosis case in Spain has been defined as approximately $ 8,000. It was found that the disease caused an average of 13 days of hospitalization and 102 days of job loss (3, 29). A study conducted in Southern Israel was revealed that the costs for patients with brucellosis were $ 57 higher before diagnosis and $ 947 one year after diagnosis compared to non-brucella cases (7).

According to a study conducted in the USA, while the outpatient cost per patient with anthrax is between 422-810 dollars and the inpatient costs are between 4,541-5,380 dollars per person (13). According to the study conducted by Zacchia and Schmitt, it was reported that the total health expenditures due to only anthrax cases reached 177 million dollars in 2001 (30), Similarly, according to a study conducted in the USA, the outpatient cost per patient with tularemia is 722-1.120 dollars and the inpatient costs are between 6.338-7.582 dollars per person (13).

In a study conducted in Italy between 2008 and 2014, it was reported that the cost per patient with cystic echinococcosis cases treated surgically was between 5.874 and 23.077 (median 11.033) dollars (20). In the study conducted by Kreindel et al., the total cost of the treatment applied for prophylactic purposes against rabies risky contact in the state of Massachusetts, the USA for the year was between 2.4 million to 6.4 million dollars, and the cost per patient with rabies risky contact was determined between 632 and 3.435 dollars (14). It is seen that vaccines and immunoglobulin treatments applied for prophylactic purposes in contacts with rabies can be costly. The annual average hospital costs of toxoplasma cases due to different reasons was calculated in a study conducted in Canada. Accordingly, it was calculated as $ 1,971 per person in cases of congenital origin, $ 763 in cases seen in adults,

and $ 5,744 in cases associated with HIV infection and the annual total hospital costs of toxoplasma cases was found to be 1,686,860 Canadian dollars for 2015 (25).

Moradi et al.'s study on the DALY burden of Crimean-Congo Hemorrhagic fever in Iran revealed that CCHF in Iran created a DALY burden ranging from 483 to 1156 between 2009 and 2015 (19). According to WHO's report from 2017, Iran, Turkey, Uzbekistan and Russia reported more than 50 CCHF cases annually and these countries are among the countries with the highest rate of CCHF cases in the world (1).As a result, it can be asserted that the seven zoonoses included in this research have an increasing financial burden on Turkish society between 2016-2018. It is required interdisciplinary cooperation to prevent zoonoses and their related losses as well as a collection of more detailed epidemiological evidence in both humans and animals; reporting of diseases; economic impact studies, including the cost-effectiveness of control programs; conducting protective and preventive vaccination programs; management of infected animals; increasing laboratory capacities and ensuring quality standards. Awareness-raising activities aimed at farmers, healthcare professionals and the general public about zoonoses and risks are also recommended. It was emphasized that the success in the control of zoonoses depends on the harmony between the institutions and related units within the country, the connection with local stakeholders and cooperation with the neighbour countries and international organizations (4, 15). Therefore, it has been suggested that zoonotic control should be handled from a global perspective; it should be carried out with global institutions such as World Organisation for Animal Health (OIE), FAO and WHO by considering global control standards (31).

It is very important to raise awareness of people who provide for livestock farming in terms of prevention on zoonotic diseases and it should be ensured that stakeholders who play a role in human and animal healthcare in a timely and effective way of communication and cooperation. In anti-zoonoses programs; to protect the society and risk groups from zoonoses-related conditions, it is important to spread awareness studies, to ensure the continuity of informative training programs about the zoonoses which are common in Turkey, and to keep them informed about new threats that may develop. To prevent and reduce social costs caused by zoonoses to society, determining the duties and responsibilities among the sectors and realizing actions within these rules are among the basic rules for achieving success in the implemented policies. For this purpose, carrying out joint scientific studies on zoonoses by the relevant stakeholders under the "One Health" concept will not only contribute to both human and animal health but will also reduce the negative socio-economic effects (17).

Burden of disease and cost analysis studies on disease are of great importance because they allow comparison, support the efficient and productive use of limited resources, and contribute to financial sustainability. Cost-oriented studies for policymakers in the field of health are important in determining priorities, distribution of resources, budget management, and agendas. Animal protection and control measures should be given higher priority across the country, as zoonotic diseases such as brucellosis incur heavy financial losses on the animal stock throughout the country (2). It should be taken into consideration that the total cost may actually reach a much higher level by adding the resources to be allocated for the activities to be carried out for this purpose and other indirect costs such as waste and by-product losses that may occur in other industries based on animal husbandry. Most infectious diseases that threaten human and community health are of zoonotic origin and difficulties in predicting when, where or how the zoonotic disease will occur, all sectors should continue to carefully monitor events related to zoonoses and work together to develop defense strategies.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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An investigation on the supplementation of rosemary volatile oil to the laying quail diets

Gülay DENİZ[1,a,✉], Mukaddes Merve EFİL[1,b], Şerife Şule CENGİZ[1,c], Kerem ATAMAY[1,d], Bahadır ANAR[1,e]

[1] Uludağ University Faculty of Veterinary Medicine Department of Animal Nutrition and Nutritional Diseases, Bursa, TURKEY
[a]ORCID: 0000-0003-3817-4359; [b]ORCID: 0000-0003-0646-9777; [c]ORCID: 0000-0003-0708-3833;
[d]ORCID: 0000-0002-6891-4613; [e]ORCID: 0000-0002-6783-0346

✉Corresponding author: denizg@uludag.edu.tr
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Abstract: The purpose of the current study was to detect the effects of different levels of rosemary volatile oil (VO) supplemented to laying quail diets on performance, egg quality parameters, and egg yolk oxidative stability. In this study, a total of 105 laying quails (*Coturnix coturnix Pharaoh*) at the age of 6 weeks were used and 3 main groups were formed, each containing 35 quails. Also, each main group was separated into 5 replicates containing an equal number of quails. Quails were fed a control diet without rosemary VO or diets which were containing at different levels (200 or 400 mg/kg) of rosemary VO supplemented to the control diet. At the end of the study, which was continued for a total of 56 days, no significant difference was found in the feed intake, feed efficiency and egg weights among the control and experimental groups. The supplementation of rosemary VO at the levels of 200 and 400 mg/kg to the diets caused an increase in quails' egg production (P<0.01). However, there was no effect on the exterior and interior egg quality parameters. A significant decrease in malondialdehyde (MDA) levels in egg yolk were observed with both levels of rosemary VO at storage (+4 0C) for 7 (P<0.001) and 28 (P<0.01) days. Considering these data, it was concluded that rosemary VO can be supplemented to laying quail diets as a natural antioxidant without negatively affecting performance and egg quality parameters.
Keywords: Antioxidant, egg quality, laying quail, malondialdehyde, rosemary volatile oil.

Biberiye uçucu yağının yumurtacı bıldırcın rasyonlarına ilave edilmesi üzerine bir araştırma

Özet: Bu çalışmanın amacı, yumurtacı bıldırcın rasyonlarına farklı düzeylerde ilave edilen biberiye uçucu yağının performans, yumurta kalite parametreleri ve yumurta sarısının oksidatif stabilitesi üzerindeki etkilerini belirlemekti. Araştırmada, 6 haftalık toplam 105 yumurtacı bıldırcın (*Coturnix coturnix Pharaoh*) kullanılmış ve her biri 35 hayvan içeren 3 ana grup oluşturulmuştur. Ayrıca, her ana grup eşit sayıda bıldırcından oluşan 5 tekrar grubana ayrılmıştır. Bıldırcınlar, biberiye uçucu yağı içermeyen kontrol rasyonu veya kontrol rasyonuna ilaveten biberiye uçucu yağının farklı düzeylerini (200 veya 400 mg/kg) içeren deneme rasyonları ile beslenmiştir. Toplam 56 gün sürdürülen araştırmanın sonunda, kontrol ve deneme gruplarının yem tüketimi, yemden yararlanma ve yumurta ağırlıklarında önemli bir fark bulunmamıştır. Biberiye uçucu yağının rasyona 200 ve 400 mg/kg düzeylerinde ilave edildiği deneme gruplarının yumurta üretiminde artış (P<0,01) saptanmıştır. Yumurtaların buzdolabında depolanmasının (+4 0C) 7. (P<0,001) ve 28. (P<0,01) günlerinde, biberiye uçucu yağının her iki dozu da yumurta sarısı malondialdehit seviyelerinde önemli bir düşüşe yol açmıştır. Bu veriler göz önüne alınarak; biberiye uçucu yağının bıldırcınların performans ve yumurta kalite parametrelerini olumsuz etkilemeksizin, rasyonlara doğal bir antioksidan olarak ilave edilebileceği sonucuna varılmıştır.
Anahtar sözcükler: Antioksidan, biberiye uçucu yağı, malondialdehit, yumurtacı bıldırcın, yumurta kalitesi.

Introduction

The ban on the supplementation of antibiotics to poultry feeds has inspired interest in natural feed additives such as probiotics, prebiotics, aromatic herbs and their essential oils which are not causing residue in animal products and resistance in microorganisms. The antimicrobial (18, 20, 41, 49), antioxidant (10, 35, 51), antifungal (33, 39), antiparasitic (44, 45), antiviral (8), anti-inflammatory (1), hypolipidemic (54), immunostimulatory (1, 54), digestive system stimulator (46) and digestive enzyme activity enhancer (54) effects of herbal volatile oils are proved with many scientific studies. Rosemary (*Rosmarinus officinalis L.*), aromatic herb specific to the Mediterranean climate, is now widely grown all over the world. It is known as the herb with the highest antioxidant activity among aromatic herbs (19,

38). This effect of rosemary is derived from the phenolic compounds, carnosic acid, carnosol, camphene, ursolic acid, rosmarinic acid, 1,8-cineole, α pinene and other VO components that give the herb its characteristic odour (3, 6, 48, 50). Viuda-Martos et al. (57) tested volatile oils obtained from five aromatic herbs (oregano, thyme, clove, sage and rosemary) commonly used in the Mediterranean diet for their antioxidant properties using different in vitro methods and concluded that rosemary VO has the highest iron-chelating ability.

Recently, there has been a strong debate about the safety aspects of chemical preservatives, as they are thought to be responsible for a number of residual toxicity, carcinogenic and teratogenic cases. Therefore, there has been a heavy demand for foods containing natural preservatives around the world. In line with this demand, the researchers focused on studies to determine the protective effect of natural additives, such as extracts from different herbs, to extend the shelf life of foods. Several studies have identified many beneficial effects of rosemary, including its potent antioxidant activity (48, 57) as well as antibacterial, anti-inflammatory and immunomodulatory effects (15, 23, 24, 60). Our hypothesis was that the strong antioxidant effect of rosemary VO would extend the shelf life of quail eggs and improve the quail's performance due to the general effects of herbal volatile oils (increasing the flavor of the feed and the activity of digestive enzymes, etc.). Thus, the purpose of the current study was to investigate the effects of different levels of rosemary VO on performance, exterior-interior egg quality parameters in laying quails and also to prove the antioxidant activity by determining the effect on the oxidative stability of the eggs.

Materials and Methods

Quails, housing and management: This research was conducted within the framework of the rules determined by the Uludağ University Ethical Committee (decision no. 2018-15/04). In this study, a total of 105 quails (*Coturnix coturnix Pharaoh*) at the age of 6 weeks were used and 3 main groups were constituted, each containing 35 quails. Also, in order to compare the feed intake and feed efficiency parameters of the experimental groups, each main group was divided into 5 replicates containing 7 quails. The quails were placed in cages at 112.5 cm2 per quail and kept there until the end of the study. Feed and water were provided *ad libitum* to quails. They were exposed to 16 h of light and 8 h of darkness a day. Average ambient temperature was 22 °C and the relative air humidity was 65 %. The study was continued for 56 days.

Experimental diets and measurements: The quails were fed a corn and soy-based basal diet formulated to meet the NRC nutrient requirements of the laying quails (42). The diet did not contain any antioxidant other than a basic level of vitamin E (Table 3). Quails were fed a control diet without rosemary VO or diets containing rosemary VO at levels of 200 mg/kg (Group 1) or 400 mg/kg (Group 2) in addition to the control diet. While deciding on the levels of rosemary VO supplemented to the treatment group, the information in the literature (22, 59) were taken into consideration. The specific gravity value of rosemary VO was detected by the supplier company (Semi Etheric Oil Ind.trade.co.ltd., Mersin /Turkey). This value were considered for calculating the amount of rosemary VO to add to the diets. The nutrient composition of the basal diet was detected according to AOAC procedures (4). Spectrophotometric determinations of calcium and total phosphorus were made according to the methods of Farese et al. (21) and, Gericke and Kurmies (26), respectively. Carpenter and Clegg equation (14) was used to calculate the metabolizable energy level: ME, kcal/kg = 53+ 38 [(CP, %) + (2.25 x ether extract, %) + (1.1 x starch, %) + (sugar, %)].

Performance, exterior and interior egg quality: Eggs were picked up and recorded each day, and the egg production percentage was calculated on a replicate basis. Every 2 weeks, feed intake (g/quail/day) was determined by weighing the remaining feeds from replicates. Also, all eggs collected from replicates were weighed separately to determine egg weight and averaged. Feed efficiency was calculated as consumed kg feed per kg egg and for a dozen eggs. Fifteen eggs from each main group (3 eggs from per replication group) were randomly taken to detect egg quality parameters every 2 weeks. Eggshell thickness was measured using an eggshell thickness gauge (Orka Technology Ltd, USA) from the top, middle and bottom parts of the eggshell after the shell membrane was removed. Eggshell breaking strength was detected with a cantilever system (5) and the values were recorded in Newton (N) unit. The heights of the albumen were measured in the egg samples, and then the haugh units belong to all groups were calculated by placing these values in the formula (13).

Measuring of chemical composition of the rosemary VO: The pure rosemary VO (Origin; Mersin /Turkey, Extraction Type; Hydro distillation, Density; 0.895 at 20 °C) used in this study were obtained from Semi Etheric Oil Industry and Trade Limited Company. Gas chromatography analysis of rosemary VO was carried out on an MS-Thermo Polaris Q GC-Thermo Trace GC (Thermo Ficher inc, MA, USA) ultra-fitted with a fused HP5-MS capillary column (Thermo Ficher inc, MA, USA) (30 x 0.25 x film thickness 0.5 µm). The temperature was programmed to rise from 95 °C to 240 °C at 4 °C /min. Samples were injected in split mode at 250 °C. Helium gas was used as the carrier at a pressure of 1.3610 atm.

Determination was made with FID (250 °C) and the injection volume was the same for all samples (8.1 µl). Chromatograms were detected using MS or MS/MS. Internal standards were taken into account in the calculation of data (43).

Thiobarbituric acid (TBA) analysis of egg yolk: At the end of this study, 45 egg yolk samples (15 from each main group) were measured for MDA levels. The lipid oxidation value of egg yolk samples stored at 4 °C in the refrigerator was determined on 1, 7 and 28 days. MDA levels in eggs from experimental groups were measured as a secondary oxidation product according to the TBA method which is a spectrophotometric method defined by Ke et al. (34). A modification of the 2-thiobarbituric acid method was used, and the results were expressed as the amount of 2-thiobarbituric acid reactive substances (mg MDA). This method is based on the observation of a red colour that is created by the oxidation of unsaturated fatty acids with TBA after heating MDA.

Statistical analysis: Statistical analysis was performed using the Statistical Package for the Social Sciences version 22.0 (SPSS, Chicago, IL, USA). One way ANOVA was used to evaluate the effects of rosemary VO on the performance parameters (egg weight, egg production, feed intake, feed efficiency), egg quality (shape index, eggshell thickness, eggshell breaking strength, haugh unit, yolk color) and egg oxidative stability (MDA levels). Values were expressed as arithmetic means ± standard error of the mean (SEM). Tukey test was used as a post hoc test and the level of significance used in all of the tests was $P<0.05$ (55).

Results

In the study, the analysed VO components of rosemary supplemented to the quail diets are shown in Table 1. As can be seen from the table, the main active ingredients of rosemary VO were 1,8-cineol (43.96 %), α-pinene (25.33 %), camphene (11.09 %) and 3-carene (10,7 %), respectively. Specific gravity value and levels of rosemary VO are summarised in Table 2. The specific gravity value of rosemary VO was 0.895 g/mL. The amounts of rosemary VO supplemented to the quail diets were calculated according to the specific gravity value of the VO. Ingredients and nutrient composition of the basal diet are given in Table 3. The analyzed dry matter, ether extract, ash, total phosphorus, calcium values and the calculated metabolizable energy value of the basal diet was within the normal range for laying quails reported by NRC (42). In this study, no significant difference was found on the feed intake, feed efficiency and egg weights among the control and experimental groups. The supplementation of rosemary VO at the levels of 200 and 400 mg/kg to the diets caused an increase in quails' egg production ($P<0.01$). However, there was no effect on the

Table 1. Analysed chemical composition of rosemary VO.

Rosemary VO	
Components	***(%)***
1,8-cineole (Eucalyptol)	43.96
α-Pinene	25.33
Camphene	11.09
3-carene	10.70
D-limonene	2.5
Limonene	1.77
Ocimen	1.68
β-pinene	1.40
Camphor	0.73
Bornyl acetate	0.04
Borneol	0.03
Isoborneol	0.02
Caryophyllene	0.02

Table 2. Specific gravity value and amounts of rosemary VO supplemented to quail diets.

Specific Gravity*	**Control**	**Group1**	**Group2**
0.895 g/mL	-	200 mg/kg	400 mg/kg
	-	0.22 ml	0.44 ml

*The amounts of rosemary VO supplemented to the treatment diets were calculated using the specific gravity value of VO.

Table 3. Ingredients and nutrient composition of the basal diet (as fed basis).

Ingredients, %	
Corn	45.00
Soybean Meal	23.15
Full Fat Soybean Meal	13.00
Wheat	4.00
Sunflower Meal	3.00
Vegetable Oil	3.80
Calcium Carbonate	6.40
Dicalcium Phosphate	1.00
NaCl	0.30
DL-Methionine	0.10
Vitamin-Mineral Premix[a]	0.25
Analyzed values, %	
Metabolisable Energy, Kcal/kg[b]	2919.69
Dry matter	89.00
Crude Protein	20.60
Ether Extract	7.94
Ash	10.84
Calcium	2.53
Total Phosphorus	0.60
Available Phosphorus[c]	3.55

[a] Provides per kg diet: α-tocopherol acetate 20 mg, retinol 2.4 mg, cholecalciferol 0.075 mg, riboflavin 3 mg, thiamin 3 mg, pyridoxal 3.5 mg, niacin 20 mg, cyanocobalamin 0.01mg, folic acid 1mg, biotin 0.03 mg, pantothenic acid 4mg, choline 600 mg, Fe 60 mg, Cu 5 mg, Zn 60 mg, Mn 80 mg, I 1, Se 0.15 mg, Co 0.2 mg..

[b] Carpenter and Clegg equation (35) was used to calculate the ME level.

[c] Calculated value.

Table 4. Effects of rosemary VO supplementation on the performance parameters[1] of laying quails from 1 to 56 days of age.

Rosemary VO (mg/kg)	Control 0	Group 1 200	Group 2 400	P
Feed intake, g/d	34.31 ± 1.04	36.96 ± 1.94	37.63 ± 0.17	NS
Egg production, %	73.47 ± 2.60 [b]	83.98 ± 1.96 [a]	82.19 ± 1.93[a]	<0.01
Egg weight, g	11.00 ± 0.10	11.11 ± 0.09	11.60 ± 0.10	NS
Feed efficiency, kg feed/kg egg	4.35 ± 0.21	4.05 ± 0.14	4.02 ± 0.13	NS
Feed efficiency, kg feed/ a dozen eggs	0.58 ± 0.03	0.54 ± 0.02	0.56 ± 0.01	NS

[a, b]: Different letters within the same row indicate significant differences among the groups.
NS: Not significant (P > 0.05).
[1]Egg weight refers to the average of all eggs collected from 5 replicates. Values of egg production, feed intake and feed efficiency measured on the basis of group are means of 5 replicates, n = 5.

exterior (shape index, eggshell thickness, breaking strength) and interior (haugh unit, egg yolk colour) egg quality parameters. No difference was determined in the 1st day egg yolk MDA values of the eggs obtained from the control and treatment groups and stored in a refrigerator at +4 °C. However, a significant decrease in MDA levels in egg yolk was observed on the 7th (P <0.001) and 28th (P <0.01) days in storage with both doses of rosemary VO.

Discussion and Conclusion

The main active phenolic compounds of rosemary VO supplemented to quail feeds in this study were 1,8-cineol (43.96 %), α-pinene (25.33 %), camphene (11.09 %) and 3-carene (10,7 %), respectively. Barakat and Ghazal (6) and, Santana-Méridas et al. (50) stated that carnosic acid, carnosol, camphene, caffeic acid, ursolic acid, rosmarinic acid, 1,8-cineole, α pinene are the most active antioxidant components found in rosemary VO. However, the VO composition of aromatic herbs may vary depending on the seasons, soil structure and climate factors.

In this study, dietary rosemary VO significantly increased egg production of laying quails (83.98 and 82.19 vs 73.47, P<0.01), but did not affect egg weight, feed intake and feed efficiency parameters (Table 4). There are contradictory results about the effect of dietary herbal extracts on performance parameters of broiler, laying hen and quails. While some researchers reported that herbal extracts positively affect performance parameters such as body weight gain, egg production, feed intake and feed efficiency (7, 27, 58), the others stated that they had no significant effect on these parameters (11, 32, 37, 52). Yesilbag et al. (59) investigated the effects of rosemary VO (200 mg/kg), oregano VO (200 mg/kg) and their mixtures (40 mg/kg rosemary VO + 160 mg/kg oregano VO) on performance and egg quality parameters in laying quails. The authors reported that there was no significant difference between the groups in body weight and egg weight. However, they determined that the rosemary VO significantly improved egg production and feed efficiency, while the mixtures of volatile oils caused a significant increase in feed intake. Simsek et al. (53) investigated the effects of dietary rosemary VO (200 mg/kg), cinnamon VO (200 mg/kg) and a mixture of these volatile oils (100 mg/kg rosemary VO + 100 mg/kg cinnamon VO) on quails. At the end of the study, there was no difference between the groups in feed intake. While the highest egg weight was obtained from the rosemary VO group, the highest egg production and feed efficiency belonged to the cinnamon VO group. In a study conducted by Hernandez et al. (29) to determine the effect of two herbal extract mixtures (5000 mg/kg sage + thyme + rosemary and 200 mg/kg oregano + cinnamon + pepper) on broiler performance, a little performance enhancer effect of herbal extract mixtures as in this study was seen, but none of the supplementations caused significant effects. Jamroz and Kamel (30), and Jang et al. (31) reported that herbal extracts or their volatile oils caused an increase in broiler performance by stimulating the secretion of digestive enzymes and increasing the digestibility of nutrients in feed. It has been suggested that the inconsistency in the effectiveness of herbal extracts on the performance of different poultry species may depend on many factors such as the composition of the basal diet, feed intake and environmental conditions or harvesting time of the herbs, extraction methods, conservation conditions and the differences in the levels of extracts supplemented to the diets (12, 16, 28, 40).

No significant effect of rosemary essential oil on eggshell breaking strength, eggshell thickness, shape index, haugh unit and egg yolk colour were determined in groups fed diets containing rosemary VO at levels of 200 mg/kg and 400 mg/kg (Table 5). Similarly, rosemary VO supplementation to the laying hen diets did not have any significant effects on shape index, eggshell weight,

eggshell thickness, eggshell breaking strength, haugh unit and yolk colour (9, 23, 47). On the other hand, Cufadar et al. (17) stated that the eggshell breaking strength and eggshell thickness significantly increased in laying hens fed with 250 mg/kg rosemary VO supplemented diet when compared to the control group. Alagawany and Abd El-Hack (2) also demonstrated that performance and egg quality parameters were improved by supplementation of 3000 mg/kg rosemary powder to laying hen diets. The number of studies examining the effects of rosemary VO on egg quality parameters in quails is limited. In a study conducted on laying quails, it was determined that rosemary VO significantly increased the egg white and yolk index values and affected the egg yolk colour positively (59). In a word, the supplementation of rosemary VO at different levels to the laying quail diets in the current study did not significantly affect the egg quality parameters.

The effect of rosemary VO on lipid oxidation of egg yolk stored in the refrigerator (+4 °C) for 1, 7 and 28 days was determined by measuring the MDA level which is secondary oxidation product according to the TBA method. As the degree of oxidation in yolk lipids increases during eggs storage, the level of MDA measured in egg yolk also increases. There was no difference in the 1st day egg yolk MDA (TBA-reagent) values of the eggs obtained from control and treatment groups and stored in a refrigerator at +4 °C in the present study. However, a significant decrease was determined in MDA levels of egg yolk in both levels of rosemary VO (200 and 400 mg /kg diet) on the 7 (0.05 and 0.07 vs 0.44, P <0.001) and 28 (2.97 and 3.03 vs 6.59, P <0.01) days of storage (Table 6). Our data are in agreement with previous results reported on the antioxidant effect of rosemary VO. Radwan et al. (47) propounded that the supplementation of 1 % rosemary VO to the diet of hens significantly reduced MDA values in eggs stored at room temperature compared to the control diet. Lopez-Bote et al. (38) and Tang et al. (56) also found that the dietary rosemary VO had the potential to increase the antioxidant capacity of chicken meat. It is well known that major phenolic compounds, carnosic acid, carnosol, camphene, caffeic acid, ursolic acid, rosmarinic acid, 1,8-cineole, α pinene and other phenolic compounds are the most active antioxidant components found in rosemary VO (3, 6, 50). Galobart et al. (25) reported that there was a delay in iron-induced lipid oxidation in hen eggs fed on diets containing rosemary extract, and carnosic acid in rosemary could act as an effective antioxidant when included in the diet at levels of 500 mg /kg or above. Krause and Ternes (36) established that if the hen diets contain 500 or 1000 mg/kg of carnosic acid, the rate of carnosic acid transfer from diet to egg is 0.0025 %. It was determined that the rosemary VO used in the present study contains 1,8-cineol (43.96 %), α-pinene (25.33 %), campfene (11.09 %) and 3 carene (10.7 %) (Table 1), among the main phenolic compounds in rosemary mentioned above. In this study, the significant decrease in MDA levels of egg yolk stored in the refrigerator can be explained by the fact that phenolic hydroxyl groups in rosemary VO delay the formation of hydroxyl peroxide by giving hydrogen to peroxide radicals at the beginning of lipid oxidation (19, 20).

Table 5. Effects of rosemary VO supplementation on egg quality parameters[1] of laying quails.

Rosemary VO (mg/Kg)	Control	Group 1	Group 2	P
	0	200	400	
Shape index	77.28 ± 0.29	77.72 ± 0.24	76.22 ± 0.56	NS
Eggshell thickness, μm	0.13 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	NS
Eggshell breaking strength, N/cm2	13.17 ± 0.23	13.05 ± 0.24	13.11 ± 0.29	NS
Haugh unit	90.62 ± 0.54	90.12 ± 0.60	89.77 ± 0.53	NS
Yolk color	11.72 ± 0.06	11.65 ± 0.06	11.80 ± 0.06	NS

NS: Not significant (P > 0.05).
[1]Values are means of 15 eggs (3 eggs per replicates), n=15.

Table 6. Effects of rosemary VO supplementation on MDA levels1 (mg MDA/kg sample) in egg yolk samples at different storage time (+4 ºC).

Rosemary VO (mg/kg)	Control	Group 1	Group 2	P
	0	200	400	
Day 1	0.05 ± 0.01	0.04 ± 0.01	0.03 ± 0.01	NS
Day 7	0.44 ± 0.10[a]	0.05 ± 0.01b	0.07 ± 0.01[b]	<0.001
Day 28	6.59 ± 0.52[a]	2.97 ± 0.52b	3.03 ± 1.00[b]	<0.01

[a, b]: Different letters within the same row indicate significant differences among the groups.
NS: Not significant (P > 0.05).
1Values are means of 15 eggs (3 eggs per replicates), n=15.

In conclusion, supplementation of 200 mg/kg and 400 mg/kg of rosemary VO to the laying quail diets increased egg production without adversely affecting other performance parameters and egg quality, and reduced egg yolk MDA values during storage in the refrigerator. Based on the data obtained from this research and the information provided from the literature, it has been concluded that rosemary VO can be used as a natural antioxidant in laying quail diets to extend eggs shelf life and to minimize quality losses during storage, considering its strong antioxidant effect.

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Ethical Statement

This study was approved by the Uludag University Animal Experiments Local Ethics Committee (Decision no. 2018-15/04).

Conflict of Interest

The authors are declared that there is no conflict of interest.

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Marker-assisted introgression of myostatin from Texel to Ramlıç sheep: Growth and real-time ultrasound carcass traits in F_1 and BC_1 lambs

Koray ÇELİKELOĞLU[1,a,✉], Mustafa TEKERLİ[1,b], Metin ERDOĞAN[2,c], Serdar KOÇAK[1,d], Ebubekir YAZICI[3,e], Özlem HACAN[1,f], Zehra BOZKURT[1,g], Samet ÇİNKAYA[1,h], Mustafa DEMİRTAŞ[1,i]

[1]Department of Animal Science, Faculty of Veterinary Medicine, Afyonkarahisar, TURKEY; [2]Department of Veterinary Biology and Genetics, Faculty of Veterinary Medicine, Afyonkarahisar, TURKEY; [3]Department of Obstetrics and Gynecology, Faculty of Veterinary Medicine, Afyonkarahisar, TURKEY.

[a]ORCID: 0000-0002-1610-2226; [b]ORCID: 0000-0002-8634-1193; [c]ORCID: 0000-0003-0975-1989; [d]ORCID: 0000-0002-7832-887X; [e]ORCID: 0000-0002-1219-4370; [f]ORCID: 0000-0001-6340-1117; [g]ORCID: 0000-0001-8272-7817; [h]ORCID: 0000-0003-0736-4571; [i]ORCID: 0000-0002-8349-9649

[✉]Corresponding author: kcelikeloglu@aku.edu.tr
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Abstract: The aim of the study was to evaluate live weights, body measurements, and real-time ultrasound carcass traits of F_1 and BC_1 genotypes in a marker assisted introgression (MAI) process. Effects of some factors on live weight and body measurements including ultrasonographic assessments in *Musculus longissimus dorsi* (MLD) were investigated at the F_1 (n=51) and BC_1 (n=99) cross lambs born in 2015 – 2017 period. Variance analysis showed that genotype had significant ($P < 0.001$) effect on pre- and post-weaning growth characteristics. The birth type had significant ($P < 0.05$) effect on pre- and post-weaning growth traits. Live weights at twelve months of age were higher in heavier born lambs. The growth performance of F_1 lambs was between Texel and Ramlıç. Myostatin heterozygous BC_1 lambs had been potentially heavier than the non-carriers and pure Texel. MLD depth and area were also statistically higher ($P < 0.05$) in BC_1 lambs carrying myostatin than non - carriers. As a result, the beginning phase of introgression processes implemented was found to be successful.

Keywords: Crossing, introgression, myostatin, Ramlıç, Texel.

Miyostatin mutasyonunun Teksellerden Ramlıçlara belirteç yardımlı aktarımı: F_1 ve G_1 kuzularda büyüme ve gerçek zamanlı ultrasonografik karkas özellikleri

Özet: Bu çalışma, miyostatin mutasyonunun belirteç yardımlı aktarım yöntemi ile Teksellerden Ramlıç koyunlarına geçirilmesi sırasında F_1 ve G_1 kuzularda büyüme ve ultrasonografik karkas özelliklerindeki değişimi belirlemek amacıyla yapılmıştır. 2015 - 2017 sezonlarında doğan F_1 (n = 51) ve G_1 (n = 99) kuzularda canlı ağırlık, vücut ölçümleri ve belgözü kasının (*Musculus longissimus dorsi*) ultrasonografik değerlendirmeleri üzerine kimi faktörlerin etkileri araştırılmıştır. Varyans analizi sonuçları, genotipin sütten kesim öncesi ve sonrası büyüme özellikleri üzerinde önemli ($P<0,001$) bir etkiye sahip olduğunu göstermiştir. Sütten kesim öncesi ve sonrası büyüme özellikleri üzerine doğum tipinin istatistiki olarak önemli ($P<0,05$) olduğu saptanmıştır. Doğum ağırlığı yüksek olan kuzuların 12. ay canlı ağırlığı da yüksek olmuştur. F_1 kuzularda büyüme performansı bulguları Teksel ve Ramlıç kuzular için belirlenen değerler arasında bulunmuştur. Tek kopya miyostatin mutasyonu taşıyan G_1 kuzuların taşıyıcı olmayan G_1 ve saf Teksel kuzulardan ağır olma kapasitesi bulunduğu görülmüştür. MLD derinliği ve alanı bakımından mutasyonu taşıyan G_1 kuzular, taşımayanlara göre önemli ($P<0,05$) düzeyde üstün bulunmuştur. Sonuç olarak uygulanan introgresyon programının başlangıç aşamasının başarılı olduğu görülmüştür.

Anahtar sözcükler: İntrogresyon, melezleme, miyostatin, Ramlıç, Teksel.

Introduction

The Ramlıç sheep carrying 65% American Rambouillet and 35% indigenous Dağlıç genotypes has become a native breed in the ancient Phrygian region of Anatolia for the last decades. Birth weight, daily live weight gain, weights at weaning and six months of age for

Ramlıç lambs were reported in the ranges of 4.04 - 4.63, 0.270 - 0.300, 22.44 - 30.92 and 29.04 - 37.40 kg, respectively (1, 7, 11, 12, 15, 21, 34). Same traits except for weight at six-months of age for Texel and its crosses in different conditions were found between 3.04 - 5.03, 0.17 - 0.32 and 14.86 - 34.42 kg, respectively (2, 9, 10, 13, 22, 23, 26 - 28, 33). Meanwhile, the live weight in this breed has reached to 45.2 - 46.7 kg at the age of 12 months according to McMillan et al. (28). The depth of Musculus longissimus dorsi in British Texel was reported to be 27.5 mm at 140 days of age according to ultrasonographic measurements by Wolf and Jones (32). Additionally, same researchers observed that the body length, wither height, chest circumference and rump width measurements as 58.0, 55.8, 80.9 and 24.6 cm respectively. The mean live weight obtained in the study was expressed as 43.6 kg. The chest circumference in weaning lambs carrying 50% Texel genotype was also 61.56 cm according to Koritiaki et al. (23). Above mentioned growth characteristics were reported to be affected by different environmental factors such as sex, birth type, month of birth, year, and weaning age (15).

Texels are known as homozygous carriers of myostatin (Mstn +/+) mutation leading to the double muscling phenotype in sheep market throughout the World, while Ramlıç is one of the meat - wool sheep breeds in Turkey. Breeding of leaner and fast-growing lambs and the production of high - quality carcasses are key components to compensate for the meat production deficiency in Turkey. Genetic progress can be accelerated by identifying and using the Quantitative Trait Loci (QTL) or Nucleotides (QTN) in marker - assisted selection (19). Myostatin mutation (g+6723G>A) reported in Texel sheep is located in 3'untranslated regions of Growth differentiation factor 8 gene (*GDF8* or *Myostatin*) (8, 24). Valuable alleles such as myostatin are transferred from foreign breeds to natives by introgression which is the procedure whereby a marker gene is swapped. Thus, a new allele will have been added to the gene pool of indigenous breed (25, 31). This method has been used to pass the Myostatin allele to Lacaune breed successfully Grasset et al. (16). This mutation can be used to increase muscle development and growth of lambs in the selection programs of developing countries.

The aim of the study was to evaluate the growth characteristics of F_1 and BC_1 genotypes during the transfer of myostatin mutation (g+6723G>A) from Texel into the Ramlıç by introgression.

Materials and Methods

In this study, animals were treated following the guidelines of the experimental animal ethics committee of the Afyon Kocatepe University (Decision no.49533702-26). This research was carried out in Afyonkarahisar province Sheep and Goat Breeders Association's Stud Animals Breeding and Test Station situated at 39°02'59.3"N 31°20'23.9"E.

Eight myostatin homozygous pure Texel rams were mated with pure Ramlıç ewes to produce F_1 (Mstn +/-) (Texel X Ramlıç) lambs (n = 51) in the summer of 2015. Then, a total of 100 pure Ramlıç ewes were bred with F_1 ram lambs (n = 20) carrying single - copy myostatin. BC_1 (F_1 X Ramlıç) lambs (n = 99) were born in 2016 - 2017 lambing season.

Eighty-eight BC_1 lambs were genotyped to detect carriers or non - carriers for the aspect of myostatin mutation. The weaning was realized after the overwhelming majority of lambs arriving the age of 120 days. The non - carrier BC_1 lambs were culled after weaning according to the selection program conducted on the farm. Daily live weight gain was estimated using the birth and weaning weights. Also, the weights at six and 12 months of age were calculated by interpolation from the weights recorded periodically.

Records at the time of weaning for some body measurements (wither height, body length, rump width and heart girth) and ultrasonographic values (area and depth) of *Musculus longissimus dorsi* (MLD) and backfat thickness (BF) in F_1 and BC_1 lambs were also used to evaluate the progress in the project.

The ultrasonographic inspections (Mindray DP10, China) were carried out from the midst of 12 and 13th ribs with a 5 MHz convex probe (23). The images were recorded with the MP4 player (Orite® PMP500, Australia) connected to the USG via cable and transferred to a computer. These images were monitored with GOM Player (Gretech Corporation, South Korea) and frozen at the most appropriate time and measured using ImageJ software (National Institute of Health, Bethesda, USA). ImageJ values were calibrated before measurements as described by Bracken et al. (5).

Blood samples for genetic analyses were collected from the *Vena jugularis* of the animals to vacuum tubes with EDTA and were brought to the laboratory in the cold chain and kept at + 4ºC until the DNA isolation stage. DNA extraction was made with a modified method described by Boom et al. (4). DNA samples were checked for their integrity on the agarose gel and their amounts were measured using a spectrophotometer. DNA quantity and quality were controlled and stored at –20 ° C.

g+6723_F_5'-GGT TCG TGA TGG CTG TAT AAT GTG A-3' and g+6723_R_5'-GAT TTC AGA TAA TAG AGT TAA ATC ATT TTG GTT TGC TT-3' primers designed with FastPCR 6.1.2 program (20) were used to determine the SNP (g+6723G>A) in the 3'UTR region of GDF8 gene according to NC_019459.2 reference sequence of NCBI (National Center for Biotechnology Information). A region of 136 bp is amplified with PCR.

Then, a band of 136 bp for AA genotype, 136 and 68 bp bands for AG genotype and 68 bp band for GG genotype were obtained by cutting the PCR product with Tail Restriction Enzyme. For this purpose, a total of 10 µl PCR mixture including 1 µl genomic DNA, 0.8 µl $MgCl_2$ (50 mM), 0.25 µl dNTP, 0.25 µl for each primer (10 pmol each), and 0.0625 µl Platinium Taq Polymerase (Thermo) were prepared. Reactions were performed on the ABI Veriti PCR instrument. Amplification was carried out by using denaturation for 120 seconds at 95 °C and 40 cycles of 95 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds. PCR samples were confirmed with 2% agarose gel electrophoresis. For RFLP, a mix consisted of 5 µl PCR product, 1 µl 10x FastDigest Green buffer, 0.5 µl FastDigest *Tai*I restriction enzyme (Thermo, FD1144) and 3.5 µl ddH_2O incubated for 15 minutes at 65 °C and monitored on 2% agarose gel electrophoresis for genotyping.

Data were analyzed by GLM procedure of MINITAB 18 (29) with the following statistical models: $Y_{ijklmn} = \mu + G_i + S_j + BT_k + BM_l + DA_m + e_{ijklmn}$ for birth weight and daily live weight gain; $Y_{ijklmnop} = \mu + G_i + S_j + BT_k + BM_l + DA_m + WA_n + BW_o + e_{ijklmnop}$ for weaning weight, weight at six months of age, weight at 12 months of age, morphometric measurements and ultrasonographic MLD characteristics at weaning.

Where Y = observation value (morphometric and ultrasonographic measurements), µ = overall mean, G = effect of genotype (Ramlıç, F_1, BC_1 (+/-), BC_1 (-/-) and Texel),

S = effect of sex (male and female), BT = effect of birth type (multiple or single), BM = effect of birth month (December, January and February), DA = effect of dam age (≤730 d, >730 and ≤1095 d and >1095 d), WA = effect of weaning age (≤130 d, >130 and ≤145 d and >145 and ≤180 d), BW = effect of birth weight (≤4.5 kg and >4.5 kg) and e = random error N (0, σ^2) related with each observation. The Tukey procedure provided by in Minitab 18 (29) was used for multiple comparisons.

Results

The least squares means for morphometric and ultrasonographic measurements of Ramlıç, F_1, BC_1 and Texel lambs are presented in the Tables 1, 2, 3 and 4.

Table 1. Least squares means for growth traits of Ramlıç, F_1 and Texel lambs born in 2015 - 2016.

Factor		n	Birth weight (kg)	Average daily weight gain (kg)	Weaning weight (kg)	n	Weight at six mo. of age (kg)	n	Weight at twelve mo. of age (kg)
	µ	143	3.870±0.153	0.17460±0.00577	30.488±0.930	126	34.382±0.944	106	49.03±1.47
Genotype			***	***	***		***		***
	Ramlıç	21	4.100±0.252[a]	0.18760±0.00950[a]	33.89±1.550[a]	19	39.360±1.560[a]	19	59.07±2.39[a]
	F_1 (+/-)	51	4.147±0.159[a]	0.18868±0.00600[a]	30.013±0.869[b]	47	34.403±0.862[b]	42	50.41±1.34[b]
	Texel (+/+)	71	3.364±0.189[b]	0.14751±0.00712[b]	27.56±1.190[b]	60	29.380±1.240[c]	45	37.61±2.04[c]
Sex			**	‡	‡		**		**
	Female	83	3.667±0.171[b]	0.16970±0.00645	29.74±1.03	75	33.12±1.04[b]	66	44.86±1.63[b]
	Male	60	4.073±0.169[a]	0.17950±0.00635	31.24±0.98	51	35.64±1.00[a]	40	53.20±1.59[a]
Birth type			***	***	**				
	Multiple	44	3.569±0.201[b]	0.16177±0.00756[b]	28.93±1.18	40	32.860±1.180	33	47.83±1.85
	Single	99	4.172±0.144[a]	0.18742±0.00544[a]	32.048±0.86	86	35.899±0.887	73	50.23±1.40
Birth month			*	*					
	December	7	3.236±0.347[b]	0.1620±0.01310[b]	32.09±2.270	6	34.091±0.884	6	50.02±1.36
	January	63	4.204±0.154[a]	0.17359±0.00578[ab]	30.296±0.896	55	33.719±0.930	47	50.04±1.46
	February	73	4.171±0.145[a]	0.18818±0.00547[a]	29.083±0.892	65	35.340±2.370	53	47.03±3.69
Dam age (month)			‡						
	≤24	12	3.578±0.291	0.1798±0.01100	31.51±1.600	11	34.06±1.60	10	46.15±2.51
	>24 and ≤ 36	34	4.175±0.184	0.17766±0.00693	30.47±1.030	30	34.45±1.08	27	49.72±1.70
	>36	97	3.857±0.168	0.16635±0.00634	29.48±1.070	85	34.63±1.08	69	51.22±1.65
Weaning age (day)									
	≤130	46	-	-	29.690±1.350	44	34.200±1.390	36	48.24±2.21
	>130 and ≤145	51	-	-	30.390±1.160	44	34.940±1.190	38	48.64±1.92
	>145 and ≤180	46	-	-	31.386±0.959	38	34.001±0.986	32	50.21±1.54
Birth weight (kg)					***		‡		‡
	≤4.5	93	-	-	28.886±0.892[b]	81	32.847±0.900	69	47.78±1.36
	>4.5	50	-	-	32.090±1.150[a]	45	35.920±1.170	37	50.29±1.90

‡: P<0.10; *:P<0.05; **:P<0.01; ***:P<0.001.

[abc]: Least squares means with different superscript in each fixed effect are significantly different (P<0.05).

Table 2. Least squares means for growth traits of Ramlıç, BC_1 and Texel lambs born in 2016 – 2017.

Factor		n	Birth weight (kg)	Average daily weight gain (kg)	Weaning weight (kg)	n	Weight at six mo. of age (kg)	n	Weight at twelve mo. of age (kg)
	μ	224	4.7943±0.0877	0.20528±0.00431	32.274±0.616	121	35.933±0.876	109	49.37±1.19
Genotype				**	**		***		***
	Ramlıç	98	4.647±0.108	0.21151±0.00530[a]	33.359±0.735[a]	53	38.73±1.09[a]	53	54.86±1.41[a]
	Ramlıç BC_1 (-/-)	48	4.859±0.136	0.21019±0.00667[a]	32.569±0.941[ab]	-	-	-	-
	Ramlıç BC_1 (+/-)	40	4.962±0.135	0.21301±0.00664[a]	33.468±0.937[a]	40	36.91±1.04[a]	34	51.40±1.44[a]
	Texel (+/+)	38	4.708±0.156	0.18640±0.00769[b]	29.700±1.070[b]	28	32.16±1.41[b]	22	41.85±1.98[b]
Sex			***	***	***		***		***
	Female	120	4.5911±0.0994[b]	0.18969±0.00489[b]	30.472±0.687[b]	73	33.44±1.00[b]	66	44.13±1.36[b]
	Male	104	4.9980±0.1060[a]	0.22087±0.00523[a]	34.077±0.752[a]	48	38.43±1.05[a]	43	54.61±1.44[a]
Birth type			***	***	**		‡		‡
	Multiple	66	4.4040±0.1290[b]	0.19332±0.00634[b]	31.005±0.876[b]	36	34.75±1.23	32	47.89±1.63
	Single	158	5.1851±0.0798[a]	0.21723±0.00392[a]	33.543±0.602[a]	85	37.12±0.86	77	50.85±1.20
Birth month				‡	**				
	December	67	4.8280±0.1240	0.21106±0.00609	33.786±0.903[a]	39	36.15±1.24	36	48.06±1.66
	January	157	4.7611±0.0859	0.19949±0.00423	30.762±0.751[b]	82	35.72±1.06	73	50.68±1.45
Dam age (month)			*						
	≤24	65	4.9010±0.1260[ab]	0.20798±0.00621	32.536±0.873	42	36.230±1.100	40	49.43±1.47
	>24 and ≤ 36	21	4.4750±0.1870[b]	0.20005±0.00922	32.00±1.260	11	36.260±1.930	9	49.09±2.61
	>36	138	5.0064±0.0732[a]	0.20781±0.00360	32.283±0.552	68	35.306±0.770	60	49.59±1.08
Weaning age (day)					***				
	≤130	65	-	-	29.430±0.960[b]	34	33.750±1.330	29	46.74±1.87
	>130 and ≤145	123	-	-	33.209±0.718[a]	67	36.056±0.980	60	48.48±1.30
	>145 and ≤180	36	-	-	34.180±1.180[a]	20	38.000±1.650	20	52.88±2.17
Birth weight (kg)					***		***		*
	≤4.5	63	-	-	29.824±0.818[b]	35	33.490±1.170[b]	29	47.22±1.64[b]
	>4.5	161	-	-	34.725±0.671[a]	86	38.376±0.916[a]	80	51.52±1.21[a]

‡: P<0.10; *:P<0.05; **:P<0.01; ***:P<0.001.
[abc]: Least squares means with different superscript in each fixed effect are significantly different (P<0.05).

Table 3. Least squares means for body and real-time ultrasonographic measurements at weaning in F_1 lambs born in 2015 - 2016.

Factor		n	Wither height (cm)	Body length (cm)	Rump width (cm)	Hearth girth (cm)	MLD depth (cm)	MLD area (cm^2)	Backfat thickness (cm)
	μ	51	58.917±0.831	59.797±0.800	18.530±0.424	80.91±1.99	2.4788±0.0829	9.601±0.451	0.5467±0.0268
Sex								*	
	Female	29	58.617±0.940	59.306±0.905	18.763±0.479	81.72±2.25	2.5202±0.0937	10.192±0.510[a]	0.556±0.0303
	Male	22	59.218±0.947	60.288±0.912	18.297±0.483	80.11±2.27	2.4375±0.0944	9.010±0.514[b]	0.5372±0.0306
Birth type			*		‡	‡	‡		
	Multiple	9	57.490±1.330[b]	59.14±1.28	17.890±0.680	78.19±3.20	2.372±0.133	9.089±0.724	0.5291±0.0431
	Single	42	60.341±0.618[a]	60.453±0.595	19.170±0.315	83.64±1.48	2.5854±0.062	10.114±0.335	0.5644±0.0199
Birth month			‡	*	‡				
	December	5	64.40±2.48	65.87±2.39[a]	20.910±1.270	90.05±5.96	2.549±0.248	10.22±1.35	0.6203±0.0802
	January	35	57.05±1.21	58.14±1.16[ab]	17.940±0.617	78.52±2.90	2.449±0.121	9.548±0.656	0.5147±0.0390
	February	11	55.31±1.80	55.38±1.73[b]	16.738±0.919	74.17±4.32	2.438±0.180	9.038±0.978	0.5052±0.0582
Dam age (month)									
	≤24	12	59.110±1.250	60.75±1.210	18.863±0.639	81.60±3.00	2.4650±0.1250	10.025±0.680	0.5464±0.0405
	>24 and ≤ 36	17	59.770±1.140	60.07±1.100	18.137±0.581	81.64±2.73	2.5500±0.1130	9.566±0.618	0.5485±0.0368
	>36	22	57.878±0.836	58.567±0.805	18.590±0.426	79.50±2.00	2.4207±0.0833	9.213±0.453	0.5453±0.0270
Birth weight (kg)								‡	
	≤4.5	26	58.672±0.891	59.131±0.858	18.242±0.454	79.91±2.14	2.4254±0.0888	9.100±0.483	0.5360±0.0288
	>4.5	25	59.160±1.030	60.463±0.990	18.818±0.525	81.92±2.47	2.5320±0.1030	10.102±0.558	0.5575±0.0332
Weaning age (day)									
	≤130	23	61.21±1.31	61.33±1.26	19.476±0.666	84.36±3.13	2.459±0.130	9.538±0.709	0.5587±0.0422
	>130 and ≤145	22	60.51±1.42	60.67±1.36	19.170±0.723	84.10±3.40	2.381±0.141	8.995±0.769	0.5852±0.0458
	>145 and ≤180	6	55.03±2.40	57.39±2.31	16.940±1.220	74.28±5.75	2.596±0.239	10.270±1.30	0.4963±0.0775

‡: P<0.10; *:P<0.05; **:P<0.01; ***:P<0.001.
[abc]: Least squares means with different superscript in each fixed effect are significantly different (P<0.05).

Table 4. Least squares means for body and real-time ultrasonographic measurements at weaning in BC_1 lambs born in 2016 – 2017.

Factor	n	Wither height (cm)	Body length (cm)	Rump width (cm)	Hearth girth (cm)	MLD depth (cm)	MLD area (cm^2)	Backfat thickness (cm)
μ	80	63.080±0.609	60.903±0.704	21.604±0.289	84.27±1.08	3.461±0.124	16.087±0.911	0.7740±0.0258
Genotype						*	*	
Ramlıç BC_1 (+/-)	37	62.714±0.686	60.930±0.793	21.633±0.326	83.89±1.22	3.618±0.140[a]	17.16±1.03[a]	0.7589±0.0290
Ramlıç BC_1 (-/-)	43	63.445±0.684	60.876±0.791	21.574±0.325	84.64±1.21	3.304±0.139[b]	15.01±1.02[b]	0.7890±0.0289
Sex		*						
Female	39	62.320±0.680[b]	60.325±0.786	21.423±0.323	83.92±1.21	3.482±0.138	16.34±1.02	0.7739±0.0287
Male	41	63.839±0.698[a]	61.480±0.806	21.784±0.331	84.61±1.24	3.440±0.142	15.83±1.04	0.7741±0.0295
Birth type					*			
Multiple	24	63.086±0.837	60.405±0.968	21.708±0.398	82.55±1.49[b]	3.326±0.170	15.26±1.25	0.7559±0.0354
Single	56	63.073±0.568	61.401±0.657	21.499±0.270	85.98±1.01[a]	3.596±0.116	16.908±0.851	0.7920±0.0240
Birth month			‡				**	
December	34	63.597±0.768	61.737±0.888	21.784±0.365	84.40±1.36	3.498±0.156	17.63±1.15[a]	0.7958±0.0325
January	46	62.562±0.692	60.069±0.800	21.423±0.329	84.13±1.23	3.424±0.141	14.54±1.04[b]	0.7521±0.0292
Dam age (month)		‡						
≤24	8	64.23±1.110	61.44±1.28	21.933±0.528	83.60±1.97	3.335±0.226	15.91±1.66	0.7687±0.0469
>24 and ≤ 36	14	63.058±0.875	60.790±1.01	21.718±0.416	85.18±1.55	3.563±0.178	16.04±1.31	0.8113±0.0370
>36	58	61.951±0.460	60.478±0.532	21.160±0.219	84.021±0.817	3.4849±0.0937	16.305±0.689	0.7419±0.0194
Birth weight (kg)		***	*	**	*	*	**	
≤4.5	21	61.436±0.771[b]	59.883±0.892[b]	21.059±0.367[b]	82.67±1.37[b]	3.315±0.157[b]	14.29±1.15[b]	0.7684±0.0326
>4.5	59	64.723±0.624[a]	61.922±0.722[a]	22.148±0.297[a]	85.86±1.11[a]	3.607±0.127[a]	17.881±0.934[a]	0.7796±0.0264
Weaning age (day)				*	‡			
≤130	8	63.00±1.150	60.85±1.33	20.694±0.548[b]	81.83±2.05	3.578±0.235	15.06±1.73	0.8065±0.0488
>130 and ≤145	54	62.571±0.541	60.750±0.625	21.569±0.257[ab]	83.576±0.960	3.307±0.110	16.775±0.810	0.7601±0.0229
>145 and ≤180	18	63.665±0.932	61.110±1.08	22.548±0.443[a]	87.40±1.650	3.498±0.190	16.430±1.39	0.7553±0.0394

‡: P<0.10; *:P<0.05; **:P<0.01; ***:P<0.001.
[abc]: Least squares means with different superscript in each fixed effect are significantly different (P<0.05).

Findings showed that birth weight was significantly (P<0.05) affected by genotype, sex, birth type and birth month. The birth weight was found to be highest in F_1 lambs whereas Texel lambs were lowest in this trait. The effect of genotype, birth type and birth month on average daily live weight gains were statistically significant (P<0.05) and, Ramlıç and F_1 lambs were found to be superior to Texel. Weaning weight was also significantly (P<0.05) affected by genotype, birth type and birth weight. Weaning weight of Ramlıç lambs had higher than the others, while F1 genotype was between the Ramlıç and Texel. The effects of genotype and sex on weights at the six and 12 months of age were also found to be significant (P<0.01). Ramlıç lambs had the highest values in both traits, followed by F_1 and Texel. The effects of birth type on wither height, sex on MLD area and, birth month on body length at weaning were significant (P<0.05) in F_1 lambs.

Analysis of variance showed that sex, birth type and dam age had a significant (P<0.05) effect on birth weights of all genotypes. The effect of genotypes on birth weight was not significant, but the perusal of the least-squares means revealed that BC_1 lambs carrying the mutation tend with the highest birth weight. Daily live weight gains significantly (P<0.01) affected by genotype, sex and birth type. The impact of genotype, sex, birth type, birth month, weaning age and birth weight on weaning weight were statistically significant (P<0.05). Live weights at six and 12 months of age significantly (P<0.05) affected by genotype, sex and birth weight. Differences between Ramlıç and BC_1 genotypes were not significant in these traits. The influences of sex on wither height, weaning age on rump width, birth type on chest circumference, genotype on MLD depth and genotype and birth month on MLD area were found to be statistically significant (P<0.05) in BC_1 lambs. The birth weight was also found to be significant (P<0.05) in all traits, except MLD depth (P<0.10) and backfat thickness.

Discussion and Conclusion

There was no significant difference in birth weight between pure Ramlıç and F_1 lambs in first lambing season and BC_1 lambs in second lambing season. The least-squares means showed that crossbreds carrying myostatin mutation tended to be heavier in birth. Similar findings were reported in Romneys (17) and Iranian Makoei sheep

(14). The birth weight values determined for BC_1 lambs are just above the range of 4.00 to 4.95 kg reported for Ramlıç and Rambouillet in the literature (6, 7, 12, 15, 18, 21, 30, 34, 35). The birth weight values in F_1 and Texel lambs are consonant with the findings (3.7 - 5.1 kg) of literature (3, 13, 22, 23, 26, 33) for lambs carrying different level of Texel genotype. Sex, type of birth and month of birth had a significant effect ($P<0.05$) on birth weight in consistent with the results of various researchers (11, 15, 34, 35). The daily live weight gain of Ramlıç, F_1 and BC_1 lambs were found to be convenient with the literature (1, 6, 18, 19, 30) reported for Ramlıç, Rambouillet and its crossbreeds. Average daily live weight gain of Texel lambs were slightly short of the 0.190 - 0.318 kg limits reported in other studies (2, 10, 26). The differences may be due to the variations in feeding and management, weaning times, and statistical models used in various researches. The weaning weights of Ramlıç, F_1 and BC_1 lambs determined in this study were found to be higher than the values amongst 24.1 and 28.6 kg reported in previous literature (11, 12, 15, 34, 35). Likewise, to Ramlıç and its crosses of our study, Bromley et al. (6) notified that the weaning weights for Columbia, Polypay, Rambouillet and Targhee lambs as 36.4 kg, 33.7 kg, 32.7 kg and 33.8 kg, respectively. The weaning weights of Texel lambs in this study were in the range of 20.9 – 31.2 kg reported by McEwan et al. (27) and McMillan et al. (28) for purebred and crossbred Texel lambs. It was also found just above 26.8 kg that was obtained by Wuliji et al. (33) in Texel x Romney crossbred and below the value of 34.42 kg reported by Khusro et al. (22). The weight findings at six months of age for Ramlıç and BC_1 lambs were higher than that of Yalçın et al. (34) and Ceyhan et al. (7) for Ramlıç and Rambouillet. The MLD depth of F_1 lambs were lower than the British Texel lambs, while higher in BC_1 lambs (32). The significant difference between myostatin carriers and non - carriers for MLD depth and MLD area may have been derived from the effect of myostatin mutation. The body length of F_1 and BC_1 lambs and chest girth in BC_1 lambs were also higher than the results of the same researchers. This situation suggested that repeated backcrossing has no harmful effect on body size.

It was determined that the factors such as genotype, sex, type of birth, maternal age, weaning age and birth weight may have significant effects on the pre- and post-weaning growth characteristics of lambs. These factors should be taken into consideration in selection and backcrossing studies in some special situations such as detecting of myostatin mutation. The trend in least-squares means for weaning weight showed that the myostatin heterozygous BC_1 lambs had been potentially heavier than the non-carriers and pure Texel. Their weights at six and twelve months of age were not statistically significant from Ramlıç lambs. MLD depth and area were significantly higher ($P<0.05$) in BC_1 lambs carrying myostatin than non – carriers. Consequently, findings revealed that the beginning phase of introgression processes practiced in this study has been succeeded.

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Ethical Statement

This study was approved by the animal ethics committee of the Afyon Kocatepe University (AKUHADYEK) Decision no.49533702-26.

Conflict of interest

All authors declare there are no conflicts of interest among them and with any other people or corporations.

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Comparison of growth and developmental stability traits of Japanese quails reared in conventional and enriched cages

Doğan NARİNÇ[1,a,✉], Kübra Melis SABUNCUOĞLU[1,b]

[1]Akdeniz University, Faculty of Agriculture, Animal Science Department, Antalya, Turkey
[a]ORCID: 0000-0001-8844-4412; [b]ORCID: 0000-0001-6553-1345

✉Corresponding author: dnarinc@akdeniz.edu.tr
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Abstract: This study was carried out to determine the effects of different rearing systems on some performance and welfare characteristics in Japanese quail. For this purpose, a total of 630 birds were housed in battery-type conventional cages, conventional floor cages and enriched floor cages. There were statistically significant differences between the experimental groups and the sexes in terms of the average body weight of the quails at 42 and 56 days of age (both P<0.01). Accordingly, it was determined that the average body weight of the quails raised in the conventional battery cages were higher than those raised both in the conventional and enriched floor cages. Gompertz growth curve model parameters of β_0, β_1, β_2 were estimated as 262.05, 3.76, 0.050 for birds housed in battery-type conventional cages, 228.12, 3.57, 0.042 for birds housed in conventional floor cages, and 252.53, 3.86, 0.045 for birds housed in enriched floor cages, respectively. Among the bilateral traits, the highest mean value of relative asymmetry was found for wing length of birds reared in conventional battery type cages (P<0.01). As a result, it was determined that quails raised in conventional battery type cages are more advantageous for economic production. However, it may be recommended to use enrichment objects for an animal welfare oriented production system.

Keywords: Animal welfare, bilateral symmetry, enriched cage, growth curve.

Konvansiyonel ve Zenginleştirilmiş Kafeslerde Yetiştirilen Japon Bıldırcınlarında Büyüme ve Gelişim Dengesi Özelliklerinin Karşılaştırılması

Özet: Bu çalışma farklı yetiştirme koşullarının bıldırcınların bazı performans ve refah özelliklerine etkilerini belirlemek amacıyla gerçekleştirilmiştir. Bu amaçla toplam 630 Japon bıldırcını batarya tipi konvansiyonel kafeslerde, konvansiyonel yer kafeslerinde ve zenginleştirilmiş yer kafeslerinde barındırıldı. Çalışmada kullanılan bıldırcınların canlı ağırlık ortalamaları bakımından hem deneme grupları hem de cinsiyetler arasında istatistiksel olarak önemli farklılıklar belirlenmiştir (P<0.01). Buna göre konvansiyonel katlı kafeste yetiştirilen bıldırcınların canlı ağırlık ortalamalarının hem yer kafesinde yetiştirilenlerden, hem de zenginleştirilmiş kafeste yetiştirilenlerden daha yüksek olduğu belirlenmiştir. Gompertz büyüme eğrisi modeli parametreleri olan β_0, β_1, β_2 ortalamaları sırasıyla konvansiyonel kafes için 262,05, 3,76, 0,050, yer kafesi için β_0, β_1, $\beta_2$parametre ortalamaları ise sırasıyla 228,12, 3,57, 0,042 ve zenginleştirilmiş kafes için β_0, β_1, $\beta_2$parametre ortalamaları sırasıyla 252,53, 3,86 ve 0,045 olarak bulunmuştur. Bilateral özelliklerden kanat uzunluğu için en yüksek oransal asimetri ortalamaları konvansiyonel batarya tipi kafeste saptanmıştır (P<0,01). Sonuç olarak konvansiyonel katlı kafeslerde yetiştirilen bıldırcınların ekonomik üretim için daha avantajlı olduğu belirlenmiştir. Fakat hayvan refahı odaklı bir üretim için ise zenginleştirme objelerinin kullanılması tavsiye edilebilir.

Anahtar sözcükler: Büyüme eğrisi, hayvan refahı, iki yönlü simetri, zenginleştirilmiş kafes.

Introduction

In order to reduce the stress level and create welfare in poultry, it is recommended that the birds be housed in areas similar to their natural habitats and to add some environmental enrichment objects to the area where they can exhibit their natural behaviors. Environmental enrichment is defined as changing the environment offered to animals in order to improve biological functions and increase behavioral opportunities (7, 8). Environmental enrichment is a widely used technique to improve animal welfare and has a wide range of behavioral and physiological implications. In the cage and floor systems where poultry are housed, nest, perches, sand bath, walking path, playing equipment, relaxing sound or lighting equipment can be used as environmental enrichment elements (39).

There have been a number of studies of the effects of particular enrichment types on poultry performance and behavior.It has been reported by many researchers that environmental enrichment practices in broiler chickens have no effect on body weight gain, feed efficiency and carcass characteristics (5, 22, 37). Jones (16) reported that the level of fear was reduced in broilers raised in the enriched environment, and the adaptation ability of birds against different objects increased. Martrenchar et al. (23) reported that the aggressive pecking behavior was reduced in turkeys raised in an enriched environment. In another study investigating the effect of environmental enrichment and music stimulation on behavior in broilers, it was reported that the fear behavior towards different objects was significantly lower (15).

Studies on environmental enrichment in poultry have generally been carried out in chickens (9, 22, 37, 39). There are limited number of studies on environmental enrichment practices in Japanese quail (26, 36). In developed countries, 80% of the total poultry production is obtained from chicken species while 20% of the production is from other poultry species. For this reason, it is necessary to work on the breeding practices of minor poultry species such as goose, duck, partridge and quail to improve the product quality. The commercial production of quails is intensively carried out due to the fact that quails reach sexual maturity and slaughter age in a very short time, they are reared in large numbers per unit area due to their small size and they are resistant to diseases. In addition, Japanese quails have been used as model animals in various scientific studies for years (29). The production, which is made without distinguishing the meat or egg yield in quails, is carried out both in battery cages and in the deep litter system. However, there are a limited number of studies on rearing systems which promote behaviors that are important for metabolic and mental health in quails and their relationship with productivity. Miller and Mench (26), who examined the effects of different environmental enrichment methods on behavior and productivity in Japanese quails, reported that live weight was not affected by environmental enrichment. In another study conducted by Miller and Mench (25), Japanese quails were reared in trial units with four different enriched environments, and the effects of these housing types on aggressiveness, feather pecking, feather loss, feed wasting and some performance characteristics were investigated. In a study using conventional battery cage and enriched cage (36), some behavioral characteristics of Japanese quails were compared. The aim of this study is to compare growth and developmental stability characteristics of Japanese quails housed in different rearing system. Thus, it was targeted to determine the effect of an enriched environment on growth and developmental stability characteristics of Japanese quails.

Materials and Methods

The care and use of animals were in accordance with laws and regulations of Turkey and approved by Ministry of Food, Agriculture and Livestock (decision number 22875267-325.04.02-E.3211771) and Animal Experiments Local Ethics Committee of Akdeniz University. The study was conducted using three in-time replicates over the course of 56 days. A total of 630 randombred Japanese quail (*Coturnix coturnix Japonica*) chicks were hatched at Avian Sciences facility of Akdeniz University. Chicks with wing numbers were randomly distributed at one day old among 2 battery type cages (conventional battery cages=CBC) and 4 floor pens with deep litter (2 conventional floor pens=CFP, 2 enriched floor pens=EFP). Weekly live weights and developmental stability measurements were performed by matching individual records during the trial. A total of 240 chicks assigned to the conventional environment treatment were housed in the fattening type of battery cages (12.63 kg BW/m^2 at slaughter age). A total of 240 chicks assigned to the conventional environment treatment were housed in the floor pen cages with deep litter (12.63 kg BW/m^2 at slaughter age). A total of 150 chicks assigned to the enriched environment were placed in the floor pen cages with deep litter (6.87 kg BW/m^2 at slaughter age). Chicks in the floor groups (CFP and EFP) were placed on 4-cm fresh wood-shavings litter in an environmental chamber. Chicks in the cage group (CBC) were placed in a 2-cage stainless steel battery (five levels of two cages, cup-type automatic waterers and trough feeders). Perches, sandboxes, walking paths, nail and beak scrubbers and various game objects were used as enrichment equipment in the EFC trial units.

Chicks in all chambers were brooded at 32 °C on days 1 to 5, 29 °C on days 6 to 10, 27 °C on days 11 to 17, and 21 °C thereafter. Both chambers shared the same ventilation system, and the ventilation rate was equal for both groups. Water and a corn-soybean meal quail ration (24% CP, 2900 kcal ME/kg) formulated to meet or exceed the minimum NRC standards for all ingredients were provided ad libitum. The lights were on for 24 h/d through day 5, and for 23 h/d thereafter.

Four bilateral traits (face, wing, shank diameter, shank length) were measured in millimeters by using calipers to determinate the developmental stability in Japanese quail at 56 days of age. The recorded morphological traits were right (R) and left (L) lengths and diameters of shank (metatarsus), right (R) and left (L) lengths of wing (radius), and face lengths. Relative asymmetry (RA) of bilateral traits was defined as $(|L-R|/((L+R))/2)\times100$. A series of steps (30) were

followed before identifying exhibited asymmetry as fluctuating asymmetry. First, the presence of directional asymmetry (normal distribution with a mean of not zero) and antisymmetry (nonnormal distribution) was tested by inspection of the distribution of signed right minus left differences (R−L). The presence of directional asymmetry was tested for using one sample t-test. Departures from normality were assessed using Shapiro Wilktest. If differences in (R−L) exist, asymmetry should be leptokurtically distributed: the greater the magnitude of differences, the greater the leptokurtosis. Second, the fluctuating asymmetry and measurement errors are normally distributed about a mean of zero (9).

To obtain the estimates of individual growth curve parameters, all quail were weighed weekly from hatching to 8 weeks of age. In many studies fitted to model of the growth samples of Japanese quails, it was determined that the best model in terms of goodness of fit criteria was the Gompertz model (3, 11, 19, 31, 33). For this reason, the Gompertz non-linear regression model (I) was used to estimate growth curve of each quail.

$$\boldsymbol{y_t = \beta_0 e^{(-\beta_1 e^{-\beta_2 t})}} \text{ (I)}$$

where $\boldsymbol{y_t}$is the weight at age *t*, $\boldsymbol{\beta_0}$ is the asymptotic (mature) weight parameter, $\boldsymbol{\beta_1}$ is the scaling parameter (constant of integration) and $\boldsymbol{\beta_2}$ is the instantaneous growth rate (per day) parameter (4, 31, 33). The Gompertz model is characterized by an inflection point in a manner such that β_0/e of the total growth occurs prior to it and the remainder occurring after. The coordinates of the point of inflection, age and weight at inflection point (IPW and IPA, respectively), were obtained as follows:

$$\boldsymbol{IPW = \beta_0/e} \text{ (II)}$$

$$\boldsymbol{IPA = ln(\beta_1)/\beta_2} \text{ (III)}$$

The nonlinear regression analyses for growth curves of birds were performed using NLIN procedure of SAS 9.3 statistics software. The descriptive statistics, Shapiro Wilk normality tests and hypothesis tests of the traits were obtained using UNIVARIATE procedure of SAS 9.3 statistics software. Data was subjected to two-way ANOVA to test the effects of treatment groups and gender on weekly body weights, individual growth curve parameters, and values of relative asymmetry using the GLM procedure of the SAS 9.3 statistics software. Means were compared using Duncan's multiple range test. The level of significance for all statistical analyses was based on $P < 0.01$.

Results

The descriptive statistics and results of statistical analyses (analyses of variance and Duncan multiple range tests) for body weight at 42 and 56 days of age, parameters of Gompertz growth curve and its point of inflection coordinates were presented by treatment groups and gender in Table 1. In studies using Japanese quails and in commercial production, generally six and eight week periods are accepted as the fattening period. Therefore, in

Table 1. The results of statistical analysis for traits of body weight and growth curve by treatment groups and gender.

Treatment		BW 42 (g)	BW 56 (g)	β_0	β_1	β_2	IPA (days)	IPW (g)
CBC		131.09±1.78^{a}	169.58±2.17^{a}	262.05±8.15^{a}	3.76±0.04^{a}	0.050±0.001^{a}	27.88±0.75^{c}	83.93±2.62^{c}
CFC		112.18±2.04^{c}	155.89±2.49^{c}	228.12±7.12^{c}	3.57±0.04^{b}	0.042±0.001^{b}	34.37±0.86^{a}	96.41±3.00^{a}
EFC		125.73±2.51^{b}	163.72±3.07^{b}	252.53±10.06^{b}	3.86±0.05^{a}	0.045±0.001^{ab}	32.97±1.06^{a}	92.91±3.70^{a}
Sex								
♀		126.84±1.78^{a}	168.24±2.17^{a}	253.00±7.11	3.76±0.04	0.046±0.001	31.44±0.75	93.08±2.62
♂		119.16±1.70^{b}	157.88±2.08^{b}	242.14±6.82	3.71±0.03	0.045±0.001	32.04±0.72	89.09±2.51
Interaction								
CBC	♀	136.40±2.46	176.61±3.01	269.70±11.49	3.82±0.05	0.050±0.001	28.24±1.04	88.56±3.63
	♂	125.77±2.56	162.55±3.13	254.41±11.56	3.70±0.05	0.049±0.001	27.53±1.08	79.30±3.78
CFC	♀	114.07±2.87	159.11±3.51	240.72±9.87	3.60±0.06	0.042±0.001	34.50±1.21	99.23±4.23
	♂	110.28±2.89	152.66±3.53	215.53±10.27	3.54±0.06	0.041±0.001	34.24±1.22	93.60±4.25
EFC	♀	130.04±3.75	169.01±4.58	248.58±15.01	3.85±0.07	0.046±0.002	31.58±1.58	91.46±5.52
	♂	121.43±3.35	158.43±4.09	256.48±13.40	3.87±0.07	0.044±0.002	34.36±1.41	94.36±4.93
Source of Variation					P values			
Treatment		<0.001*	<0.001*	0.005*	<0.001*	<0.001*	<0.001*	0.005*
Sex		0.002*	0.001*	0.271	0.286	0.389	0.563	0.271
TreatmentxSex		0.443	0.515	0.406	0.511	0.900	0.384	0.406

CBC= Conventional battery cages, CFC= Conventional floor cages, EFC= Enriched floor cages, BW42 and BW56= Body weight at 42 and 56 days of age; β_0= Asymptotic BW parameter; β_1= Shapeparameter; β_2= Instantaneous growth rate parameter; IPT and IPW = age and weight at inflection point, *=Statistically significance, P<0.01, ^{a,b}= Means with in the same effect and column with no common superscript differ (P<0.01).

this study, live weight averages of both ages were focused on. The average body weights of the quails housed in conventional battery cages at 42 and 56 days of age were found as 131.09 g and 169.58 g, respectively, while the average body weights of those raised in the floor cages were 112.18 g and 155.89 g, and 143.80 g and 163.72 g for those grown in the enriched floor pens. The highest live weights at 42 and 56 days of age were measured in quails housed in a battery-type cage ($P<0.01$). Females had higher averages than males in terms of live weight values for both weeks ($P<0.01$). The coefficients of determination were found between 0.9978 and 0.9999 in the growth curve analyzes performed individually for all quails. In terms of the mature weight parameter (β_0) of the Gompertz growth curve model, similar to the live weight averages, CBC group had a higher mean value ($P<0.01$), while no significant difference was found between female and male quails in terms of this parameter ($P>0.01$). Similar situations were valid for the other parameters of Gompertz growth model and the mean values of weight and time of the inflection point. The growth curves obtained as a result of the analysis with Gompertz model using the live weights of the quails in the experimental groups, and the graphs of these growth curves plotted according to the sex in each experimental group were presented in Figure 1.

The mean values and statistical analysis results for the relative asymmetry measurements of the face, wing, shank length and diameter determined at 56 days of age in Japanese quails were presented according to the experimental groups and sexes in Table 2. A statistically significant difference was found only for the wing in terms of the relative asymmetry averages of the bilateral traits ($P<0.01$). In the study, the mean value of relative asymmetry for the wing was found to be 10.85% in quails reared in battery type cages, and it was higher than the average values (CFC: 6.68% and EFC: 7.87%) of those raised on the floor pens ($P<0.01$). In the study, no significant difference was found between genders in terms of relative asymmetry values determined for bilateral characteristics.

The symmetry conditions determined at the age of 8 weeks for the face, wing, shank length and shank diameter of the Japanese quails in the experimental groups are presented in Table 3. In terms of symmetry status determined according to Shapiro Wilk and One Sample T-test results, antisymmetry for face length and wing length and directional symmetry for shank length and diameter were determined in CBC group. While fluctuating asymmetry was determined only for the face length among the bilateral traits of the CFC group quails, it was determined that the symmetry status was directional for the other bilateral characteristics. While the fluctuating asymmetry conditions were determined for wing and shank length traits of quails housed in the enriched floor cage, antisymmetry for the face length and directional asymmetry for shank diameter were determined.

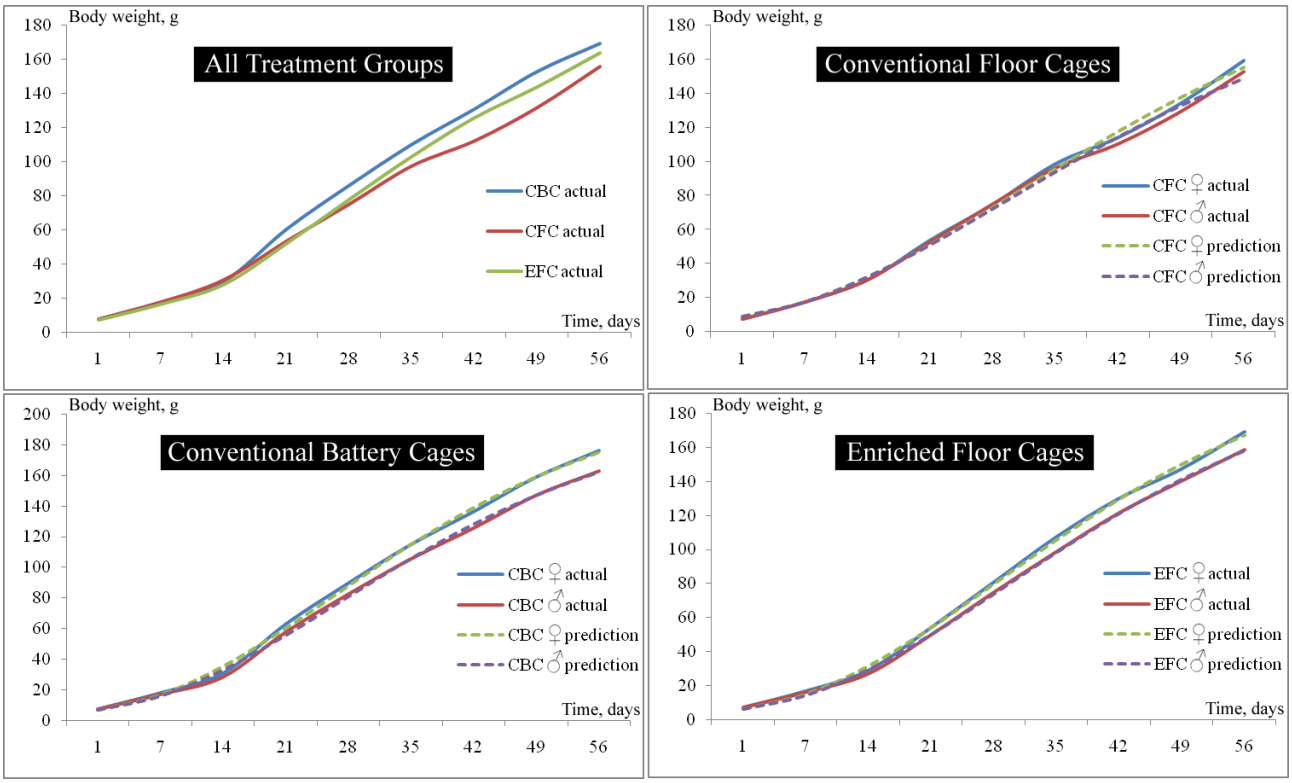

Figure 1. Growth curves by treatment groups.

Table 2. The results of statistical analysis for relative asymmetry means of bilateral traits by treatment groups and gender.

Treatment		Face Length (%)	Wing Length (%)	Shank Length (%)	Shank Diameter (%)
CBC		9.49±0.46	10.85±0.46[a]	5.60±0.31	15.71±0.84
CFC		7.59±0.84	6.68±0.85[b]	5.25±0.57	15.06±1.54
EFC		8.14±0.58	7.87±0.59[b]	4.71±0.39	17.98±1.07
Sex					
♀		8.39±0.54	8.51±0.54	5.23±0.37	16.88±0.99
♂		8.42±0.51	8.43±0.52	5.14±0.35	15.62±0.95
Interaction					
CBC	♀	8.71±0.62	10.74±0.62	5.79±0.42	14.31±1.14
	♂	10.27±0.67	10.96±0.68	5.41±0.46	17.11±1.23
CFC	♀	8.46±1.26	7.50±1.28	4.61±0.86	18.27±2.32
	♂	6.72±1.10	5.87±1.11	5.88±0.75	11.86±2.02
EFC	♀	8.00±0.80	7.27±0.81	5.28±0.54	18.06±1.47
	♂	8.28±0.85	8.46±0.86	4.14±0.57	17.90±1.56
Source of Variation			P values		
Treatment		0.061	<0.001*	0.208	0.167
Sex		0.962	0.920	0.863	0.359
Treatment x Sex		0.211	0.391	0.220	0.032

CBC= Conventional battery cages, CFC= Conventional floor cages, EFC= Enriched floor cages, *=Statisticallysignificance, P<0.01, [a,b]= Means with in the same effect andcolumn with no common superscript differ (P<0.01).

Table 3. Asymmetry status in bilateral traits according to treatment groups.

Treatment	Criteria	Face Length	Wing Length	Shank Length	Shank Diameter
CBC	Shapiro Wilk	0.005*	0.006*	0.099	0.314
	One Sample T-test	0.597	<0.001*	<0.001*	<0.001*
	Status	Antisymmetry	Antisymmetry	Directional Asymmetry	Directional Asymmetry
CFC	Shapiro Wilk	0.349	0.373	0.686	0.110
	One Sample T-test	0.057	<0.001*	<0.001*	<0.001*
	Status	Fluctuating Asymmetry	Directional Asymmetry	Directional Asymmetry	Directional Asymmetry
EFC	Shapiro Wilk	0.005*	0.379	0.558	0.964
	One Sample T-test	0.676	0.086	0.014	0.003*
	Status	Antisymmetry	Fluctuating Asymmetry	Fluctuating Asymmetry	Directional Asymmetry

CBC= Conventional battery cages, CFC= Conventional floor cages, EFC= Enriched floor cages, *=Statistically significance, P<0.01.

Discussion and Conclusion

The mean values of body weight in all birds at 6 and 8 weeks of age were found between 112.18 g and 143.80 g, and between 155.89 g and 169.58 g, respectively. Similar results (from 91.63 to 114.76 g) for 6 weeks of age have been reported by Aggrey et al. (2), Raji et al. (38) and Rocha et al. (40). Yalçın et al. (45) and Daikwo et al. (10) who reported the average body weight of eight weeks in accordance with the results of this study, reported that these values ranged between 133.76 and 182.00 g. In a study conducted by Sadjadi and Becker (41), live weights of males and females at 8 weeks of age were determined as 107 g and 114 g, respectively. On the contrary, live weight values at 6 weeks of age were found to be quite high (from 181.52 g to 231.6 g) in studies conducted by some researchers (1, 6, 28, 43). Minvielle (27) reported that the reasons for the weekly live weight values of Japanese quail to be very different are the result of the adaptation of these birds to cage conditions from immigrant life and effects of genetic improvement studies. The difference in body weight of male and female quails in favor of females is a species-specific dimorphism opposite to that of other birds. A similar situation has been reported by Akbaş and Yaylak (4).

In this study, the quails in the battery-type cage were heavier than the other groups in terms of the average values at 6 and 8 weeks of age. However, when the floor cages were compared among themselves, it was observed that the quails in the enriched ones at both weeks of age had higher live weight averages. Miller and Mench (26) argued that as a potential result of environmental enrichment there should be an increase in growth and other yield traits. However, in their study, it was determined that none of the various environmental enrichment practices had a positive or negative effect on growth. Jones (18) claimed that environmental enrichment practices that reduce problematic behaviors can increase productivity. However, there are very limited and empirical literature reports for this view in poultry. Jones et al. (17) reported that environmental enrichment using new objects increased body weight gain and feed conversion rate in broilers and laying hens. In the studies conducted by Von Borell et al. (44) and Mendl et al. (24), it was revealed that the averages of aggression, fear, and stereotype behaviors were higher in impoverished environments, and they were associated with poor growth and low yield traits. However, in a study conducted by Nicol (35), it was reported that intermittent music and new objects did not affect performance characteristics in broilers. There are very different results in the literature on the subject. In this study, remarkable differences found in the weekly average body weight of the quails in the enriched floor pens compared to the quails housed in the conventional ground cages.

In studies investigating the growth of Japanese quails with the Gompertz model, the adult weight parameter (β_0) was estimated by Akbaş and Oğuz (3), Narinç et al. (32), Fırat et al. (11), and Kaplan and Gürcan (19) in the range of 208.3-287.7 g. In the study, the averages of the β_0 parameter obtained from all three experimental groups were found between 228.12 and 262.05 g, consistent with the adult weight parameter values reported in the other studies. In addition, Grieser et al. (14) reported that the β_0 parameter in a flock selected to increase live weight was estimated in the range of 275-369 g, and the β_0 parameter for quails from different genotypes in the same study was estimated between 131 and 215 g. Environmental and genetic manipulations greatly affect growth curve parameters. In the study, the integration coefficient parameter (β_1) of Gompertz growth curve model was estimated between 3.57 and 3.86 for growth samples of Japanese quails. Similarly, this parameter was estimated as 3.89 and 3.82 in nonselected-randombred Japanese quail flocks by Akbaş and Oğuz (3) and Kızılkaya et al. (20), respectively. The β_2 parameter representing the instantaneous growth rate was estimated in the range of 0.042-0.050. These means were consistent with the values (0.039-0.046) reported by Aggrey et al. (2) and Raji et al. (38). Estimating small values for the β_2 parameter indicates late maturity and high adult weight. On the other hand, high β_2 values represent early maturity and lower adult weight (32). The ages and weights of the inflection point of the Gompertz growth curve model of all quails in the study were between 27.88 and 34.37 days, and between 83.93 and 96.41 g, respectively. According to the results of many studies in which growth samples of Japanese quails were analyzed with the Gompertz function, the values obtained for the inflection point age of the curve were reported to be between 14.76-34.58 days of age. In these studies, it was reported that the growth curve inflection point weight was between 76.22-124.56 g (3, 19, 32, 38). The mean values for the age and weight of inflection point of Gompertz growth curve determined for the Japanese quails in this study were found to be compatible with the averages reported in these studies.

As in weekly live weights, a statistically significant difference was found between the experimental groups in terms of the β_0 parameter of the Gompertz growth curve which represents the asymptotic body weight. The mean value of β_0 parameter of quails raised in conventional battery cages was higher than the other experimental groups ($P<0.05$). In addition, the mean value of β_0 parameter of the quails housed in the enriched floor cages was also higher than the average of those housed in the conventional floor pens ($P<0.05$). Statistically significant differences were found between the experimental groups in terms of the mean values of the β_1 parameter which is a biological constant, and the β_2 parameter which represents the instantaneous growth rate of the Gompertz growth curve model ($P<0.05$ for both). In the current literature, there is no study on the analysis of the effects of environmental enrichment applications on the growth of poultry using non-linear regression models. In addition, Genç et al. (13) who carried out the growth curve analyzes using Gompertz function in Japanese quails housed in different types of cages (individual and colony) and in different stocking densities, reported that the average value of β_0 parameter of the quails housed in individual cages was higher. In the same study, it was reported that inflection point age and weight were also affected by both cage type and stocking density. The fact that environmental manipulations caused differentiation in the growth curves of quails in the study conducted by Genç et al. (13) was found to be compatible with the results of this study.

In terms of the relative asymmetry values, only one difference was found between the groups for wing length characteristics ($P<0.05$). Accordingly, the mean value of relative asymmetry (10.85%) of the quails housed in the conventional battery cages was higher than the averages (6.68% and 7.87%, respectively) of those housed in conventional and enriched floor cages. There are

contradictions regarding the application of the procedure to reveal the relationship of developmental stability-welfare in poultry using symmetry of bilateral traits. Garcia (12) claimed that stress in poultry can cause asymmetry in all parts of the body, but to reveal this, many bilateral traits need to be evaluated and these characteristics require correction because they are not numerically homogeneous. Besides, Knierim et al. (21) claimed that fluctuating asymmetry is specific of a trait and that correction can prevent the detection of bilateral characteristics sensitive to symmetry. One of the first studies to measure the developmental stability using bilateral symmetrical traits in Japanese quails was carried out by Nestor et al. (34), and the effects of long-term divergent selection for live weight on the balance of development were revealed. In the current study, no statistically significant difference was found in terms of the mean values of relative asymmetry for the length of face, wing, shank and shank diameter in female and male quails ($P>0.05$ for all). Although there is no study on the effect of environmental enrichment or alternative rearing systems on developmental stability in Japanese quails, there are some studies investigating the effect of some environmental manipulations on bilateral symmetry (30). Sarıca and Özdemir (42) revealed that quails exposed to heat stress lead to differences in terms of left-right difference averages of some bilateral traits (beak, finger, nostril and eye). Campo et al. (9) reported that chickens reared under conventional conditions had higher mean relative asymmetry than those raised in the alternative system, and they claimed that alternative breeding systems or environmental enrichment prevented the impairment of symmetry in bilateral traits. The results of both studies support that the quails that were enriched in this study had lower values in terms of proportional asymmetry values regarding the wing length than those raised in the battery type cage. The results of both studies support that the relative asymmetry values of the wing length of the EFC quails were lower than those grown in the battery type cage in current study.

The type of symmetry observed in the bilateral characteristics of creatures that are not exposed to genetic and environmental stress is fluctuating asymmetry (the mean of the difference between the right and left sides is equal to zero and has a normal distribution). Directional asymmetry (the mean of the difference between the right and left sides is not equal to zero and shows a normal distribution) occurs usually due to genetic stress. The situation defined as asymmetrical or antisymmetry (the differences between right and left sides do not show normal distribution) occurs as a result of environmental stress (21). In the study, a total of 4 symmetry types were determined regarding the length of face, wing, shank, and shank diameter in the quails of each experimental group (Table 3). Two fluctuating asymmetry, one directional asymmetry, and one antisymmetry were identified for the quails housed in the enriched floor cages. Two directional asymmetry and two antisymmetry were determined for the quails housed in the conventional battery cages. In the conventional floor cages, one fluctuating asymmetry and three directional asymmetries were detected. As it can be seen in Table 3, the fluctuating asymmetry type, which is the indicator of welfare, were mostly found in the enriched floor cages, while the highest detection in terms of antisymmetry, which is accepted as the indicator of environmental stress, was found in the quails raised in the conventional battery cages.

As a result, it is possible to obtain higher yields by using conventional battery cages in a production system where higher live weight is targeted and there are economic concerns. However, it was determined in this study that for a production meeting the criteria for goodanimal welfare, promising results can be obtained in case of using enriched floor cages. Even if it is not included in this study, it is thought that the use of some enrichment objects in conventional battery cages may produce positive results in terms of animal welfare, and more studies are needed on the subject.

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Ethical Statement

It was determined by Akdeniz University Animal Experiments Local Ethics Committee that this study does not need ethics committee approval with the document numbered B.30.2.AKD.0.05.07.00/50.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Some inherited disorders in pacing horses in Turkey

Ceyhan ÖZBEYAZ[1,a], Banu YÜCEER ÖZKUL[1,b,✉]

[1] Ankara University, Faculty of Veterinary Medicine, Department of Animal Husbandry, Ankara, Turkey
[a]ORCID: 0000-0002-3748-9992; [b]ORCID: 0000-0002-7036-6230

✉Corresponding author: byuceer@ankara.edu.tr
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Abstract: This study was carried out to detect the presence of mutant alleles of polysaccharide storage myopathy (PSSM) and severe combined immunodeficiency (SCID) disorders in pacing horses raised in different regions of Turkey. Blood/hair samples from 264 (182 Indigenous, 31 Iranian, 24 Afghan, and 27 Bulgarian) pacing horses aged 4 and over were used. As a result of the study, a mutation-heterozygosis (GA) in the GYS1 gene related to PSSM disease was detected in one of the pacing horses (Bulgarian horse). No deletions were observed in the DNA-PKcs gene region in the pacing horses for SCID disease. This study has been carried out to identify the status of two inherited disorders in pacing horses in Turkey. It was determined that there are no mutant genes in indigenous pacing horses, in terms of PSSM and SCID disorders, which are the major hereditary disorders in horses.
Keywords: Horse, pacing, PSSM, SCID, Turkey.

Türkiye'deki Rahvan atlarında bazı kalıtsal bozukluklar

Özet: Bu çalışma, Türkiye'de farklı yörelerde halk elinde yetiştirilen ve Rahvan koşularına katılan atlarda polisakkarid depolama miyopatisi (PSSM) ve şiddetli bileşik immun yetersizlik (SCID) hastalıkları bakımından mutant allel varlığının araştırılması amacıyla yapılmıştır. Bunun için 4 yaş ve üzeri 264 (182 Yerli, 31 İran, 24 Afgan ve 27 Bulgar) rahvan atın kan/kıl örnekleri kullanılmıştır. Çalışma sonucunda, incelenen Rahvan atlarından bir tanesinde (Bulgar rahvan atında) PSSM hastalığı ile ilgili GYS1 geninde mutasyon-heterozigotluk (GA) tespit edilmiştir. SCID hastalığına ilişkin, çalışmada kullanılan rahvan atlarında DNA-PKcs gen bölgesinde delesyon gözlenmemiştir. Sonuç olarak bu çalışma, Türkiye'deki rahvan atlarında bu iki kalıtsal hastalığın durum tespitine yönelik olarak gerçekleştirilmiştir ve bu çalışmada, at yetiştiriciliğinde önemli kalıtsal bozukluklardan ikisi olan PSSM ve SCID hastalıkları açısından yerli rahvan atlarında zararlı genlerin bulunmadığı tespit edilmiştir.
Anahtar sözcükler: At, PSSM, rahvan, SCID, Türkiye.

Introduction

Horse breeding was mainly carried out to use horses as an energy source before mechanization. Nowadays, it is limited to breeding horses for riding and working. However, it has become more widespread in horse racing, show jumping, sports riding, pacing, and local sports such as javelin throwing. Horses that are successful in such competitions are generally used in breeding. Under these circumstances, it is possible that hereditary diseases are passed to the generations and the frequency of the relevant mutant gene also increases. Techniques developed in recent years can explain the inheritance of hereditary diseases, and the carriers can be identified and the eradication of the disease is facilitated. While some hereditary diseases are detected only in certain breeds, others can be found in many breeds. However, when a crossing is done, it is always possible that mutant alleles are passed to other breeds.

Genes that cause hereditary disorders and thus patient and carrier animals can be detected by molecular techniques and these diseases can be removed more easily from the herd (10, 21).

Hereditary diseases have been reported in horses such as Hyperkalemic Periodic Paralysis (HYPP), Polysaccharide Storage Myopathy (PSSM), Malignant Hyperthermia (MH), Glycogen Branching Enzyme Deficiency (GBED), Severe Combined Immunodeficiency (SCID), Junctional Epidermolysis Bullosa (JEB), Hereditary Equine Regional Dermal Asthenia (HERDA), Gray Horse Melanoma, Lavender Foal Syndrome (LFS) (6, 9).

PSSM is a muscle disease and autosomal dominant glycogen storage disorder that occurs when sugar is stored in the muscles in the form of polysaccharides, which is an abnormal form of sugar instead of glycogen. As a result of a mutation in the Glycogen synthase 1 gene (GYS1), the

clinical symptoms of PSSM appear and are seen in warm-blooded horses, especially in Quarter horses and other breeds (11, 15). The main clinical sign of the disease is cramps and involvement in the muscles. Muscle pain, stiffness, fatigue even at the lightest exercise, reluctance to move, muscle atrophy, high serum CK and AST levels are also observed. Muscle glycogen concentration is four times higher than the normal value (7, 31).

PSSM disease was first identified in Quarter horses in the USA (27), then it was detected in Ponies, Morgan, Arabian, Thoroughbred, Standardbred and Warm-Blooded horse breeds as well as many other horse breeds (17, 29).

In 8% of 94 horses slaughtered in a slaughterhouse in Britain, 22% of 46 horses presented to the clinic (19) and by histological examination of skeletal muscle biopsies of 1426 horses from different breeds 572 horses (40.1%) were diagnosed with PSSM (15). Horses (588 [34%] of 1714) from different breeds were found positive for GYS1 mutation and most of them were heterozygote (28). Haflinger horses (9 [18%] of 50) in Austria were heterozygote (25); 250 (62%) of 403 draft horses of 13 breeds in Belgium, France, Germany, Netherlands, Spain and Sweden carried the mutant allele (3). The prevalence of genetic susceptibility to Type 1 PSSM in Shire, Morgan, Appaloosa, Quarter, Paint, Exmoor Pony, Saxon-Thuringian Coldblood, South German Coldblood, Belgian, Rhenish German Coldblood and Percheron horses varied between 0.5 and 62.4%. Thoroughbred, Akhal-Teke, Connemara, Clydsdale, Norwegian Fjord, Welsh Pony, Icelandic, Schleswig Coldblood and Hanoverian horses were free for the mutation (18). The prevalence of GYS1 mutation has been reported to be high in Draft (87%) and Quarter (72%) horses and low in Warmblood (18%) and other light horse breeds (24%) (16).

SCID is an autosomal recessive disorder and was first reported in Arabian horses in 1973 (20). This disease is caused by the DNA-protein kinase (DNA-PKcs) catalytic subunit gene on the 9th chromosome which is a 5 base pair deletion (frameshift mutation) (2, 23). The DNA-PKcs enzyme is necessary for the gene that regulates antigen receptor on B and T lymphocytes (22).

Affected foals are normal at birth, but secondary infections develop immediately after. The number of B and T lymphocytes is insufficient, they can't mature and there is no antibody synthesis, thymus and peripheral lymphoid tissues are hypoplastic. The pathogenesis of this disease in horses is completely related to the deficiency of B and T cells. In this disease, sufficient antigen-specific immune response for protection from infectious diseases has not occurred. Therefore, foals that fail to produce enough antibodies after vaccination or infection are very susceptible to diseases and usually die within the first few months after birth (9, 12, 14, 32).

The frequency of SCID carriers in Arabian horses in the United States is 8.4% (4); this rate is 2.3% for the foals of Arabian breeding stock (24) while no carriers have been found among Iranian Arabian horses (26); 16 of 88 Arabian horses (18%) in Morocco have been reported as carriers (23); in the state farm in Turkey, no mutant allele has been encountered in 239 Arabian horses (13) while 44 of 508 Arabian horses (8.7%) in the USA were reported to be carriers of SCID (8).

The pacing horses bred in Turkey are local and imported horses from Iran, Afghanistan, and Bulgaria. Some of the local pacing horses may have been influenced by Arabian horses. Hereditary disorders that are reported only in Arabian horses can also be seen in indigenous horses. This study aimed to detect the presence of mutant alleles that determine the inherited diseases PSSM and SCID in pacing horses in Turkey.

Materials and Methods

This research was conducted within the scope of the decision of the ethics board dated 27/08/2010 and no. 2010-96-337 of Ankara University Animal Experiments Local Ethics Board. The present number is not known, as there is no registration system-based breeding of pacing horses. Animals used in the study are formed, in 7 different geographic regions of Turkey were kept under extensive conditions, pacing horses 4 years and older. The samples of the pacing horses were taken by going to the relevant city and towns on the dates of the pacing horseraces, and by reaching the place where the horse was found in places where the races were not (for example, Ankara, Antalya, Aydın, Bursa, Erzincan, Erzurum, Eskişehir, İzmir, Konya, Kütahya, Mardin, Samsun, Trabzon, etc.). The animal material of the research is formed by a total of 264 pacing horses (blood/hair samples [from mane] of them) some of which are indigenous (n=182) and imported [Iranian (n=3), Afghan (n=24), Bulgarian (n=27)]. Some samples in the projects no 110 0 824 supported by TUBITAK were also used as materials.

To make DNA isolation from the hair samples, the section where the root parts of these samples were cut for 0.5 cm and 4 or 6 hair samples were left to incubation at 56 ^{o}C for a night with Proteinase-K. Following the incubation, 200 µl was taken from these samples and DNA isolation was performed according to MagAttract DNA Blood Mini M48 Kit protocol by using the Qiagen BioRobot M48 device (1).

Following DNA isolation, the relevant samples were subjected to PCR by using ABI 7500 thermal cycler, and PCR was performed according to the relevant kit protocol.

Determination of Genotypes: PSSM (GYS1) SNP genotyping; it was aimed to determine the Arg309His

mutation in the glycogen synthase (GYS1) gene (GenBank: NC_009153.2). Arginine amino acid (CGT) in the 309th position of the gene turns into histidine (CAT) amino acid as a result of the G>A mutation. The study was carried out according to the 5 'nuclease method and genotyping was done by working two PCR mixes, as wild-type and mutant for each sample. The primer sequences used to replicate the gene region and to detect the mutation were; for Wild-Type PCR mix, P1: 5' CCGAATCCAGGAGTTTGTGTG 3', P2: 5' CATTGTTCTGACGCTCAGGAAC 3', for Mutant PCR mix, P1: 5' CCCGAATCCAGGAGTTTGTGTA 3', P2: 5' CATTGTTCTGACGCTCAGGAAC 3'. In the study, 5 'FAM TATGGGTATGTGGGCCAGATACCCA BHQ 3' sequence was used as the TaqMan® probe sequence for both Wild-Type and Mutant PCR mix. 5 µl 10X buffer, 4 mM MgCl2, 1.2 pmol P1 and P2, 0.6 pmol Probe, 100 ng DNA, 0.3 µl HotStart Taq DNA Polymerase was used and PCR Grade Water was added to be 25 µl total volume of the mix used for the Wild-Type and Mutant PCR mixes used in the PCR. The PCR program was used to replicate the GYS1 gene region, consisting of 35 cycles including 10 min at 95 °C for initial denaturation, 15 sec of denaturation at 95 °C, and 1 min at 60 °C of bonding. The PCR process was performed with the ABI 7500 device and on which PCR mix is irradiated were determined by looking for fluorescent radiation revealed by the FAM dye in the probe.

SCID (DNA-PKcs) SNP Genotyping; it was aimed to detect 5 base pair deletions (TCTCA) in the DNA-Dependent Protein Kinase, catalytic subunit (DNA-PKcs) gene region (GenBank: AF448228.1). Frame-shift occurs as a result of the 5 base pair deletions in codon 9480 of the gene, and as a result, unstable protein is synthesized. The study was performed according to the 5 'nuclease method and genotyping was performed for each sample with Wild-Type (no deletion) and Mutant (with deletion) PCR mix. The primer sequences used to replicate the gene region and to detect the mutation were; for the Wild-Type PCR mix, P1: 5' ATAAGGAAACAAGGTAATTTATCA TCTCA 3', P2: 5' GAAACATCGATTTGTGATGATGT CATC 3', for the Mutant PCR mix, P1: 5' TAAGGAAACAAGGTAATTTATCAAATTCC 3', P2: 5' GAAACATCGATTTGTGATGATGTCATC 3'. In the study, 5 'FAM CTTCTAAAAACCTGGACAAACAG ATATCCGG BHQ 3' sequence was used as the TaqMan® probe sequence for both Wild-Type and Mutant PCR mixes. 5 µl 10X buffer, 4 mM $MgCl_2$, 1.8 pmol P1 and P2, 1.0 pmol Probe, 100 ng DNA, 0.3 µl HotStart Taq DNA Polymerase was used for Wild-Type and Mutant PCR mixes and PCR Grade Water was added to be 25 µl total volume of the mix. A PCR program consisting of 40 cycles including 10 min at 95 °C for initial denaturation, 15 sec of denaturation at 95 °C and 1 min at 60 °C of bonding was used to replicate the DNA-PKcs gene region. The PCR process was performed with the ABI 7500 device and on which PCR mix is irradiated were determined by looking for fluorescent radiation revealed by the FAM dye in the probe.

Results

The presence of the GYS1 mutant allele which determines to PSSM and DNA-PKcs mutant allele which determines to SCID disorder in pacing horses were studied in Turkey. This is the first study regarding inherited disorders in pacing horses in Turkey.

In this study, alleles were evaluated for PSSM and SCID diseases by looking for fluorescent radiation after PCR amplification.

Polysaccharide Storage Myopathy: In terms of PSSM disease, if radiation was observed only in the Wild-Type PCR mix, the sample was considered Wild-Type (GG), if radiation was observed in both Wild-Type and Mutant PCR mixes, the sample was considered heterozygote (GA) and if radiation was observed only in the Mutant PCR mix, the sample was considered homozygote mutant (AA). The GYS1-GG homozygote of a normal individual in Figure 1, the GYS1-GA heterozygote of a mutant carrier individual in Figure 2, and the SNP images of all investigated horses for GYS1 are given in Figure 3. A GYS1 mutant allele in a horse of Bulgarian origin was detected as a heterozygote. The GYS1 gene of all pacing horses (Indigenous, Iranian, and Afghan origins) was found to be homozygote for Wild-Type (Table 1).

Severe Combined Immunodeficiency: In terms of SCID disease, if radiation was observed only in the Wild-Type PCR mix, the sample was considered Wild-Type (no deletion in both alleles), if radiation was observed in both the Wild-Type and Mutant PCR mixes, the sample was considered heterozygote (deletion in only one allele) if radiation was observed only in the Mutant PCR mix, the sample was considered as a homozygote mutant (deletion in both alleles). In terms of DNA-PKcs allele, there were images of the homozygote Wild-Type allele and Wild-Type genotypes of all individuals (Figures 4 and 5). All samples of pacing horses have been homozygote Wild-Type in Turkey. No mutant allele or heterozygote genotypes were detected in the horses studied (Table 1).

While the frequency of the GYS-1 mutant allele was 3.71% in Bulgarian horses, this rate was 0.38% in all horses. In terms of the GYS-1 allele, 263 horses were homozygote, only 1 horse was a heterozygote. The DNA-PKcs wild-type allele frequency was found at 100%. Therefore, all genotypes were wild-type homozygote.

Table 1. Distribution of GYS-1 (PSSM) and DNA-PKcs (SCID) allele frequencies (%) and genotypes in pacing horses from different origins.

Origin	n	GYS-1 genes frequencies		DNA-PKcs genes frequencies		GYS-1		DNA-PKcs	
		Wild-Type	Mutant	Wild-Type	Mutant	Homozygote Wild	Heterozygote Genotypes	Homozygote Wild	Heterozygote Genotypes
Indigenous	182	100.00	0.00	100.00	0.00	182	-	182	-
Iranian	31	100.00	0.00	100.00	0.00	31	-	31	-
Afghan	24	100.00	0.00	100.00	0.00	24	-	24	-
Bulgarian	27	96.29	3.71	100.00	0.00	26	1	27	-
Total	264	99.62	0.38	100.00	0.00	263	1	264	-

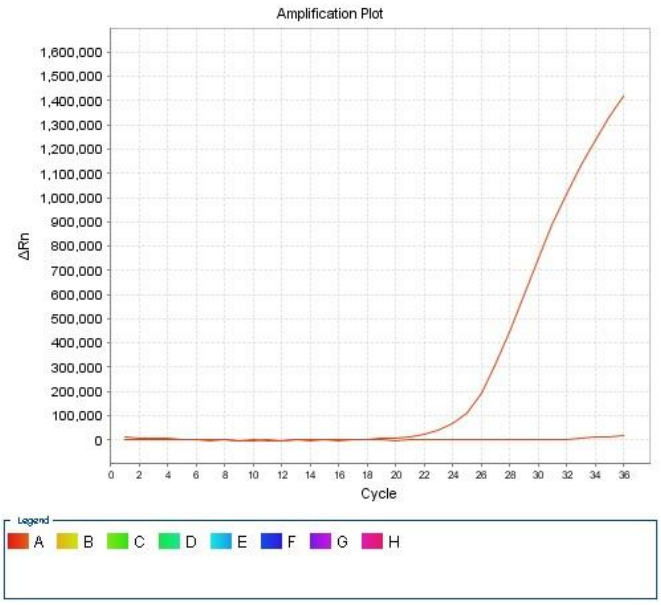

Figure 1. GYS1-GG (normal-wild type homozygote).

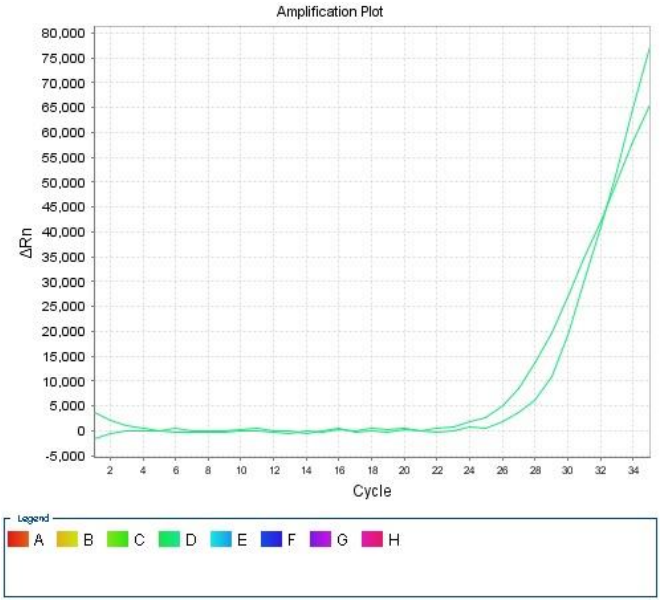

Figure 2. GYS1-GA (wild type heterozygote).

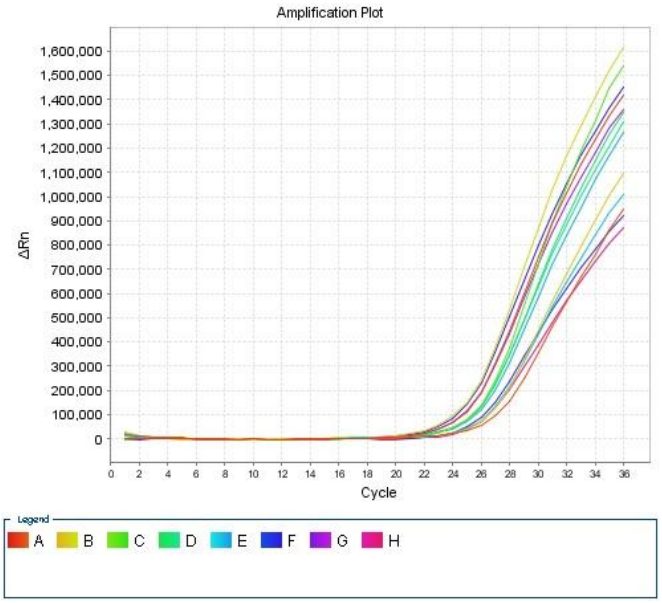

Figure 3. Peak image for GYS1 in all horses.

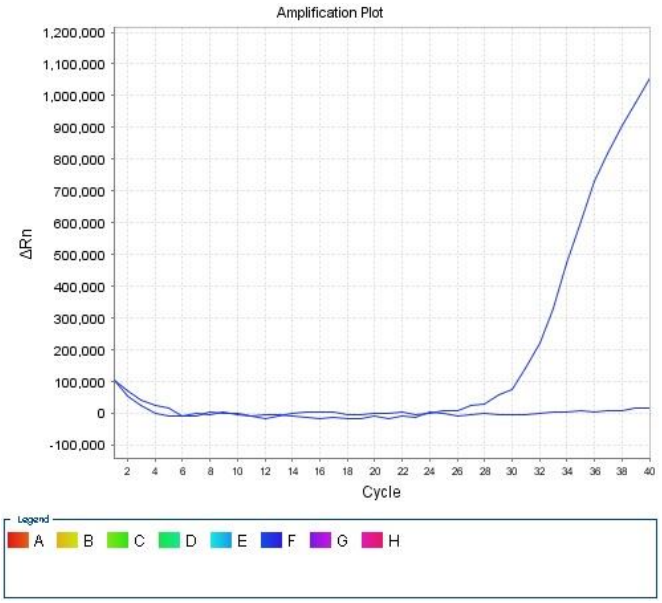

Figure 4. DNA-PKcs (wild type homozygote-normal).

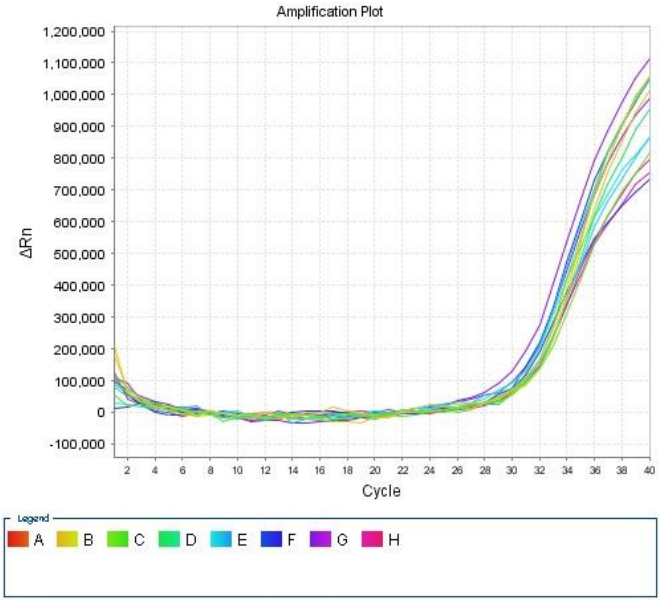

Figure 5. Peak image for DNA-PKcs in all horses.

Discussion and Conclusion

The existence of mutant alleles leading to the occurrence of polysaccharide storage myopathy and severe combined immunodeficiency which are hereditary diseases has been investigated in pacing horses from different origins.

Polysaccharide storage myopathy disease has occurred as a result of a point mutation in the GYS-1 gene, which converts the amino acid of arginine to histidine (17). This mutation increases the enzymatic activity, leading to the accumulation of polysaccharides such as glycogen in the skeletal muscles. The inheritance of the mutation is autosomal dominant (11). This mutation is formed as a result of a 10 base pair substitution in the GYS-1 gene located on the 10th chromosome (16).

The frequency of the GYS-1 mutant allele was found to be 3.71% in the pacing horses of Bulgarian origin, and the frequency of the GYS-1 mutant allele was 0.38% in all horses in the study. These frequencies are much lower than the reported mutant allele frequencies (45-66%; 8-22%; 40.1%; 34%; 18%; 62%) in some studies (3, 15, 19, 25, 28, 30). In these studies, in which the mutant allele frequencies are reported higher in terms of PSSM may be due to different horse breeds and different geographies, and also PSSM is mostly seen in Quarter, Draft horses and related breeds. In this study, only one out of 27 horses of Bulgarian origin was found with the GA genotype for PSSM disorders, while all other (n=263) horses were determined with the GG genotype (wild-type). Analyses were repeated to ensure that of heterozygote GA genotype was not caused by a false reading, however, no errors were detected in the repeated analyses. Other researchers (5, 18) have reported that horse breeds such as Thoroughbred, Arabian, Akhal Teke, Icelandic Clydesdale are wild-type like all the Indigenous, Iranian and Afghan pacing horses. On the other hand, Bulgarian pacing horses are larger than the other pacing horses and have been derived through crossbreeding. Therefore, it is thought that the PSSM mutant allele may have been transferred to Bulgarian pacers from heavy horse breeds.

Severe combined immunodeficiency is an autosomal-simple recessive inherited that is generally seen in Arabian horses and their crosses. Heterozygote animals appear healthy, but homozygote recessive foals die shortly after birth as a result of secondary infections. If the herds' genetic status is not known, the mutant gene can subsist in the herd for centuries without the disorder being seen. Therefore, the disease can be eradicated and animal losses can be prevented, especially by which are carriers predetermined in breeding animals (4, 20, 26).

In this study, all samples (n= 264), were determined as wild type. The findings obtained in this study differed from the results of some research (4, 8, 23, 24) on SCID disorder. The horse breed in these reported studies is Arabian horses, and the mutant carrier rates have been reported to be high 2.3%, 8.4%, 8.7% and 18%, respectively in the above literature. However, 120 Iranian Arabian horses (26) and 239 Turkish Arabian horses (13) are reported to be not carriers. The fact that all of the wild types sampled Arabian horses from Iran and Turkey suggests that there was no mixture from outside especially the West of Iran and Turkey. The absence of mutant allele in pacing horses, even if they received blood from Arabian horses could be attributed to the fact that lacking the SCID mutant gene in Arabian horses in Turkey.

The existence of mutant alleles causing SCID and PSSM hereditary disorders in pacing horses in Turkey (Indigenous, Iranian, Afghan, Bulgarian) has been investigated in this study. While no mutant allele was detected for SCID, only one horse was detected as heterozygote with a mutant allele for PSSM. Except for one pacing horse originating from Bulgaria, genes that cause PSSM and SCID hereditary disorders were not found in the other horses. It is evident that pacing horses in Turkey are clean for these disorders and that hereditary disorders are also transferred through imports such as infectious diseases. Therefore, it is necessary to check for hereditary disorders as well as infectious diseases when animals are imported. It is suggestible that both pacing horses as well other horse breeds whose immune system has collapsed and which has died at an early age are checked for carrying the mutant allele for SCID and those that get tired quickly and have frequently stiffness of muscle are checked for carrying the mutant allele for PSSM.

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Ethical Statement

This study was approved by the Ankara University Animal Experiments Local Ethics Committee (2010-96-337).

Conflict of Interest

The authors are declared that there is no conflict of interest.

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Chemical, rheological, and organoleptic analysis of cow and buffalo milk mozzarella cheese

Abdul Rauf BHAT[1,a], Atta Hussain SHAH[1,b], Mansoor AYOOB[1,c], Muhammad Faisal AYOOB[2,3,d,✉], Farrukh SALEEM[3,e], Muhammad Mohsin ALI[3,f], Muhammad FAYAZ[3,g]

[1]Department of Animal Product Technology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tando Jam-70060, Pakistan.[2]Department of Veterinary Pathology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tando Jam-70060, Pakistan. [3]National Veterinary Laboratories, Ministry of National Food Security and Research Islamabad-44000, Pakistan

[a]ORCID: 0000-0002-1624-0338; [b]ORCID: 0000-0003-4558-3660; [c]ORCID: 0000-0003-4991-8069; [d]ORCID: 0000-0002-4514-5911; [e]ORCID: 0000-0002-5571-7207; [f]ORCID: 0000-0002-8823-6055; [g]ORCID:0000-0001-6374-4849

✉Corresponding author: ayoob.faisal@yahoo.com
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Abstract: This research was aimed to determine the impact of milk source on mozzarella cheese's chemical, rheological, and organoleptic characteristics. A total of 10 lots of cow and buffalo milk mozzarella cheese each were prepared. Prominent milk source influence on the chemical characteristics and calorific values of mozzarella cheese was determined. A decrease in moisture content and an increase in fat, protein, ash, and calorific values were noted in mozzarella cheese against their respective milk. Moisture content in cow milk mozzarella cheese (CMM) was considerably higher than buffalo milk mozzarella cheese (BMM). Average fat, ash content and calorific values appeared considerably high in BMM cheese compared to CMM cheese but protein content was recorded inverse and statistically non-significant (P>0.05). The average meltability was slightly higher in CMM cheese and the stretchiness of CMM cheese appeared significantly high from that of BMM cheese. BMM cheese was remarkably high in yield in contrast to that of CMM cheese. Appearance, flavor, and body/texture scores were slightly higher in CMM cheese as compared to BMM cheese. Regardless, the average benefit computed for BMM cheese was high in comparison to CMM cheese, the differences among them were statistically non-significant. The present study concludes that buffalo milk is better to produce mozzarella cheese with higher calorific values, better yield, and with more economic values, while cow milk is better to produce mozzarella cheese with the more proteinous, enhanced stretchiness, and better organoleptic quality.

Keywords: Buffalo milk, cow milk, Mozzarella cheese, organoleptic, rheological.

Introduction

Mozzarella cheese is popular around the world due to its use for pizza topping. Its rheological (melting and stretching) properties, softness and appearance are best for making pizzas. Mozzarella cheese belongs to 'pasta filata' class that requires a skillful stretch of the coagulated milk in lukewarm water to achieve smoothness. It is a product made from buffalo-milk, by coagulating the milk casein by direct addition of lactic acid or adventitious micro-organisms with rennet or a similar enzyme from which moisture was extracted by incising, cooking or pressing, and then matured at appropriate temperatures and humidity (19). Bacterial culture is used to convert milk sugar (lactose) into lactic acid. The production rate of acid is important in assessing the consistency of the cheese (7). Mozzarella cheese is a savory and nutritious milk product. However, its quality depends on the physical properties, especially the ability to melt, elasticity, color, and free oil production. Differences in processing conditions, temperature, storage time, and milk quality can affect the functional properties of mozzarella cheese (2). The fat and casein contents contained in a curd during processing are typically high in Mozzarella cheese, which contains reasonably little quantities of water-miscible components (whey proteins, milk sugar, and water-miscible vitamins) that are primarily found in the wey. Mozzarella cheese is a rich source of nutrients, proteins, vitamins, fat, and carbohydrates (16). It provides many health benefits and can protect against gout, which causes uric acid crystals to develop in the joints (17). The calcium present in mozzarella also contributes to weight loss and protects from breast cancer and metabolic disorders that raise the

risk of cardiovascular disease or attack (15). For mozzarella cheese, buffalo milk is favored due to its high fat, vitamin A, calcium, and low cholesterol content. Rheological properties (meltablility and stretchiness), oil-free production and browning are the chief functional attributes of mozzarella cheese which are greatly reliant on the basic formation and composition of the cheese. The resulting cheese functionality is determined by the moisture content, fat percentage, salt, and mineral especially calcium, pH, and proteolysis. These properties perform a key part in consumer expectations about the consistency of the cheese. Eating habits are changing very rapidly through out the world. This has resulted in more pizza consumption and other similar fast foods. As a result, pizza-usable cheese has become increasingly relevant commercially in the dairy industry, where various types of cheeses with different labels are available on the local market. Besides, preparation and quality assessment of mozzarella cheese from two different milk sources can improve dairy industries to select the milk that may be the best for producing mozzarella cheese. This study had been aimed to examine the chemical, rheological and organoleptic attributes of mozzarella cheese produced from buffalo and cow milk. Also, its calorific values, yield, and economic impact have been measured.

Materials and Methods

Collection of milk samples and chemicals required for mozzarella cheese making: Buffalo and cow milk collected from the Livestock Experiment Station, Sindh Agriculture University Tandojam. It was first strained through a muslin cloth as soon as the milk was collected, and the volume was measured. Approximately 250 ml of milk was transferred for analysis to sample bottles; the rest of the milk was used for making mozzarella cheese. During mozzarella cheese production, the tablet rennet enzyme (Lahore Farmer Cheese Company), sodium chloride (NaCl) of laboratory-grade (E. Merck Darmstadt Company) for preparing Brine solution and Artisan starter culture prepared at the Animal Products Technology Department were used.

Analysis of milk samples

Moisture content: Association of Official Analytical Chemists (5) procedure was adopted to calculate moisture percentage with the help of the following formula.

$$\text{Moisture\%} = \frac{W_2 - W_3}{W_2 - W_1} \times 100$$

Where,

W1 = Empty plate weight

W2 = plate weight + sample weight

W3 = plate weight + dried sample weight

Total protein content: Protein content was determined according to the method of British standards Institution (9). The nitrogen percentage was calculated using the following formula.

$$\mathbf{N\%} = \frac{1.4(V_1 - V_2) \times Normality\ of\ HCl}{Weight\ of\ sample\ taken \times weight\ of\ diluted\ sample} \times 250$$

Where,

V1 = value of titration

V2 = value of the blank sample

By considering the nitrogen percentage present in the milk as protein, the protein content was calculated by conversion of nitrogen percentage using the following formula.

Protein percentage = N% × Conversion Factor (CF)

(CF = 100/N%, the protein of milk and dairy products (i.e. 15.66).

Fat content: Abegao et al. (1) explained the Gerber method to determine the fat content present in the milk and other dairy products by using a butyrometer, which was used in the present study.

Ash content: Ash content was determined according to the method of AOAC (5) by applying the following formula.

$$\text{Ash content\%} = \frac{Ashed\ sample}{Sample\ taken} \times 100$$

Preparation of starter culture: Starter culture (Artisan) was prepared in buffalo skimmed milk by inoculation of a portion of the previously rendered Dahi (3 percent) and purified by repeating it several times. The milk was heated for 10 minutes (90 °C), cooled to 45 °C, and inoculated with previously made Dahi (3%) incubated (40±2 °C) until the pH fell to 4.5 or 4.7. During the time of experimental research, this culture was then preserved as a mother culture.

Preparation of brine solution (2.5% NaCl): 2.5% (w/v) Sodium chloride solution was prepared by dissolving 25 g NaCl into 1000 ml of distilled water. The brine solution was kept at 4 °C in a refrigerated till use.

Preparation of mozzarella cheese: Dave et al. (12) method for mozzarella cheese preparation was used and the basic manufacturing steps are illustrated in Figure 1.

Analysis of mozzarella cheese

Preparation of cheese sample: The cheese sample (100 g) was mashed in a pestle mortar or grinder, to make homogeneous and bring it to a temperature of 25 °C.

Moisture, total protein, fat, and ash content: Moisture, total protein, fat, and ash contents were examined according to the method as described in this section earlier, respectively. However, a minor modification has been made in the case of protein and fat content determination. The sample weight was decreased to 2 g against 5 g in total protein assay and for fat 3 g instead of 11 ml. The strength of sulfuric acid was also reduced to a 65% level.

Figure 1. Flow chart showing the basic methodology of Mozzarella cheese making.

Meltability: The method described by Cais-sokolinska and Pikul (10) with slight alteration was used to examine the meltability of mozzarella cheese. Small pieces of cheese samples were made and measured their initial diameter with the help of a vernier caliper. Then cheese specimen was heated in the microwave oven at 60 °C for 5 minutes. After that cheese specimen was cooled to room temperature for 30 min and measured melted cheese diameter. Meltability was determined by using the following formula.

Meltability = Melted cheese diameter – Initial diameter of cheese specimen

Stretchiness: The cheese was removed from the refrigerated storage (4 °C) and cut into small pieces, then ground at ambient temperature in a blender (Model No. T.S. 696. E G. Anex Germany product gmbH). The size of the particle in the cheese was around 5 mm. 200 g of ground cheese was placed in a glass container and heated at 60 °C for 5 minutes. The cheese specimen was manually stretched with a finger until the string of cheese was almost to split. At that time measured the cheese string length with the measuring scale. Stretchiness was measured by using the following formula.

Stretchiness = stretched cheese string length – initial cheese particle length

Organoleptic Properties: A jury of six judges conducted the organoleptic analysis to determine the cheese sample. The judges included teachers and M. Phil students from the Animal Products Technology Department. The vocabulary used for the product definition consisted of appearance, color, flavor, and body/texture with the hedonic scale of 15, 10, 45, and 30 respectively (22).

Cheese yield: Cheese yield was determined by the following formula as reported by Sulieman et al (24).

$$\text{Yield\%} = \frac{\textit{weight of cheese}}{\textit{weight of milk}} \times \mathbf{100}$$

Calorific values: Calorific values of cheese samples were calculated by using energy conversion factors of major components like 4 for each protein and carbohydrates and 9 for fat as reported by Al-Amiri et al. (3).

Economics: The total per-unit cost of ingredients and unforeseen expenses during mozzarella cheese making were gathered and based on those expenditures, the expected benefit was computed by the following formula.

Per unit current marketing cost of the product- per unit manufacture cost of the product.

Statistical analysis: The result data gathered were processed, collated, and analyzed using Student Edition of Statistics (SXW), Version 8.1 (copyright 2005 Analytical Software, U.S.A). A descriptive statistics of summary statistics and one-way analysis of variance tests were used to detect group differences, and the results were further analyzed with the least significant difference (LSD) test at 0.05 levels in case of substantial variation between groups.

Results

Influence of milk source on the chemical composition of mozzarella cheese

Moisture content: The minimum moisture content of BMM cheese was observed as 44% and the maximum 51%, while the minimum moisture content of CMM cheese was recorded as 51% and the maximum 58%. A remarkable ($P<0.05$) variation was recorded between the moisture content of cow milk and buffalo milk as cheese base milk. The average moisture content (87.40 ± 0.27%) in cow milk appeared considerably high compared to that of buffalo milk (84.0 ± 0.34%). A similar trend of moisture content was noted in mozzarella cheese prepared from each cow and buffalo milk. CMM cheese remained significantly ($P< 0.05$) high in moisture content (54.10 ± 0.64%) in contrast to that of BMM cheese (46.70 ± 0.69%). Further results revealed that average moisture content in both BMM and/or CMM cheese decreased significantly from that of their corresponding milk (Figure 2, Table 1).

Table 1. Mean values of chemical, rheological and organoleptic attributes of buffalo and cow milk mozzarella cheese (Mean±SEM).

Attributes	Buffalo Milk	Cow Milk	Buffalo Milk Mozzarella Cheese	Cow Milk Mozzarella Cheese	P
Chemical Analysis					
Moisture contents (%)	84.0±0.34^{b}	87.40±0.27^{a}	46.70±0.69^{d}	54.10±0.64^{c}	1.476
Total Protein (%DMB)	26.92±0.92^{b}	23.80±0.49^{b}	42.87±1.39^{a}	45.27±1.65^{a}	3.44
Total Fat (%DMB)	36.34±1.36^{c}	28.18±0.61^{d}	46.32±1.02^{a}	41.82±1.31^{b}	3.204
Ash contents (%DMB)	4.27±0.21^{b}	4.67±0.27^{b}	6.52±0.24^{a}	6.23±0.28^{a}	0.72
Rheological Analysis					
Meltability (cm)	-	-	2.87±0.08^{b}	3.41±0.13^{a}	0.002
Stretchiness (cm)	-	-	6.45±0.36^{b}	7.65±0.33^{a}	0.027
Organoleptic Analysis					
Appearance (max score 15)	-	-	10.81±0.36^{a}	11.86±0.41^{a}	0.074
Color (max. score 10)	-	-	6.80±0.30^{b}	7.70±0.28^{a}	0.041
Flavor/ Taste (max score 45)	-	-	34.80±0.79^{a}	35.10±1.13^{a}	0.825
Body/ Texture (max score 30)	-	-	24.56±0.77^{a}	24.70±0.54^{a}	0.883
Mozzarella Cheese Yield (%)	-	-	16.95±1.29^{a}	12.68±0.93^{b}	0.028
Calorific value (Kcal/100g)	89.79±1.14^{c}	65.72±1.33^{d}	318.49±6.42^{a}	268.83±5.08^{b}	12.01

^{a,b,c,d} Values within a row with different superscripts differ significantly at $P<0.05$.

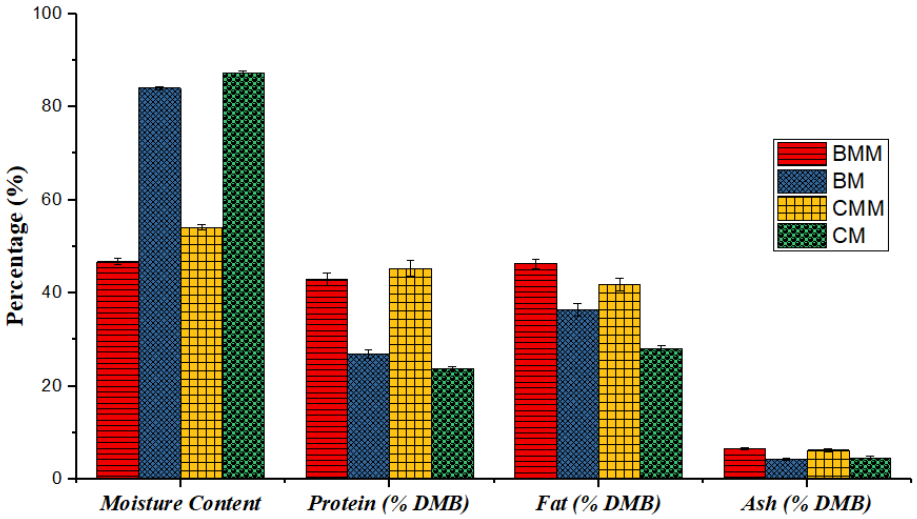

Figure 2. Influence of milk source on the chemical composition of mozzarella cheese.

Protein content: The total protein content of BMM cheese was in a range between 37.00 and 49.10%, while in the case of CMM cheese it varied between 35.70 to 50.00%. The average total protein content on Dry Matter Base (% DMB) of BMM cheese (42.87 ± 1.39%) was lower than that of CMM cheese (45.27 ± 1.65%) and the difference among them was insignificant (P>0.05). The average protein content in buffalo milk (26.92 ± 0.92%) was recorded although high but not statistically different from that of cow milk (23.80 ± 0.49%) and depicted in Figure 2 and Table 1. However, a significant increase in the protein content was noted of both types of mozzarella cheeses against their corresponding milk, nevertheless, this increase in mozzarella cheeses was recorded inverse.

Fat content: The average fat content of CMM cheese (41.82 ± 1.31%) was recorded substantially (P<0.05) lesser than that of BMM (46.32 ± 1.02%). The minimum fat content in BMM cheese was examined as 40.60% and the maximum 51.80%, whereas the minimum fat content in CMM cheese was observed as 36.20% and the maximum 46.90%. Fat content was observed considerably (P<0.05) higher in buffalo milk (36.34 ± 1.36%) in contrast to that of cow milk (28.18 ± 0.61%) and it was increased considerably in mozzarella cheese with a similar trend to that of their corresponding milk (Figure 2, Table 1).

Ash content: The minimum and maximum ash content of BMM cheese were 5.30 and 7.80%, and in the case of CMM cheese, it was 4.50 and 7.20%, respectively. Regardless, the average ash content of CMM cheese (6.23 ± 0.28%; DMB) was found lower than that of BMM cheese (6.52 ± 0.24%; DMB), the least significant difference test (LSD 0.05) revealed non-substantial variations among them. However, the concentration of ash content in both mozzarella cheeses were appeared considerably high against their corresponding controls i.e. buffalo milk (4.27 ± 0.21%) and cow milk (4.67 ± 0.27%) and presented in Figure 2 and Table 1.

Rheological attributes of mozzarella cheese

Meltability: The meltability of CMM cheese was observed between 3.00 and 4.00 cm and that of BMM cheese, it was in a range of 2.40 and 3.20 cm. The average meltability of CMM cheese (3.41 ± 0.13) remained statistically high (P<0.05) than that of BMM cheese (2.87 ± 0.08) and is depicted in Figure 3 and Table 1.

Stretchiness: The minimum and maximum stretchiness of mozzarella cheese made from cow milk was observed as 6.00 and 9.00 cm, whereas in BMM cheese, it was ranged between 4.00 and 8.00 cm, respectively. The average stretchiness of mozzarella cheese made from cow milk (7.65 ± 0.33 cm) was noticeably (P<0.05) greater than that of BMM cheese i.e. 6.45 ± 0.36cm (Figure 3 and Table 1).

Organoleptic analysis of mozzarella cheese

Appearance: The score for BMM cheese ranged between 9.00 and 13.00 and averaged 10.81 ± 0.36. Whereas the appearance score of CMM cheese was between 10 and 14 with an average score of 11.86 ± 0.41 over a score of 15 (Figure 4 and Table 1). Statistically, non-significant (P>0.05) variation among both types of mozzarella cheeses were found, regardless of the appearance score rated high for cow milk mozzarella cheese.

Color: The color score of BMM cheese ranged between 5.00 and 8.00 with an average score of 6.80 ± 0.30, whereas it was between 6 and 9, and averaged 7.70 ± 0.28 for CMM cheese over a score of 10 (Figure 4 and Table 1). The average score perceived by CMM cheese was found substantially higher (P<0.05) than the BMM cheese.

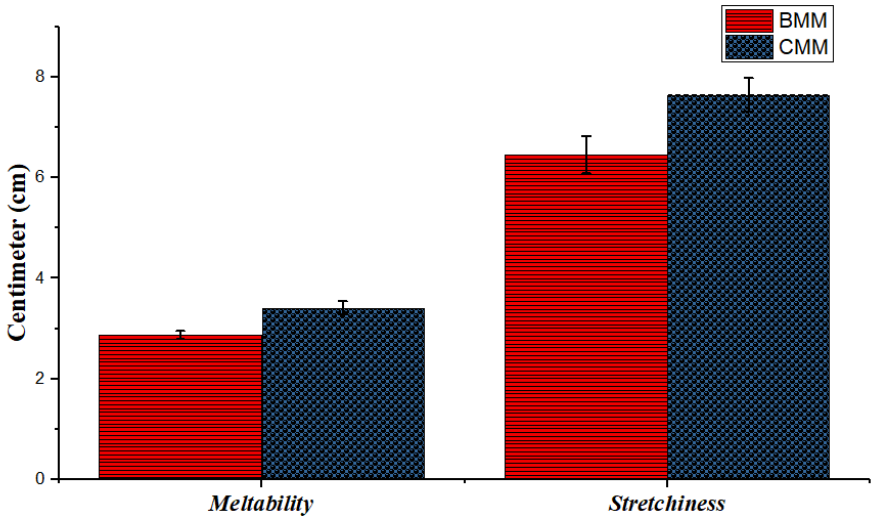

Figure 3. Influence of milk source on rheological properties of mozzarella cheese.

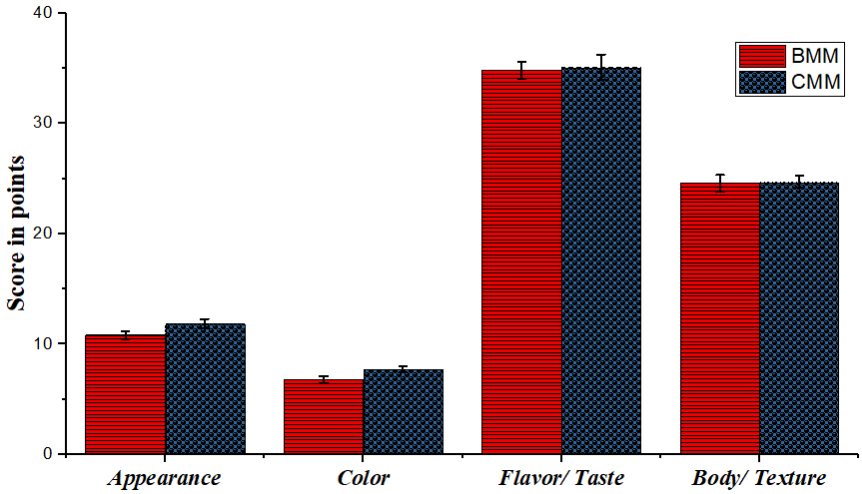

Figure 4. Influence of milk source on organoleptic attributes of mozzarella cheese.

Flavor/Taste: The score evaluated for the flavor/taste of BMM cheese varied between 30.50 and 38.50, whereas that of CMM cheese, was between 29.00 and 40.00 over a score of 45. BMM cheese (34.80 ± 0.79) perceived a slightly lower score as compared to that of CMM cheese (35.10 ± 1.13), but the difference is not significant (Figure 4 and Table 1).

Body/Texture: The score rated for body/texture of BMM cheese varied between 20.00 and 28.00, while that of rated for CMM cheese, it was in between 21.00 and 27.50 over a score of 30. Mean score of body/texture of BMM cheese (24.56 ± 0.77) was found comparatively analogous ($P>0.05$) to that perceived by CMM cheese i.e. 24.70 ± 0.54 (Figure 4 and Table 1).

The yield of mozzarella cheese: The minimum yield of BMM cheese was observed as 13.75% and the maximum 20.00%, while the minimum yield of CMM cheese was recorded as 9.00% and the maximum 14.20%. Buffalo milk on average yielded considerably ($P<0.05$) higher quantity of mozzarella cheese (16.95 ± 1.29%) than that made from cow milk i.e. 12.68 ± 0.936% (Figure 5 and Table 1).

Calorific values of mozzarella cheese: The range of calorific values (kcal /100 g) of BMM cheese was between 283.40 and 351.00, while in CMM cheese, it varied between 241.00 to 299.00 kcal /100 g. The average calorific value (kcal /100 g) of BMM cheese (318.49 ± 6.42 kcal /100 g) was considerably ($P<0.05$) higher than the CMM cheese (268.83 ± 5.085 kcal /100 g). However, both cheeses appeared considerably ($P<0.05$) high in calorific values from that of their corresponding milk i.e. buffalo milk (89.79 ± 1.14 kcal /100 g) and cow milk (65.72 ± 1.33 kcal /100 g), presented in Figure 6 and Table 1.

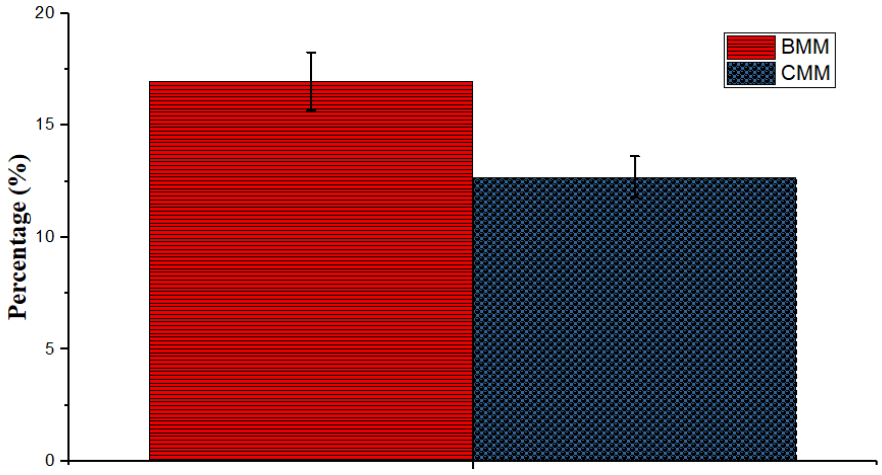

Figure 5. Yield (%) of mozzarella cheese made from buffalo and cow milk.

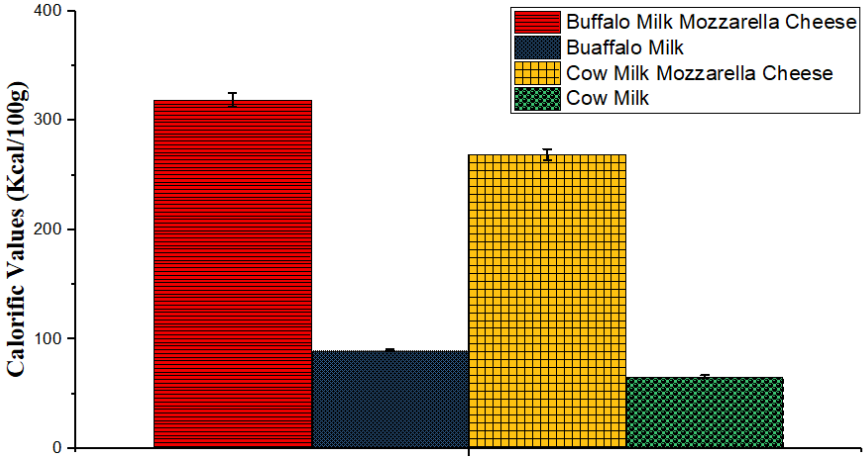

Figure 6. Calorific values of mozzarella cheese made from buffalo and cow milk.

Table 2. Economic Analysis of mozzarella cheese made from cow and buffalo milk.

Economic Markers	Component	BMM cheese			CMM cheese			
		Quantity	Rate (Rs)	Amount (Rs)	Quantity	Rate (Rs)	Amount (Rs)	P
Cost (Rs)								
	Milk	4 kg	70	280.0	4 kg	60	204.00	-
	Rennet	1/8 tablet	250/ tablet	31.25	1 tablet	250/ tablet	31.25	-
	Starter Culture	120 g	-	5.46	120 g	-	4.76	-
	Miscellaneous	-	-	183.35	-	-	163.005	-
	Total (A)	-	-	550.06	-	-	489.01	-
Income (Rs)								
	Cheese (B)	0.67/ kg	1400/ kg	935.20	0.507 kg	1400/ kg	710.92	0.050
Benefit (Rs)								
	(B-A)	-	-	385.14	-	-	221.91	0.217

Economic of Mozzarella cheese: The total expenditure to produce BMM cheese (Rs 550.06) appeared slightly higher than that of CMM cheese (Rs 489.01). However, the income was computed considerably high in BMM cheese (Rs 935.20) than that of CMM cheese (Rs 710.92). Regardless, the remarkable variation occurred between the incomes of BMM cheese and CMM cheese, the expected benefit was not significant among them i.e. Rs 385.14 ±81.0 and 221.91 ± 52.67, respectively (Table 2).

Discussion and Conclusion

Chemical analysis of BMM and CMM cheeses: The significant influence of milk source was noted on the moisture content of mozzarella cheeses. Mozzarella cheese prepared from cow milk appeared high in moisture content compared to that of BMM cheese. This is because of the composition of their corresponding milk from which these were manufactured. A similar trend of moisture content was recorded in the milk of buffalo and cow whereby cow milk appeared high in moisture content than that of buffalo milk in the current study. However, a remarkable decrease in the moisture content of mozzarella cheese was noted either prepared from cow milk or/buffalo milk. This decrease in moisture content could be associated with the removal of whey from coagulated curd, and consequently, the increase in total solids content was observed in the final product. The majority of mozzarella cheese batches were consistent with the findings described by Sameen et al. (21) and Sulieman et al. (24), i.e. 52.44 and 50.49%, respectively.

In the present study, comparatively high protein content was noted in mozzarella cheese from that of their corresponding milk. However, protein content in mozzarella cheese was found inverse compared to that observed in milk. CMM cheese appeared slightly higher in protein content than that of BMM cheese, whereby their corresponding milk appeared opposite in protein content i.e. marginally better protein content in buffalo milk. Nevertheless, the variation between the protein content of both types of cheeses and/or kinds of milk existed statistically non-significant ($P>0.05$). It is noteworthy that a slight variation in protein content of mozzarella cheese manufactured from two different milk sources may be attributed to the casein content of milk that may have not been properly recovered from buffalo milk and/or whey proteins during pre-heat treatment of buffalo milk which may have not been denatured. Several factors have been reported in different studies, for instance, protein degradation may occur as heat treatment increases, and that the rate of amino acid liberation decreases as heat treatment increases (23). Variation in mozzarella cheese protein content may also occur either due to crude enzyme extract and/or withholding of whey in the finished product (cheese) that might enhance the protein content (16).

A significant influence of milk sources was noted on the fat content of mozzarella cheese. BMM cheese appeared markedly high in fat content than the CMM cheese, is due to the composition of the corresponding milk from which they were manufactured. A similar trend of fat content was recorded in the milk of buffalo and cows. However, a considerable decrease in the fat content of BMM and CMM cheeses was noted. It is interesting to note that an increase in milk component among total solids content may occur if one of the components decreases in proportion from its original proportion, and in the current study whey protein along with lactose content may have been drained in whey liquid during the manufacturing of mozzarella cheese, and in a consequence increase in other components might have been occurred. Similar sorts of outcomes were reported by Bhattarai and Acharya (8), Mijan et al. (18) and Sameen et al. (21) i.e. 42.81 to 52.9%, (on DMB).

The ash content of BMM cheese appeared higher in contrast to that of CMM cheese. The variation in ash content in both types of mozzarella cheese existed non-significant. This could be due to the composition of the corresponding milk from which these were manufactured. A similar trend of ash content was recorded in the milk of buffalo and cow, whereby cow milk appeared slightly high in ash content than that of buffalo milk but statistically non-significant ($P>0.05$). However, the average value of BMM and CMM cheeses were (6.52 ± 0.238 and 6.23 ± 0.278% DMB) in accordance with the outcomes described by Bhattarai and Acharya (8) and Mijan et al. (18), i.e. 7.7% and 8.1% DMB 4.8% and 5.1% DMB.

Rheological analysis of BMM and CMM cheeses: The amount of cheese flow and spreading upon exposure to heat is the chief functional attribute of mozzarella cheese termed as "meltability" (21). This property although varied between the cow and buffalo milk mozzarella cheese, the difference among them was found significant. It is noteworthy that variation in meltability of BMM cheese and CMM cheese is due to a decrease in calcium content and higher moisture content present in cow milk, while it might have been improved through the removal of the para-casein matrix (19). Nevertheless, current results of meltability agreed with the results observed by Bertola et al. (6) and Bhattarai and Acharya (8), i.e. 3.4 cm, and 3.06, 4.33 cm, respectively.

The stretchiness of CMM cheese was significantly higher than the BMM cheese. The increased stretchiness of CMM cheese could be attributed to the reduction in the calcium level that causes a decrease in the structural strictness of the cheese matrix subsequently enhancing the stretchiness (8). During ripening, proteolysis results in increased porosity of the casein matrix, and therefore resistance to stretching has reduced (6). The current results are in agreement with the outcomes reported by Bhattarai and Acharya (8) which suggests that cheese with greater meltability had higher stretchiness.

Impact of milk source on yield and calorific values of BMM and CMM cheeses: BMM cheese appeared although high in yield contrast to CMM cheese, the difference among them existed non-significant. This is possibly due to the composition of their corresponding milk from which these were manufactured, whereby buffalo milk was significantly high in TS content than that of cow milk. It is of interest to note that the yield of cheese

is dependent on the recovery percentage of solids, whereby the greater the number of recovered solids, would yield a higher amount of cheese, and this has also been reported in a previously conducted study (13), whereby the recovered amount of cheese was recorded highest from buffalo milk and the lowest from cow milk. Another factor that might involve yield is the use of rennet that enhances the whey separation from the curd and prevent the excess loss of casein protein (7, 14).

A significant influence of milk source was noted on calorific values of mozzarella cheese. Calorific values of BMM cheese were significantly higher than the CMM cheese. The difference in calorific values of cheeses in the present study corresponds to the milk source from which those were prepared, whereby buffalo milk appeared considerably high in energy value than the cow milk. A similar trend in the energy value of buffalo and cow milk was reported (4). Moreover, researchers had confirmed the present finding, who reported a remarkable proportion of calories in buffalo milk (3450 KJ /Kg) than the cow milk (3169 KJ /Kg). Moreover, it has been described that in terms of calories per unit weight, buffalo milk is superior to cow milk due to its better chemical composition (20).

Organoleptic analysis of BMM and CMM cheeses: Organoleptic attributes like appearance and flavor/taste are the most important factor in determining consumer's responses. It has been described that the flavor of mozzarella cheese made from cow milk is liked more by most of the panelists (11). Moreover, in the present study appearance, flavor/taste, and body/texture score rated for CMM cheese and BMM cheese varied slightly, although the difference among them appeared non-significant. A significant difference was noted in the color of BMM cheese and CMM cheese. The CMM cheese appeared yellowish-white as compared to BMM cheese which was white. The color differences are possible due to carotene content which is rich in cows' milk (18).

Economic value of BMM and CMM cheeses: Economics is a knowledge concerned with the production, consumption, and transfer of wealth. The cost of BMM cheese was marginally higher as compared to the CMM cheese. This variation in cost may be due to the higher market price of buffalo milk. The difference between the income and benefit of BMM cheese and CMM cheese was insignificant. The income and profit were somewhat better in BMM cheese as compared to CMM cheese and is due to a higher yield in BMM cheese. The better chemical composition of buffalo milk renders it extremely appropriate for cheese and dairy processing. 1 kg of cheese production consumes 5 liters of buffalo milk as compared to 8 kg of cow milk which makes buffalo milk more valuable for processors (4).

It is concluded from the present study that milk sources had a prominent influence on the chemical characteristics and calorific values of mozzarella cheese. Fat, mineral/ash, and calorific values were found considerably high in BMM cheese in contrast to that of CMM cheese. The protein content and stretchiness of CMM cheese appeared significantly better compared to that of BMM cheese. CMM cheese although received a better organoleptic score, but statistically not different from the BMM cheese. Buffalo milk yielded more quantity of mozzarella cheese as well as economic values compared to that of cow milk.

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

Ethical Statement

This study does not present any ethical concerns.

Conflict of interest

The authors declared that there is no conflict of interest.

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Effects of aromatic plant extract mixture on laying efficiency, egg quality and antioxidant status in laying quails

Deniz KARAKÇI[1,a], İsmail ÇETİN[2,b], Ece ÇETİN[3,c], Derya YEŞİLBAĞ[4,d,✉]

[1]Tekirdağ Namık Kemal University, Faculty of Veterinary Medicine, Department of Biochemistry, Tekirdağ, Turkey; [2]Tekirdağ Namık Kemal University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Tekirdağ, Turkey; [3]Tekirdağ Namık Kemal University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Tekirdağ, Turkey; [4]Bursa Uludağ University, Faculty of Veterinary Medicine, Department of Animal Nutrition and Nutritional Diseases, Bursa, Turkey
[a]ORCID: 0000-0002-1884-1874; [b]ORCID: 0000-0001-7589-4852; [c]ORCID: 0000-0002-8783-5507; [d]ORCID: 0000-0003-1816-2684

✉Corresponding author: dyesilbag@uludag.edu.tr
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Abstract: This study aimed to evaluate the effects of commercial plant extract mixture added to laying quail rations on performance, egg quality, plasma antioxidant and blood biochemical parameters. In this experiment, 160 Japanese quails aged twelve weeks were equally divided into four groups of 40 (five replicates of 8 quails each). The study included a control and three treatment groups. The treatment groups were as follows: (1) 100 mg/kg aromatic plant extract mixture-group 1; (2) 150 mg/kg aromatic plant extract mixture-group 2 (3) 200 mg/kg aromatic plant extract mixture-group 3, respectively. When examined the egg parameters at the end of the study, significant differences (P<0.05) were found in the parameters of egg weight, Haugh unit (HU), albumen index, egg yolk color and eggshell thickness. Egg weight and eggshell thickness values were measured the highest (P<0.05) in group 1. HU, egg yellow color and albumen index values were found significantly higher (P<0.05) in all experimental groups compared to the control group. Significant differences (P<0.05) were determined in superoxide dismutase (SOD) and malondialdehyde (MDA) values of plasma antioxidant parameters in the study. In the study, High density lipoprotein (HDL) value, one of the blood (plasma) parameters, was found to be higher (P< 0.001) in the experimental groups. Consequently, we can say that the addition of herbal extract mixture to quail diets increases egg quality and has a positive effect on cholesterol and plasma antioxidant parameters.

Keywords: Antioxidant, egg quality, MDA, phytobiotic, quail.

Aromatik bitki ekstrakt karışımının yumurtacı bıldırcınlarda yumurta verimi, yumurta kalitesi ve antioksidan durum üzerine etkileri

Özet: Bu çalışmada, yumurtacı bıldırcın rasyonlarına ilave edilen ticari bitkisel ekstrakt karışımının performans, yumurta kalitesi, plazma antioksidan ve biyokimyasal parametreler üzerine olan etkilerini değerlendirmek amaçlanmıştır. Deneysel çalışmada, 160 adet 12 haftalık Japon bıldırcını, her grupta 40 adet olacak şekilde eşit olarak dört gruba ayrıldı (5 alt grup her grupta 8 bıldırcın). Araştırma 1 kontrol ve 3 deneme gruplarından oluşmuştur. Deneme grupları sırasıyla: (1) 100 mg/kg aromatik bitkisel ekstrakt karışımı- Grup 1; (2) 150 mg/kg aromatik bitkisel ekstrakt karışımı- Grup 2 ve (3) 200 mg/kg aromatik bitkisel ekstrakt karışımı- Grup 3 olarak oluşturuldu. Araştırma sonunda, yumurta parametreleri incelendiğinde yumurta ağırlığı, Haugh birimi, albumin indeksi, yumurta sarı rengi ve yumurta kabuk kalınlığı parametrelerinde önemli farklılıklar (P<0,05) tespit edildi. Yumurta ağırlığı ve yumurta kabuk kalınlığı değerleri en yüksek (P<0,05) grup 1'de ölçüldü. Haugh birimi, yumurta sarı rengi ve albumin indeksi değerleri kontrol grubuyla kıyaslandığında tüm deneme gruplarında anlamlı derecede (P<0,05) yüksek bulundu. Araştırmada kan plazma antioksidan parametrelerinden SOD ve MDA değerlerinde önemli düzeyde (P<0,05) farklılıklar belirlendi. Araştırmada, kan parametrelerinden HDL değeri deneme gruplarında daha yüksek olarak (P<0,001) tespit edildi. Sonuç olarak bıldırcın diyetlerine bitki ekstrakt karışımı ilavesinin yumurta kalitesini artırdığını, kolesterol ve plazma antioksidan parametreleri üzerinde olumlu etkisi olduğunu söyleyebiliriz.

Anahtar sözcükler: Antioksidan, bıldırcın, fitobiyotik, MDA, yumurta kalitesi.

Introduction

Today, poultry breeding contributes to meeting the needs of people with meat and egg products and animal protein with its suitability for industrial production and low price. Most of the poultry production is more economical than the production of livestock species. Antibiotics, which are used as growth promoters in poultry diets, have ensured that the increasing demands for poultry products are met rapidly and resulted in efficiency. However, the use of antibiotics in rations as feed additives was prohibited in 2006 due to the fact that they leave residues in animal products and cause the development of resistant bacteria. After the prohibition of antibiotics used as growth and health promoters in livestock, feed additives that can be used as an alternative to antibiotics have been investigated in order to minimize the incurred losses of the poultry and livestock sector (6). As in this research, aromatic plants and plant extracts defined as phytobiotics are added to animal feeds as feed additives. Phytobiotics with biological and aromatic properties can be added directly or used in the form of extract acquired from these plants. In addition to their aromatic effects, phytobiotics have antioxidative, antifungal, anticarcinogenic, anti-inflammatory, and antimicrobial effects. Besides, these plants have been utilized for centuries to extend the shelf life of foods (43).

Phytobiotics, which are added to poultry feeds as additives, are supplemented to increase the digestibility and consumption of feeds, to improve performance or the quality of the products obtained (42). In the study, a new generation plant extract mixture with the active ingredients of Sanguinarine, Honokiol and Magnolol was used in order to benefit from its biological activities. Sanguinarine is a plant extract obtained from the plants *Macleya cordata* and *Sanguinaria Canadensis* (1, 17). Sanguinarine is a quaternary benzo [c] phenanthyridine (QBA) alkaloid belonging to the *Papaveraceae* family (14, 26). The pure alkaloid form of this active ingredient was used as a traditional medical drug in North America, Europe, and China it was isolated several years ago. It was found that sanguinarine which has antibacterial (18), anti-inflammatory (27), antiviral (10), antifungal (33), antitumoral (12), and immunomodulator (15) effects, reduces amino acid degradation in small intestines with its addition to poultry feed (16). However, Banerjee et al. (4) reported that serum sanguinarine levels were positively correlated with serum MDA levels in their study. In the study conducted by Vieira et al. (38), it was found that the addition of sanguinarine to broiler rations increased their live weight in the 3rd week. *Magnolia officinalis* is a plant belonging to the *Magnoliaceae* family, also called 'houpu' (shell part is 'hou', the remaining part is 'pu') which has been used traditionally in China for a long time (31, 37). This herb is used in China for the treatment of acute pain, diarrhea, cough, and urinary system problems (13). The main active compounds of *M. officinalis* are Magnolol and Honokiol. These compounds act for lipid peroxidation and inhibition of free radicals (25). It also provides bacteriostatic, anti-inflammatory, antitumoral, and antimicrobial activities (9, 40). In addition, it is also used in the medical, pharmaceutical, and cosmetic industry (20).

This study aimed to investigate the effects of the combined use of the plant extracts in question. For this purpose, the effects of sanguinarine, honokiol and magnolol extract mixture added to laying quail rations on egg quality, plasma antioxidant and biochemical values and performance parameters were examined.

Materials and Methods

Animals and Diets: A total of 160 Japanese quails (*Coturnix coturnix japonica*) aged twelve weeks were housed in cages and were randomly allocated to four treatment groups each consisting of 40 quails. Each group was divided into 5 subgroups. The quails were kept in laying cages (100 cm wide, 45 cm deep, 21 cm high in front, and 17 cm high in the rear, 112.5 cm 2 per quail) and housed there until the end of the study. Feed and water were provided ad libitum. The quails received a basal diet (maize and soya bean based; 16.8 % crude protein; 2720 kcal/kg Metabolisable energy-ME) that was formulated to meet the NRC (National Research Council) (28) requirements for nutrients, including vitamins and minerals. The diet did not contain antibiotics, coccidiostats or growth promoters. The content of the basal diet is presented in Table 1. Group feeding was used in all treatment groups. The one control group and 3 treatment groups were treated as follows: 0 mg additive of diet (control); (1) with the addition of 100 mg/kg aromatic plant extract mixture (2) with the addition of 150 mg/kg aromatic plant extract mixture (3) with the addition of 200 mg/kg aromatic plant extract mixture. The levels of product were decided as per the manufacturer recommendations. The aromatic plant extract Filopower, a natural growth promoter, was supplied by Yem-Vit A.Ş (İzmir, Turkey). The aromatic plant extract mixture included 50% wheat middlings, 24% mixing of flavoring compounds (Magnolia, Sanguinarina), 23.5% calcium carbonate, 2% of products and by-products of tubers and roots and 0.5% barley meal (unit given by the manufacturer). During the experimental period, the 24-hour constant lighting program was maintained at 16 hours light and 8 hours dark. The experiment was conducted for 10 weeks. The study protocol was approved by Ethics Committee of Tekirdağ Namık Kemal University Approval number: T 2018-3/1). The nutritional composition of the diets was determined according to the AOAC (Association of Official Analytical Chemists) (3).

ME levels of diets were estimated using the equation created by Carpenter and Clegg (21): ME, kcal/kg = 53+ 38 [(CP, %) + (2.25 x ether extract, %) + (1.1 x starch, %) + (sugar, %)].

Performance Parameters: The quails were weighed twice, at the beginning and at the end of the experiment, and their live weight was determined. The feeds were weighed every two weeks and the feed consumption was recorded as the group average. The feed conversion rate (FCR) was calculated as the amount of feed consumed for a dozen egg production. In order to calculate the egg production, the number of eggs was noted every day at the same time. Eggs collected every 15 days were kept for 24 hours, and then the egg weight was determined by weighing them on a precision scale (Sartorius, Model BSA224S-CW, Germany). After the weight of the eggs and egg yield percentage obtained from the groups were determined, the egg mass was calculated using these values.

Egg mass = Egg weight x Egg yield/100

Antioxidant and Biochemical Parameters: During the 70th day of the study, the animals were slaughtered for blood collection. During the slaughter, blood samples of 40 animals in total, 10 randomly from each group, were taken into tubes containing 2 ml anticoagulant (K_3 EDTA). The blood samples were centrifuged at 3000 rpm for 10 minutes, transferred to plasma microtubes and stored at -20ºC until the day of analysis. The changes of antioxidant parameters in plasma including SOD (Superoxide Dismutase) (Catalog no: K-335-100 Biovision, USA) GSH-Px (Glutathione Peroxidase), (Catalog no: K762-100 Biovision, USA) TAC (Total Antioxidant Capacity) (Rel Assay Diagnostic, Turkey) TOC (Total Oxidant Capacity) (Rel Diagnostic Assay, Turkey), MDA (Malondialdehyde) (Catalog number: 201-16-0161 Sunred Biological Technology Co., Ltd., China), CAT (Catalase) (Catalog number: 201-16-0159 Sunred Biological Technology Co., Ltd, China) were measured by using commercial kits using ELISA method and biochemical parameters such as Total Protein, Albumin, Total Cholesterol, HDL, LDL (Low Density Lipoprotein), Triglyceride were evaluated using spectrophotometric and colorimetric methods by a microplate reader (Thermo Scientific Multiskan Go, USA).

Egg Quality Parameters: On the 15th, 30th, 45th, 60th, and 70th days of the study, 8 eggs (total 40 egg per groups) of the last two days from each subgroup were collected and egg weight, shape index, shell thickness, shell weight, egg shell breaking strength were determined in order to examine the external quality characteristics while the albumen index, yolk index, yolk color, and HU were defined to examine the internal quality characteristics (2). In determining the egg weight and shell thickness, the eggs were weighed (g) and recorded after taring on a scale with 0.0001 precision (Sartorius, Model BSA224S-CW, Germany). The shape index value is measured by placing the width and length of each egg, the widest and longest points of the egg on a digital caliper (Caliper, Mitutoyo Code No.500-181-20, Model CD-15CPx, Japan), and then the shape index value was calculated by adding these two values to the formula = egg width (mm) / egg length (mm) x100. In determining the shell thickness of the eggs, the outer and inner membranes of three separate pieces, one from each of the two ends and middle parts of the egg, were peeled, and each was measured by placing it in a digital caliper. Average shell thickness was found by taking the arithmetic mean of these three values. In order to determine the egg shell breaking strength of the eggs, each egg was placed vertically and with the blunt end upwards on the egg shell breaking strength measuring device (Push Pull Scale, Imada, Model No. SU-05, Japan). Then the upper vice of the device was slid downwards and tightened so that there was no gap between the vice and the egg. The vice was then gently pushed further down to the breaking point of the egg and the value read on the scale at the breaking point was recorded as Newton / cm2 (N/cm2). In determining the albumen index, firstly, after each egg was broken without dispersing on a glass plate, the albumen height and albumen length were measured with a digital caliper and the albumen height was recorded with a tripod micrometer. The formula of albumen index = (albumen height (mm)/average of length and width (mm)) x100 was used to calculate the index. HU in egg was determined using the values of egg weight-g (G) and albumin height-mm (H). HU = 100log (H + 7.57-1.70G0.37). In order to assign the yolk index, first, yolk height and yolk width were measured with a digital caliper after breaking each egg without spreading on a plate. The calculation was made using the formula yolk index = yolk height (mm) / yolk width (mm) x 100. Roche color scale consisting of 15 slices was used to measure the color of egg yolk (DMS, 2005-HMB, 51548, Switzerland).

Statistical Analysis: In the statistical analysis of live weight, feed consumption, FCR, biochemical parameters, egg yield and quality, a One Way ANOVA was applied for the variables meeting the parametric test assumptions while when statistical differences were found between the groups, a Duncan multiple comparison test was used to identify in which group or groups the difference was (35). Statistical significance was determined at $P<0.05$. Linear and quadratic effects of dietary plant extract mixture inclusion level were studied using polynomial contrasts. Statistical analyses were performed with the SPSS 21 package program (SPSS Inc., Chicago, IL, USA). Statistical analysis was carried out using the SPSS 10.00 (36) software package for Windows (SPSS Inc., Chicago, IL, USA).

Results

The ingredient and chemical composition of the diets is shown in Table 1. The effects of aromatic plant extract mixtures on some quail performance parameters are presented in Table 2. In the study, no difference (P> 0.05) was found in terms of starting and ending live weights of quails. But plant extract mixture addition increased finally live weight quadratically (P< 0.05). When the results were examined, it was seen that the egg weight data in the first group were higher than the control and group 2 (P<0.001) and quadratic (P<0.01). There was no statistically significant difference between the control and experimental groups in terms of egg mass, feed intake and FCR (P>0.05). The effects of dietary treatments on some egg quality characteristics are given in Table 3. The data clearly show that although the difference in shape index, yolk index, egg shell breaking strength and eggshell weights were not significant between groups (P>0.05), HU, albumen index, yolk colour and egg shell thickness were significantly different (P<0.05). Among these parameters, the albumen index was not found quadratically significant. The highest values in these parameters (HU, albumen index, yolk color and eggshell thickness) were measured in experimental groups. The HU and albumen index values, measures of egg quality, were found to be high in the experimental groups. These values show us that additives can contribute to improving egg quality. Yolk colour was significantly improved in the all treatment groups compared with the control group. The effect of dietary treatments on plasma antioxidant parameters are shown in Table 4. In the study, while plasma SOD and MDA values differed (P<0.05) between control and experimental groups, no difference was observed

Table 1. Ingredients and chemical composition of basal diet.

Ingredients	g/kg
Maize, Grain	590
Soybean meal	208
Full fat soybean	110
Sunflower meal	46
Wheat	27
Monocalcium phosphate	8.5
Salt	2.5
Vit-Min premix[a]	3.5
DL-methionine	1.6
Sodium bicarbonate	1
Choline choloride	0.8
Phytase	0.6
Toxin binder	0.5
Analyse concentrations, g/kg	
Metabolisable energy[b], kcal/kg	2720
Crude protein	168.2
Crude fat	42.8
Ash	118
Dry matter	885.2
Calcium	3.73
Phosphorus	0.64

[a]Provides (per kg diet): Vit A (retinol), 2.4 mg; Vit D_3 (cholecalciferol), 0.075 mg; Vit E (α-tocopherol acetate) 20 mg;Vit B_1 (thiamine), 3mg; Vit B_2 (riboflavin), 3 mg; Vit B_6 (pyridoxal), 3.5 mg; Vit B_{12} (cobalamin), 0.01 mg; Niacine, 20 mg; Calcium d-pantothenate, 4 mg; Folic acid, 1 mg; Choline, 600 mg; Biotin, 0.03 mg; Mn, 80 mg; Fe, 60 mg; Zn, 60 mg; Cu, 5 mg; I, 1 mg; Co, 0.2 mg; Se, 0.15 mg

[b]Metabolisable energy content of diets was estimated using the equation devised by Carpenter and Clegg.

Table 2. Effects of aromatic plant extract mixture on performance parameters.

					Statistical analyses[1]	
					Polynominal contrasts	
Parameters	**Control**	**Group 1**	**Group 2**	**Group 3**	***P-linear***	***P- quadratic***
Live weight (initially),g	234.27±4.56	239.89±4.56	236.59±4.61	232.71±5.97	0.720	0.340
Live weight (finally),g	249.33±5.99	266.78±6.32	256.42±5.72	268.92±8.28	0.101	0.033
Feed intake, g/day	41.80±0.77	42.98±1.08	39.86±0.96	40.89±1.14	0.209	0.940
Egg yield, %	91.28±1.22	86.61±2.00	89.00±1.49	90.07±1.03	0.311	0.109
Egg weight, g	11.96±0.10[c]	12.62±0.07[a]	12.26±0.07[b]	12.42±0.12[ab]	0.024	0.011
Feed conversion rate[2]	0.55±0.007	0.59±0.02	0.54±0.01	0.54±0.015	0.191	0.188
Egg mass, g/quail/day	10.93±0.24	10.97±0.32	10.91±0.25	11.11±0.18	0.679	0.753

[a,b,c]:Different superscripts in each row shows the significant difference between the groups p<0.05. [1]: The statistical analysis tests the differences between treatment groups (ANOVA) and the linear-quadratic effect of plant extract mixture inclusion levels (polynominal contrasts) [2]:kg feed/12 eggs.

in other plasma parameters. The plasma SOD level was measured in the group 2 with the most different (P<0.05) and the highest value, while the lowest plasma MDA value was measured in the group 1 (P<0.05). However, the plasma SOD value did not constitute a quadratic significance. In the study, biochemical plasma albumin, total protein, LDL, HDL, triglyceride and total cholesterol parameters are presented in Table 5. When the HDL value was examined in the study, it was found that there was a statistically significant difference between the groups and the HDL values were at the highest level in the group 2 and group 3 groups (P<0.05).

Table 3. Effects of aromatic plant extract mixture on egg quality parameters.

					Statistical analyses[1]	
					Polynominal contrasts	
Parameters	**Control**	**Group 1**	**Group 2**	**Group 3**	***P-linear***	***P- quadratic***
Shape index	81.84±0.26	81.16±0.28	81.75±0.29	81.81±0.28	0.161	0.197
Haugh unit	90.03±0.41^{b}	92.00±0.36^{a}	91.38±0.31^{a}	91.58±0.36^{a}	0.007	0.04
Albumen index	12.14±0.20^{b}	12.87±0.15^{a}	12.80±0.15^{a}	12.68±0.16^{a}	0.035	0.344
Yolk index	49.88±0.39	49.63±0.19	49.26±0.20	49.27±0.28	0.826	0.701
Yolk colour	3.18±0.06^{c}	3.51±0.06^{ab}	3.69±0.07^{a}	3.42±0.07^{b}	0.004	0.001
Eggshell thickness, µm	14.83±0.11^{d}	16.83±0.11^{a}	16.05±0.12^{b}	15.65±0.12^{c}	0.002	0.001
Eggshell breaking strength, N	12.72±0.18	13.36±0.24	13.23±0.19	13.10±0.16	0.253	0.063
Eggshell weight, g	1.84±0.02	1.88±0.02	1.84±0.02	1.86±0.02	0.817	0.857

$^{a, b, c, d}$: Different superscripts in each row shows the significant difference the groups p<0.05. n: 40
^{1}: The statistical analysis tests the differences between treatment groups (ANOVA) and thelinear- quadratic effect of plant extract mixture inclusion levels (polynominal contrasts)

Table 4. Effects of aromatic plant extract mixture on plasma antioxidant parameters.

					Statistical analyses[1]	
					Polynominal contrasts	
Parameters	**Control**	**Group 1**	**Group 2**	**Group 3**	***P-linear***	***P- quadratic***
GSH-Px (mmol/min/ml)	93.83±6.33	114.70±7.92	105.87±7.01	112.26±6.27	0.142	0.303
SOD (U/ml)	96.14±6.05^{b}	93.64±5.71^{b}	123.41±8.24^{a}	101.26±5.13^{b}	0.123	0.133
CAT (ng/ml)	44.90±1.75	48.61±1.60	51.01±1.77	50.62±1.83	0.500	0.248
MDA (nmol/ml)	20.81±0.71^{a}	18.16±0.72^{b}	19.15±0.57^{ab}	19.88±0.56^{ab}	0.021	0.013
TAS (mmol/L)	0.68±0.06	0.65±0.05	0.85±0.07	0.86±0.09	0.420	0.770
TOS (µmol/L)	4.05±0.36	3.64±0.46	5.11±0.46	4.70±0.51	0.102	1.00

$^{a, b}$: Different superscripts in each row shows the significant difference between the groups p<0.05. n: 10
^{1}:The statistical analysis tests the differences between treatment groups (ANOVA) and the linear-quadratic effect of plant extract mixture inclusion levels (polynominal contrasts)

Table 5. Effects of aromatic plant extract mixture on biochemical parameters.

					Statistical analyses[1]	
					Polynominal contrasts	
Parameters	**Control**	**Group 1**	**Group 2**	**Group 3**	***P-linear***	***P- quadratic***
Albumin (g/dl)	2.20±0.08	2.26±0.09	2.37±0.04	2.23±0.06	0.568	0.207
HDL (mg/dl)	53.32±3.21^{c}	62.83±2.89^{b}	74.13±3.61^{a}	72.40±3.23^{a}	0.001	0.092
LDL (mg/dl)	61.87±4.43	44.76±6.75	48.49±5.86	40.81±4.25	0.295	0.390
Triglyceride (mg/dl)	276.10±17.05	259.78±24.93	245.04±13.59	243.79±10.10	0.082	0.862
Total Cholesterol (mg/dl)	139.41±5.02	129.96±12.07	142.64±7.09	129.36±4.38	0.618	0.807
Total Protein (g/dl)	5.78±0.29	5.24±0.24	5.26±0.39	5.58±0.44	0.793	0.830

$^{a, b, c}$: Different superscripts in each row shows the significant difference between the groups P<0.05. n:10
^{1}:The statistical analysis tests the differences between treatment groups (ANOVA) and the linear-quadratic effect of plant extract mixture inclusion levels (polynominal contrasts).

Discussion and Conclusion

In this study, no statistically significant difference was found between the groups in terms of performance parameters, feed intake, egg yield, egg mass and FCR. Only significant difference was detected in egg weight. The lowest egg weight was measured in the control group. Studies on the use of aromatic plant or plant mixtures have found various effects on performance parameters. In parallel with the results of the study, Kozlowski et al. (19) reported that the addition of *Macleaya cordata* to broiler diets had no significant effect on feed intake, live weight gain and FCR. In another study, it was concluded that the addition of sanguinarine to the ration generally had no effect on body weight and FCR during the initial or development periods (15). Unlike this study, Vieira et al. (38) revealed that sanguinarine added to broiler diets increased the live weight of animals in the 3rd week. In addition, Yesilbag et al. (45) found that the addition of aromatic plant extract mixture (sanguinarine, magnolol, honokiol) to broiler ration at the level of 100 ppm caused a significant increase in total feed consumption. It shows that the increase in feed consumption is due to the positive effect of the aromatic plant used on the palatability of the feed. In the study, egg weight in group 1 was statistically higher than the control and other groups. We can say that the increase in the weight of the eggs collected from this group in the study may be due to this group's consumption of more feed. In another study, it was observed that egg weight increased with the addition of 200 mg/kg of resveratrol, which also has antioxidant properties, to the laying hen rations (46).

The effects of dietary treatments on internal and external quality parameters of eggs are shown in Table 3. In the study, it was found that the addition of aromatic plant extract mixture to quail diets significantly increased in terms of HU, albumen index, egg yolk color and eggshell thickness parameters compared to the control group, resulting in improvements in egg quality in these groups. According to the results of the literature review, no data could be found on the use of the herbal extract mixture we used in the study in poultry laying animals. However, there are studies on the use of other aromatic plant and extracts in layers, and it has been found that the addition of aromatic plant extract mixtures generally can a positive improvement in egg quality, especially yolk color (44). It was found that the essential oil mixture of rosemary and thyme added in high doses to quail rations increased the egg yolk color, yolk and albumen index (44). In another study in which ginger was added to the ration as a feed additive, when the egg quality parameters were examined, it was seen that there was no statistically significant difference (41). In the research, the highest HU value among the egg quality parameters was determined in the experimental groups. The HU is known as an indicator of egg freshness and is related to shelf life. The HU results in this study were similarly affected positively. The improvement in this characteristic shows that the phytobiotic mix can improve the egg quality parameter on shelf life. Similarly, it has been reported that the addition of 300 mg/kg antioxidant juniper oil to the laying hen ration increases the HU value (8). Botsoglou et al. (7) reported a significant effect on egg weight, egg shape index, yolk index, HU and eggshell thickness from rosemary, oregano, saffron and α-tocopheryl acetate supplementation of hen diets. Another study found that dietary thyme oil supplementation did not make a significant difference in egg weight, egg shape index, yolk diameter, yolk height, yolk color, HU, and egg shell thickness (11).

Some active compounds extracted from plants can be used as an alternative to oxidative stress control due to their capacity to neutralize free radicals. These compounds include honokiol and its isomer magnolol and sanguinarine, which are extracted from the plants of *Magnolia officinalis* and *Macleaya cordata*, respectively (22). When SOD values were examined in the study, it was seen that the SOD value of group 2 has a higher value compared to the control and other groups. In a study conducted on ducks, it was found that magnolol increased the mRNA levels in hepatic SOD, CAT and GSH-Px (23).

Magnolol and honokiol were found to have a protective effect through an antioxidant effect against liver damage caused by D-galactosamine which is a hepatotoxic agent. Thus, magnolol and honokiol inhibit intracellular GSH-Px depletion (30). In parallel with this situation, in the study of Pang et al. (29) conducted in mice, it was determined that CAT, GSH-Px and SOD activities were higher in the group given 100 mg/ml through gavage than the control and other groups.

Reactive oxygen species (ROS), hydroxyl (OH^-) radical and superoxide radical (O^{-2}) are formed from the partial reduction of the oxygen molecule. Endogenous sources in ROS production (free radicals produced by the body's own functioning), especially the radicals leaking from electron transport systems in unhealthy mitochondria are the most important free radical source. In lipid peroxidation, it causes a decrease in the ratio of unsaturated fatty acids. As a result of this reaction, fatty acid hydroperoxides and aldehyde compounds are formed and tissue destruction occurs. The thiobarbituric acid (TBA) reaction used to measure this lipid peroxidation is the most used method due to its ease and sensitivity. TBA is used to detect lipid peroxidation products that are converted to MDA (34). In the study, the plasma MDA values which determine the amount of lipid peroxidation were found to be at the lowest levels in the group 1. This

may indicate that the aromatic plant extract mixture given at this dose prevents lipid peroxidation and provides an antioxidant effect metabolically. In addition, in the study conducted by Bavarsadi et al. (5) it was found that the MDA ratios were the lowest in the group which 7.5 mg/kg of sanguinarine was added to laying hens feed. Many studies have proved that the addition of polyphenol compounds, into poultry and livestock feeds protect many tissues and organs against diseases and show antioxidant activity (24, 39). It has been demonstrated by Shen et al. (32) that Magnolol, a polyphenol compound, has antioxidant activity. In this study, no statistically significant differences were found among the groups in terms of CAT, TOS (Total Oxidant Status), TAS (Total Antioxidant Status) plasma antioxidant parameters. As it is known, HDL prevents the presence of high amounts of cholesterol in the blood by carrying cholesterol from tissues and vessels to the liver. High levels of cholesterol in the blood damage blood vessels. As HDL removes excess cholesterol from the blood, it is commonly called 'good cholesterol'. When the plasma HDL lipoprotein value, one of the biochemical parameters, was examined, the HDL values of group 2 and group 3 were measured at the highest level. Contrary to this finding, no change in serum HDL and triglyceride levels was found in a study where different doses of sanguinarine were added to the feed (5). In order to support the study findings, studies are needed to examine the relationship between the aromatic plant mixture containing the same active ingredients and the blood HDL level.

It was determined that the use of extracts of *Macleaya Cordata* and *Magnolia* tree together on laying quail rations caused statistically significant differences between groups in egg quality parameters, plasma antioxidant and biochemical parameters. According to the results of the literature reviews, there are not many studies investigating the effect of the new generation aromatic plant extract mixture, also called phytobiotic. For this reason, evaluating the subject of the study together with the performance and blood parameters can add value to future studies in terms of discussion. However, more research is needed on this subject in order to make more meaningful interpretations. As a result, it is thought that the new generation plant extract mixture has natural, residue-free, egg quality enhancing and antioxidant properties that are introduced to the market as an alternative to antibiotics. Due to these properties, it is concluded that the use of phytobiotic extract mixture in rations of laying quail may provide beneficial effects.

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Ethical Statement

This study was approved by the Tekirdağ Namık Kemal University Animal Experimentation Local Ethics Committee (T 2018-3/1).

Conflicts of Interest

The authors declare no conflicts of interest.

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High mortality rate of shipping fever cases in cattle caused by bovine herpesvirus type 1 (BoHV-1)

Eda Baldan TOKER[1,a], Kadir YEŞİLBAĞ[1,b,✉], Özer ATEŞ[1,c], Berfin KADİROĞLU[1,d], Gizem AYTOĞU[1,e]

[1]Bursa Uludağ University, Faculty of Veterinary Medicine, Department of Virology, Turkey
[a]ORCID: 0000-0003-2468-3945; [b]ORCID: 0000-0003-1793-6879; [c]ORCID: 0000-0001-7676-9033;
[d]ORCID: 0000-0001-5969-6127; [e]ORCID: 0000-0002-3411-081X

[✉]Corresponding author: kyesilbag@uludag.edu.tr
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Abstract: This study reports the high prevalence and molecular characterization of BoHV-1 infection in imported cattle with respiratory system disease after international transport. A high mortality rate of 14.16% (51/360) was reported in a group of animals imported from Hungary to Turkey in 2019. A total of 17 samples were evaluated (3 lung tissue and 14 nasal swab samples) from 15 cattle aged 6 to 9 months not vaccinated against BoHV-1. Virus isolation, polymerase chain reaction (PCR) and restriction endonuclease analysis (REA) procedures were performed within the scope of this study. By virus isolation in MDBK cells, cytopathologic effects was detected in 8 samples (3 lung tissue and 5 nasal swabs samples). The same eight samples were also found positive by BoHV-1 PCR targeting gC (UL44) gene region. According to the sequencing result, the sample (ID: 10054) dropped into a cluster of BoHV-1.1. The REA was applied to the samples to confirm the results of phylogenetic analysis. All of the isolates were identified in the subgroup BoHV-1.1 by REA. These results showed a high mortality risk for imported animals and the possibility for BoHV-1 entering the receiving country via imported animals after transport. This event is a serious problem both for the control of BoHV-1 as well as for animal health and welfare.
Keywords: Animal transport, BoHV-1, PCR, sequencing, shipping fever.

Bovine herpesvirus tip 1'in (BoHV-1) neden olduğu yüksek ölüm oranlı nakil hastalığı vakaları

Özet: Bu çalışmada, uluslararası nakil sonrası solunum sistemi hastalığı tespit edilen ithal sığırlarda BoHV-1 enfeksiyonunun yüksek prevalansını ve etkenin moleküler karakterizasyonunun bildirilmesi amaçlanmıştır. 2019 yılında Macaristan'dan Türkiye'ye ithal edilen bir grup sığırda yüksek ölüm oranı (%14,16; 51/360) tespit edildi. 6-9 ay yaş aralığında ve BoHV-1'e karşı aşılanmamış 15 hayvandan sağlanan toplam 17 örnek (3 akciğer dokusu ve 14 burun svabı) değerlendirildi. Çalışma kapsamında virus izolasyonu, PCR ve restriksiyon endonükleaz analizi (REA) işlemleri uygulandı. MDBK hücrelerindeki virus izolasyonu çalışması sonucunda 8 örnekte (3 akciğer dokusu ve 5 burun svabı) sitopatolojik etki tespit edildi. BoHV-1'in gC (UL44) gen bölgesini hedefleyen PCR metodu aracılığıyla aynı 8 örnekte pozitif sonuç alındı. Nükleik asit dizi analizi için seçilen 1 izolatın (ID: 10054) filogeni analizinde BoHV-1.1 alt tipinde yer aldığı tespit edildi. Filogenetik analiz sonuçlarını doğrulamak için numunelere REA uygulandı ve tüm izolatların BoHV-1.1 alttipi içerisinde yer aldığı teyit edildi. Bu sonuçlar, nakliye sonrasında ithal hayvanlarda BoHV-1 kaynaklı yüksek mortalite oranının görülebileceğini ve uluslararası hayvan ticaretinde uygulanan tüm test protokollerine rağmen BoHV-1'in ithal hayvanlar aracılığıyla yeni bölgelere rahatlıkla taşınabileceğini göstermektedir. Bu durum hem BoHV-1'in kontrolü hem de hayvan sağlığı ve refahı için ciddi bir sorun teşkil etmektedir.
Anahtar sözcükler: BoHV-1, hayvan nakli, PCR, sekanslama, nakil hastalığı.

Introduction

Bovine herpesvirus-1 (BoHV-1), which causes infectious bovine rhinotracheitis (IBR) infection, leads to significant economic losses in the cattle industry. BoHV-1 (renaming to *bovine alphaherpesvirus-1*) is a member of the genus *Varicellovirus* in the *Alphaherpesvirinae* subfamily, which belongs to the *Herpesviridae* family (13). The viral genome with a length of around 135 kb contains about 70 proteins. Based on viral peptide models and genomic analysis, BoHV-1 comprises three subgroups namely BoHV-1.1, BoHV-1.2a, and BoHV-1.2b. While BoHV-1.1 is associated with respiratory and genital system infections, BoHV-1.2 has been mostly associated with genital system infections (19). BoHV-1.1 and BoHV-

1.2 subtypes contain differences in glycoprotein C (gC) epitopes (25).

The virus can lead to fever (40.5–42ºC), anorexia, increase in respiratory rate, dyspnea, persistent severe cough, depression, and severe reduction in milk production in dairy cattle. IBR, the respiratory form of the infection can form subclinical, mild, or severe disease and have a very high morbidity rate (20). BoHV-1 settles in the sensory neurons of the trigeminal ganglion and germinal centers of the pharyngeal tonsils following acute infection and causes lifelong latency. Latent infection is reactivated under stressful conditions such as bad care, crowded pens, weaning, and transport (15, 27). Subsequently, the virus spreads and leads to transmission between housemates. Also, BoHV-1 accelerates apoptosis in $CD4^+$ T cells and causes the immune system to be suppressed (15). Immune suppression by BoHV-1 can lead to the formation of pneumonia caused by secondary bacterial agents i.e. *Mannheimia haemolytica*, *Pasteurella multocida,* and *Histophilus somni*. IBR is not a lethal disease in general, but it can cause significant economic losses due to abortion, loss of weight and milk yield, die of newborn calves, infertility, insufficient feed conversion, secondary viral/bacterial pneumonia, and treatment costs. While the morbidity rate is approximately 30% due to IBR in dairy herds, losses ranging from $ 25 to $ 55 per cow can occur (29). Bovine respiratory disease (BRD) complex, also known as shipping fever, has been reported to cause at least 1 billion $/year of economic loss for the US cattle industry (15). Not only latent infection and immunosuppression of BoHV-1 but also economic losses create an important problem for herd health. The presence of latent BoHV-1 infection causes the continuous circulation of the infection in the herd. For this reason, it is important that the quarantine applications before animal entry, screening of regularly herd for the presence of BoHV-1, and removing infected cattle from the herd. In the risk assessment study for biosecurity, it was reported that the high probability of transmission of BoHV-1, especially through animal transport by local movement or by introduction from another country (3).

Turkey had a cattle population of approximately 18 million and about 700,000 live cattle were imported only in 2019 (31). Although health control practices are applied in the origin countries, quarantine and screening test applications are important for diseases that may develop during and after transportation. However, if the animal is not acutely infected and the latent infection is not reactivated, negative results would be obtained in virological tests (33). The rules and test protocols to be applied during animal import to Turkey are determined by the Ministry of Agriculture and Forestry. For BoHV-1 controls in import cattle from European Union member states to the Republic of Turkey, different veterinary health control procedures such as allowing the import of either unvaccinated and sero-negative animals or animals vaccinated with EU approved killed marker vaccines and to be detected negative by IBR-gE ELISA, are implemented (23). However, there can be mistakes existed in the selected method or during the practices. This situation causes great problems for both the individual herds and national interest, especially in BoHV-1 infections that generally flow latently. Therefore, re-testing or bringing the existing applications to a better level, imported animals for suspected diseases after transportation can contribute to both the importance of controlling BoHV-1 infections for international trade and the identification of the infection source. The aim of this study was to point out the risk of BoHV-1 infection for imported cattle showing signs of the respiratory system and high mortality rate after transport.

Materials and Methods

Case record of the herd: In the study, cattle imported to Southeastern Turkey (Adıyaman province) from Hungary were examined for the presence of BoHV-1. Imported cattle were 6 to 9 months old and no vaccine was applied before transport. All the imported animals were reported to be in a healthy appearance during the transport and kept under quarantine conditions in the same facility as a single import party. A total of 51 (14.16%) from 360 imported animals were recorded to be died after transportation due to respiratory problems. Random sampling was performed in affected animals and sent to our laboratory for virological analysis. In addition, the isolation of *Pasteurella multocida* and *Mannheimia haemolytica* were recorded in these animals.

Field samples*:* A total of 17 samples (3 lung tissue and 14 nasal swab samples) obtained from 15 cattle (3 dead and 12 live cattle) were tested. The collected nasal swab samples were placed into tubes containing 2 mL sterile phosphate-buffered saline (PBS) and brought to the laboratory under a cold chain. Tissue samples were collected from the lungs of dead animals and they were delivered to the laboratory under the cold chain conditions. Approximately 1 gram of lung tissue material was homogenized in 9 mL sterile PBS. Both the swab samples and tissue homogenates were vortexed and centrifuged at + 4ºC, 3000 rpm for 20 minutes. The supernatants were collected into a stock tube by passing through a 220 nm filter and stocked at -80 °C until testing.

Cell culture and virus isolation: Madin Darby Bovine Kidney (MDBK) cell line (100,000 cells/mL) was used for virus isolation from samples and the production of test viruses. In the preparation of MDBK cell cultures, Dulbecco's Modified Eagle's Medium (DMEM) (Sigma,

D7777, USA) supplemented with 10% fetal calf serum (FCS) (Capricorn, FBS-11A, South America), 100 UI/mL Penicillin/Streptomycin (BI, 03-031-1B, Israel) and 250 µL/mL Amphotericin B solution (BI, 03-028-1B, Israel) were used. The cell line and FCS were free of bovine viral diarrhea virus and mycoplasma.

For virus isolation, 24-well plates coated with MDBK cells were used. All of the 17 samples were subjected to virus isolation. Two hundred microliters of inoculum were added onto MDBK and plates were incubated for 1 h at 37°C for virus adsorption. After incubation, 1 ml of DMEM was added to the plate wells and the cells were examined under a microscope for 7 days for the presence of cytopathogenic effects (CPE). On the 7th day, the cells were harvested by freezing at –80 °C and thawing at 37°C. All these steps were repeated with three blind passages.

Viral DNA extraction and PCR amplification: Viral DNA extracted from all the samples (supernatants of a nasal swab, lung homogenates, and cell culture supernatants) using the commercial nucleic acid isolation kit (Macherey-Nagel Nucleospin Virus, Germany). The collected DNA was subjected to PCR targeting the partial gC gene of BoHV-1. The primers PF: 5'-CGGCCACGACGCTGACGA-3' and PR: 5'-CGCCGCCGAGTACTACCCT-3' (11) were used in the reaction. The PCR master mix was performed in a volume of 50 µL: 22 µL DNase/RNase-free water, 25 µL Maxima hot start Green Taq PCR master mix (2×, Thermo scientific, K1061), 1 µL PF primer (50 pmol), 1 µL PR primer (50 pmol) and 1 µL DNA. The thermal protocol profile was 95ºC for 4 min; 35 cycles of 95ºC for 30 s, 58ºC for 30 s and 72ºC for 35 s, and a final extension at 72ºC for 10 min. The 575-bp PCR product was detected by electrophoresis.

The samples obtained from the imported animals were also screened against common viruses of the respiratory system (BCoV, BVDV, BRSV, and BPIV-3) by RT PCR as described by Chou et al. for BCoV (9) and Toker et al. for BVDV, BRSV, and BPIV-3 viruses respectively (28).

Sequencing and phylogenetic analysis: One of the samples that were strongly positive in electrophoresis was selected. Sequence analysis was performed using Sanger dideoxy sequencing by a commercial company (Macrogen, South Korea). The gC gene sequences were aligned using the BioEdit program. Phylogenetic analysis was carried out by the Maximum likelihood method with 1000 bootstrap replication using MEGA-X. The reference sequences of the phylogenetic tree were obtained from GenBank.

Restriction endonuclease analysis (REA): For typing of the viral strains, a restriction endonuclease analysis was applied as described (17). Firstly, a multiplex PCR was applied to the nucleic acids of all the field isolates via the RS1 primer pair, 493-bp (UL39, F: 5′-TCGTCGAAGAGCGTCCACACA-3′, and R: 5′-ACCGCGCTGTACCGGCAGCT-3′) and RS2 primer pair, 700-bp (US3, F: 5′-TACAAATCGGCGGCGCC AAA-3′ and R: 5′-TTGTTGACGGCCAAGTATAA-3′). The RS1 and RS2 primers were replicated in UL39 open reading frame and US3 upstream intergenic region of BoHV-1, respectively, and have one HindIII restriction site (sequence AAGCTT). Briefly, the PCR reaction contained 1 µL DNA, 1 µL of both RS1 and RS2 sense and antisense primers (50 pmol), 25 µL Maxima hot start Green Taq PCR master mix (2×, Thermo scientific, K1061), DMSO (6%), and nuclease-free water up to 50 µL. The thermal protocol profile was 95ºC for 5 min; 40 cycles of 95ºC for 30 s, 54ºC for 1 min and 72ºC for 1 min, and a final extension at 72ºC for 5 min. Subsequently, the PCR products obtained were incubated with a fast digest HindIII enzyme (Thermo scientific, FD0504) at 37ºC for 15 minutes. The REA products were visualized on the 1% agarose gel with Safe View Classic.

Results

Virus isolation and PCR: During the virus isolation, a total of 8 samples (3 lung tissue and 5 nasal swabs) produced CPE on MDBK cell monolayers on the different day of the incubation (Fig 1). By the applied PCR method according to partial gC gene region, the same 8 samples were found positive to produce a 575 bp product. In addition, since both lung tissue and nasal swab samples of two cattle were positive, BoHV-1 was found in 6 out of 15 cattle.

Besides, other common viruses of the respiratory system (BCoV, BVDV, BRSV, and BPIV-3) were also investigated in these cattle and found negative.

BoHV-1 sequencing and phylogenetic analysis: After sequencing of the isolate BoHV-1/10054, which was strongly positive in PCR, phylogenetic analysis was performed with previously reported gC gene sequences of the BoHV-1 genome. The isolate 10054 was in the cluster with BoHV-1.1 (Fig 2). The obtained sequence has been deposited to GenBank (Accession number: MW316648).

Restriction endonuclease analysis: For further confirmation of the phylogenetic classification of the isolate BoHV-1 10054, the REA was performed. The REA results were evaluated as for the criteria: (i) BoHV-1.1 subtype shows no cleavage both in RS1 and RS2 fragments; (ii) BoHV-1.2a subtype is not cleaved in the RS1 fragment, while the RS2 fragment is divided into 2 sub-fragments; (iii) BoHV-1.2b subtype was also cleaved into 2 sub-fragments, both in RS1 and RS2 fragments. According to this assay, all of the tested eight isolates dropped in the group of BoHV-1.1 (Fig 3).

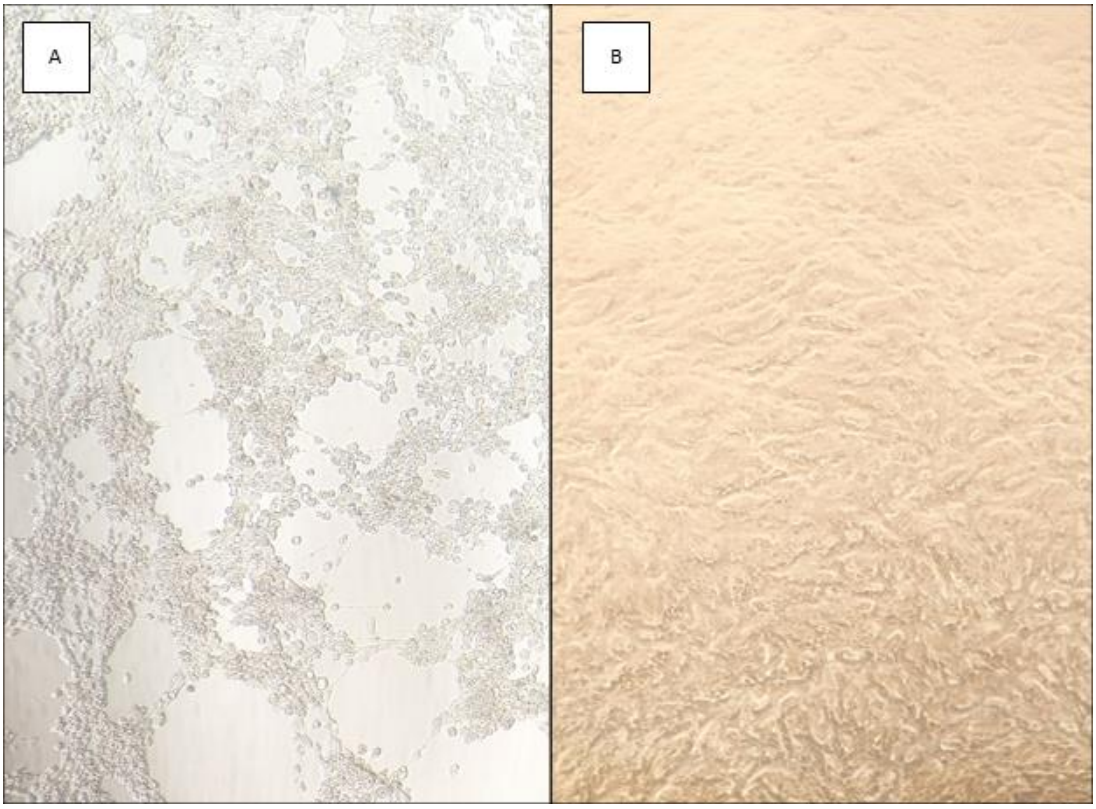

Figure 1. Microscope images were obtained in virus isolation (x20 magnification).
A: Cytopathologenic effect of nasal swab sample 10054 (1st passage level) at 96 h p.i. B: Uninfected MDBK cell culture.

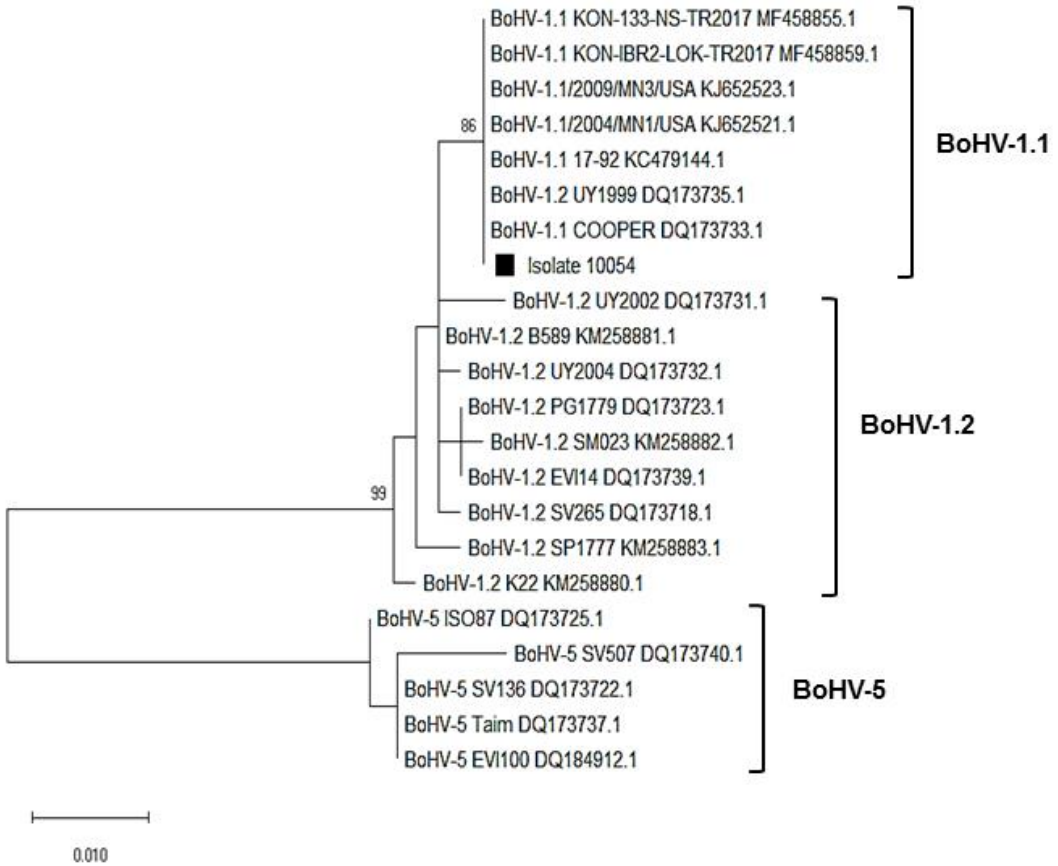

Figure 2. Phylogenetic typing of BoHV sequences based on partial gC (UL44) gene coding sequences. Field strain is marked with a square.

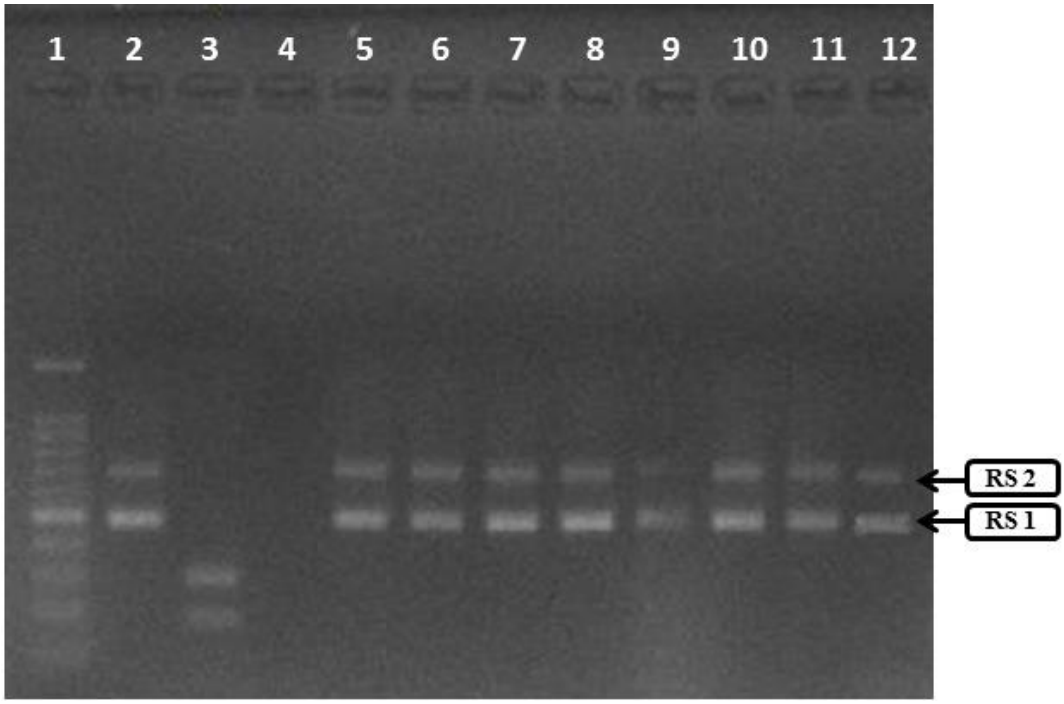

Figure 3. Restriction endonuclease analysis of BoHV-1 isolates.
Line (1): DNA ladder (100bp, GeneAll, GA-010, Korea); (2): BoHV-1 Cooper (BoHV-1.1); (3): BoHV-1.2b isolate (unpublished); (4): Negative control (ddw); (5-12): Samples 10047, 10048, 10049, 10051, 10054, 10059, 10060, 10061.

Discussion and Conclusion

After primary infection, BoHV-1 enters the sensory ganglia, becomes latent, and causes the animals to remain seropositive throughout their lifetime. Since the chance of detecting acute BoHV-1 infection is low, serological methods are used more often in the diagnosis of BoHV-1 (21). The agent BoHV-1 was detected for the first time in dairy cows in California, USA in 1953 (35), while it was isolated from cattle in Turkey in 1987 (6). The presence of BoHV-1 infection in Turkish cattle has been demonstrated by various studies (4, 22, 30, 36). Although BoHV-1 infection is common worldwide, there are differences in prevalence and incidence found in different territories. Despite the infection has been eradicated from some European countries including Austria, Denmark, Finland, Sweden, Switzerland, and Norway (1), it still exists in other countries including Belgium, Scotland, Ireland, Lithuania, and Hungary (5, 10, 14, 18, 26).

In the present study, eight BoHV-1 isolates were obtained from severe respiratory cases of imported cattle after transport from Hungary to Turkey. We found that these 8 isolates were included in the BoHV-1.1 subtype using sequencing, phylogenetic analysis (Fig 2), and REA assay (Fig 3). These data indicate the compatibility between phylogenetic analysis and REA assay results as previously offered (17). In light of this result, BoHV-1 subtyping can be carried out more quickly and easily without the need for nucleic acid sequencing.

In addition to the virological results, *Pasteurella multocida* and *Mannheimia haemolytica* have been identified in the suspected cases (data not shown). Although mixed infections are frequently detected in the etiology of BRD; the primary cause of BRD is usually viral agents (2, 24). Since BHV-1 suppresses the immune system of the host by various mechanisms (7, 12, 32), it can cause mixed infection and severe pneumonia especially with secondary bacterial infections (35). This situation leads to death in affected animals and serious economic problems for the enterprise.

The cattle that were tested in the study, were imported from Hungary to Turkey. This means approximately 2500 kilometers of road distance for imported cattle. Many previous studies have reported an increase in the level of corticosteroids in animals even after 4-6 hours of transport (16, 34). Significantly, another research has been reported that transportation stress increases serum concentrations of oxidative stress biomarkers that are related to episodes of BRD and mortality in calves (8). It has also been reported in previous studies that BoHV-1 is reactivated after transport (27) and has a high probability of transmission through animal transport (local movement or entry from other European countries) (3). In the present study, the detection of BoHV-1 in transported animals supports the formation of respiratory tract diseases (shipping fever) in animals after transport. The detected mortality rate of 14.16%

(51/360) represents a very important economic impact of BoHV-1 induced shipping fever to beef cattle establishments.

In this study, BoHV-1 infection was detected in imported 6 to 9 months old animals possibly due to reactivation of the latency. Having clinical disease soon after arrival when the animals were in quarantine period also supports this hypothesis. The data of this study indicate that imported animals can still act as a source of BoHV-1 infection for cattle populations in the target country. This is particularly crucial for disease free regions and countries. When Turkey's high quantities of live animal import are considered, especially the entry of persistent or latent diseases can pose a serious problem for the country's economy. Official rules for animal importation described by the local authority should be strictly applied in the field. Any case, both the countries having IBR control program and individual enterprises in the countries with no official control program should be aware of BoHV-1 transmission risk by animal transport despite official protocols applied. Importantly, it is indisputable that those of infected animals will be the BoHV-1 reservoir throughout their lives.

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Ethical Statement

All of the samples were sent to the laboratory by field veterinarians by sampling according to officials' ethical rules for animal welfare.

Conflict of Interest

The authors declared that there is no conflict of interest.

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The negative effects of cold conditions on pregnancy rates in dairy cows

Mehmet CENGİZ[1,a,✉], Armağan HAYIRLI[2,b], Bülent BALLİ[2,c]

[11]Atatürk University, Faculty of Veterinary Medicine, Departments of Obstetrics and Gynecology, Erzurum, Turkey; [2]Atatürk University, Faculty of Veterinary Medicine, Animal Nutrition & Nutritional Disorders, Erzurum, Turkey.
[a]ORCID: 0000-0001-9913-3468; [b]ORCID: 0000-0002-4446-0848; [c]ORCID: 0000-0001-7542-4519

[✉]Corresponding author: mehmet.cengiz@atauni.edu.tr
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Abstract: This study was conducted to determine the pregnancy rate in response to two sexual synchronization protocols in 70 healthy multiparous Fleckvieh dairy cows during the cold and warm seasons. After pairing by the actual parturition date [cold seasons (-38 to 0 ºC) (Nov - Feb) *vs.* warm seasons (0 to 32 ºC) (Mar-Oct)], cows were randomly subjected to either Co-Synch (CoS) or double $PGF_{2\alpha}$ injection (dPG) on d 35 postpartum. Data were subjected to Chi-Square (χ^2) analysis to determine the pregnancy rate at the first service. Overall pregnancy rates (%) at the first service were 57.1 and 40.0 for Groups CoS and dPG, respectively ($\chi^2 = 2.03$, P = 0.15) and 35.0 and 66.7% during cold and warm seasons, respectively ($\chi^2 = 6.78$, P = 0.009). The protocol effect on the pregnancy rate at the first service was insignificant during cold ($\chi^2 = 1.17$, P = 0.28) and warm ($\chi^2 = 1.62$, P = 0.20) season. In summary, the cold conditions negatively affected the pregnancy rate at the first service. The synchronization protocol methods did not differ to improve the pregnancy rate at the first service when cows were exposed to cold conditions.
Keywords: Cold condition, dairy cow, first service, pregnancy rate, synchronization protocol.

Soğuk şartların süt ineklerinde gebelik oranları üzerindeki olumsuz etkileri

Özet: Bu çalışma, soğuk ve ılık mevsimlerde 70 adet sağlıklı Fleckvieh sütçü ineklere uygulanan iki seksüel senkronizasyon programı sonrası gebelik oranlarını değerlendirmek amacıyla gerçekleştirildi. Doğum sonrası 35. günde, inekler gerçek doğum tarihine göre eşleştirdikten sonra [soğuk mevsim (-38 to 0 ºC) (Kasım - Şubat), ılık mevsim (0 to 32 ºC) (Mart - Ekim)], Co-Synch (CoS) veya çift doz $PGF_{2\alpha}$ enjeksiyon (dPG) protokol gruplarına rastgele dağıtıldı. İlk tohumlamada gebelik oranının belirlenmesi için χ^2 analizi uygulandı. İlk tohumlamada toplam gebelik oranı, sırasıyla Grup CoS ve dPG için , 57,1 ve 40,0 ($\chi^2 = 2,03$, P = 0,15) ve soğuk ve sıcak mevsimler için % 35,0 ve 66,7 ($\chi^2 = 6,78$, P = 0,009) olarak bulundu. Soğuk ($\chi^2 = 1.17$, P = 0,28) ve sıcak mevsimler ($\chi^2 = 1,62$, P = 0,20) için ilk tohumlamada gebelik oranı üzerine protokol etkisi belirgin değildi. Özetle, soğuk şartlar ilk tohumlamada gebelik oranını olumsuz etkiledi. İnekler soğuk şartlara maruz bırakıldığında senkronizasyon protokolüne bağlı olarak gebelik oranı değişmedi.
Anahtar sözcükler: Gebelik oranı, ilk tohumlama, inek, senkronizasyon protokolü, soğuk iklim.

Introduction

Despite advancements in dairy cattle reproduction, the adverse effects of extreme ambient temperatures remain to be one of the major limitations in cattle fertility. The thermoneutral zone is an environmental temperature interval between - 5 and 25 ºC, in which lactating cows do not require additional energy for heating or cooling (19). An increase in both heat and humidity disturbs physiological processes at the cell and organ levels. The negative effect of heat stress on fertility is well documented, spanning from clinical signs of estrus, follicular development, and oocyte competence to embryonic survival in cows (15, 18, 20).

Although the cows tolerate cold stress more than heat stress (16), cold stress can be an issue of herd management during winter in cold regions depending on farm construction type. Because cows are warm-blooded animals, they need to maintain a constant core body temperature (38 ºC). The cut-off temperature (lower critical temperature) initiating cold stress depends on hair coat, wind, and rain (29, 30). The cold stress is associated with behavioral changes, which include huddling, reduced body condition, decreased feed and water consumption, and limited mobility behaviors (17, 25). Moreover, cows experiencing cold stress have lower respiration, urination, and heart rates (37), leading to dehydration, which can be

accompanied by increased serum concentrations of non-esterified fatty acids, adrenalin, and cortisol as well as rates of hepatic glycogenolysis and extra-hepatic tissue lipolysis (4, 35). Blood is diverted from body extremities to protect vital organs. Decreased intake due to cold stress may further aggravate energy deficit, especially in transition dairy cows, because extra 2% energy is needed for every 1 ºC drop beyond the lower critical temperature (24). If the energy requirement is not compensated, the catabolic profile further jeopardizes reproductive physiology.

Major adaptive changes occurring in cold stress include 1) thermal insulation manifested by activity and hair coat, 2) an increase in resting metabolic rate, and 3) an increased rate of digestive passage (37). There may not be a reduction in lactation yield (18), but in lactation efficiency (38). This is because the priority of nutrients is diverted to maintenance from productive processes (39). Thus, reproductive parameters (*i.e.*, delayed resumption of cyclicity, mild estrus sign, ovulation problem, and embryonic loss) may be compromised in prolonged exposure to cold stress (40).

The synchronization of the estrus cycle and/or ovulation is one of the reproductive strategies to overcome the adverse effects of extreme ambient temperature on reproductive performance (10). This experiment was conducted to compare the pregnancy rate at first service upon two commonly used sexual synchronization protocols in Fleckvieh cows reared under below critical temperature.

Materials and Methods

Environmental conditions of the study location: The cows were housed in double-row free-stall barn with a concrete surface and covered by a plastic tent (15 m height) that protected against wind, rain, and snow, without providing insulation. The barn is located in Erzurum, the Northeastern Turkey (39º54′31″N, 41º17"E), where is a highland (altitude of 1853 m) and ruled by continental climate conditions (long and harsh winter - short and mild summer), with an average low temperature of -8.6 ºC and the average high temperature of 12 ºC. The average annual precipitation is 453 mm. Snow falls on an average of 80 days and remains for about 150 days. This study was approved by Atatürk University Animal Experiments Local Ethical Committee with decision number 2017/92.

Animals, management and experimental groups: This experiment involved 70 Fleckvieh cows that were in the second lactation without reproductive problems (*i.e.*, dystocia, retained placenta, and subinvolution) in early postpartum and that showed at least one estrus and had at least one palpable corpus luteum before the initiation of the experiment. They were milked twice daily and fed once daily *ad libitum* consumption of TMR consisting of 65% roughage (10% sainfoil hay, 50% orchard grass hay, and 40% corn silage) and 35% compound pelleted feed on a DM basis. The average milk production was 23.7 ± 5.6 (13.2 - 34.8) kg in 35 days in milk.

After pairing according to the actual parturition date [cold season (- 38 to 0 ºC) (November - February) *vs*. warm season (0 to 32 ºC) (March - October)] cows were subjected to either Co-Synch (CoS, n = 35) or double $PGF_{2\alpha}$ injection (dPG, n = 35) protocols (Table 1). The cows in Group CoS were injected with 2 mL GnRH analogue (Ovarelin®, 50 µg/mL, gonadoreline diacetate tetrahydrate, Ceva Animal Health, Istanbul, Turkey) at 06:00 a.m. on d 35 postpartum, 2 mL $PGF_{2\alpha}$ analogue (250 µg/mL cloprostenol, PGveyx Forte®, Veyx-Pharma, Schwarzenborn, Germany) at 06:00 a.m. on d 42 postpartum, and then inseminated 60 hrs later. The cows in Group dPG were injected with 2 mL cloprostenol by the 11-day interval at 06:00 a.m. on d 35 and 46 postpartum and inseminated 60 hrs later. Immediately after insemination, all cows in both groups were injected with 200 µg of GnRH.

Table 1. Synchronization protocols.

Day Relative to Calving	Co - Synch (CoS)	Double $PGF_{2\alpha}$ injection (dPG)
35	100 µg GnRH analogue	500 µg $PGF_{2\alpha}$ analogue
42	500 µg $PGF_{2\alpha}$	-
46	-	500 µg $PGF_{2\alpha}$ analogue
+ 60 hrs	Insemination with 100 µg GnRH analogue	Insemination with 100 µg GnRH analogue

Ovulatory follicles were monitored and measured by ultrasonography (Wed 3000®, Shenzen WELLD Medical Electronics Co. Ltd., Shenzen, China) before all inseminations and the cows which had follicle diameter larger than 16 mm were inseminated, whereas the cows, which had smaller follicles were not inseminated. Pregnancies were monitored on d 28, 35, and 60 relative to insemination by ultrasonography. The cows were confirmed pregnant after the detection of the amniotic vesicle, amniotic fluid, and heartbeat of the embryo. The same protocol was applied in subsequent services until all cows were confirmed to be pregnant.

Statistical analysis: To improve the pregnancy rate by 10% at an alpha error of 0.05 and beta error of 0.95, 24

cows were needed per group (PS: Power and Sample Size Calculation, Version 3.1, Nashville, TN). Cows were assigned to experimental groups prospectively within blocks of calving season. Data were subjected to the cross-tabulation to evaluate the pregnancy rate at the first service in response to the sexual synchronization protocol and the insemination season using Chi-Square analysis (Version 13.2.2; MedCalc, Ostend, Belgium). The effect of the synchronization protocol was also separately investigated during cold and warm seasons. Moreover, 2-way ANOVA (main effect of the sexual synchronization protocol and the insemination season as well as their interaction) was employed to determine the total number of insemination to achieve pregnancy. Statistical significance was declared at $P< 0.05$.

Results

The pregnancy rate was calculated according to examination results on the 60th d of gestation. The pregnancy rate at the first service in the cold season was much lower than that in the warm season (35.0%, 14/40 *vs*. 66.7%, 20/30; $\chi^2 = 6.78$, $P = 0.009$) (Table 2). The pregnancy rate at the first service in response the synchronization protocol was insignificant (57.1%, 20/35 and 40.0%, 14/35 for cows in Group CoS and dPG, respectively; $\chi^2 = 2.03$, $P = 0.15$) (Table 3). The synchronization protocol type affected the pregnancy rate at the first service during neither cold ($\chi^2 = 1.17$, $P = 0.28$) nor warm ($\chi^2 = 1.62$, $P = 0.20$) season (Table 4). No embryonic loss was detected following to first insemination.

Non-pregnant cows were subjected to the same synchronization protocol until achieving pregnancy. The total number of insemination was affected by the insemination season ($P = 0.04$), but not by the synchronization protocol ($P = 0.80$). The total number of insemination to achieve pregnancy was 1.52 ± 0.20 (1.13/1.92, 95% CI) for cows inseminated in cold and warm seasons and 1.61 ± 0.19 (1.23/1.98, 95% CI) and 2.00 ± 0.18 (1.63/2.37, 95% CI) for cows in Groups CoS and dPG and 2.09 ± 0.17 (1.74/2.43, 95% CI).

Table 2. Effect of season on the conception rate at the first service ($\chi^2 = 6.78$, $P = 0.009$).

Pregnancy status	Cold	Warm	n (%)
Non-pregnant	26	10	36 (51.4)
Pregnant	14	20	34 (48.6)
n (%)	40 (57.1)	30 (42.9)	

Table 3. Effect of synchronization protocol on the conception rate at the first service ($\chi^2 = 2.03$, $P = 0.15$).

Pregnancy Status	Cold	Warm	n (%)
Non-pregnant	15	21	36 (51.4)
Pregnant	20	14	34 (48.6)
n (%)	35 (50.0)	35 (50.0)	

Table 4. Effect of synchronization protocol on pregnancy at first insemination in the cold and warm seasons.

Season	Protocol	Pregnancy (n)	P
Cold	CoS	9	($\chi^2 = 1.17$, $P = 0.28$)
	dPG	5	
Warm	CoS	11	($\chi^2 = 1.62$, $P = 0.20$)
	dPG	9	

Discussion and Conclusion

Stress is defined as the inability of an animal to cope with its environment, a phenomenon that is often reflected in a failure to achieve genetic potential (11). Reproduction is an important physiological system that is fragile to stressors (9), including exposure to severe cold temperatures (13, 41, 42). Although cold stress is considered a subjective phenomenon, the animal evokes the sympathetic nervous system to overcome cold stress through increasing metabolic heat production, cardiac output, and mobilization of body reserves (4). Exposure to cold stress activates the hypothalamic-pituitary-adrenal axis, resulting in increased rates of lipolysis and glycogenolysis as well as elevations in hematological and metabolic parameters (14, 37). Cold stress affects cow behaviors, such as seeking shelter and shivering (5), decreased laying and feeding times (37). In agreement with previous studies (17, 25), some behavioral changes, such as immobility and crowding at standing position together and showing no signs of estrus, were observed. These were associated with the frozen and slippery floor and effort to decrease energy loss. These physiological and behavioral efforts as well as shifting priority of energy utilization to maintain body temperature can affect the pregnancy rate. Thus, the farm management decided to artificially inseminate the cows by estrus synchronization.

In actual environmental temperatures ranging from -12 to -23 ºC, the cow needs 1.2-1.4 fold energy. In a relationship with energy and protein intake at the time of the reproductive stage, the animal can gain/lose weight, which affects reproductive outcome (31). Exposure to cold stress causes a reduction in feed intake, which induces body condition loss and negative energy balance

(5). This leads to an abnormal uterine environment and decreased circulating steroidal hormones and IGF concentrations, which are associated with the suppression of LH secretion, reduced ovarian responsiveness to LH and delayed ovulation (6). Transition cows exposed to cold stress may have delayed return to estrus and prolonged days open, resulting in poor reproductive performance. Below the low critical temperature, reproduction is compromised as reflected by suppression of estradiol - 17β synthesis and pre-ovulatory LH release, follicular and oocyte development, ovulation, and embryonic survival (33). It was shown that the pregnancy rate was the lowest in February and the highest in July in Canada (22). The adverse effect of cold stress is independent of daylight length (8). In comparison with the season average of 25 ºC, there was a poor development of larger follicles in cows exposed to cold stress (27). In the presented study, pregnancy rate decreased in the cold season as compatible with previously described (8, 22). Moreover, lower circulating P_4 concentration could be one of the causes of decreased pregnancy rate and embryonic mortality during extreme winter conditions (13). The serum progesterone level was not measured in the presented study. However, embryonic loss was not observed in the pregnant cows until the 60^{th} day of gestation. This result was associated with that the cold conditions could not be harmful on embryonic survival as heat stress (16). Additionally, due to the cows, which had larger follicles than 16 mm, were inseminated, this election might support the quality of the oocytes and embryos (7).

Environmental temperature (13, 31) environment and barn infrastructure affect sexual behavior (12, 18, 26). The relationship between maximum environmental temperature and mounting activity is curvilinear fashion. At temperatures below -10 ºC, mounting activities were low (~3 hours), and increased as the temperature rose to 25 ºC (~ 8 hours), and then decreased as temperature rose above 30 ºC (~ 7 hours) (34). In cold seasons, it is reported that cows in free stall housing with a dry lot have more mounting activity and exhibit more pronounced sexual activity than hot weather (26). However, during the extremely cold season, slippery surface negatively affects mobility and estrus behaviors (17, 25). Cows on the concrete surface showed less mounting activity than those on the dirt surface in winter conditions (32, 36). Platz et al. (28) reported 19 collapsing and slipping incidences out of 23 mounting activity on the concrete surface. In the present study, none of the behavioral and metabolic response variables were measured.

Many studies showed that the pregnancy rate at the first service was reported to be high during the cool-cold seasons (1, 2, 23). Depending upon barn infrastructure inside temperature may not reach below the lower critical temperature (27). The warm season in this study lays within the thermoneutral zone, which does not interfere with metabolic status, estrus sign, ovarian activity, and managerial protocol for crew and animals. It is quite rare that research deals with cold stress in dairy cattle kept in barns equipped with curtain walls (4). This may be due to the assumption that curtain-sided barns provide suitable protection against weather conditions. The tent cover did not warm barn enough to avoid the alleys being frozen. This study was not designed to evaluate the accuracy of estrus detection, in terms of duration and intensity of estrus behaviors. Cold stress itself and frozen concrete surface could cause worsened welfare and limit sexual activity, and consequently lower pregnancy rate during the cold season.

Synchronization of estrus and ovulation become standard components in reproductive management, especially in large dairy operations (21). Under extreme cold conditions, timed-artificial insemination may help avoid injuries associated with estrus behavior on the slippery surface through separating sexually active cows. The pregnancy rate at the first service did not differ in response to the CoS and dPG protocols. Previous studies dealing with similar protocols also achieved a similar pregnancy rate at the first service (3, 23, 28).

In summary, this preliminary experiment investigating the cold stress effect on pregnancy rate has a number of limitations. These include lacking measurements of daily changes in temperature, humidity, and wind speed in the barn, data of feed intake, observations on behavioral changes, and measurements for physiological/biochemical parameters. Seasonal temperature elicited a more notable effect on the pregnancy rate than sexual synchronization protocol. Extreme cold conditions adversely affected the pregnancy rate probably by directly affecting the cow's metabolic status or indirectly affecting the cow's behavior in relation to swamped barn conditions. Employment of the CoS or dPG protocol was not advantageous to each other to improve pregnancy rate at the first service during the extreme cold conditions.

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Ethical Statement

This study was approved by Atatürk University Animal Experiments Local Ethical Committee with decision number 2017/92.

Conflict of interest

The authors declared that there is no conflicts of interest. The authors are responsible for the content and writing of the paper.

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IPARD supports positively affect hematological parameters, milk production, and welfare indicators of dairy cows

Hikmet ARI[1,a], Recep ASLAN[2,b,✉], Mehmet Şükrü GÜLAY[3,c]

[1]Freelance Veterinarian, Afyonkarahisar, Turkey; [2]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Physiology, Afyonkarahisar, Turkey, [3]Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department Physiology, Burdur, Turkey.
[a]ORCID: 0000-0003-2517-1247; [b]ORCID: 0000-0002-7541-0405; [c]ORCID: 0000-0002-4960-1152

✉Corresponding author: raslan@aku.edu.tr
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Abstract: The study investigated body condition score (BCS), hematological parameters, metabolic profile indicators, total oxidant-antioxidant status, and milk yield (MY) of cows in non-funded (NF) and funded (F+) dairy farms by the European Union funds (under the Instrument for Pre-accession Assistance for Rural Development-IPARD) to support rural development. Sixty multiparous Holstein cows (110 to 209 days of lactation) from 3 NF and 3 F+ dairy farms were used in the experiment. Hematological parameters were within physiological limits in both dairy farms. Total erythrocyte count and percent hematocrit were higher in the F+ group. Serum non-esterified fatty acids (NEFA) level and aspartate aminotransferase (AST) activity were higher in NF farms compared to the supported ones. The serum triglyceride, total cholesterol, glucose levels, and gamma-glutamyl transferase (GGT) activity were higher for the cows in F+ farms. The average daily MY and BCS of cows in NF and F+ dairy farms were 15.8 L and 2.83, and 24.4 L and 3.11, respectively ($P<0.01$). The total antioxidant status (TAS) of cows in NF farms was significantly lower than F+ farms, whereas total oxidant status (TOS) was significantly higher for cows in NF farms. In conclusion, the grant support is important in establishing a more capable, more efficient, and more suitable infrastructure for animal welfare, and positively affects the physiological health and efficiency of animals in these dairy farms.

Keywords: Animal welfare, body condition score, metabolic profile, physiological parameters, oxidant-antioxidant status.

IPARD destekleri süt ineklerinde hematolojik parametreleri, süt verimini ve hayvan refah göstergelerini olumlu etkilemektedir

Özet: Çalışma, kırsal kalkınmayı desteklemek için Avrupa Birliği fonları tarafından verilen "Kırsal Kalkınma İçin Katılım Öncesi Yardım Aracı-IPARD" kapsamındaki hibelerin süt ineklerinde vücut kondüsyon skoru (VKS), hematolojik parametreleri, metabolik profil göstergeleri, toplam oksidan-antioksidan durumu ve süt verimine etkilerini araştırmayı hedeflemektedir. Bu çalışmada IPARD desteği alan (F+) üç çiftlik ve destek almamış (NF) üç çiftlikte 110-209 günler arası laktasyon periyodundaki toplam 60 multipar Holstein ineği rastgele seçildi. Bulgulara göre, hem NF hem de F+ çiftliklerindeki ineklerin hematolojik parametreleri fizyolojik sınırlar içerisindedir. Ancak NF grubunda F+ grubuna göre serum NEFA düzeyi ve AST aktivitesi daha yüksek, F+ grubu ineklerin ise serum trigliserid, toplam kolesterol, glikoz seviyeleri ve GGT aktivitesi, eritrosit ve hematokrit seviyeleri NF grubuna göre daha yüksek bulunmuştur. NF grubunda günlük ortalama süt verimi ve VKS değerleri 15,8 L ve 2,83, F+ grubunu oluşturan süt çiftliklerinde ise 24,4 L ve 3,11'dir ($P<0,01$). NF çiftliklerinde ineklerin toplam antioksidan durumu F+ çiftliklerindeki ineklerden önemli ölçüde düşük, önemli bir risk faktörü olan toplam oksidan statü ise önemli ölçüde yüksekti. Sonuç olarak, hibe desteğinin hayvanlarda fizyolojik göstergeleri olumlu olarak etkilediği, bu hayvanların barınma, bakım ve besleme koşullarının sürekli kontrol ediliyor olması nedeniyle hayvan refahına daha uygun olduğu izlenmiştir. Hayvanların daha uygun ve izlenebilir bir altyapıya sahip olmaları açısından IPARD hibe desteklerinin sürdürülebilir hayvan sağlığı ve verimliliği için önemli olduğu düşünülmüştür. Sonuç olarak bu süt çiftliklerindeki hayvanların fizyolojik sağlığı, kondüsyonları ve verimliliklerinin IPARD desteklerinin sağladığı koşullardan olumlu yönde etkilendiği görülmüştür.

Anahtar sözcükler: Fizyolojik göstergeler, hayvan refahı, metabolik profil, oksidan-antioksidan statü, vücut kondüsyon skoru.

Introduction

The supports for rural development have increased significantly in the last couple of decades and several support programs are in effect. The main objectives for such support programs are protecting and developing genetic resources at their original places, updating the recording systems, improving farming and healthier production, ensuring sustainability, increasing the effectiveness of farming policies, preventing animal diseases, and supporting animal health and welfare (33).

A fast and accurate instrument to understand the welfare, physiological status and productivity of animal are not only important for businesses and researchers but it is also significant for the country's economy and global animal husbandry research. Recently, efficient, safe, and sustainable agriculture and livestock practices have become the common agenda of the international community. Animal health and welfare should be prioritized for activities with these qualifications (7). For this reason, increasing and updating the quality standards of animal life and animal products has become a basic agricultural policy, and has increased in the agenda of the researchers. It is generally accepted in the international community that the most appropriate step in this area is to support rural development (2). One of these support programs was founded by the European Union to aid candidate and/or potential candidate countries by the IPA Council Regulation (EC) No 1085/2006, the Instrument for Pre-Accession Assistance (IPA). This support has different components and Turkey as a candidate country has the right to benefit from all components (8, 33). Thus, the Instrument for Pre-Accession Rural Development (IPARD) grant support program was started to implement as IPARD-I for the first time in Turkey, covering the years 2007-2013 (17). Considering that 87% of the enterprises in Turkey are small businesses, the contribution of this grant support to production and quality to these agricultural enterprises is very important (33). Thus, this study was carried out to compare funded (F+) and non-funded (NF) small-medium sized dairy cattle enterprises that were established within the scope of the IPARD-I program during 2007-2013 in Afyonkarahisar province. For this purpose, hematological parameters, metabolic indicators, oxidant-antioxidant status, body condition score (BCS), and milk yield (MY) characteristics of cows in F+ and NF dairy farms were evaluated during the experiment.

Materials and Methods

The research was carried out with the approval of Afyon Kocatepe University, Experimental Animals Ethics Committee (AKÜHADYEK - 467-15). Data from 60 Holstein cows at 110-209 days of lactation (2 to 5 years old) were studied in the experiment. The cows were randomly selected from the six dairy farms [three IPARD funded (F+, n=30) and three non-funded (NF, n=30) dairy farms]. All dairy farms were the members of the Breeders Association in the Afyonkarahisar region. The median days in lactation for the cows in F+ and NF dairy farms were 145 and 156 days, respectively. The median number of lactation was 3 in both groups. The rations given to cows during our study period were in Table 1. MY values were acquired from the individual farm records.

Table 1. Rations used in IPARD supported (F+) and not supported (NF) dairy farms.

Nutrients	F+	NF
Dry Matter, kg/day	28.10	24.19
NEL, Mcal/day	41.58	35.55
Crude Protein, %	15.35	13.18
Rumen Degradable Protein, %	36.01	58.44
Bypass Protein, %	63.99	41.56
NDF, %	36.69	41.67
ADF, %	22.16	26.24
NFC, %	38.39	36.30
Fat, %	4.50	3.78
Ca, %	0.92	0.93
P, %	0.39	0.32

NEL=Net energy for lactation, NDF=Neutral detergent fiber, ADF=Acid detergent fiber, NFC= Nonstructural carbohydrates.

General characteristics of the farms: All F+ farms had more than 60 milking cows, the udders were cleaned before and after the milkings, and milking was performed twice a day with an automated milking system. On the other hand, the NF farms had 18 to 25 milking cows. These farms had mobile milking machines, the udders were cleaned usually only before (50%) or after (30%) the milkings and the milking frequencies were twice a day. The cows in F+ farms received regular veterinary service, the manure was removed regularly from the stalls by automated scrapers, and the manure was stored in storage facilities. The hoof care was performed once every six months. The cows in NF farms mostly received veterinary service when there was a major health problem, the manure was collected manually and transferred to the field or stored next to the barns, and hoof care was performed once a year.

Blood samples and analyses: Vena jugularis was used for blood collection and the collection procedure was performed between 06:00 and 07:00 am before the morning feeding or milking. The blood was collected directly into anticoagulant free and EDTA coated vacutainer tubes (Vacutest, Arzergrande, Italy). In the EDTA coated tubes, hematological parameters were measured by using an auto-analyzer (Mindray BC-2800

Vet blood count, Shenzhen, China). The remaining blood samples and other anticoagulant free tubes were centrifuged at 4 ºC for 10 minutes (NF 1000R, at 1500xg). Serum and plasma samples were kept at -20 ºC until used for total antioxidant status (TAS), total oxidant status (TOS), and metabolic profile analyzes.

A fully automated ELISA reader (Chemwell 2910, Awareness Tech. Inc., USA) was used for the analysis of metabolic and biochemical parameters from the serum samples. While serum non-esterified fatty acid (NEFA) and beta-hydroxybutyrate (BHBA) values were measured using species-specific bovine kits (Randox Laboratories®, Crumlin, UK), glucose (GLU), total cholesterol (CHOL), triglyceride (TG) levels, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), and gamma glutamyl transferase (GGT) activities were determined using species-nonspecific kits (Biolabo SA®, France). Total antioxidant status and TOS levels were measured in serum samples of dairy cows with the specific ELISA kit (Assay Rel Diagnostics, Turkey).

Body condition scoring: Body condition scoring was performed according to the guidelines described by Edmonson et al. before the morning feeding or milking (11). The amount of fat covering the tail head, vertebrae, loin, and rump were evaluated with a score from thin to fat (1 to 5; 1 referred to extreme emaciation and 5 referred to obese; in increments of 0.25). The same individual judge evaluated the cows. The most critical areas evaluated were vertebrae at the middle of the back, rear view of the hook bones, side view of the line between the hook and pin bones, and a cavity between the tail head and pin bones.

Statistical analysis: The data obtained in the study were evaluated using the SPSS 17.0 statistical program. First, the normality tests of the data were performed for the farms that constitute F+ and NF groups. Independent two samples t-test procedure was used for comparison of F+ and NF farms. For statistical significance, $P<0.05$ value was chosen and values were expressed as mean ± standard deviation.

Results

In general, the parameters tested in the current study were homogeneous for the farms that constitute F+ and NF groups. In addition, there was no statistical difference among the 3 farms that represent the F+ group, and the 3 farms that represent the NF group for hematological parameters, biochemical and oxidant-antioxidant indicators, BCS, or MY.

Comparisons of the hematological parameters such as red blood cell counts (RBC), hematocrit (HCT), hemoglobin (HMG), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and platelet counts (PLT) between F+ and NF dairy farms were in Table 2. There was a statistically significant difference between F+ and NF farms in terms of RBC ($P<0.05$). Although HMG and MCV did not significantly differ, HCT ($P<0.05$) and MCH ($P<0.05$) were different between F+ and NF farms (Table 2). Moreover, PLT ($P<0.001$) was higher for cows in F+ farms (Table 2).

Table 2. Hematological parameters of dairy cows in 3 IPARD supported (F+, n=30) and 3 not supported (NF, n=30) dairy farms.

Parameters	NF	F+	P
RBC (10^{12}/L)	6.14 ± 0.70	6.60 ± 0.91	0.02
HMG (g/dL)	10.0 ± 1.03	10.3 ± 0.92	NS
HCT (%)	27.5 ± 3.39	30.1 ± 2.64	0.002
MCV (fL)	44.8 ± 3.29	45.7 ± 4.85	NS
MCH (pg)	16.4 ± 1.15	15.7 ± 1.60	0.05
MCHC (g/dL)	22.5 ± 2.34	22.9 ± 3.19	NS
WBC (10^9/L)	8.6 ± 2.60	10.3 ± 4.37	NS
PLT (10^9/L)	296.4 ± 143.1	438.5 ± 109.9	0.001

RBC = red blood cell, HMG = hemoglobin, HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, WBC = white blood cell, PLT = platelet, NS = not significant.

The data on biochemical indicators such as AST, ALT, and GGT activities, BHBA, GLU, CHOL, and TG levels for F+ and NF groups were shown in Table 3. Although serum ALT activity, BHBA and BUN concentrations did not differ significantly, considerable alterations were apparent for serum activities of AST ($P<0.001$), GGT ($P<0.05$), NEFA ($P<0.001$), GLU ($P<0.001$), CHOL ($P<0.01$), and TG ($P<0.001$) levels between the F+ and NF groups (Table 3).

Table 3. Biochemical parameters of dairy cows in 3 IPARD supported (F+, n=30) and 3 not supported (NF, n=30) dairy farms

Parameters	NF	F+	P
BHBA (mmol)	0.43 ± 0.34	0.36 ± 0.12	NS
NEFA (mmol)	0.85 ± 0.62	0.22 ± 0.18	0.001
AST (U/L)	111.3 ± 18.0	92.20 ± 7.97	0.001
ALT (U/L)	26.0 ± 7.44	27.9 ± 8.60	NS
GGT (U/L)	22.4 ± 8.34	28.0 ± 7.73	0.02
GLU (mg/dL)	52.8 ± 2.98	58.5 ± 4.25	0.001
BUN (mg/dL)	17.9 ± 4.59	20.1 ± 6.47	NS
CHOL (mg/dL)	129.0 ± 28.1	157.5 ± 40.1	0.003
TG (mg/dL)	15.2 ± 4.13	19.4 ± 3.71	0.001

BHBA = beta hydroxy butyrate, NEFA = non-esterified fatty acid, AST = Aspartate Aminotransferase, ALT = Alanine Aminotransferase, ALP = Alkaline Phosphatase, GGT = Gama Glutamyl Transferase, GLU = glucose, BUN = blood urea nitrogen, CHOL = cholesterol, TG = triglyceride, NS = not significant.

Table 4. Oxidant-antioxidant status, body condition score (BCS) and milk yield (MY) of dairy cows in 3 IPARD supported (F+, n=30) and 3 not supported (NF, n=30) dairy farms.

Parameters	F+	NF	P
TAS (μmol Trolox equivalent/L)	0.98 ± 0.33	0.53 ± 0.45	0.001
TOS (μmol H_20_2 equivalent/L)	1.50 ± 1.41	3.16 ± 1.35	0.001
BCS (1 to 5)	3.11 ± 0.41	2.83 ± 0.47	0.01
MY (kg)	24.4 ± 5.18	15.8 ± 3.23	0.01

TAS = total antioxidant status, TOS = total oxidant status.

The oxidant-antioxidant indicators and BCS-MY values are in Table 4. As an indicator of antioxidant status, TAS values were higher in F+ dairy farms compared to NF dairy farms ($P<0.001$). On the contrary, TOS values were significantly lower for cows in the F+ group ($P<0.001$; Table 4). In addition, both BCS and MY values were significantly higher in the F+ group than the NF group ($P<0.01$).

Discussion and Conclusion

There are several studies on the biochemical and hematological indicators, antioxidant-oxidant status, MY, and BCS status of dairy cows in different lactation periods (5, 22, 31). However, studies on how IPARD support might affect biochemical and hematological indicators, antioxidant-oxidant status, MY, and BCS of cows in dairy enterprises are not available in the current literature. It is important to discuss the indicators and productivity of animals in F+ and NF dairy farms, considering that the grant-supported farms are better equipped and have a higher concern for animal health and well-being (33). It has been observed that the F+ farms sampled in the current study had a better environment for their cows compared to NF. The higher consideration for animal health and well-being of the cows in the F+ farms were generally reflected in hematological profiles, biochemical indicators, oxidant-antioxidant status, BCS scores, and MY values.

Avidar et al. (3) and Rowlands (24) established a link between the hematological and biochemical profiles of animals and their milk production. In the current study, RBC, platelet, and HCT values were higher in the F+ group. It is known that blood cells may vary depending on factors such as age, race, gender, geographical altitude, and environment. Moreover, animals living in better conditions tended to have higher blood parameters (35). Considering that the cows used in the current study were in the same race, gender, altitude, and similar geographical conditions, it can be said that the improved hematological values for dairy cows in F+ farms might be positively affected by the care and feeding conditions. In addition, it was evaluated that the blood cells may be increased due to the increased metabolic and immunological needs of animals with higher MY. Apart from our study, no study investigating hematological indicators of dairy cows in IPARD supported farms has been encountered. In our study, the levels of RBC were within physiological limits in both F+ and NF groups. However, the higher RBC levels for the cows in the F+ group supports increased oxygen-carrying and utilization capacity and therefore the metabolic activity for these cows which, in turn, augments efficiency (30).

The serum CHOL level is an important indicator because it gives information about lipid metabolism (34). In our study, when F+ and NF groups were compared, the CHOL level was significantly higher for cows in the F+ group. Different studies stated that serum CHOL levels were affected by MY at different lactation stages. Rowlands (24) also suggested that there was a link between MY and serum CHOL. There was a positive relationship between serum CHOL level and MY after calving and serum CHOL levels increased with the advancement of lactation (23, 26). In another study, Gueorguieva (15) emphasized that there was an increase in CHOL levels, especially in the early lactation phase, so there was a positive correlation between MY and serum CHOL levels.

The serum TG level may be an important marker for dairy cows, especially during the transition period and it is an important parameter that gives information about liver fat metabolism (16). Turgut (29) stated that serum TG level should be in the reference range of 0 to14 mg/dL. In the current study, the serum TG levels for the cows in the F+ group were slightly above this reference value, and also higher than for the cows in the NF group. The elevation seen in serum TG levels in the F+ group might be related to higher metabolism and MY. Sevinç and Aslan (27) stated that serum CHOL and TG levels declined not before, but during the fatty liver syndrome in dairy cows. High-yielding dairy cattle are at risk of the fatty liver syndrome. Relatively high levels of serum TG can be an important buffer against fatty liver syndrome when the levels were within the reference range (27). Thus, relatively higher serum TG levels for cows in the F+ group could be a positive factor that protects them against fatty liver.

Fatty liver is an important metabolic disorder, mostly seen postpartum. Serum NEFA and BHBA levels are important parameters that give vital information about body fat mobilization level, the status of carbohydrate metabolism, and some metabolic disorders such as ketosis and fatty liver (14). It has been emphasized that there will be an increase in serum BHBA and NEFA levels in dairy cows following parturition, but cows may find it difficult to balance the energy input and output after calving (13). Serum NEFA and BHBA can give information about the dairy cows' energy metabolism during the transition period through mid-lactation. Accordingly, the elevated serum levels of these two parameters are good indicators that animals are fed inadequately (14). Consequently, the improved biochemical parameters, such as CHOL, TG, BHBA, and NEFA support the conclusion that the monitoring processes, better rations, and higher standards provided by IPARD support were beneficial to protect animal health and physiology in the supported farms.

When energy intake is limited, it causes excessive fatty acid mobilization to support milk production. Increased serum levels of NEFA can lead to an increased NEFA uptake and TG storage by the liver. Thus, postpartum high serum levels of NEFA can cause fatty liver (14). In the current study, serum NEFA levels were higher in NF farms than F+ farms. This suggests that dairy cows in F+ were less affected by the negative energy balance expected after calving and these cows overcame the harmful effects of negative energy balance in a shorter period when compared to the cows in the NF farm.

The physiological status of the liver can be determined by looking at the serum levels of metabolites produced in the liver. For example, during the breakdown of liver cells, ALT enters the blood. When there is damage to liver tissues, the amount of ALT in the blood increases. Therefore, ALT is often used in detecting liver damage. Serum AST levels, on the other hand, increase during the cellular damage in the RBC or tissue damage in the liver, heart, skeletal muscles, pancreas, and kidneys (28). In dairy cows, serum ALT and AST ranges were reported as 14-38 and as 78-132 IU/L, respectively (9). There was a correlation between serum ALT-AST activities and milk production (3, 24), and the serum ALT and AST activities increased in lactating dairy cows (18). In the current study, the serum activities of ALT and AST in both groups were within normal physiological limits, suggesting that the related tissues such as liver, heart, skeletal muscle, pancreas, and kidneys were in healthy conditions.

Kweon et al. (21) stated that oxidant-antioxidant status affects vital metabolism. Furthermore, oxidative stress is one of the primary risks for physiological processes and provides a predisposition for many diseases (5). It was emphasized that cows with high MY tend to have oxidative stress and it can be an important risk factor for dairy cows during early and mid-lactation (4, 5, 9). As a result, antioxidant additives may affect lactation period performance in dairy cows (9). The sum of oxidative indicators is generally measured and determined by TOS levels (10). In the current study, higher TOS and lower TAS levels for cows in NF farms imply that the oxidative stress at calving probably continued through the mid-lactation in this group at a higher level. Accordingly, the lower TOS and higher TAS values for the cows in the F+ farms suggest that there were fewer oxidative factors present after calving. Therefore, improved environmental factors such as noise, dust and contamination, and better welfare could lower the stress, improve TAS and TOS levels, and support the antioxidant status of the cows in F+ farms.

Body condition scoring is one of the indicators that have a direct effect on MY and fertility rates of dairy cows. Although live weight can vary from cow to cow, BCS should be comparable and within the ideal limits in a given lactation period since ideal BCS is an important criterion in determining the energy and nutritional needs of dairy cows (12). Thus, BSC is an important tool to predict whether animals are being fed properly and BCS monitoring is very important in cases where MY increases (12). When the ideal BCS cannot be achieved, infertility problems are usually experienced in over and underweight cows (6, 31). In the current study, the BCS of F+ and NF farms were 3.11 and 2.83, respectively. Overall, studies usually stated that a BCS of 3.0 to 3.5 is ideal in the mid-lactation period for dairy cows (20). Compared to the previous studies, the average BCS of cows in NF farms fell below the ideal BCS of 3.0, whereas the BCS of cows in F+ farms were in the desired range. Losses can occur in BCS when dairy cows cannot meet their energy needs. BCS is an important follow-up tool on animal welfare and cows should be fed with well-balanced rations to prevent the loss in BCS (32). In the current study, the ration content and energy-protein balance were better in F+ than in NF farms. Moreover, it seems that the IPARD supports helped to improve physical, managerial, and psychological conditions in the F+ farms and this could explain the better BCS in F+ farms.

Paralleled with BCS, MY was also affected by IPARD support. There was a difference in MY between F+ and NF dairy farms. While the mean daily MY of cows in NF farms was 15.0 L, that of cows in F+ farms was 24.4 L. Therefore, MY in dairy farms that were supported by IPARD was significantly higher. Alpan and Arpacık (1) stated that MY in dairy cows can be affected by hereditary and environmental factors. Nutritional factors are also effective in MY (19, 25). Better environmental factors and nutritional status of cows in F+ dairy farms could explain

the higher MY and better BCS for the cows in these farms. Moreover, better animal care and the environment could also decrease the possible metabolic disorders. Thus, it is expected that the daily MY productions of IPARD supported farms are likely to be high because nutritional imbalances and metabolic disorders are known to affect MY (35).

Overall, the farms supported by IPARD had better animal care and feeding systems, the sensitivity of farmworkers was higher, animal health and welfare was monitored better, and these farms had more modern equipment. The improved conditions could help to minimize stress in the farms supported by IPARD. In addition, the fact that these farms were checked periodically by the Agricultural and Rural Development Support Institution experts and these regular controls could have a positive impact on the farm management to act more scientifically. In this sense, the health practices, animal care and feeding conditions, and farming equipment in family-owned medium-sized enterprises were far behind compared to IPARD-supported farms.

In conclusion, IPARD grant support positively affected the physiological processes, yield qualities, and animal welfare of the dairy cows because of its positive contributions to the maintenance, care, feeding, and animal material, which provided important contributions to the national economy and global economic processes as well as human, animal and environmental health. Considering that IPARD grants can possibly lead to creating sustainable systems for animal health-welfare, as well as safer and stable products, this support program can be recommended to small and medium-sized businesses in countries with high potential to improve their agricultural production.

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Ethical Statement

The research was carried out with the approval of Afyon Kocatepe University, Experimental Animals Ethics Committee (AKÜHADYEK - 467-15).

Conflict of Interest

The authors declare no competing interests.

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Wound healing effect of Anzer origin propolis specimens on rats' intestinal incision

Zülfükar Kadir SARITAŞ[1,a,✉], Musa KORKMAZ[1,b], H. Hüseyin DEMİREL[2,c], Aziz BÜLBÜL[3,d], Tuba Berra SARITAŞ[4,e], Fatma GÖRÜCÜ[1,f], Yusuf KOÇ[1,g]

[1]Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Surgery, Afyonkarahisar, Turkey; [2]Afyon Kocatepe University, Bayat Laborant and Veterinary Health Division, Afyonkarahisar, Turkey; [3]Muğla Sıtkı Koçman University, Milas Veterinary Faculty, Department of Physiology, Milas, Muğla, Turkey; [4]Afyonkarahisar Health Sciences University, Faculty of Medicine, Department of Anesthesiology and Reanimation, Afyonkarahisar, Turkey.
[a]ORCID: 0000-0002-7659-6635; [b]ORCID: 0000-0002-7646-0009; [c]ORCID: 0000-0002-4795-2266; [d]ORCID: 0000-0003-0995-3986; [e]ORCID: 0000-0002-3206-6851; [f]ORCID: 0000-0001-7630-0788; [g]ORCID: 0000-0002-6342-5466

✉Corresponding Author: zksaritas@hotmail.com
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Abstract: This study was investigated the wound healing effect of Anzer origin propolis specimens on rats' experimental intestinal incisions. Thirty-four male Wistar albino rats have been divided into five groups, weighing 250-300 gr. Water solution of Anzer propolis (PW3 [n=6], PW7 [n=6]) or ethanolic solution of Anzer propolis (PE3 [n=6], PE7 [n=6]) were administered. Control groups (K3 [n=5], K7 [n=5]) received no treatment. The surgical procedure (day 0) consisted of a laparotomy under general anesthesia, during which the colon was incised and repaired with sutures. The Anzer propolis specimen diluted with physiologic saline was administered by gavage (100 mg/kg/day) for 3 and 7 days before and after surgery to PW3 and PW7 groups. Ethanol dissolved Anzer propolis was administered by gavage (100 mg/kg/day) for 3 and 7 days before and after surgery to PE3 and PE7. Experimental animals have been humanly euthanized on the 3rd and 7th days for biochemical and histopathological evaluations. Serum total oxidant status, total antioxidant status, interleukin-1, interleukin-6, tumor necrosis factor-alpha, myeloperoxidase, hydroxyproline and nitric oxide, levels were measured by ELISA. The incision line sampled to evaluate inflammatory cells, fibroblastic activity, neovascularization, and collagen level. The study indicates that the ethanolic Anzer propolis solution's oral administration has a beneficial effect on intestinal wound healing for seven days pre-and postoperatively (PE7).

Keywords: Anzer propolis, intestinal incision wound healing, rat, total oxidant status, total antioxidant status.

Anzer menşeli propolis örneklerinin sıçanların bağırsak kesisi üzerindeki yara iyileştirici etkisi

Özet: Bu çalışmada, Anzer menşeli propolis örneklerinin sıçanların deneysel bağırsak kesisi üzerindeki yara iyileştirici etkisinin araştırılması amaçlanmıştır. 250-300 gr ağırlığındaki toplam 34 erkek Wistar Albino sıçan beş gruba ayrıldı. Anzer propolis su solüsyonu (PW3 [n=6], PW7 [n=6]) ve Anzer propolisin etonolik solüsyonu (PE3 [n=6], PE7 [n=6]) uygulandı. Kontrol gruplarına (K3 [n=5], K7 [n=5]) tedavi uygulanmadı. Cerrahi prosedür (0. gün) genel anestezi altında laparotomi ile kolon enzize edildi ve dikiş uygulanarak kapatıldı. Fizyolojik tuzlu su ile seyreltilmiş Anzer propolis örneği PW3 ve PW7 gruplarına ameliyat öncesi ve sonrası 3 ve 7 gün süreyle gavaj (100 mg/kg/gün) ile uygulandı. Etanolde çözdürülmüş Anzer propolisi, PE3 ve PE7 gruplarına ameliyat öncesi ve sonrası 3 ve 7 gün sonda ile (100 mg /kg/gün) uygulandı. Deney hayvanlarına biyokimyasal ve histopatolojik değerlendirmeler için 3. ve 7. günlerde ötenazi yapılmıştır. Serum total oksidan seviye, total antioksidan seviye, interlökin-1, interlökin-6, tümör nekroz faktör-alfa, miyeloperoksidaz, hidroksiprolin ve nitrik oksit seviyeleri ELİSA yöntemi ile ölçüldü. Ensizyon hattı, yangı hücreleri, fibroblastik aktivite, neovaskülarizasyonu ve kollajen seviyesini değerlendirmek için örneklendi. Bu çalışma, etanolik Anzer propolis solüsyonunun oral uygulamasının, operasyon öncesi ve sonrası yedi gün boyunca bağırsak yara iyileşmesi üzerinde faydalı bir etkiye sahip olduğunu göstermektedir (PE7).

Anahtar sözcükler: Anzer propolis, bağırsak enzisyonu yara iyileşmesi, rat, total oksidant seviye, total antioksidan sseviye.

Introduction

The wound healing process is similar in various tissues; however, it has some distinctive gastrointestinal tract features (21). Unlike skin wounds, smooth muscle cells and fibroblasts make collagen synthesis and tensile strength occur much faster in the intestine. Knowledge related to local and systemic factors affecting gastrointestinal anastomosis healing has increased; however, anastomotic leakage and separation are serious problems that occur at a high frequency and result in high mortality (2, 15). Propolis, a resinous enzymatic substance enriched by bees' salivary glands, is used to fill beehive walls, cracks, or voids (10). Most of the compounds in propolis can be identified, verified, and classified after the purification step (3, 6).

The composition of propolis varies by the vegetation, climate, season, and environmental condition of the area where it is collected. Still, it mainly consists of resin (50%), beeswax and fatty acids (30%), essential oils (10%), polyphenols and flavonoids (10%), pollen (5%), and vitamins and minerals (5%) (24).

Propolis has been shown to have antitumor (1), antioxidant (4), antibacterial (11), antiviral (34), antifungal (35), and anti-inflammatory properties; however, the effect of propolis on oxidative stress is not well-documented. Anzer propolis is a regional propolis specimen unique to the Eastern Black Sea Region of Turkey. This study investigated the wound-healing effect of Anzer propolis 'specimen in rats' intestinal incisions using histopathologic and biochemical techniques.

Materials and Methods

A total of 34 Wistar albino male rats weighing 250-300 g were used in the study. Rats were housed in standard cages with a 12 h light/dark cycle. The animals were fed ad libitum up to 2 hours before the study and allowed free access to water.

Anesthesia Protocol: General anesthesia was provided as a combination of 13 mg/kg Xylazine hydrochloride (Rompun, 50 ml Fl. 23.32 mg/ml, Bayer-Germany) and 87 mg/kg Ketamine hydrochloride (Alfa, 10% Ala-Phe, Izmir, Turkey) via intramuscularly.

Surgical Protocol: After carefully shaving the abdomen of the rats, the area was cleaned with Povidone-iodine. A laparotomy was performed under general anesthesia and sterile conditions. After reaching the descending colon, a longitudinal incision was made in the antimesenteric region and sutured with double bowel sutures of 6-0 Prolene. The anastomosis line was checked for leaks and once hemostasis was confirmed, the incision was repaired with 6-0 polyglactin 910 Vicryl suture. In all groups, the abdominal wall and outer skin were closed with standard suture techniques. Postoperative wound care was performed following skin sutures and isofix bandaging. Gentamycine 4 mg/kg/day was administered IM for 5 days.

Anzer Propolis Extract Preparation: Commercially available Anzer Propolis Extract containing 95% propolis, 5% ethanol, from the Anzer province of the East Black Sea Region in Turkey was used in the study. The ethanol content of the Anzer Propolis Extract was removed by vaporization, at the degree of 78.4 C in a dry heat oven. The purified propolis was then grounded and homogenized into powder using a homogenizer. Prior the gastric gavaged to the animals, purified propolis (100 mg/kg body weight) was diluted with saline to make a total volume of 1 mL for the PE3 and PE7 groups,

Study Groups: A total of 34 male Wistar albino rats weighing 250-300 g were randomly divided into the following groups:

Group 1: Propolis water-based 3 (PW3, n=6): 100 mg/kg/day Anzer propolis was diluted in saline and administered by gastric gavage from day -3 before surgery and continued for 3 days postoperatively. On the third day, the rats were euthanized.

Group 2: Propolis water-based 7 (PW7, n=6): 100 mg/kg/day Anzer propolis was diluted in saline and administered by gastric gavage from day -7 before surgery and continued until postop day 7 at which time the rats were euthanized.

Group 3: Propolis ethanol-based 3 (PE3, n=6): 100 mg/kg/day Anzer propolis was diluted in ethanol and administered by gastric gavage from day -3 before surgery and continued until the 3rd postoperative day at which time the rats were euthanized.

Group 4: Propolis ethanol-based 7 (PE7, n=6): Anzer propolis diluted in ethanol was gastric gavaged at a dose of 100 mg/kg/day from day -7 before surgery and continued until the 7th postoperative day at which time the rats were euthanized.

Control groups: (K3, n=5) and (K7, n=5): Propolis was not administered to this group pre- or postoperatively. On day 0, a colon anastomosis was performed following general anesthesia and laparotomy. Rats were euthanized on the 3rd (K3) and 7th day (K7) postop.

In all cases at necropsy, a sample was taken from the anastomosis line for histopathological examination, and blood samples were collected by cardiac puncture for biochemical measurements.

Biochemical Measurements: Serum levels of total oxidant status (TOS), total antioxidant status (TAS), interleukin -1 (IL-1), interleukin -6 (IL-6), tumor necrosis factor alpha (TNF-α), myeloperoxidase (MPO),

hydroxyproline (HYP), nitric oxide (NO), and antioxidant activity were measured by commercial ELISA kits with an MVGt Lambda Scan 200 (Bio-Tek Instrument, Winooski, VT, USA). The following Biont kits were used: **TOS** (Rat Total Oxidant Status, Catalog No: YLA1392RA, China)**, TAS** (Rat Total Antioxidant Status, Catalog No:YLA1389RA, Chaina), **IL-1** (Rat IL-1, Catalog No: YLA0153RA, China), **IL-6** (Rat IL-6, Catalog No: YLA0031RA, China), **TNF-α** (Rat TNF- α, Catalog No:YLA0118RA, China), **MPO** (Rat MPO, Catalog No:YLA0046RA, China), and **HYP** (Rat HYP, 96 TEST, Catalog No: YLA0068RA, China).

Histopathological Examination: Colon samples were stored in 10% neutral buffered formaldehyde solution. After 48 hours, specimens were trimmed and placed into cassettes, passed through an alcohol and xylene series, and blocked in paraffin. Blocks were cut 4-5 microns thick with a microtome and made into slides. Hematoxylin-eosin stained sections were examined under a light microscope and changes in the incision line were evaluated.

Statistical Analysis: The results were acquired by applying one-way ANOVA tests using the SPSS 16.0 Statistics package program. A Duncan test was applied to results with statistical differences and the data were expressed as mean ± standard deviation. Statistical significance was accepted as $P<0.05$.

Results

Histopathological evaluation is detailed in Table 1. Serum levels of NO, TAS, TOS, MPO, IL-1, IL-6, TNF-α, and HYP were measured by ELISA (Table 2).

Histopathological examination results of tissue sections from the anastomosis line and inflammatory cells, fibroblastic activity, neovascularization and collagen levels were evaluated (Figure 1).

Table 1. Effects of Propolis on Bowel Wound (Mean ± Standard Deviation).

	Histopathological findings				
Groups	**Inflammatory cells**	**Fibroblastic activity**	**Neovascularization**	**Collagen**	**P value**
K3	1.43±0.43^{c}	1.27±0.52^{b}	1.43±0.43^{c}	1.27±0.52^{c}	0.000
K7	1.61±0.51^{c}	1.62±0.49^{b}	1.57±0.53^{c}	1.21±0.26^{c}	0.000
PW3	3.05±0.37^{b}	3.38±0.75^{a}	3.07±0.38^{b}	3.37±0.41^{b}	0.000
PW7	4.05±0.42^{a}	3.55±0.83^{a}	3.93±0.49^{a}	3.55±0.54^{b}	0.000
PE3	3.75±0.53^{a}	3.92±0.52^{a}	3.62±0.53^{a}	3.58±0.52^{b}	0.000
PE7	4.03±0.41^{a}	3.87±0.51^{a}	4.11±0.41^{a}	4.26±0.52^{a}	0.000

$^{a, b, c:}$ Inflammatory cell, fibroblastic activity, neovascularization and collagen values bearing different letters in the same column are statistically significant ($P<0.05$).
K: control, PW: Water-based propolis, PE: Ethanol-based propolis.

Table 2. Group Biochemical Parameters (Mean ± Standard Deviation).

	K3	**K7**	**PW3**	**PW7**	**PE3**	**PE7**	**P**
TOS	5.5760±.28574^{a}	4.7060±.10801^{c}	5.2680±.11097^{ab}	4.8400±.16211^{bc}	5.3333±.11618^{a}	4.1567±.09691^{d}	0.000
TAS	12.1820±.62056^{b}	13.0720±1.15246^{ab}	12.3480±.48555^{b}	14.0340±1.32785^{b}	15.1167±.60039^{ab}	16.5869±1.11400^{a}	0.015
IL-1	18.2660±1.72875	19.3460±.40078	20.1780±1.09896	19.5440±.61191	18.5733±.88499	17.4683±1.22009	0.538
TNF-α	53.6660±7.95570	47.9740±6.30029	52.6200±7.07548	38.8400±2.87064	48.1250±5.32121	55.6917±8.76147	0.574
IL-6	38.5400±4.44505^{ab}	21.4500±1.63133^{c}	37.8620±2.68475^{ab}	22.0588±1.98869^{c}	45.9617±5.02943^{a}	29.0517±3.63555^{bc}	0.000
MPO	21.8840±1.23079^{ab}	18.2920±.63377^{c}	23.2700±1.31721^{a}	19.3680±.93094b^{c}	22.1117±1.70461^{ab}	14.8417±.59530^{d}	0.000
HYP	271.8000±15.11092	285.6000±9.38936	289.2000±9.74372	297.8000±6.56049	302.5000±4.07226	317.6667±13.50473	0.071
NO	13.4200±.49639^{a}	11.8400±.37497a^{b}	13.4400±.20640^{a}	12.0000±.34785^{ab}	12.0167±.30705^{ab}	10.7500±1.05822^{b}	0.023

^{a,b,c}: Values with different letters in the same line are statistically significant ($P<0.05$). K: Control, PW: Water-based propolis, PE: Ethanol-based propolis, TOS: total oxidant status, TAS: total antioxidant status, IL-1: interleukin -1, IL-6: interleukin -6, TNF-α: tumor necrosis factor alpha, MPO: myeloperoxidase, HYP: hydroxyproline, NO: nitric oxide.

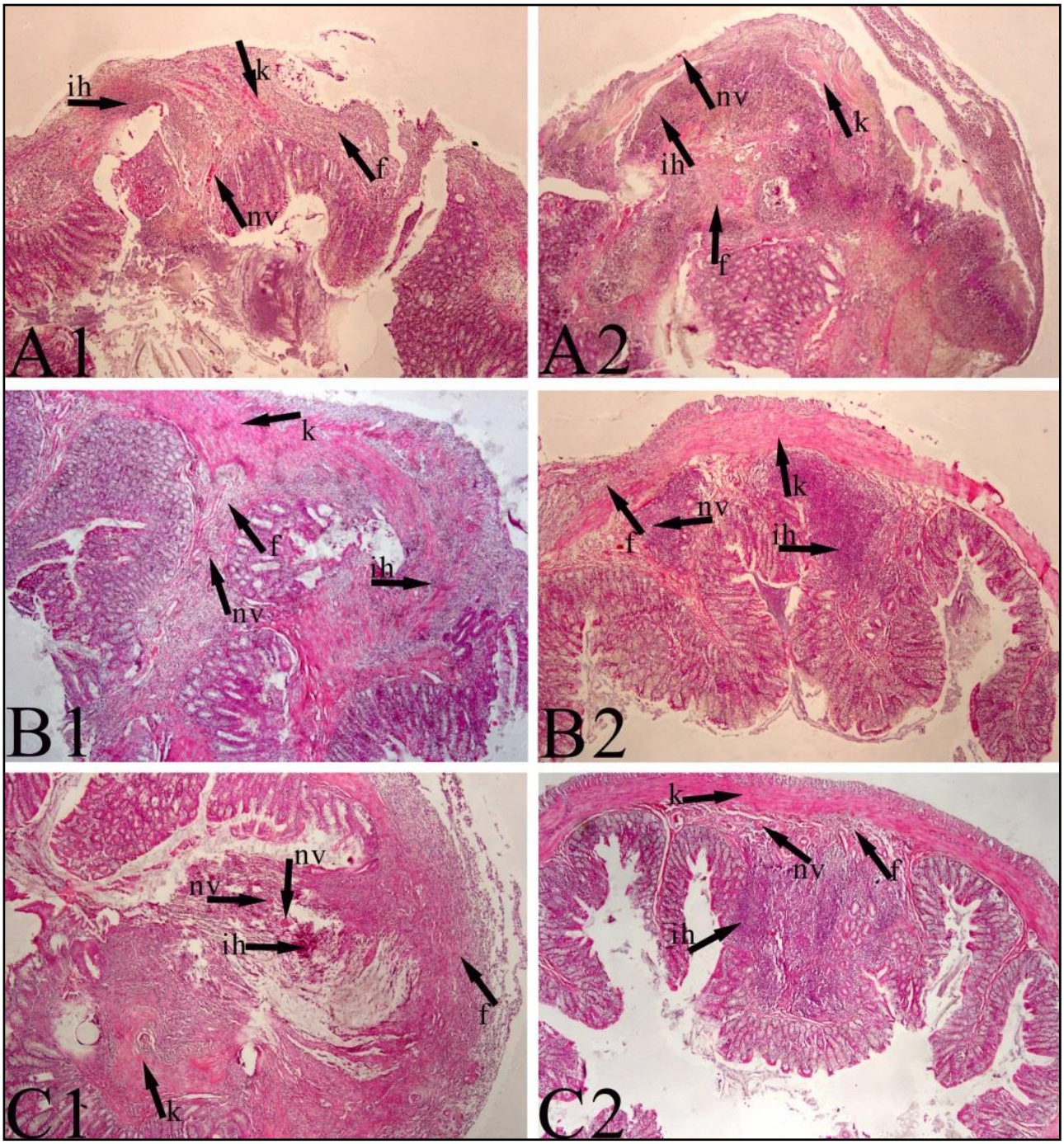

Figure 1. Histopathologic views of the groups.
A1:K3, A2:K7, B1:PW3, B2: PW7
C1: PE3, C2: PE7
K: Control, PW: Water-based propolis, PE: Ethanol-based propolis,
nv: neovascularization, f: fibroblastic activity, ih: inflammatory cell, k: collagen.

When inflammatory cell measurement results and fibroblastic activity of all groups were compared, values for PW7, PE3, and PE7 groups were found to be statistically significantly higher than the other groups ($P<0.001$). The highest values for fibroblastic activity were determined in the PE3 and PE7 groups. Neovascularization measurements were highest in the PE7 and PW7 groups when compared to the other groups ($P<0.001$). The PE7 group had the highest arithmetic mean with statistical significance ($P<0.001$). The PE7 group was also statistically higher compared to the other groups with regard to collagen measurement ($P<0.001$) (Table 1).

There were no statistical differences between groups with regard to TNF-α values ($P>0.05$). The increase in IL-6 in the PE3 group was statistically significant when

compared to the other groups (P<0.001). The PW7 group IL-6 value was the same as that measured in the K7 group (P<0.001) (Table 2).NO, TOS, and TAS values in serum samples are detailed in Table 2.

The PE7 group TAS level was found to be higher than the other groups with a statistical significance (P<0.001) while the TOS level was lower than all other groups (P<0.001) (Table 2).

Discussion and Conclusion

While colon surgery is a common procedure, anastomosis leaks are a frequently encountered complication with high morbidity which has accelerated associated studies and research. This study aimed to histopathologically and biochemically evaluates the effects of orally dosed Anzer propolis on wound healing in rats following colon anastomoses.

Wound healing is a complex process in which the tissue repairs itself (23, 36). The wound healing process is similar in various tissues; however, it has some distinctive features in the gastrointestinal tract, for example, in bowel wounds, stretching time develops much earlier than for the skin (23) and collagen is synthesized by smooth muscle cells in intestinal wounds (23, 36).

Bowel wound healing includes inflammation, proliferation, fibroplasia, and maturation stages. Inflammation begins with vasodilation, secretion of vasoactive substances, increased vascular permeability, and neutrophil infiltration within 3 hours following vasoconstriction of the wound edges. Macrophages and fibroblasts then migrate to the wound area. Macrophages regulate inflammation by releasing cytokines (5, 16, 23).

In addition, systemic and local factors play a role in the healing of bowel wounds (13, 23). Important factors in the extracellular matrix are collagen fibers, fibroblasts, and immune cells that regulate wound strength in the early postoperative healing process (8, 13, 23).

Collagen fibrils synthesized in the submucosa form bridges in the anastomosis line. Even though in the early period the force that holds the wound edges together are the sutures, after 7-14 days they lose their importance with the increase of collagen bridges. In the maturation and restructuring phase, cross-links in collagen fibrils increase. At this stage, the wound becomes less cellular and granulation tissue is replaced by the tightening tissue (37).

Our findings obtained from the tissue sections taken from the suture line were compared statistically and PW7, PE3, and PE7 groups were found to be statistically higher than other groups. For fibroblastic activity PW7, PE3, and PE7 groups were statistically significantly higher than the other groups. The highest values were found in the PE3 and PE7 groups. For neovascularization measurement results, when the findings obtained in all groups were compared statistically, PE7 and PW7 groups were the highest.

The PE7 group had the highest arithmetic mean with statistical significance. The PE7 group measurement was found to be statistically higher for collagen when compared to other groups. The quantitative measurement of HYP, an amino acid found only in collagen, is directly proportional to the formation of collagen, and its prediction aids in understanding the rate of progression in which the healing process occurs clinically. It is known that collagen accumulation is the sum of synthesis and destruction, and both occur simultaneously in the wound healing process (26).

While the HYP value was highest in the PE7 group serum samples, statistical significance between groups was not determined, although it was the lowest in the K3 group. The significantly high level of collagen in the PE7 group is supported by the literature.

A common complication in bowel operations is adhesion formation. It has been reported that in 12% to 17% of patients who have undergone abdominal surgery for various reasons, subileus occurs due to serosal adhesions developed in the early or late postoperative period (18, 20).In the current study, adhesion development was negligible at both the 3rd and 7th days.

MPO is an enzyme used in the creation of toxic agents that neutrophils use to break down the agents they phagocytize; thus, it is used as an indicator of neutrophil infiltration in tissues. Koç et al. (22) reported that MPO was significantly lower in a group in which 4% icodextrin was used following anastomoses. The low value detected indicated that less adhesions occurred due to a less severe inflammatory response (18).

In our study, the MPO level was found to be statistically significant in 3-day groups, while it was lowest in the PE7 group. In the study, adhesion development was evaluated according to the frequency (0-3+) at necropsy on the 3rd and 7th postoperative days. Adhesion development at a 0-1+ level was observed in all groups, but adhesion formation to the peritoneum and intra-abdominal organs was not observed at the anastomosis site. Our findings support the literature data.

It is reported that a complex reaction referred to as an acute phase inflammatory response begins immediately after surgical trauma (30) and the production of acute phase proteins increases immediately after surgical interventions (33). The acute phase protein response regulator is IL-6 (20, 32) which stimulates the secretion of other inflammatory cytokines such as TNF-α and IL-1 secretion of IL-6 (12, 20, 38).

Immunomodulatory effects have also been reported for honey proteins. MRJP-3 has been found to suppress IL-2, IL-4, and IFN-γ production by antigen-stimulated T cells (29). NO is produced by glycopeptides and

glycoproteins, zymosan-activated human neutrophils and murine macrophages, ranging from 2 to 450 kDa. NO is also produced by phagocytosis, LPS-activated murine macrophages, and TNF-a production by phagocytosis and human monocytic cells (25).

In this study, no statistical difference in serum TNF-α values was determined between groups. A rise in serum IL-6 levels in the PE3 group was statistically significant compared to other groups. The value in the PW7 group remained at the same level as that of the K7 group.

The level of IL-6 may have increased due to the effect of ethanol. It is also noteworthy that the PW7 IL-6 value, is approximately the same as that of the K7 group and shows that Anzer propolis dissolved in water causes less reaction. When the IL-1 serum results were compared statistically, there was no difference between groups.

Heinrich et al. (20) reported that the acute phase response peaked after trauma, and that mediators increased after surgery (9, 17, 28). Harada et al. (19) determined a statistically significant relationship between IL-6 and TNF-α levels. In light of these data, an increase of the parameters in the PW3 and PE3 groups was determined. This result was interpreted as proof that the level of inflammatory mediators decrease after day 3 in parallel with the literature.

Italian multifloral honey with daidzein, apigenin, genistin, luteolin, kaempferol, quercetin, and chrysin as major components has been reported to inhibit the release of LPS-stimulated N13 cells from microglia, (7). Given the role of neuroinflammation in neurodegenerative diseases, these data confirm the possible use of honey-flavonoid fraction against disorders such as Alzheimer's or Parkinson's.

Honey protein apalbumin-1, called MRJP-1, has been found to block the mannose receptors of human phagocytic cells, thereby inhibiting phagocytic activities. This inhibitory effect appears to be increased in honey containing methylglyoxal (MGO) due to apalbumin glycation (27).

Generally, phenolics are considered to contribute significantly to the antioxidant capacity of honey. Given that the phenolic composition of oral sources are highly variable, honey is expected to exhibit a wide variety of antioxidant properties (14, 31).

With regard to the TAS values, since the PE7 group was found to be higher than the other groups with a statistical significance it was considered evidence that Anzer propolis has an antioxidant effect. This finding supports the studies conducted with other propolis, honey, and honey derivatives. And considering that the PE7 group had the lowest level of TOS compared to the other groups, it is seen as evidence that the application of Anzer propolis shows considerable antioxidant activity.

We have confirmed with biochemical and histopathological findings that the oral administration of Anzer propolis dissolved in ethanol has positive effects on intestinal wound healing.

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Ethical Statement

This study was approved by the Afyon Kocatepe University Animal Experiments Local Ethics Committee (11.06.2018 dated and AKUHADYEK 93-18 numbered).

Conflict of Interest

The authors declare that there are no conflicts of interest.

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Case Report / *Olgu Sunumu*

Pericardial Abscess Associated with *Mycoplasma arginini*: A Rare Case from a Cat

Özlem ŞAHAN YAPICIER[1,a]✉, Aybars AKAR[2,b], Yusuf Sinan ŞİRİN[3,c]

[1]Republic of Turkey Ministry of Agriculture and Forestry, Veterinary Control Central Research Institute, Bacteriology Diagnostic Laboratory, Ankara, Turkey; [2]Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Internal Medicine, 15030, Burdur, TURKEY; [3]Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Surgery, 15030, Burdur, TURKEY

[a]ORCID ID: 0000-0003-3579-9425; [b]0000-0001-7530-8761; [c]0000-0003-1322-7290

✉Corresponding author: ozlem.sahanyapicier@tarim.gov.tr
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Abstract: A two and half-year-old, mixed breed, male cat was admitted to Burdur Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Animal Teaching Hospital with respiratory distress and coughing lasting for a month. During the initial examinations of ultrasound and radiography, a pericardial abscess was detected near the right side of the heart and was drained with pericardiocentesis. Percardial biopsy material was screened for the bacterial isolation and identification. Bacteriological methods based on colony morphology, sugar fermentation tests, and molecular confirmation using 16S rRNA-23S rRNA specific primers were performed. Result on conventional and molecular analysis, *Mycoplasma arginini* (*M. arginini*) were detected. The patient was treated with azithromycin and enrofloxacin. Full recovery was observed during follow-up examination after a month. The findings of this case increase awareness of pathogen *M. arginini* in cats and zoonotic importance has been emphasized for pet owners.
Keywords: Cat, *Mycoplasma arginini*, pericardial abscess.

Mycoplasma arginini ile İlişkili Perikardiyal Apse: Kedide Nadir Görülen Bir Olgu

Özet: Bir aydır süren solunum sıkıntısı ve öksürük şikayeti olan 2,5 yaşında, melez erkek bir kedi Burdur Mehmet Akif Ersoy Üniversitesi Veteriner Fakültesi Hayvan Hastanesi'ne getirildi. Ultrason ve radyografi incelemesinde, kalbin sağ tarafında bir perikardiyal abse tespit edildi ve perikardiyosentez ile drene edildi. Perikardiyal biyopsi materyali, bakteriyel izolasyon ve identifikasyon için tarandı. Koloni morfolojisi, şeker fermentasyon testlerine dayalı bakteriyolojik yöntemler ve 16S rRNA-23S rRNA'ya özgü primerler kullanılarak moleküler doğrulama gerçekleştirildi. Konvansiyonel ve moleküler analiz sonucunda *Mycoplasma arginini* (*M. arginini*) tespit edildi. Hasta azitromisin ve enrofloksasin ile tedavi edildi. Bir ay sonraki kontrol muayenesinde tam iyileşme görüldü. Bu vakanın bulguları, kedilerde patojen olan *M. arginini* konusundaki farkındalığımızı arttırmakta ve evcil hayvan sahipleri için zoonotik önemi vurgulanmaktadır.
Anahtar sözcükler: Kedi, *Mycoplasma arginini*, perikardiyal apse.

Mycoplasma species are cell wall-less bacteria that survive on mucosal surfaces where they act as both primary and commensal opportunistic pathogen of the conjunctiva and upper respiratory system in cats (1, 11). Especially *Mycoplasma felis* (*M. felis*), *Mycoplasma gateae* (*M.* gateae), *Mycoplasma arginini* (*M. arginini*), and *Mycoplasma felininutum* (*M. feliminutum*) are regarded as fairly host-specific species that are isolated from the upper respiratory tracts of cats (3, 8, 11, 12).

Since its first description in 1968 (2), *M. arginini* has been recovered from tissues and secretions of various animals such as cattle, camel, sheep and goat (6, 10). *M. arginini* also was implicated in zoonotic transmission, especially for immune-compromised humans (23, 25).

Pericardial diseases are uncommon conditions in cats. Previous studies report that the prevalence of pericardial diseases ranging between %1.0-2.3. Among the pericardial diseases, infectious pericarditis and pericardial abscess are even rarely reported in cats (5, 20).

A two and half-year-old, male castrated mix breed cat, weighing 3.4 kg brought to the Animal Hospital, Burdur Mehmet Akif Ersoy with respiratory complaints

including breathing difficulties and ongoing coughs for a month Multiple courses of antibiotic, i.e. amoxicillin and clavulanic acid (Synulox, Zoetis, 20 mg/kg, SC, BID for 7 days) had been prescribed previously in a private veterinary clinic. However, according to the owner, cat's condition worsened despite the medical treatment and anorexia and lethargy developed.

On physical examination of the cat, it was observed that open mouth, abdominal breathing, sitting in the sternal position were present. Pathological changes in lung sounds were further detected during auscultation. The heart sounds taken from the left side of the patient were clearly heard, while the sound of the heart from the right was muffled and dull. The patient had normothermia (38.7°C), tachycardia (158 / min), and tachypnea (68 / min).

Blood was collected from the cat for hemogram and serum biochemistry. Hematological parameters were identified leukocytosis (27.11 x 10^9/l) with granulocytosis (19.88 x 10^9/l). Other parameters were unremarkable (Table 1). The patient's serum biochemistry values were also normal (Table 2). For further examination, the patient was referred to the relevant units for radiography and echocardiography.

Table 1. Hematological values of the cat before the treatment.

Parameter	Value	Range	Evaluation
White Blood Cell(WBC)	24.11 x 10^9/l	5.5-19.5	High
Lymphocytes (Lym)	5.54 x 10^9/l	1.5-7	Normal
Monocytes (Mon)	1.69 x 10^9/l	0-1.5	Normal
Granulocytes (Gra)	16.88 x 10^9/l	2.5-14	High
Lym%	23.0 %	20-55	Normal
Mon%	7.0 %	1-3	High
Gra%	70.0 %	35-80	Normal
Red Blood Cell (RBC)	10.37 x 10^{12}/l	5-10	High
Hemoglobin (HGB)	11.0 g/dl	8-15	Normal
Haematocrit (HCT)	33.32 %	24-45	Normal
Mean Cell Volume (MCV)	32 fl	39-55	Low
Mean Cell Hemoglobin (MCH)	10.6 pg	12.5-17.5	Low
Mean Cell Hemoglobin Concentration (MCHC)	33.0 gr/dl	30-36	Normal
Red Cell Distribution Width (RDW)	30.7 %	-	
Platelets (PLT)	228 x 10^9/l	300-800	Low
Platelet Crit (PCT)	0.22 %	-	
Mean Platelet Volume (MPV)	9.5 fl	12-17	Low
Platelet Distribution Width (PDW)	28.7 %	-	

Table 2. Serum biochemistry values of cat before the treatment.

Parameter	Value	Range	Evaluation
Blood Urea Nitrogen(BUN)	37 mg/dl	19-34	High
Creatinine(Cre)	0.59 mg/dl	0.9-2.2	Low
Alanine Transaminase (ALT)	90 mg/dl	25-97	Normal
Gamma-Glutamyl Transferase (GGT)	13 u/l	1.8-12	High
Alcaline Phosphatase(ALP)	121 u/l	0-45	High
Glucose(Glu)	123.6 mg/dl	60-120	High
Total Protein(TP)	7.2 mg/dl	6-7.9	Normal
Albumin(Alb)	2.45 g/dl	2.8-3.9	Low
Total Bilirubin(TBil)	0.04 mg/dl	0-0.26	Normal
Calcium(Ca^+)	12.95 mg/dl	9.3-11.2	High
Potasium(K^+)	5.37 mEq/l	3.8-4.5	High

Thoracic ventrodorsal radiography revealed a mass on the right caudal lobe, next to the right ventricle (Figure 1). To evaluate the mass and its effect on the heart, an echocardiography examination was performed. There was no evidence of any kind of myocardial dysfunction due to the abscess's formation is towards to right caudal lobe of the lung (Figure 2). According to the ultrasound examination, the mass had an echoic wall and anechoic area (Figure 3). To confirm the suspicion, it was determined to do a pericardiocentesis. Following aseptic preparation of the right hemithorax, pericardiocentesis was performed under ultrasound guidance. Biopsy material was taken and submitted for microbiological analysis. The bacteriological culture was performed as described (17). Briefly, the abscess swap was streaked on 5% defibrinated sheep blood, Mac Conkey agar, and PPLO agar supplemented mycoplasma G maintained in aerobic conditions at 37°C for 24-48 h and microaerophilic condition at 37°C for 48-72 h respectively. Mycoplasma suspected colonies were examined with stereomicroscopic (20-60x). Molecular identification of isolate was done with 16S rRNA sequence

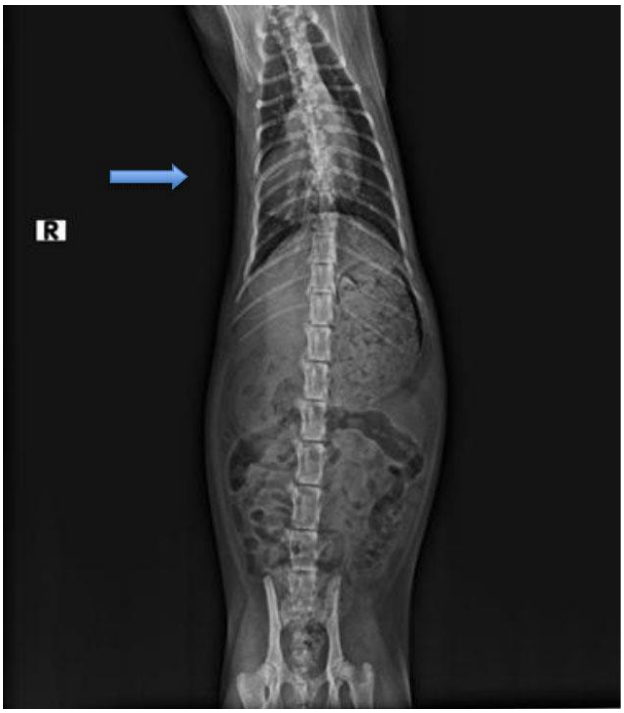

Figure 1. X-ray of the patient's chest.
A mass next to the right ventricle (blue arrow).

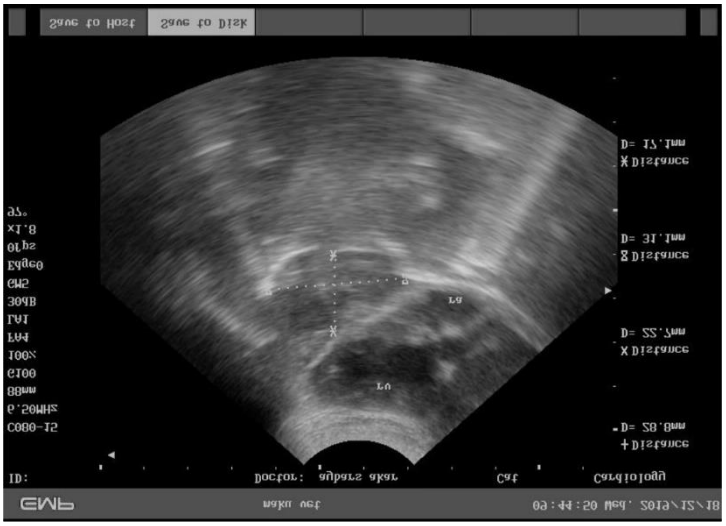

Figure 2. Pericardial mass near the right side of the heart. Left apical four chamber view. ra = right atirum, rv = right ventricle.

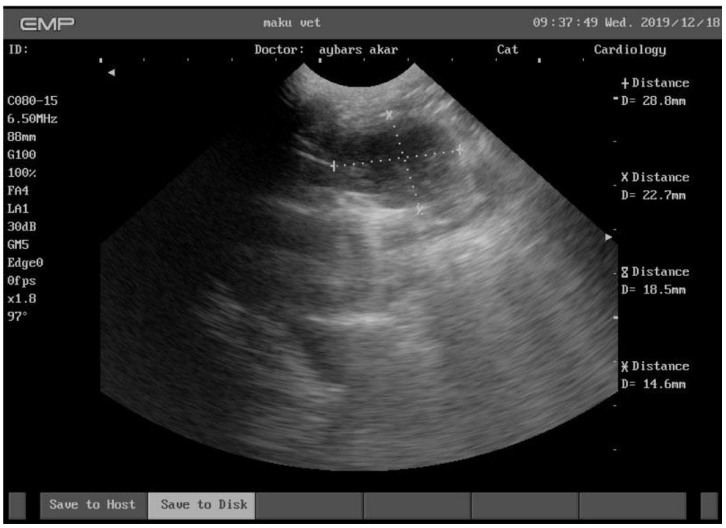

Figure 3. Hyperechoic walls and anechoic area of the mass.

analysis (ABI 3130; Applied Biosystems, USA) using universal primers 27F(5′-AGAGTTTGATCCTGG CTCAG-3'), 1492R(TACGGCTACCTTGTTACGACTT-3') which were used for both amplification and sequencing. Sequences were analyzed in the National Center for Biotechnology Information database using the Basic Local Alignment Search Tool (BLAST).

Analysis of the obtained 16S rRNA gene sequence with BLAST revealed that the isolate had a 98% sequence similarity with *Mycoplasma arginini* strain. The nucleotide sequences obtained in the study were deposited in the NCBI database with the following GenBank accession numbers: MT740481 and MT740482.

Culture of the abscess resulted in isolated of *Mycoplasma* sp. administration of amoxicillin and clavulanic acid had no effect against the infection, it was prescribed azithromycin (10 mg/kg, twice a day, per os, 7 days, Deva Holding, Turkey) and enrofloxacin (2,5 mg/kg, twice a day, subcutaneous, 7 days, Bayer, Germany) without waiting for sequence result. The cat had a complete response to treatment with no recurrence of clinical signs at 1-month post-procedure and continued to improve during the following days. Therefore the owner reported the cat was completely normal at home (Table 3, Table 4).

Table 3. Hematological values of the cat after the treatment.

Parameter	Value	Range	Evaluation
White Blood Cell(WBC)	14.06 x 10^9/l	5.5-19.5	Normal
Lymphocytes (Lym)	4.64 x 10^9/l	1.5-7	Normal
Monocytes (Mon)	0.69 x 10^9/l	0-1.5	Normal
Granulocytes (Gra)	8.74 x 10^9/l	2.5-14	Normal
Lym%	33.0 %	20-55	Normal
Mon%	4.9 %	1-3	High
Gra%	62.1 %	35-80	Normal
Red Blood Cell (RBC)	10.34 x 10^{12}/l	5-10	High
Hemoglobin (HGB)	15.1 g/dl	8-15	High
Haematocrit (HCT)	43.99 %	24-45	Normal
Mean Cell Volume (MCV)	43 fl	39-55	Low
Mean Cell Hemoglobin (MCH)	14.6 pg	12.5-17.5	Low
Mean Cell Hemoglobin Concentration (MCHC)	34.3 gr/dl	30-36	Normal
Red Cell Distribution Width (RDW)	22.4 %	-	
Platelets (PLT)	162 x 10^9/l	300-800	Low
Platelet Crit (PCT)	0.22 %	-	
Mean Platelet Volume (MPV)	13.4 fl	12-17	Normal
Platelet Distribution Width (PDW)	27.3 %	-	

Table 4. Serum biochemistry values of the cat after the treatment.

Parameter	Value	Range	Evaluation
Blood Urea Nitrogen(BUN)	48 mg/dl	19-34	High
Creatinine(Cre)	1.65 mg/dl	0.9-2.2	Normal
Alanine Transaminase (ALT)	30.7 mg/dl	25-97	Normal
Gamma-Glutamyl Transferase (GGT)	5 u/l	1.8-12	Normal
Alcaline Phosphatase(ALP)	34 u/l	0-45	Normal
Glucose(Glu)	83.16 mg/dl	60-120	Normal
Total Protein(TP)	8.02 mg/dl	6-7.9	High
Albumin(Alb)	3.29 g/dl	2.8-3.9	Normal
Total Bilirubin(TBil)	0.24 mg/dl	0-0.26	Normal
Calcium(Ca^+)	11.84 mg/dl	9.3-11.2	High
Potasium(K^+)	4.44 mEq/l	3.8-4.5	Normal

Mycoplasmas can be caused by numerous etiologies that have various clinical presentations and can often present cases associated with conjunctivitis (11, 15), upper respiratory disease (9), bronchial disease (7), and arthritis (14, 26) in cats. However, there is limited knowledge about the characteristics and clinical manifestations of mycoplasmas in domestic animals, such as horses, dogs, and cats (24). To date, pericardial abscess related to *M. arginini* has been an abnormal finding that stated in previous studies (16, 18). However, the mechanism and etiology of *M. arginini* in cats with this disease is complex.

Pericardiocentesis is effective in the management of septic pericarditis and has been reported with idiopathic pericardial effusion cases. Removing pericardial effusion is important from both a diagnostic and a therapeutic standpoint. When cardiac tamponade is diagnosed in such cases, pericardiocentesis should be performed as soon as possible (4, 13, 22). Unsurprisingly in our case, pericardiocentesis appeared necessary and, if not done, it is associated with and poor diagnosis of *M. arginini* and especially prognosis of the diseased cat.

Diagnosing *M. arginini* from cats may not be initially considered by a laboratory. This is because firstly, feline mycoplasmas may not cause enough economic loss or morbidity as other companion animals, secondly routine isolation procedures from cats do not commonly include mycoplasma-specific. Alternatively, mycoplasmas frequently detected from clinically normal cats and respiratory disease' common clinical features may make it difficult to distinguish from several primary pathogens such as feline herpesvirus 1 (FHV-1), feline calicivirus (FCV) (15, 19).

More recent advances in molecular and genetic technologies have been more effective and specific for the characterization of the 16S rRNA gene nucleotide sequence, which has provided a molecular basis for species identification and phylogeny construction of feline mycoplasmas (1, 11, 15, 21). In our case, these techniques have been applied to *Mycoplasma* isolate for the identification of *M. arginini.*

To date, appropriate treatments for *M. arginini* infections have been treated with long-term administration of anti-mycoplasma drugs, including macrolides (23). In the present case, the patient recovered promptly after administration of azithromycin and enrofloxacin treatment without waiting for the sequence result of *Mycoplasma* spp. isolate.

Here we detected for the first time *M. arginini* associated with pericardial abscess in a cat in Turkey. It was concluded that this case raises awareness in selecting the appropriate imaging and sampling for microbiological and molecular diagnosis in the diagnosis of feline mycoplasmosis. The findings of this case increased our understanding of the pathogen *M. arginini* in cats and highlighted its zoonotic importance in particular for pet owners.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Case Report / *Olgu Sunumu*

Pulmonary Veno-Occlusive Disease in a Cat with Lymphoma

Gözde YÜCEL TENEKECİ[1,a,✉], Osman Safa TERZİ[2,b], Mehmet ŞAHAL[2,c], Rıfkı HAZIROĞLU[1,d]

[1]Department of Pathology, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey; [2]Department of Internal Medicine, Faculty of Veterinary Medicine, Ankara University, Ankara, Turkey.
[a]ORCID: 0000-0002-2586-8346; [b]ORCID: 0000-0002-7877-8897; [c]ORCID: 0000-0003-3189-5778;
[d]ORCID: 0000-0002-1134-3581

[✉]Corresponding author: gozdeyucel@gmail.com
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Abstract: Pulmonary veno-occlusive disease (PVOD) is an uncommon disease in humans and animals caused by pulmonary hypertension (PH). The aim of this study was to investigate pathomorphological findings of PVOD in a cat. A mass was detected in the right atrium with echocardiography in an 11-year-old male cat. Following treatments, necropsy of the cat was performed after death due to respiratory failure. Macroscopically, masses were detected in mesenteric lymph node, mediastinal lymph node and, heart. Congestion and consolidated areas with edema were seen in the examination of the lung. Histopathologically, neoplastic lymphoid cells were observed in intestine, lymph nodes and heart. In lung, vein lumens were almost completely occluded by mostly loose intimal fibrosis. Near affected veins, increasing in number of the capillaries was observed. While intimal fibrosis was detected by Masson's trichrome staining, the occluded veins were demonstrated by Verhoeff-van Gieson staining. In this case report, pathomorphological evaluation of PVOD was performed and as a result of clinicopathological examinations; PVOD, intestinal lymphoma, and cardiac metastasis were described in a male cat.
Keywords: Cardiac, histopathology, intestinal, lymphoma, Pulmonary Veno-Occlusive Disease.

Lenfomalı Bir Kedide Pulmoner Veno-Oklüzif Hastalık

Özet: Pulmoner Veno-Oklüzif Hastalık (PVOH) pulmoner hipertansiyonun (PH) neden olduğu, insanlarda ve hayvanlarda nadir görülen bir hastalıktır. Bu olgu ile bir kedide gözlenen Pulmoner Veno-Oklüzif Hastalık'ın patomorfolojik yönden incelenmesi amaçlanmıştır. 11 yaşlı erkek kedinin ekokardiyografisinde sağ atriumda kitle gözlendi. Tedavilerin ardından solunum güçlüğüne bağlı ölen kedinin nekropsisi yapıldı. Makroskobik olarak, mezenteriyal ve mediastinal lenf yumrularında ve kalpte kitleler ile karşılaşıldı. Akciğer muayenesinde ödem ve konjesyon ile konsolide alanlar görüldü. Histopatolojik olarak bağırsak, lenf yumruları ve kalpte neoplastik lenfoid hücreler gözlendi. Akciğerde şiddetli intimal fibrozise bağlı venlerin lümenlerinde daralma ve tıkanmalar dikkati çekti. Etkilenen çoğu damarların çevresinde kapillarizasyon gözlendi. Masson trikrom boyamasıyla intimal fibrozis tespit edilirken, tıkalı damarlar Verhoeff-van Gieson boyamasıyla gösterildi. Bu olguda bir kedide PVOH patomorfolojik yönden değerlendirilmiş ve klinikopatolojik incelemeler sonucunda erkek bir kedide PVOH, intestinal lenfoma ve kardiyak metastazı tanımlanmıştır.
Anahtar sözcükler: Bağırsak, histopatoloji, kardiyak, lenfoma, Pulmoner Veno-Oklüzif Hastalık.

Pulmonary veno-occlusive disease (PVOD) is a rare pathological change seen in both humans and animals. Pulmonary veno-occlusive disease occurs as a severe and uncommon variant of pulmonary hypertension (PH) which remains a miscellaneous problem (8, 13, 18). According to the World Health Organization (WHO), PH is generally classified by the underlying cause: pulmonary arterial hypertension (PAH), Left Heart Disease, chronic obstructive pulmonary disease, chronic pulmonary thromboembolic disease, and unclear multifactorial mechanisms (11). In veterinary medicine, as well as the poor understanding of the determination and causes of PH; PVOD has been reported only once in dogs (18). A definite diagnosis of PVOD requires pathologic examination of lungs. And, the significant finding of PVOD is occlusion of pulmonary veins by fibrous tissue. Also, alveolar capillary congestion and capillary proliferation are observed (6, 10, 16).

Lymphoma is one of the neoplasms that occur frequently in domestic animals, especially in cats. Lymphomas have usually malignant character and consist of lymphocytes or lymphoid cells. They can be classified

grossly according to anatomical distribution such as multi-centric, alimentary, mediastinal, miscellaneous. Besides being the most encountered tumor in cats, alimentary/intestinal lymphoma is more common than other anatomical forms (12, 15, 17). While no cause can be determined for most intestinal lymphomas, the indirect role of Feline immunodeficiency virus (FIV) on lymphomagenesis and the direct role of Feline leukemia virus (FeLV) as an oncogenic retrovirus are known to be effective in the etiology of intestinal lymphomas in cats (9, 15).

The objective of this case was to evaluate clinicopathological of Pulmonary veno-occlusive disease detailed first time in a cat with metastatic intestinal lymphoma. So, owing to the pathomorphological examination, a relationship was established between the mentioned diseases.

An 11-year-old, 5,6 kg, neutered male cat living indoors was presented to the emergency room with a 3 day history of escalated respiratory distress, dyspnea folowing 20 days of anorexia. All of the mucous membranes were pale to cyanotic. During the clinical examination, the cat was found to be dyspneic and mildly dehydrated. Thoracic auscultation revealed no breath sounds from left part of the thorax.

Initial diagnosis was made with a complete blood count and biochemical profile (Erba XL-600, ERBA Diagnostics Mannheim, MA, USA), and thoracic radiographs. The blood panel showed some abnormalities including a leukocytosis of 22,2 x 109 cells/l (reference interval [RI] 5,5–19,5) elevated alanine aminotransferase (230,3 IU/l; RI 6–83), elevated aspartate aminotransferase (156,8 IU/l; RI 26–43), elevated creatinine kinase (703,3 IU/l; RI 56–130), uremia (155,6 mg/dl; RI 15–64,2), elevated creatinine (5,23 mg/dl; RI 0,8-1,8).

Severe pleural effusion was prominent in the thoracic radiopgraphs.. At this stage, an echocardiography (Hitachi ARIETTA 60 OH, USA) was performed, which revealed the presence of a large pericardial effusion, both anterior and posterior to the heart. Also a mass which was suspected to be a tumor or a thrombus was detected in the right atrium (Figure 1-a). 250 ml of serosanguinous fluid was yielded with ultrasound-guided thoracosynthesis.

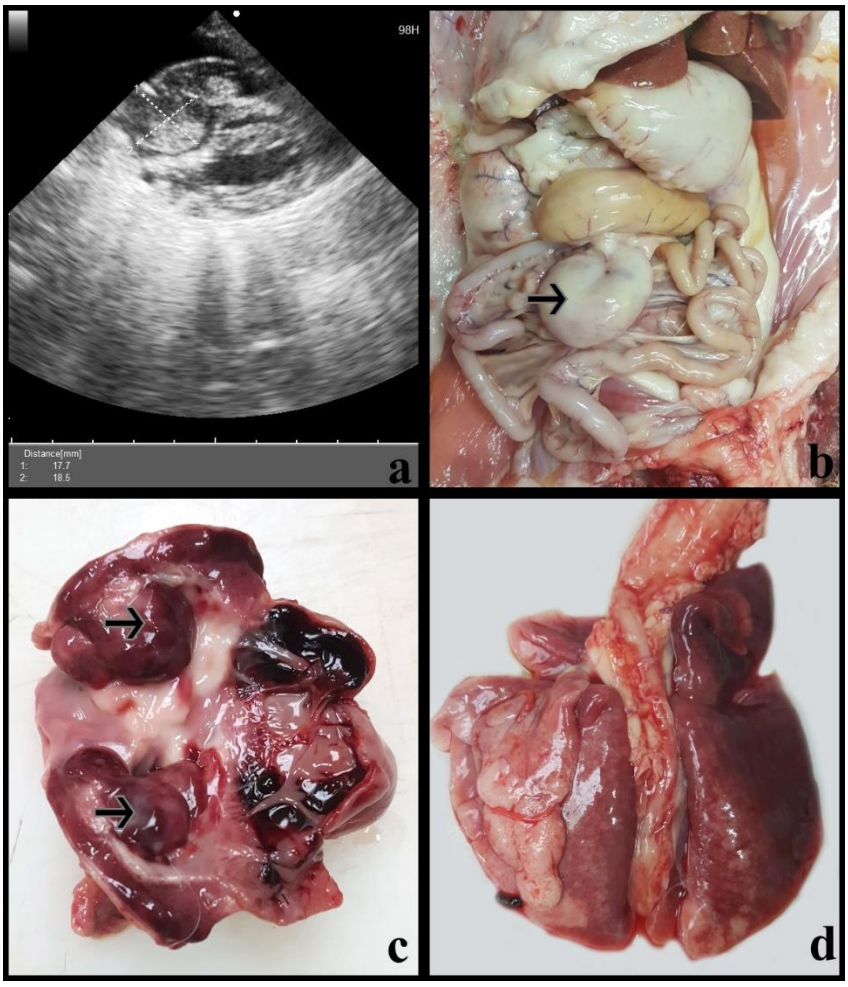

Figure 1. Echocardiogram and macroscopic findings, cat.

a. Mass in right atrium on echocardiogram. **b.** Mesenteric lymph node near caecum (arrow). **c.** Reddish mass in right atrium (arrows). **d.** Pale-coloured on the lung parietal surface.

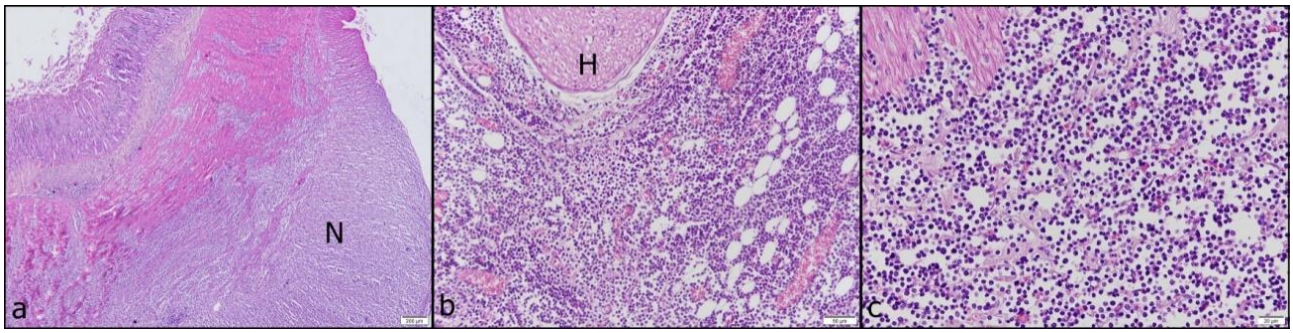

Figure 2. Microscospic findings, cat.

a. Appearance of lymphoma (N) in intestine. HE. **b.** The neoplastic cells (N) extended to His bundle (H) in heart, HE. **c.** General appearance of neoplastic cells at high magnification in heart, HE.

Furosemide 2 mg/kg IV was administered first. This was repeated 3 hours later at 3 mg/kg, and no changes were observed in the respiratory rate. Additionally, the cat was started on crystalloid fluids at 15 ml/kg/h, ampicillin sulbactam 25 mg/kg q12h IV and enalapril 1,25 mg PO. After 15 days, marked abdominal respiration, respiratory distress, lethargy and loss of appetite signs appeared again. 250 mL of fluid was removed by thoracosynthesis and pericardiosynthesis and then inspiration relaxed. After 8 days, because of severe excessive respiratory distress and heart failure, the patient died.

The necropsy was performed in Department of Pathology. Sections from lesions of organs were fixed in 10% neutral buffered formalin and routinely processed. Formalin-fixed, paraffin-embedded tissues were sectioned at 4-6 μm, and stained with haematoxylin and eosin (HE), Masson's trichrome staining, and Verhoeff van Gieson (VVG). The stainings were performed according to routine process (5).

Macroscopically, mesenteric lymph node near caecum appeared to be a huge mass with 8x3x3 cm diameter (Figure 1-b). The cut surface was necrotic. Other mesenteric lymph nodes were also increased in volume. When the chest cavity was opened, a huge mediastinal lymph node was noticed. While examining the heart, the right atrium was prominently enlarged. On the cross-section, a mass, attached from pericardium to myocardium, was seen. The mass was approximately 1 cm diameter and had reddish color and elastic consistency (Figure 1-c). In lung examination, consolidated areas with edema and congestion were seen (Figure 1-d).

Microscopically, in the mesenteric and mediastinal lymph nodes, follicular architecture was replaced by a population of uniformly lymphoid cells which were round to oval in shape. They generally had narrow cytoplasm and hyperchromatic nuclei. Not only the mesenteric lymph nodes but also the aggregate lymph follicles in intestines had malignant features (Figure 2-a). They were diagnosed as malignant lymphoma. Additionally, the centrum of the mesenteric lymph node was severely necrotic and had neutrophil leukocyte infiltration and pyknotic cells. Also, the mass in the heart showed neoplastic lymphocytic cells. These cells extended from the pericardium to the myocardium, even to the His bundles (Figure 2-b), had round to ovoid shape with generally hyperchromatic nucleus with narrow cytoplasm (Figure 2c).

In lung, vein lumens were almost completely occluded by mostly loose intimal fibrosis (Figure 3-a, b). This part of the vein is stained green/blue color with Masson's trichrome staining (Figure 3-c). With Verhoeff-van Gieson staining, in veins, single elastic lamina scattered due to fibrosis, was detected (Figure 3-d). Near affected veins, increasing in number of the capillaries was recognized. Severe edema and emphysema were seen. Also, alveolar macrophages in bronchial lumen, neutrophil leucocytes in vessel lumen were detected. In liver, severe fatty degeneration was observed.

Pulmonary veno-occlusive disease (PVOD) is one of a clinicopathological findings which is rarely seen. It was first described in 1934 by Dr. J. Hora in a 48-year-old man and until today, lots of cases were reported in human medicine about PVOD (2). According to literatures, even though some factors has been considered as possible causes like genetic factors, toxic causes, autoimmune disorders, lung diseases and heart failures; the exact cause is still unknown. But, it is acceptable as a variant of PH (7, 8). PVOD was also reported in a few dogs with hypertension (18). And, it is a known fact that prevalence of hypertension and heart failure are higher in dogs than cats (14). In the present case, PVOD was seen in a cat. The PVOD phenomenon was also reported in a Persian cat, and although that case was associated with cardiomyopathy, the main reason also remained confidential (3). In the presented case, the cause of heart failure that indirectly leads to PVOD was a cardiac metastasis of alimentary lymphoma.

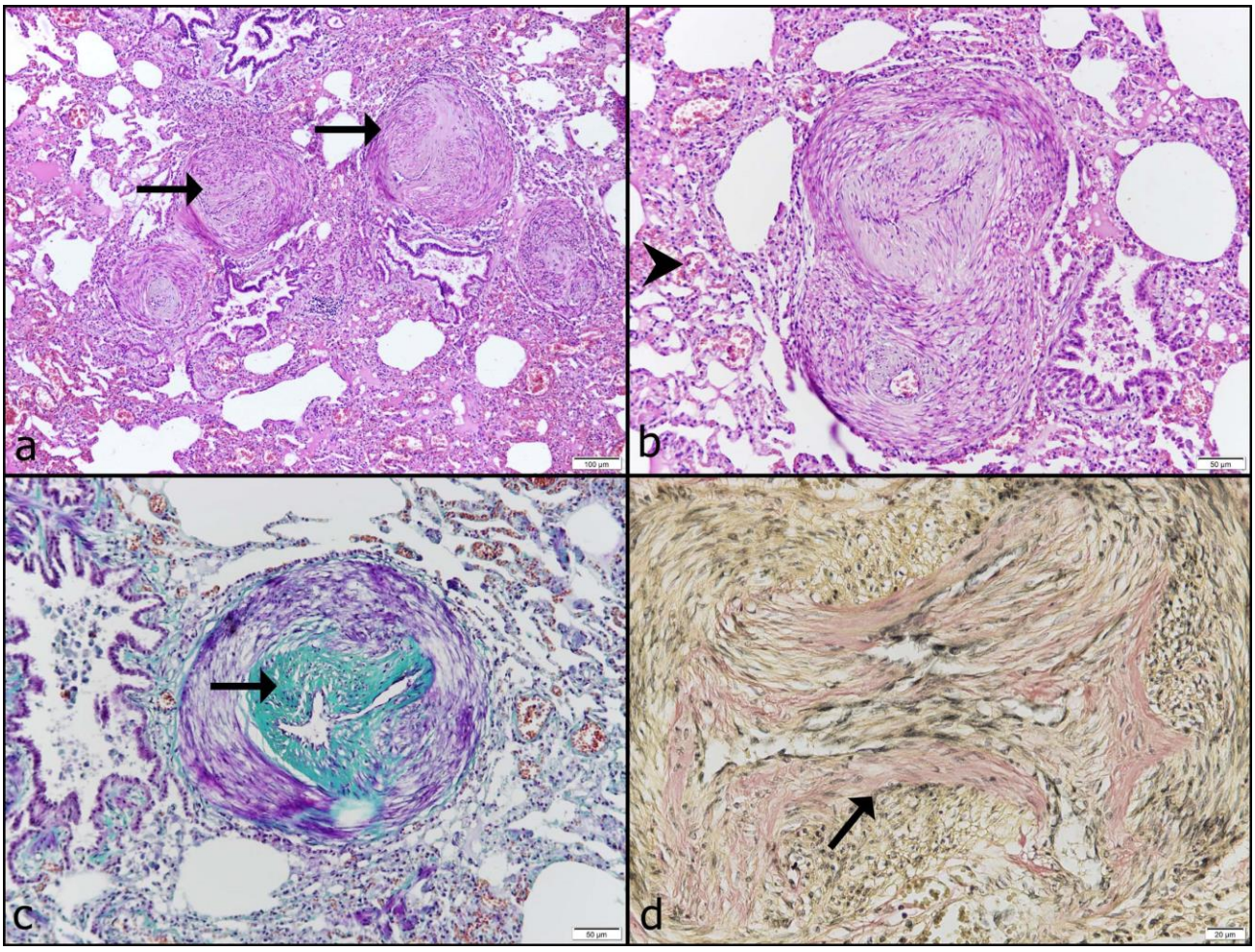

Figure 3. Microscospic findings, cat.

a. Occluded veins in lung (arrows), HE. **b.** Occluded vein lumens (arrows) and alveolar capillary congestion (arrow head), HE. **c.** Occluded vein lumens by intimal fibrosis (arrow) in lung, Masson's trichrome staining. **d.** Scattered single elastic lamina (arrow) due to fibrosis in lung, Verhoeff-van Gieson staining.

In patiens with PVOD, serum aspartate aminotransferase and alanine aminotransferase levels usually are within normal range (4). These values, which were high in this study, were attributed to liver fat, which was also observed histopathologically. But, presence of dyspnea and cyanosis were characteristic clinical signs of severe pulmonary disease including PVOD (18).

For a diagnosis of PVOD, histopathological examination is essential. As in human and canine PVOD, in the present case, occluded pulmonary vein lumens, capillarization and edema were evident (12, 18). Similar to the argument presented by Williams et al. (18), in the current case Verhoeff-van Gieson staining was used to distinguish veins from arteries. The occluded veins with a single elastic lamina were shown with this staining. Besides this, intimal fibrosis was detected by Masson's trichrome staining with green color of fibrous tissue.

Another thing that makes the case special is the occurrence of heart failure due to cardiac metastasis of alimentary lymphoma. Cardiac masses arising from the heart generally have poor prognosis whether diagnosed as benign or malignant. Lymphoma has been observed in the heart of cats. According to a study conducted by Aupperle et al. (1), two cases of five secondary cardiac tumors were malignant lymphoma and, regardless from the type of tumor, depending on their anatomical location in heart, cardiac tumors can result in congestive heart failure as in the presented case.

In summary, pulmonary veno-occlusive disease, which is mostly seen in both humans and dogs, was reported for the first time in a cat with lymphoma. However, details of the development mechanism keep it uncertain, in present case; it was thought that the mass indirectly led to heart failure and PVOD formation in the lung.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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Review / *Derleme*

3D Printing in Veterinary Medicine

Pınar YILGÖR HURİ[1,3,a], Çağdaş OTO[2,3,b,✉]

[1]Ankara University, Faculty of Engineering, Departmant of Biomedical Engineering, Ankara; [2]Ankara University, Faculty of Veterinary Medicine, Department of Anatomy, Ankara; [3]Ankara University, Medical Design Research and Application Center, Ankara, Turkey
[a]ORCID: 0000-0002-4912-0447; [b]ORCID: 0000-0002-2727-3768

✉Corresponding author: coto@ankara.edu.tr
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Abstract: The use of 3D printing technology in the field of medicine, which started with the millennium, continues to increase today. Depending on the technological developments in this field, the use of rapid prototyping technology in the field of veterinary medicine is becoming widespread with the development of 3D printers, increasing material variety, cheaper printing costs and being more accessible. Additive manufacturing is used in veterinary education and training, experimental research and clinical studies, and its area of use is expanding day by day. In this review, both the current usage potential will be evaluated and the expected developments in the near future will be revealed.
Keywords: Additive manufacturing, Bioprinting, Rapid prototyping, three-dimentional printing, veterinary medicine.

Veteriner Hekimlikte Üç Boyutlu Baskı

Özet: Milenyum ile birlikte başlayan 3 boyutlu baskı teknolojisinin tıp alanında kullanımı, günümüzde artarak devam etmektedir. Bu alandaki teknolojik gelişmelere bağlı olarak 3 boyutlu yazıcıların gelişmesi, malzeme çeşitliliğinin artması, baskı maliyetlerinin ucuzlaması ve daha kolay ulaşılabilir olmaları ile hızlı prototipleme teknolojisinin veteriner tıp alanındaki kullanımı da yaygınlaşmaktadır. Katmanlı üretim, veteriner hekimlikte eğitim, deneysel araştırmalar ve klinik çalışmalarda kullanılmakta, kullanım alanı her geçen gün genişlemektedir. Bu makalede hem mevcut kullanım potansiyeli değerlendirilecek, hem de yakın gelecek beklentileri irdelenecektir.
Anahtar sözcükler: Biyobaskı, hızlı prototipleme, katmanlı üretim, üç boyutlu baskı, veteriner tıp.

Introduction

A high-resolution 3-dimentional (3D) image can be created digitally in a short time through CAD programs, by either directly designing a model or by using 3D surface scanners, or by reconstruction of cross-sectional medical imaging data such as ones created with computed tomography (CT), magnetic resonance imaging (MRI), and ultrasound (USG). It has been shown in many studies that these 3D data could help in veterinary medical education, training and surgical planning (8, 11, 17, 19, 23, 28). In addition, artificial intelligence (AI), virtual reality (VR) and augmented reality (AR) applications, e-learning and long-distance learning facilities that have come into our lives with the results of digitalisation in recent years have taken the use of 3D digital data to a higher level in medicine (17). Despite all the advantages of these applications, the use of digital data in applied sciences is limited at some point. 3D printing constitutes a promising approach by which it has become possible to physically prototype these 3D digital images and to create a real representation model. After the first applications of 3D printing in the medical field in the current millenium, 3D printed models for both clinical and research purposes have also started to be used for animals. In recent years, with the innovations in both software, hardware and materials fields, the use of 3D printing in veterinary medicine is expanding (29, 30, 63).

This article will review the basic principles of 3D printing technology and its educational, experimental and clinical applications in veterinary medicine.

What is 3D Printing?

3D printing, also known as additive manufacturing or rapid prototyping, is simply a process of making 3D solid objects from a digital file.

The manufacturing process of a 3D model consists of four key steps; (1) Creating the 3D digital data, (2) Image processing, (3) Modeling, and (4) 3D printing. The first step in creating a 3D object with this workflow is the formation of the 3D digital data. There are two main methods for creating the 3D digital data including (1) creating the models with a computer assisted design (CAD) software, and (2) using scanners (surface scanners or transmissive medical scanners such as CT, MR or USG). In the second step of production cascade, 3D volumetric data is created by rendering and segmentation of 3D images and then the images are exported as a compatible file format such as Surface Tessellation Language (STL). The next step is slicing the 3D digital object into layers, then, the workflow for 3D printing is created by giving g-codes for each layer. The object can also be modified according to the printing settings. The final step of 3D printing is achieved by using additive processes performed by equipment called 3D printers. As the main principle for the additive manufacturing method, the object is created by laying down successive layers of material until the object is fully created. Each of these layers can be seen as a thinly sliced cross-section of the object.

Charles W. Hull, who was an American furniture builder, filed the first patent for Stereolithography (SLA) in 1986. Hull developed a system for creating 3D models by curing photosensitive resin layer by layer. After the first 3D printer hit the marketplace commercially in 1988, many different 3D printing techniques were developed in a short time.

Although many 3D printing technologies are offered today, it can be said that the most frequently used ones in the medical field are classified into 3 according to the print material and machining technology. These include SLA, as the oldest technique, selective laser sintering (SLS), and fused deposition modeling (FDM) (59). SLA printers work based on polymerization. They use UV light and a liquid photopolymer, referred to as resin material, that is solidified and formed by crosslinking of the resin with the activity of light. SLS printers work based on binding. They use the laser as a power source and powder material that can be either metallic, ceramic or polymeric in nature. FDM 3D printers are easily the most affordable and most popular printing device in use today. They are extrusion based systems that use either molten thermoplastics that are deposited on a built plate, or cross-linkable hydrogel-based materials that are 3D printed into crosslinker baths. In the latter, the material can be loaded with cells, therefore a bioink is used during 3D printing, which is referred to as bioprinting. Bioprinting represents a new frontier of 3D printing technology. This is the realization of robotic tissue production by combining live cells and other biological elements together with biomaterials (21, 44).

Applications on Veterinary Medicine

Education and Training: Accurate anatomic models used in learning and training facilities are essential for all levels of the veterinary education system such as undergraduate and postgraduate courses, clinical skills trainings and client education (37, 45, 53, 60). Compared to other commercial production methods such as molding and machining, 3D printing is one of the most practical and costly effective methods for production of solid replicas (12, 41). Also, these models which are produced using less toxic and more resistant materials against deterioration, contribute to human health and environmental health as well as the protection of valuable and scarce anatomical specimens (5, 33).

Bakıcı et al. (3) produced 3d replicas of the hyoid bone in domestic animals and used them in comparative anatomy education. 3d volumetric data of the bones were obtained by open source CAD software from CT images. FDM printer and polylactic acid (PLA) filament was used for printing. The authors stated that it is important for veterinary anatomy education to obtain easily and cheaply accurate replicas of the hyoid bone, which is difficult to macerate and very fragile. In other studies conducted with a similar scanning and printing method, Bakıcı et al. (4) modeled digit bones both the forelimb and hindlimb in horses, Alcântara et al. (1) also produced 3D prints of the foreleg skeleton in horses. Lima et al. (37) created anatomy and fracture models on the canine mandible. Bertti et al. (7) used dog skull prints for educational purposes. Unlike the above studies, Li et al. (36) scanned the cattle bones with a surface scanner instead of the tomography images, and obtained 3d copies. Besides the anatomy education, the 3d replicas are also used in the education of other disciplines of veterinary science with regards to a subject like orthopedic surgery (26, 46), equine podiatry (50), zooarchaeology (42), pathologic specimens (35), and forensic science (31, 32, 48). In the literature review, it was seen that bone samples were often preferred in the production of 3d anatomical models for educational purposes. The reason for this can be considered both to increase the printing quality of high resolution bone tissue images obtained with CT, and easy to find the orientation of bone structures compared to soft tissues in scanning and 3d reconstruction stages. In addition, the printing time of soft thermoplastic materials such as TPU is longer and the cost is higher. 3D brain models printed from scanning files (47, 56), dog stomach models obtained from surface scanning (24), and 3d organ

replicas (6) are examples of a small number of studies with soft tissue printing models.

All these studies emphasized that accurate anatomical replicas obtained by 3d printing can be used as an alternative to traditional methods and original specimens in veterinary education.

As known, clinical skills training and simulators are an important part of the veterinary education system. For this purpose, patients, cadavers, animal models or virtual applications are often used. The most of challenges here arise from the difficulties in obtaining models, cost and sustainability, as well as ethical responsibilities. 3D printed anatomical models are also widely used for clinical skills training and simulations. For this purpose, dog and cat intubation models, injection models (62), bronchoscopy (54) and endoscopy models, fracture bone models for simulation can be created. Our experience shows that replicas created from CT and MR images in training models are superior in detail and accuracy to copies obtained using a surface scanner.

Clinical Studies: Clinical applications of 3D printing in veterinary medicine can be grouped as preoperative planning, custom-made surgical tools, laboratory devices and special guides, patient specific surgical implants and prostheses, and client education.

Today, images obtained through medical imaging methods and virtual applications are widely used in presurgical planning and provide great advantages to surgeons. However, interpretative differences between real anatomical structures and 2D images or 3D reconstructed virtual applications still continue, especially in surgical procedures to be performed on structures with complex anatomy, and there may be problems in understanding the details. At this point, the importance of case-specific models obtained with 3D printing in preoperative planning becomes clear (20, 25, 40, 49, 57). Patient specific 3d printed models of the anatomical region that require surgical repair can provide the surgeon with better visualization of the abnormalities and thus help prepare for surgery (51). These 3D models offer advantages such as tactile sensing of an object, creating surgical routes, determining and preparing the implant or prosthesis to be used, designing surgical guides if necessary, and providing a more understandable communication with the patient owner for the operation to be performed.

The study is a good example for using 3d printed models in presurgical planning and operation management that the surgeons produced a full-size 3D model based on CT images in a 10-week-old female puppy with 5 different congenital cardio-thoracic vascular anomalies. The replica was used for planning surgical angles and routes, selection of surgical tools, stapling devices and clamp sizes, the orientation of abnormalities and team coordination (15). The authors emphasized that in the surgery of complex anatomical structures, especially in terms of intraoperative orientation success, a complete comparison can only be achieved with a physical model.

Winer JN et al. (64) studied 3D printed skull models in 28 dogs and 4 cats for preoperative planning purposes before oral and maxillofacial surgery. They produced plastic replicas created on CT images of cases for mandibular reconstruction, temporomandibular joint ankylosis, palatal defects and neo plastic surgeries. Dorbandt et al. (14) also printed 3d models of 3 dog skulls for preoperative planning in case of orbital and peri-orbital mass.

3D printing models are used for preoperative planning in orthopedic studies especially in bone fracture (2, 38), leg deformities (52), and tumor surgery (64).

The studies emphasized that using 3D printed models in presurgical planning provides shortened surgery time, improved surgical accuracy and surgical success, and reduced perioperative risks and postoperative complications. They stated that 3D printed models are also a valuable resource to improve clients' understanding of the pet's disorder and the recommended treatment. Therefore, the 3D models help the clients education and communication.

3d printing technology is used in different fields of veterinary medicine to produce custom-made equipment for both users and patients. Surgical tools and equipments (43, 51, 65) or guides (22) for surgeons or patients can be produced by 3D printer. Similarly, tailor-made equipment and custom-made drugs can be produced for use in laboratories (16, 18, 39, 58, 61). Another using area is the creation of surgical guides. The case-specific guides can be very effective in terms of the success of the operation (22).

Santos et al. (55) performed external orthoses for metatarsal fractures in a calf. The area was scanned by a surface scanner and printed with petG filament on an FDM printer. It was observed that the animal was able to walk again when the leg was supported with the orthosis.

In their experimental study, Bolanos et al. (9) scaffolded and implanted bioceramic material to heal the tuber coxae defect in the horse. The researchers demonstrated that a brushite-based implant was able to promote new bone growth and the horse as a bone model is an encouraging tool for in vivo studies of biomaterials.

Kamishina et al. (34) produced a patient-specific ed printed model which was made of titanium to support the spine and used it as a surgical implant in order to provide stabilization in the surgical treatment of atlantoaxial joint subluxations, which can be seen especially in small breed dogs. Hayes et al. (27) also created a custom-made titanium plate for the cranioplasty of a dog which was suffered from osteochondrosarcoma. The presented

studies showed that the accuracy of 3d printed implants had great importance especially the production of case specific models by printers.

The sterilization of implants placed in the body is also another limitation for using 3d printed implants. Implantation of these models has great importance, especially at the point of surgery (13). While heat-induced sterilization may be appropriate for metal implants, different sterilization methods have been tried for 3D printed implants made of thermoplastic material (10).

Conclusion

This technology may prove to be worth the investment as it will bring many creative possibilities for educational enhancement, research, professional development, and for thinking outside of the box. As the technology continues to improve, 3D printing is destined to rise in applications across all disciplines of veterinary medicine.

Conflict of Interest

The authors declared that there is no conflict of interest.

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